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Genetic variants contribute to differences in response and toxicity to drugs used in autoimmune diseases: Rheumatoid arthritis and Systemic lupus erythematosus

Cristina Lucía Dávila Fajardo

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Contents

Chapter 1

General introduction and outline of the thesis

RHEUMATOID ARTHRITIS AND OTHERS AUTOIMMUNE DISEASES

Rheumatoid arthritis (RA) is an autoimmune disease associated with progressive disability and systemic complications. RA is characterized by synovial inflammation and hyperplasia, autoantibody production (rheumatoid factor and anti-citrullinated protein antibody ACPA), cartilage and bone destruction, and systemic features, including cardiovascular, pulmonary, psychological and skeletal disorders (1). The etiology of this inflammatory disease remains unclear due to complexity of interacting factors including both genetic and environmental determinants. The long-established association with *HLA-DRB1* locus has been confirmed in patients who are positive for rheumatoid factor or ACPA (2). Smoking (3), infectious agents and female gender have been recognized as risk factors associated with RA (1). Moreover, gene-gene and gene-environment interactions increase the risk for RA. The environment-gene interactions promote loss of tolerance to self-proteins that contain a citrulline residue, which is generated by post-translational modification and detected in T-cell and B-cell compartments. Why the systemic loss of tolerance is linked to a localized onset of inflammation in the joint is still unclear (1).

Disease-modifying anti-rheumatic drugs (DMARDs), including methotrexate (MTX) in RA have the capacity of reducing or preventing damage to the joints and preserving their integrity and function by modulating the immune response. However, the results of treatment with these drugs in patients diagnosed with RA are variable and unpredictable.

The anti-inflammatory mechanism of action of MTX is explained by its ability to inhibit cellular proliferation by reducing purine and pyrimidine synthesis, particularly in the cells most pertinent to synovial inflammation, such as T lymphocytes (4). MTX is well established as a competitive inhibitor of dihydrofolate reductase, and thereby prevents the regeneration from dihydrofolate of tetrahydrofolate, which is essential for the generation of folate cofactors required for de novo purine and pyrimidine synthesis (5).

The understanding of how MTX is effective at low doses in inflammatory diseases also provides some insights into how its well-known toxicities arise. The toxic effects have been suggested to result from a depletion of hepatic folate stores and the accumulation of MTX polyglutamates in the liver (6). When no complete response is obtained with MTX, other DMARDs can be used in sequential or combined therapy, or to add a biologic agent or with targeted therapy.

The introduction of biologic agents has notably altered the treatment of RA; these agents not only reduce symptoms and signs of the disease, but also delay its radiologic progression (7).

At present, five TNF inhibitors are available for the treatment of RA, three of which are full-length monoclonal antibodies: infliximab, adalimumab and golimumab. The fourth agent, etanercept, is a fusion protein of two TNFR2 receptor extracellular domains and the Fc fragment of human immunoglobulin 1 (IgG1). Certolizumab is a humanized Fab fragment conjugated to polyethylene glycol (PEG) without IgG1 region (8).

Biologic agents exert their pharmacological effects through their variable portion (designed to block the target molecule) and their constant portion (the Fc fragment of IgG1), which specifically binds the human FcG receptors (FcGRs) (9-12). FcGRs are expressed on the surface of most immune cells. Engagement of FcGRs by TNF antagonists could affect a number of cellular functions, including phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), induction of apoptosis, cytokine release and macrophage-mediated clearance of immune-complexes (12, 13).

However, these treatments are substantially more expensive than traditional DMARDs and, unfortunately not efficacious in all patients (14). Some studies point out that between 25% and 30% of subjects with RA do not respond to anti-TNF treatment (15). There are other biologic agents used in the treatment of RA: anti-IL-6 receptor (tocilizumab, sarilumab), anti-IL-1 receptor (anakinra), anti-CD20 (rituximab) and anti-CD80/86 (abatacept). Similarly, targeted synthetic DMARDs, as baricitinib and tofacitinib, were developed to interfere with a specific molecule, Janus kinases (JAKs), based on advances in molecular and structural biology. They interfere with JAKs— intracellular signal transduction molecules that translate the effects of some cytokines to cellular responses (16).

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease of unknown cause that can affect virtually any organ of the body. Immunologic abnormalities, especially the production of a number of antinuclear antibodies (ANA), are a prominent feature of the disease. Patients present with variable clinical features ranging from mild joint and skin involvement to life-threatening renal, hematologic, or central nervous system involvement (17). The clinical heterogeneity of SLE and the lack of pathognomonic features or tests complicate the diagnostics (18).

The choice of therapy for SLE is highly individualized and depends on the predominant symptoms, organ involvement, response to previous therapy, and disease severity. A general approach to treating the predominant symptomatology include immunosuppressive agents: hydroxychloroquine or chloroquine, glucocorticoids, mycophenolate, cyclophosphamide and other agents targeting B-cell pathways as belimumab (19, 20) and rituximab (21). Belimumab has been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for use in SLE patients, whereas use of rituximab is considered off-label use.

Rituximab is a chimeric monoclonal immunoglobulin G1 antibody against the CD20 protein of B-lymphocytes promoting B cell depletion (22, 23). It has become a crucial therapy against systemic autoimmune diseases, since an aberrant B cell regulation is among the common pathogenic mechanisms of these diseases (24). FDA and EMA have approved the use of rituximab in Non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and RA in combination with MTX in adult patients with moderately to severely active RA who have an inadequate response to one or more tumor necrosis factor anti-TNF, Wegener's granulomatosis, and microscopic polyangiitis in adult patients in combination with glucocorticoids. Recent studies in other systemic autoimmune diseases show the importance of this therapy in refractory patients (25-30).

PHARMACOGENETICS OF DRUGS USED IN RA AND SLE

Pharmacogenetics is defined as the study of variability in drug responses attributed to genetic factors (31, 32). It has become one of the leading and potentially most actionable areas of the personalized medicine paradigm, as evidenced by the increased availability of clinical pharmacogenetic testing (33).

The consequences of treatment with DMARDs in patients diagnosed with RA or SLE are variable and largely unpredictable. A possible cause that explains the interindividual differences in both efficacy and adverse events can be the genetic variations in genes encoding drug metabolizing enzymes or drug transporters (34-43).

The treatment with biologic agents is substantially more expensive than use of traditional DMARDs and, moreover, are not effective and safe for everyone (14). Early identification of subjects who respond to these drugs may be helpful when establishing a (cost)effective and safe treatment with these drugs (44).

OUTLINE OF THIS THESIS

The primary objective of this thesis is to investigate the role of pharmacogenetics in predicting drug response in treatments for the autoimmune diseases: RA and SLE. For this reason, this thesis is divided in two parts: pharmacogenetics related with drugs used in RA and pharmacogenetics of rituximab used in SLE and other autoimmune diseases.

Part 1: Pharmacogenetics of drugs used in RA

MTX is the most common DMARD used in RA. However, its use is hampered by frequent adverse drug events among which gastrointestinal toxicity is most frequent. Hepatotoxicity is a relatively rare but serious adverse event related to the use of MTX and is largely unpredictable. In **chapter 2** an overview is presented of the previously performed studies concerning pharmacogenetic predictive biomarkers for MTX-induced hepatotoxicity.

Treatment with anti-TNF agents results in a reduction of disease activity in most RA patients. However, a substantial part of patients does not respond to this therapy for unknown reasons. It would be highly beneficial to be able to predict whether or not an individual patient responds to treatment. **Chapters 4** and **5** describe the investigations on the role of different candidate SNPs related to the efficacy of the treatment with different anti-TNFs in RA. In addition, in **chapter 3** a replication study is presented based on 4 polymorphisms that were found associated with anti-TNF response in RA in a previously published genome-wide association study.

Part 2: Pharmacogenetics of rituximab used in SLE and other autoimmune diseases

In **chapters 6–8** the role of different genetic variants related to the pharmacodynamics of the drug or of the diseases are evaluated to study the contribution to differences in the response to rituximab in patients with SLE and other systemic autoimmune diseases.

In **chapter 6**, the possible involvement of the *-174 IL-6* polymorphism in the clinical response to rituximab in different systemic autoimmune diseases is assessed. In **chapter 7**, the aim is to investigate the possible involvement of the *FCGR3A-158F/V* polymorphism in the clinical response to rituximab in Spanish patients with different systemic autoimmune diseases. In **chapter 8**, the role of G/T polymorphism at the *IL2–IL21* region in the rituximab response in a cohort of SLE patient and different autoimmune disorders is analyzed.

Chapter 9 provides a summary of this thesis, **chapter 10** the Dutch summary (Nederlandse samenvatting), and **chapter 11** the general discussion and future perspective of this thesis.

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Chapter 2

Genetic risk factors for drug-induced liver injury in rheumatoid arthritis patients using low-dose methotrexate

Cristina Lucía Dávila-Fajardo, Jesse J. Swen,
José Cabeza Barrera, Henk-Jan Guchelaar

Pharmacogenomics. 2013;14(1):63-73

Abstract

Low-dose methotrexate is part of the mainstay of rheumatoid arthritis treatment. Hepatotoxicity is among the most feared side effects of low-dose MTX and is associated with increased morbidity. At present, histological evaluation of liver biopsies is the gold standard to retrospectively diagnose MTX-induced liver damage. Genetic markers present an interesting opportunity to pre-emptively identify patients at risk for MTX-induced hepatotoxicity. Here, we will review the literature on candidate genetic markers for the risk of MTX-induced hepatotoxicity. These candidate genetic markers include polymorphisms in the gene encoding the enzyme MTHFR.

INTRODUCTION

Low-dose methotrexate (MTX) is part of the mainstay of rheumatoid arthritis (RA) treatment (1, 2). MTX shows efficacy in approximately 50% of patients with early RA at doses of 12.5–25 mg/week (1). In this MTX schedule, the drug is thought to act primarily as an anti-inflammatory drug, specifically through the release of adenosine, rather than as an antimetabolite drug (3, 4). Once MTX has entered the cell it is subject to polyglutamation, which inhibits several key enzymes, including MTHFR, an enzyme involved in the folic acid cycle (Figure 2.1).

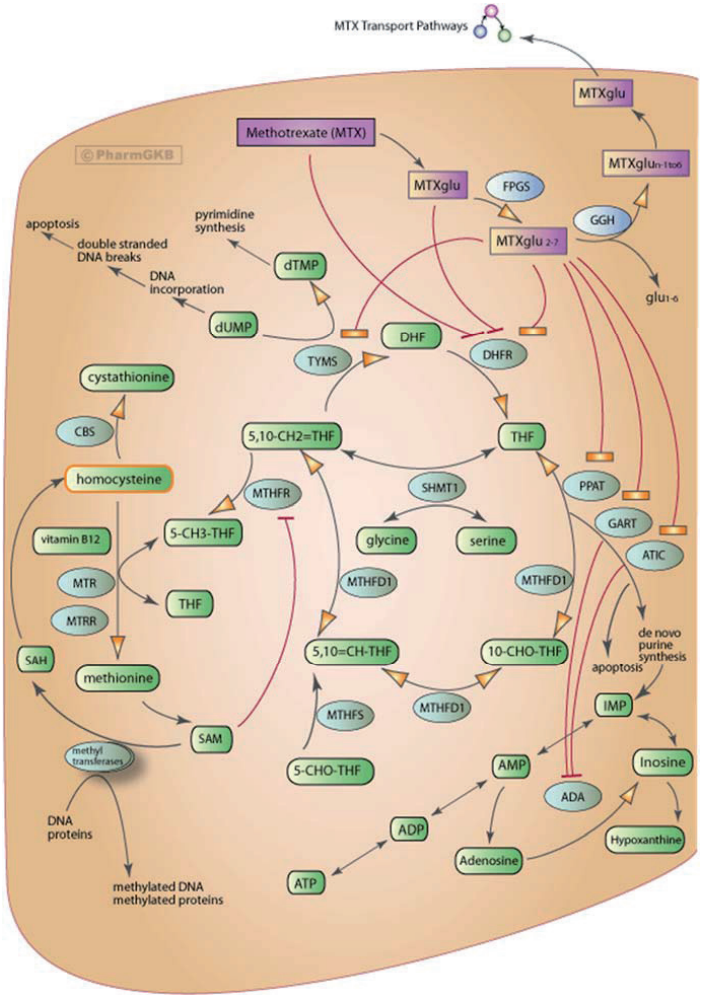

Figure 2.1: Candidate genes in the Methotrexate pathway. Copyright PharmGKB.
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MTX hepatotoxicity

MTX has a well-defined toxicity profile, including bone marrow suppression as well as gastrointestinal, pulmonary and hepatic toxicity. With the low MTX doses used in rheumatology, side effects are frequent and the cause of cessation of MTX therapy in approximately 30% of patients during the first year of treatment (5). Hepatotoxicity is among the most feared side effects of low-dose MTX. Effects of MTX on liver histology during chronic MTX use are fatty infiltration, macrovesicular steatosis, hepatocellular necrosis and fibrosis (6) with predominantly portal and periportal inflammation, which may herald the development of cirrhosis (7). However, clinically, or even biochemically, these findings may be silent for years (6).

Different mechanisms for MTX-induced hepatotoxicity have been proposed (4), including a depletion of hepatic folate stores and the accumulation of toxic MTX polyglutamates in the liver. Also, MTX has been shown to enhance the release of adenosine from hepatic slices *ex vivo*.

This event, in turn, activates and stimulates matrix protein production by fibrogenic stellate cells in the liver (Figure 2.1) (4).

Early prospective studies of MTX in patients with RA showed that its use is associated with an increase of hepatic transaminase enzymes such as ALT and AST in some patients (8). In addition, studies showed that liver enzyme abnormalities correlated with actual biopsy samples of hepatic tissue (9, 10). The incidence of MTX-induced increase of transaminases varies according to different definitions. Some investigators have defined it as elevated liver enzymes two- to three-times greater than the upper limit of the normal (ULN) range. These studies have estimated the frequency of increase of transaminases to be 7.5–26% of all patients treated with MTX. Others have shown that the incidence of abnormal ALT/AST is 48.9% with cutoff transaminase values of above ULN and 16.8% with cutoff transaminase values of >two- to three-times ULN level (11, 12).

Studies on the incidence of liver injury after long-term MTX therapy in RA patients showed that the risk of developing cirrhosis or fibrosis is less than 2% (13–16). Thus, liver enzyme elevations in RA patients on MTX are frequent but often transient and MTX-induced fibrosis/cirrhosis is rare. Whiting-O´Keefe reported a prevalence of advanced histological changes (Grade IIIb/IV) of 2.7% after 4 years on MTX in a meta-analysis of 334 RA patients (17).

The impact of MTX-induced hepatotoxicity can be serious. Two surveys, from Sweden and Spain, suggest that drug-induced liver injury (DILI) with jaundice is associated with greater mortality or the need for liver transplantation than is hepatocellular and/or mixed injury

(18, 19). Interestingly, it was shown that elevated AST and bilirubin levels were independent predictors of death and liver transplantation in patients with hepatocellular injury (18).

Assessing MTX hepatotoxicity

At present, histological evaluation of liver biopsies is the gold standard to diagnose MTX-induced liver damage (6, 20, 21). However, this procedure is invasive and uncomfortable for patients and serious complications (e.g., hemorrhage and pneumothorax) may occur incidentally (22). In addition, many trials show the incoherence of liver enzymes and histological findings (23). Therefore, noninvasive methods for detecting and monitoring liver fibrosis are highly desirable (24). Imaging methods of the liver have been evaluated but ultrasound and magnetic resonance imaging are both inadequate for detecting fibrosis since they yield morphological rather than dynamic and functional information regarding the liver (24-26). The amino terminal peptide of type III collagen in serum correlates directly with the amount of ongoing hepatic fibrogenic activity. However, the amino terminal peptide of type III collagen is not organ specific and may be raised in children and in various other pathologies, including arthritis, scleroderma and hyperthyroidism (22, 24).

The use of a standardized severity and causality score for MTX-induced hepatotoxicity is of importance as to objectively register events in patients and to compare incidences across studies. A clinical diagnostic scale was developed for the diagnosis of DILI and considered to be suitable for use in routine clinical practice (27, 28, 88). In a multinational evidence-based guideline for the use of MTX in RA (29) it is recommended that when initiating MTX treatment or increasing the dose, ALT with or without AST, creatinine and complete blood count measurements should be performed every 1–1.5 months until a stable dose is reached and every 1–3 months thereafter. MTX treatment should be stopped if there is a confirmed increase in ALT/AST > 3 ULN, but may be reinstituted at a lower dose following normalization of the liver enzymes. If ALT/AST levels are persistently elevated up to 3 ULN, the dose of MTX should be adjusted and diagnostic procedures should be considered in the case of persistently elevated ALT/AST > 3 ULN after discontinuation (29). It has been observed that cessation of MTX therapy does not always result in immediate improvement in abnormal liver enzyme values but may persist for several days or even weeks after discontinuation. Obviously, other risk indicators such as the use of nonsteroidal anti-inflammatory drugs, obesity and the use of alcohol contribute to the risk of MTX-induced hepatotoxicity (29). More recently, studies have reported that pharmacogenetic variants in genes encoding proteins involved in the mechanism of action of MTX are associated with MTX-induced hepatotoxicity. These genetic and non-genetic determinants may be useful

to predict the individual patients' risk for MTX-induced hepatotoxicity and could help to reduce the incidence and morbidity. This study aims to review the literature on potential risk factors for MTX-induced hepatotoxicity in RA patients, including the role of genetic markers that contribute to this important clinical side effect.

METHODS

In general, DILI is defined as a rise in either ALT level above ULN/more than two- to three-times of ULN, or alkaline phosphatase level more than twice ULN, or total bilirubin level more than twice ULN when associated with increased ALT or AST (30, 89). A systematic literature search of PubMed was performed in September 2012 using MESH terms 'Methotrexate', 'Rheumatoid arthritis', 'DILI and/or Risk factors and/or SNP'. Individual abstracts were reviewed for relevance related to determinants of low-dose MTX-induced hepatotoxicity. Selected papers were studied comprehensively and summarized. Cross references were screened for relevance and included when useful.

RESULTS

Our initial literature search identified 230 publications (Figure 2.2). In total, 30 of these references were excluded; eight because they were not published in English, ten because they did not include RA and 12 because they did not include MTX.

Non-genetic risk factors

An overview of the identified nongenetic risk factors is presented in Table 2.1. The cumulative MTX dose and duration of treatment play an important role in the evolution of MTX-induced hepatotoxicity (15, 31, 32). A cumulative dose of > 1.5 g and a duration of therapy exceeding 2 years in RA patients are considered risk factors for hepatotoxicity. However, long-term treatment, as long as 10 years, of weekly oral low-dose MTX, did not result in cirrhosis or severe fibrosis in RA patients who did not abuse alcohol (33) suggesting that the relationship is probabilistic rather than absolute and is only one of the potential risk factors that contributes to MTX-induced hepatotoxicity (34). Patients on average had a 6.7% (95% CI: 2.1–11.4) chance of progressing at least one histologic grade on liver biopsy for each gram of MTX taken (17).

Another possible nongenetic risk factor is the use of other hepatotoxic drugs or chemicals (35) such as alcohol (17, 34, 36-38). Patients who are heavy drinkers (at least 100 g of

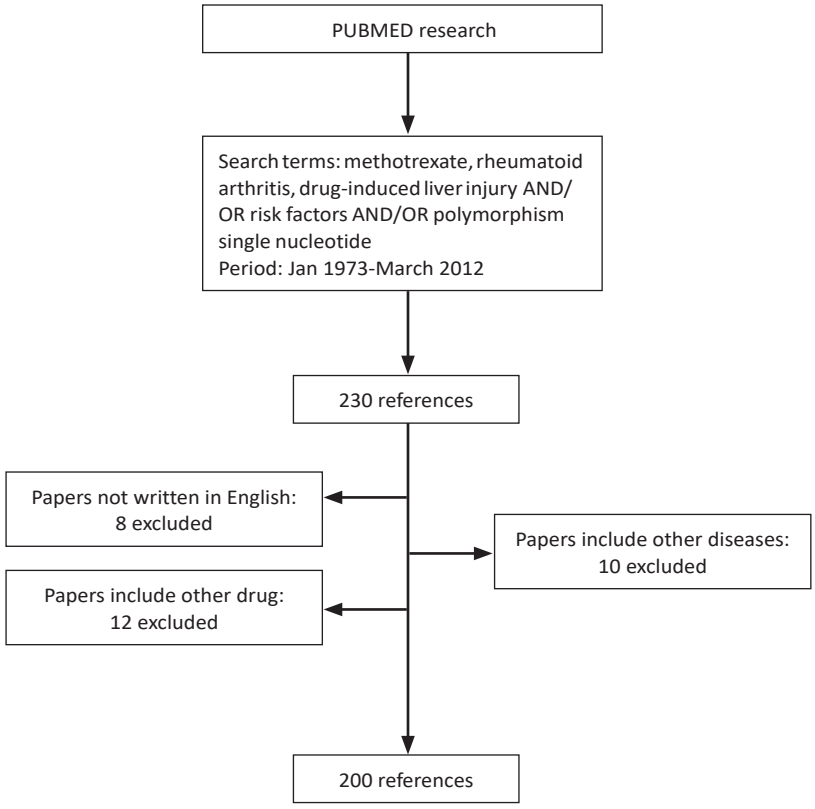

Figure 2.2: Results of the literature search.

alcohol/week) were more likely to have advanced changes on liver biopsy (17.8 vs 4.5%, p = 0.0003) (17). However, in the study by Malatjalian et al. no statistically significant correlation between alcohol consumption and MTX-induced hepatotoxicity could be demonstrated (39). Furthermore, it has been suggested that impaired renal function and concomitant use of drugs that decrease the elimination of MTX or facilitate tissue uptake by displacing MTX from plasma protein-binding sites, such as aspirin and probenecid, may pose a risk for MTX-induced hepatotoxicity (40). Curtis et al. showed that risk of developing abnormal ALT/AST levels was incrementally greater in those receiving MTX (≥ 10 mg/day) in addition to leflunomide compared with those who receive MTX only (41).

Other obvious risk factors for MTX-induced liver disease include persistent abnormal liver functions, history of liver disease (including infection with hepatitis B and C virus), and a family history of liver disease (29, 34).

Table 2.1: Non-genetic risk factors for methotrexate-induced liver injury

Study	n	Mean dose (mg/week)	Duration (weeks)	Definition of hepatotoxicity	Patients with hepatotoxicity, n (%)	Risk factors	Ref.
Kremer et al.	719	7.5–25	18–36	Transaminases level > ULN	111 (15.43)	Obesity and alcohol use	(37)
Hoekstra et al.	411 (137 non-folate and 274 use folate)	7.5–25	48	ALT > 3 × ULN	36 (26) non-folate and 11 (4) use folate	Obesity and alcohol use	(38)
van Ede et al.	236	7.5–25	48	ALT > 3 × ULN	30 (12.71)	Non-folate supplementation	(46)
Philips et al.	134	7.5–25	DNS	DNS	3 (2.24)	Obesity, diabetes and viral/ alcoholic hepatitis	(87)
Whiting O'Keefe et al.	636	7.5–25	18–48	Worsening of at least one grade on classification of Roenigk	177 (27.9)	Alcohol and cumulative dose MTX	(17)
Tilling et al.	550 RA 69 PsA	1.25–30	144	3 × ULN	41 in RA (7.5) and 10 in PsA (14.5)	Male PsA and alcohol	(82)
Morgan et al.	32	7.5	24	Elevated liver enzyme levels	4 (12.5)	Non-folate supplementation	(42)
Quintin et al.	1571	10.2–11.6	105–157.6	Transaminases three-times ULN	41 (2.61)	MTX exposure (weeks) and MTX weekly dose (mg)	(31)
Curtis et al.	1953	10–17.5	> 20	Transaminases level >1–2 ULN	429 (22)	PsA, leflunomide used concomitantly	(41)
Kent et al.	481	12.6	DNS	ULN >1	304 (63)	Non-folate supplementation, elevated BMI, untreated hyperlipidemia	(45)
Visser et al.	2199	12.5	168	ULN >1	31	Alcohol, diabetes and obesity	(16)

DNS: Data not shown; MTX: Methotrexate; PsA: Psoriasis; RA: Rheumatoid arthritis; ULN: Upper limit of the normal; BMI: body mass index.

Folate status may also be of importance since the use of at least 5 mg folic acid/week with MTX therapy reduces gastrointestinal and liver toxicity (42-46). A meta-analysis of nine studies including a total of 788 RA patients also indicated that folic acid supplementation reduces gastrointestinal and liver toxicity of MTX without reducing drug efficacy (47). Van Ede et al. (44) showed that 53% of RA patients treated with MTX and who did not take folic acid had elevated liver enzymes levels versus 13% of patients who took folic acid only.

The toxicity profile of MTX may also be dependent on the route of administration. Comparing MTX 15 mg/week subcutaneously versus orally in RA patients showed more frequent discontinuations due to toxicity after subcutaneous MTX administration without a clear difference in type of adverse event except in increase of ALT, which was lower in patients who received subcutaneous MTX (48).

Pharmacogenetic risk factors

Genetic susceptibility plays an important role in the occurrence of adverse drug effects including hepatotoxicity (49). In recent years, several genetic markers have been associated with an increased risk of developing DILI (50, 51). These genetic markers are usually not generic but highly drug specific and potentially lead to a better understanding of the hepatotoxic mechanism and preventative strategies (52). Genetic polymorphisms in several genes have been related to MTX-induced toxicity in RA patients. Particularly variants in *MTHFR* have been related to low-dose MTX-induced hepatotoxicity (Table 2.2).

MTHFR

The *MTHFR* gene is the best studied gene with respect to MTX metabolism. At least 82 polymorphisms have been described (53, 54), although functional data on these variants are available for only a few. The SNPs found to be related to MTX hepatotoxicity are C677T and A1298C (55). The C677T polymorphism leads to an alanine to valine amino acid change at codon 222 and has known functional effects, such as leading to the formation of an enzyme with reduced activity (55). There is a wide range of clinical effects associated with these polymorphisms, for example increased gastrointestinal side effects and increased liver toxicity (56), and various adverse effects (57). Approximately, 50% of the Caucasian population carries at least one copy of the *MTHFR* C677T variant allele (58). Heterozygotes (CT) retain 60% of the MTHFR enzyme activity and represent approximately 40% of the Caucasian population. The homozygous variant TT genotype represents 10% of Caucasians and confers only 30% of normal MTHFR activity (55, 59).

Table 2.2: Pharmacogenetic association studies of methotrexate-induced liver injury in *MTHR* gene

SNP	Authors	n	Mean dose MTX (mg/ week)	Duration (weeks)	Definition of hepatotoxity	Hepatotoxity, n (%)	Genotypes	OR	95% CI	p-value	Ref.
677C>T (rs1801133)	van Ede et al.	236	7.5–25	48	ALT ≥ 3 ULN	30 (12.7)	CT or TT>CC	2.38*	1.06–5.34	0.035	(46)
	Caliz et al.	468	10–25	DNS	DNS	DNS	TT>CT + CC	1.42	1.01–1.98	0.043	(64)
	Aggarwal et al.	150	10.9–11.2	104 ± 82	DNS	10 (6.7)	CC>CT + TT	8 (9.2%) vs 3 (4.8%)			(66)
	Kim et al.	385	6.3–15.3	92–335.8		48 (12.46)	CC>CT or TT	6 (4.5%) vs 42 (16.7%)			(67)
A1298C (rs1801131)	Dervieux et al.	48	7.5–25	24	AST > ULN	4 (8.3)	AC or CC>AA	DNS	DNS	DNS	(63)
	Mena et al.	70	7.5	DNS	ALT or AST > 2 ULN	13 (19)	AC + CC>AA	2.75	1.11–6.75	0.023	(65)
Total		889				105 (11.81)					

The patients included in the study by Caliz et al. were not included in the total because they do not specify the exact number and percentage of patients with MTX-induced hepatotoxity. MTHFR catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. ALT: Alanine transaminase; AST: Aspartate transaminase; DNS: Data not shown; MTX: Methotrexate; OR: Odds ratio; ULN: Upper limit of the normal. * Relative risk (RR).

The *MTHFR* A1298C polymorphism is a glutamic acid to alanine substitution at codon 429 and leads to a reduced enzyme activity (60) and also results in altered clinical effects. The reduced enzyme activity leads to high blood homocysteine levels and this disorder is connected to coronary and peripheral artery diseases (61). The C allele has a frequency of 32% in the Caucasian population. Interestingly, patients who are heterozygous for both the C677T and A1298C SNPs (approximately 15% of the Caucasian population) are clinically similar to patients that are homozygous carriers of the C677T polymorphism. The two SNPs are in very strong linkage meaning that the genotypes are strongly related and not independent (55, 58).

Several groups have investigated the effect of these *MTHFR* polymorphisms on MTX efficacy (55, 62) but this is outside the scope of this article.

van Ede et al. were the first to show an association of the *MTHFR* C677T polymorphism and elevated ALT levels during MTX treatment in patients with RA (46). Thirty patients out of 236 (12.7%) withdrew from MTX therapy because of elevated ALT values. Both the heterozygous variant genotype (677CT versus 677CC) and the homozygous variant genotype (677TT versus 677CC) were associated with an increased relative risk of MTX discontinuation owing to elevation of the ALT values, independent of folate supplementation. The authors postulated that an increase of transaminases during MTX treatment in RA patients is mediated by its effects on homocysteine metabolism (46). The ALT elevations were designated 'mild' when < 3 ULN occurring on at least two of four consecutive (every 3 weeks) evaluations, and 'moderate' when ≥ 3 ULN.

In a study by Dervieux et al. MTX naive patients were enrolled in a pharmacogenetic study where they analyzed both C677T and A1298C *MTHFR* polymorphisms. A total of 8.3% of the patients showed symptoms of hepatotoxicity (AST > ULN) and any side effects were associated, among others, with the *MTHFR* 1298AC/CC genotype, but not with *MTHFR* C677T (63).

In a study by Caliz et al. the two *MTHFR* SNPs and their haplotypes were studied in relation to MTX toxicity, including hepatotoxicity, in a retrospective cohort of 468 Spanish patients with RA (64). Eighty-four of the 468 patients (18%) experienced MTX toxicity, most commonly gastrointestinal and hepatotoxicity. The C677T polymorphism was associated with an increased risk of MTX toxicity with an odds ratio (OR) of 1.42 (95% CI: 1.01–1.98) but the A1298C SNP was not related to MTX toxicity. The haplotype 677T-1298A was nominally associated with toxicity, with an OR of 1.40 (95% CI: 1.00–1.96). Thus, the C677T polymorphism in the *MTHFR* gene was found to be associated with the composite endpoint of MTX toxicity but no specific information is given for hepatotoxicity.

Mena et al. analyzed the association of both *MTHFR* C677T and A1298C polymorphisms and the presence of transaminasemia in 70 Mexican RA patients treated with MTX. A total of 19% (13 out of 70) of the patients had an increase in the serum level of transaminases. The *MTHFR* A1298C polymorphism was associated with elevation of transaminases ($p = 0.024$) (65).

Aggarwal et al. studied the relationship between the C677T gene polymorphism and low-dose MTX toxicity and efficacy in a cohort of 150 RA patients on folate supplementation (66). Ten patients (6.7%) presented hepatotoxicity. However, there was no significant difference in overall occurrence, severity or the organ specific toxicity between patients with or without polymorphism (CC: 8 [9.2%] vs CT and TT: 3 [4.8%]).

Kim et al. enrolled 385 patients with RA who had received MTX and identified toxicity associated with *MTHFR* C677T genotypes, including hepatotoxicity. Forty-eight patients (12.5%) presented elevated transaminases levels: CT/TT: 42 patients (16.7%) versus CC: six patients (4.5%). They concluded that the *MTHFR* C677T polymorphism may be an important predictor of MTX-related toxicity in patients with RA (67).

According to these articles, MTX-induced hepatotoxicity occurred in approximately 11.81% of patients; a total of 889 patients were examined and 105 presented hepatotoxicity.

Meta-analysis

Fisher et al. conducted a meta-analysis of published studies including 1400 patients for association of the *C677T* polymorphism and over 660 for the *A1298C* variant and demonstrated that the first but not the latter variant was significantly related to toxicity of MTX, including hepatotoxicity (OR: 1.71; CI: 1.32–2.21, $p < 0.001$) (68). This analysis has several limitations. First, there is an inherent heterogeneity to meta-analysis, and there were differences in the definition of toxicity, MTX dose and folic acid supplementation among the different studies examined. Second, not all studies included in the meta-analysis discriminated between the heterozygous and homozygous genotype. Because of this, the meta-analysis was performed combining all patients who deviated from the wild-type, allowing all studies to be compared in the meta-analysis (68).

Recently a second meta-analysis was published (69) looking for possible associations of *MTHFR* polymorphisms with adverse effects, including hepatotoxicity. The findings of this analysis concerning the *C677T* polymorphism and toxicity in RA patients are consistent with Fisher's meta-analysis (68) (TT vs CC [OR: 4.191; 95% CI: 1.642–10.698]; CT vs CC [OR: 1.46; 95% CI: 0.680–3.130]).

Owen et al. examined a retrospective cohort of 309 patients with RA from the UK, for which information on MTX efficacy and toxicity was available (70). Next, 17 studies were selected from the published literature on *MTHFR* C677T and A1298C variants and response in RA, including the cohort study of Owen et al. and a meta-analysis was then performed. Nine SNPs were analyzed including *C677T* and *A1298C*. Preliminary analysis revealed an association between *C677T* and MTX toxicity, which was particularly strong in the non-Caucasian group (OR: 1.93; 95% CI: 1.47–2.55). However, after adjustment for heterogeneity between the toxicity studies by a random-effects model, the association with toxicity did not persist. The authors conclude that none of the SNPs showed association with MTX efficacy or toxicity in this cohort (70).

A fourth meta-analysis, including 1,514 patients with RA, was conducted by Lee et al. (71). They report no significant associations between the toxicity and efficacy of MTX in RA and the *C677T* or *A1298C* polymorphisms of *MTHFR* (OR for adverse effects with 1298 AA versus 1298 AC/CC were 0.942; 95% CI: 0.479–1.851; p = 0.861, and OR for adverse effects with 677CC versus 677CT/TT was 0.633; 95% CI: 0.325–1.234; p =0.180). These results are conflicting with the results Fisher (68) and Spyridopoulou et al. (69) but in line with the results of Owen et al. (70).

DISCUSSION & FUTURE PERSPECTIVE

Our review of the literature shows that there is a limited number of studies that focus specifically on the study of low-dose MTX-induced hepatotoxicity.

Several nongenetic risk factors for MTX-induced liver injury have been identified, such as the use of alcohol, exposure to hepatotoxic drugs and the cumulative dose of MTX. There are more studies for nongenetic risk factors than for genetic risk factors.

Regarding genetic risk factors, the *MTHFR* C677T polymorphism appears to be the most promising predictive genetic marker for low-dose MTX-induced hepatotoxicity. Results for *MTHFR* A1298C are less consistent and require additional studies.

The identification of genetic predictors for MTX-induced hepatotoxicity presents an important potential opportunity to pre-emptively identify individual patients at risk for this debilitating disease. Many studies reported genetic variants, for example *ADORA2a*, associated with enzymes and proteins involved in the mechanism of action of MTX and their relations with efficacy and toxicity, including abnormal liver function test (72-77). However, only a small number of studies have reported variants in genes that are predictive for MTX-induced hepatotoxicity.

The currently available evidence for genetic markers for MTX-induced hepatic injury in RA treatment is limited in two ways. First, the number of studies is low with only 19 studies identified and four meta-analyses. Moreover, many of the studies have a small sample size, typically approximately 50–500 patients. With a relative low incidence of MTX-induced hepatotoxicity of approximately 11.81% (a total of 889 patients with 105 presenting with hepatotoxicity) this limits the power to identify genetic markers. The differences in results for the *C677T* polymorphism between the meta-analysis may be primarily caused by the use of different groups of studies or different meta-analysis methods. In addition, with the current data there is a risk for overestimating the effect of genetic markers since smaller studies tend to overestimate the effect of a biomarker and results from small studies are more likely to suffer from publication bias (78). In general, many biomarkers proposed as determinants of disease, risk, prognosis or response to treatment in highly cited studies do not transform to clinical practice. In addition, to be able to correctly assess the potential clinical value of any biomarker it is essential to have the diagnostic test criteria of the related test, for example, the sensitivity, specificity, positive and negative predictive value or percentage explained variance. To date, for many genetic markers, diagnostic test criteria are not commonly reported (79). Unfortunately, this is also the case for the studies identified in Table 2.2. Some data regarding the sensitivity and specificity of abnormal hepatic values are available for predicting liver injury. The American College of Rheumatology guideline for monitoring MTX-induced hepatotoxicity (29, 37) presents diagnostic test criteria, that is, sensitivity and specificity of elevated liver enzymes, to predict hepatotoxicity. The sensitivity of elevated AST levels for predicting fibrosis/cirrhosis was 80% whereas the specificity was 82%. One study suggests that ALT alone might detect 90% of the elevated AST or paired tests (80). To be able to better assess the added value of genetic markers to the classical risk factors for low-dose MTX-induced hepatotoxicity we would, therefore, like to call for the reporting of diagnostic test criteria, such as sensitivity, specificity, positive and negative predictive value or percentage explained variance, in all pharmacogenetic association studies.

The identified studies investigating determinants of MTX-induced hepatotoxicity are very heterogeneous with regard to the methodology. An elevation of liver enzymes was generally taken as a surrogate for hepatotoxicity whereas the gold standard is considered a liver biopsy (23). Although liver biopsy provides the most reliable diagnostic procedure for MTX-induced liver injury (81), it is not without risk and has cost implications. Therefore, controversy exists on the justification of liver biopsies prior to treatment with MTX, especially because of the low absolute risk of MTX-induced liver injury in RA patients (14, 15, 82). Moreover, the use of a standardized severity and causality score for MTX-induced

hepatotoxicity is essential in order to objectify clinical observations (81). Several groups have developed methods to improve the consistency, accuracy and objectiveness in causality assessment of adverse drug reactions in general (83) but mostly they were not applied in the studies examined.

In addition to the use of different definitions of hepatotoxicity, the available evidence is also restricted by differences in MTX dose and folic acid supplementation, small sample size and the lack of replication studies. Owing to the limited sample size of many studies, power to detect the true risk and determinants of MTX hepatotoxicity may be limited. Many of the reported positive associations have either not been replicated or have shown inconsistent findings (55).

In general, from the published studies, *MTHFR* C677T appears to be the most promising genetic marker predicting low-dose MTX-induced hepatotoxicity, although we have to emphasize the limited power of currently available studies to identify genetic biomarkers for hepatotoxicity. So, conflicting results exist limiting its clinical application.

Efforts should be made to further explore genetic and nongenetic risk factors for MTX-induced hepatotoxicity. Adequately powered multicenter studies, stratified by race, are needed to clarify the muddled state that exists in MTX pharmacogenetics today. Future research should focus on a genome-wide association study (GWAS) to explore additional genetic markers. GWAS have revolutionized genetic research as they allow the discovery of multiple gene variants with individually small effects. The advantage of GWAS is that they eliminate the need to choose, *a priori*, candidate genes or variants. GWAS are highly suitable to identify genetic variants contributing to complex phenotypes such as drug-induced toxicity. The GWAS approach enables novel and less obvious genetic markers to be identified, particularly for genetic variation affecting drug pharmacodynamics, which is more complex and often less well understood than drug pharmacokinetics. Recent years have shown numerous examples of the successful application of GWAS to identify genetic markers for drug-induced toxicity, including liver toxicity, hypersensitivity, skin rash and myotoxicity (84, 85). Cooperative efforts should be encouraged to prospectively collect biological samples from well-documented cases with MTX-induced hepatotoxicity and from controls.

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Chapter 3

Lack of validation of genetic variants associated with anti-tumor necrosis factor therapy response in rheumatoid arthritis: a genome-wide association study replication and meta-analysis

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Abstract

Introduction

In this study, our aim was to elucidate the role of four polymorphisms identified in a prior large Genome-Wide Association Study (GWAS) in which the investigators analyzed the responses of patients with rheumatoid arthritis (RA) to treatment with tumor necrosis factor inhibitors (TNFi). The authors of that study reported that the four genetic variants were significantly associated. However, none of the associations reached GWAS significance, and two subsequent studies failed to replicate these associations.

Methods

The four polymorphisms (rs12081765, rs1532269, rs17301249 and rs7305646) were genotyped in a total of 634 TNFi-treated RA patients of Spanish Caucasian origin. Four outcomes were evaluated: changes in the Disease Activity Score in 28 joints (DAS28) after 6 and 12 months of treatment and classification according to the European League Against Rheumatism (EULAR) response criteria at the same time points. Association with DAS28 changes was assessed by linear regression using an additive genetic model. Contingency tables of genotype and allele frequencies between EULAR responder and non-responder patients were compared. In addition, we combined our data with those of previously reported studies in a meta-analysis including 2,998 RA patients.

Results

None of the four genetic variants showed an association with response to TNFi in any of the four outcomes analyzed in our Spanish patients. In addition, only rs1532269 yielded a suggestive association ($p = 0.0033$) with the response to TNFi when available data from previous studies were combined in the meta-analysis.

Conclusion

Our data suggest that the rs12081765, rs1532269, rs17301249 and rs7305646 genetic variants do not have a role as genetic predictors of TNFi treatment outcomes.

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the synovial joints resulting in joint destruction, polyarthritis and functional disability. This inflammatory condition affects approximately 1% of the Caucasian population, making it a significant cause of comorbidity and mortality (1).

In recent years, the use of tumor necrosis factor inhibitors (TNFi) has resulted in an improvement in the treatment of RA patients by reducing both inflammation and joint damage (2-4), and their clinical use has become widespread. However, a percentage of patients do not respond adequately to this therapy; therefore, the current use of these agents is based on a trial-and-error approach (5, 6). Given the adverse effects and the high cost of this type of therapy, the establishment of pharmacogenetic markers to predict the response to TNFi treatment is a highly desirable goal.

Recently, researchers in pharmacogenetic studies have reported several genetic variants associated with clinical response to treatment with TNFi (7-11). However, to date, only the *PTPRC* and *PDE3A-SLCO1C1* loci have been associated in more than a single study (12-14).

In 2011, Plant et al. (8) performed a genome-wide association study (GWAS) in a large number of RA patients from the United Kingdom treated with TNFi. These investigators used a three-stage study design. The meta-analysis combining stages 1, 2 and 3 cohorts yielded four single-nucleotide polymorphisms (SNPs) putatively associated with the TNFi response at 6 months, although these associations did not reach the GWAS significance level. Two of the genetic variants mapped within genes, PDZ domain-containing 2 (*PDZD2*) and eyes absent homolog 4 (*EYA4*), and two polymorphisms mapped to intergenic regions on chromosomes 1 and 12. However, two subsequent GWASs conducted in European RA patients, whose treatment response was evaluated at 14 weeks, failed to replicate association with any of the four loci (9, 10).

The aim of our study was to assess the role of the four genetic variants identified by Plant et al. (8) with regard to their association with response to TNFi using a large number of RA Spanish patients, as well as to conduct a meta-analysis including previous data.

METHODS

Patients

Two sets of RA patients of Spanish ancestry treated with TNFi (infliximab, adalimumab and etanercept) were included in the study. Collection 1 comprised 438 patients, and collection 2 included 196 patients. All patients were classified according to the 1987 American Rheumatism Association criteria (15). Informed written consent from all participants and approval from the local ethical committees (Comité Ético de Investigación Clínica de

Table 3.1: Baseline characteristics of the rheumatoid arthritis

Baseline characteristics	Collection 1 (N = 438)	Collection 2 (N = 196)
Age, mean ± SD years	61.0 ± 12.04	56.3 ± 14.77
Age at diagnosis, mean ± SD years	44.95 ± 13.03	42.99 ± 13.69
Females, n (%)	365 (83.3)	160 (81.6)
Disease duration, mean ± SD years	16.45 ± 8.34	12.93 ± 8.47
Rheumatoid factor-positive, n (%)	340 (77.8)	141 (71.94)
Anti-CCP-positive, n (%)	250 (69.6)[b]	125 (73.1)[c]
Smoking status, n (%)		
Ever-smoker	51 (16.0)[b]	20 (13.9)[c]
Never-smoker	267 (84.0)[b]	124 (86.1)[c]
Health status, mean ± SD		
DAS28 at baseline	5.86 ± 1.12	5.36 ± 1.13
Treatment, n (%)		
Concurrent DMARDs	252 (92.6)[b]	159 (81.1)
Previous biologic agents	0 (0)	14 (10.2)[c]
Anti-TNF drugs, n (%)		
Infliximab	245 (55.9)	62 (31.6)
Etanercept	113 (25.8)	21 (10.7)
Adalimumab	80 (18.3)	113 (57.7)
EULAR-defined response at 6 months, n (%)		
Responders	337 (80.4)	167 (85.2)
Non-responders	82 (19.6)	29 (14.8)
EULAR-defined response at 12 months, n (%)		
Responders	259 (82.5)	118 (88.1)
Non-responders	55 (17.5)	16 (11.9)

[a] Anti-CCP, Anti–cyclic citrullinated peptide antibody; DAS28, Disease Activity Score in 28 joints; DMARD, Disease-modifying antirheumatic drug; EULAR, European League Against Rheumatism; TNF, Tumor necrosis factor. [b] Data are from < 85% of the patients: 359 for anti-CCP status, 318 for smoking status and 272 for concurrent DMARD treatment. [c] Data are from < 70% of the patients: 171 for anti-CCP status, 144 for smoking status and 137 for previous biologic agent treatment.

Galicia and Comité de Bioética del Consejo Superior de Investigaciones Científicas) were obtained in accordance with the tenets of the Declaration of Helsinki. The characteristics of the patients enrolled in this study are shown in Table 3.1.

Treatment outcomes

The Disease Activity Score in 28 joints (DAS28) was measured at baseline and at 6 and 12 months after the first TNFi infusion. Two scales were considered to assess the efficacy of the TNFi therapy. First, the absolute change in DAS28 ($\Delta DAS28 = DAS28_{end} - DAS28_{baseline}$) at 6 and 12 months of follow-up. Second, patients were classified as responders (good and moderate) or non-responders at 6 and 12 months according to the European League Against Rheumatism (EULAR) response criteria (16).

Genotyping

Genomic DNA was extracted from peripheral white blood cells or saliva using standard procedures. Four SNPs – rs1532269 and rs17301249, intronic polymorphisms mapped within *PDZD2* and *EYA4*, respectively, and rs12081765 and rs7305646 located at intergenic regions on chromosomes 1 and 12, respectively – were genotyped using a single-base extension technology (SNaPshot Multiplex Kit; Applied Biosystems, Foster City, CA, USA) in a multiplex PCR experiment (KAPA2G Fast HotStart; Kapa Biosystems, Wilmington, MA, USA) in collection 1 and using TaqMan allelic discrimination assays on a 7900HT Fast Real-Time PCR System, both purchased from Applied Biosystems, in collection 2. A deviation from Hardy–Weinberg equilibrium (HWE) was detected for the rs1532269 polymorphism in collection 2, so that SNP was genotyped in this sample set using the same methodology used for collection 1.

Statistical analysis

Power calculations were performed using Quanto version 1.2.4 software (17). All SNPs were tested for deviations from HWE. The association between SNPs and responses to TNFi was evaluated in two ways. In the first method, linear regression analysis using ΔDAS28 as the continuous dependent variable was performed under an additive genetic model using Plink version 1.07 statistical software (18). A t-test was used to identify polymorphisms associated with the response. In the second method, genotype and allele frequencies between EULAR-defined responder and non-responder patients were compared. Plink was used to create 2 × 2 or 2 × 3 contingency tables and to perform a χ^2 test and/or Fisher's exact test. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated according

to Woolf's method. Because our present study is a replication study, no correction was applied to the obtained P-values when TNFi response was evaluated at 6 months. In the analyses involving the TNFi efficacy at 12 months, however, p-values were corrected by using the Benjamini–Hochberg step-up procedure to control for false discovery rates (FDRs) in multiple testing (19). The results were considered statistically significant when p-values were lower than 0.05.

Clinical variables previously identified as being independent predictors of efficacy of TNFi, including age, gender, smoking status, rheumatoid factor status, anti– cyclic citrullinated peptide antibody (anti-CCP) status, DAS28 at baseline, concurrent and previous treatment and TNFi, were assessed for association with treatment response in a multivariate regression analysis using STATISTICA version 7.0 software (StatSoft, Tulsa, OK, USA) and Plink software in collections 1 and 2, respectively. Only baseline DAS28, gender and TNFi were associated with the efficacy of the therapy. Accordingly, analyses were adjusted for these three variables.

The analysis of the combined data from our study and the previous reports (8-10) was performed using Plink. Heterogeneity between studies was assessed using Cochran's Q and I^2 statistics (20). Pooled analyses were performed by using the Mantel–Haenszel test under the fixed-effects model or the DerSimonian–Laird test under the random-effects model when heterogeneity was detected.

The results were considered to be statistically significant when p-values were lower than $5 \times 10^{5.00E-08}$.

RESULTS

All of the four studied polymorphisms conformed to HWE expectations ($p > 0.01$). The genotyping success rate was higher than 95%.

Replication study

First, we analyzed the association between the four tested polymorphisms and the efficacy of the TNFi therapy in the 438 RA patients of Spanish Caucasian origin in collection 1. As shown in Table 3.2, in the linear regression analysis using ΔDAS28, none of the analyzed genetic variants were associated with the clinical response at 6 months ($p = 0.570$, $p = 0.831$, $p = 0.181$ and $p = 0.244$ for rs12081765, rs1532269, rs17301249 and rs7305646, respectively) or at 12 months ($p = 0.716$, $p = 0.647$, $p = 0.416$ and $p = 0.182$ for rs12081765,

Table 3.2: Association of the four single-nucleotide polymorphisms with changes in Disease Activity Score in 28 joints at 6 and 12 months in Spanish rheumatoid arthritis patients[a]

			6 months						12 months					
			Collection 1 (N = 419)			Collection 2 (N = 193)			Collection 1 (N = 314)			Collection 2 (N = 134)		
SNP	Locus	1/2	MAF	P-value[b]	β[b]	MAF	P-value[b]	β[b]	MAF	P-value[b]	β[b]	MAF	P-value[b]	β[b]
rs12081765	Intergenic	A/G	0.415	0.570	0.055	0.458	0.995	0.001	0.411	0.716	0.043	0.408	0.677	0.062
rs1532269	*PDZ2D*	C/G	0.375	0.831	0.020	0.418	0.830	0.022	0.400	0.647	0.051	0.420	0.022	0.335
rs17301249	*EYA4*	C/G	0.121	0.181	-0.190	0.132	0.458	0.120	0.131	0.416	-0.137	0.154	0.529	0.135
rs7305646	Intergenic	T/C	0.474	0.244	-0.117	0.487	0.661	-0.049	0.486	0.182	-0.161	0.473	0.554	-0.093

[a] 1, Minor allele, 2, Major allele; MAF, Minor allele frequency; SNP, Single-nucleotide polymorphism. [b] Adjusted for gender, anti-tumor necrosis factor treatment and Disease Activity Score in 28 joints at baseline.

rs1532269, rs17301249 and rs7305646, respectively). Likewise, when allele frequencies were compared between responder and non-responder patients, no association with the EULAR-defined response at 6 or 12 months was observed for any of the analyzed polymorphisms (see Additional Tables S3.1 and S3.2).

In the subsequent analysis in collection 2, none of the tested polymorphisms showed an association with ΔDAS28 at 6 months (Table 3.2) (p = 0.995, p = 0.830, p = 0.458 and p = 0.661 for rs12081765, rs1532269, rs17301249 and rs7305646, respectively) or in the stratified analysis according to the EULAR-defined response (see Additional Table S3.1). When TNFi efficacy was evaluated at 12 months, the rs1532269 polymorphism showed an association with ΔDAS28 at that time point (Table 3.2) (p = 0.022, β = 0.335); however, statistical significance was lost after correction using the Benjamini–Hochberg step-up procedure for FDR (p_{FDR} = 0.087). No association of this SNP with the EULAR-defined response at the 12-month time point was observed (see Additional Table S3.2).

No heterogeneity was observed (p > 0.1 by Cochran's Qstatistic) before we performed the meta-analysis of the two Spanish collections. No association between rs12081765, rs1532269, rs17301249 and rs7305646 and the efficacy of the TNFi therapy was evident in this pooled analysis for any of the outcomes considered as measured by ΔDAS28 (Table 3.3) and EULAR-defined response (see Additional Table S3.3).

Table 3.3: Pooled analysis of the tested polymorphisms in the two Spanish cohorts

		Meta-analysis			
		6 months		12 months	
SNP	Locus	P-value	β	P-value	β
rs12081765	Intergenic	0.677	0.029	0.586	0.050
rs1532269	*PDZ2D*	0.762	0.021	0.074	0.158
rs17301249	*EYA4*	0.607	-0.055	0.8041	-0.033
rs7305646	Intergenic	0.246	-0.086	0.1549	-0.136

SNP, Single-nucleotide polymorphism.

Meta-analysis of all available studies

We combined our data with the results of three previous studies, in order to assess the global status of the four polymorphisms (8-10). Results corresponding to ΔDAS28 at 14 weeks from two of the studies (9, 10) were combined with results at 24 weeks from the other two studies ((8) and present meta-analysis). A total of 2,998 RA patients were included

Table 3.4: Meta-analysis of association of four single-nucleotide polymorphisms with changes in Disease Activity Score in 28 joints in all available studies[a]

		rs12081765			rs1532269			rs17301249			rs7305646		
Study		MAF	P-value	β	MAF	P-value	β	MAF	P-value	β	MAF	P-value	β
Plant et al. (8)	Cohort 1 (n = 566)	0.43	7.52^{E-04}	0.29	0.37	7.11^{E-04}	0.30	0.20	3.37^{E-04}	-0.38	0.47	9.16^{E-04}	-0.28
	Cohort 2 (n = 379)	0.46	0.062	0.19	0.35	0.079	0.19	0.19	0.045	-0.24	0.49	0.049	-0.21
	Cohort 3 (n = 341)	0.47	0.712	0.04	0.34	0.701	0.04	0.19	0.261	-0.14	0.50	0.292	-0.11
Krintel et al. (9)	Copenhagen cohort (n = 196)	0.47	0.940	0.008	0.36	0.360	0.116	0.18	0.570	0.89	0.48	0.740	0.042
Umicevic Mirkov et al. (10)	Dutch stage 1 cohort (n = 882)	0.39	0.730	-0.024	0.43	0.920	0.051	0.18	0.360	-0.05	0.50	0.390	0.05
Present study	Collection 1 (n = 438)	0.42	0.570	0.055	0.38	0.831	0.020	0.12	0.181	-0.190	0.47	0.244	-0.117
	Collection 2 (n = 196)	0.46	0.995	0.001	0.42	0.830	0.022	0.13	0.458	0.120	0.49	0.661	-0.049
Meta-analysis	N = 2,998		0.102	0.068		0.0033	0.107		0.063	-0.138		0.085	-0.095

[a] MAF, Minor allele frequency.

in the meta-analysis, which had > 90% power to detect a difference of ≥ 0.6 units in ΔDAS28 at the GWAS significance threshold ($p \leq 5.0^{E-08}$) for allele frequencies ≥ 10%. Only one of the polymorphisms, rs1532269, showed a suggestive association (fixed-effects model: p = 0.0033, β = 0.107) (Table 3.4), although it did not reach the GWAS significance level. The other three were not associated with ΔDAS28 at 3 to 6 months (random-effects model: p = 0.102, β = 0.068; p = 0.063, β = -0.138; and p = 0.085, β = -0.095, for rs12081765, rs17301249 and rs7305646, respectively) (Table 3.4). When data derived from the four studies were pooled, heterogeneity for the rs12081765, rs17301249 and rs7305646 variants was evident (Cochran's Q-statistic $p < 0.05$, $I^2 > 40\%$).

DISCUSSION

Our results make it unlikely that any of the four polymorphisms identified by Plant et al. (8) could be used as genetic predictors of TNFi treatment outcomes, because they were not associated in our large Spanish RA study. This lack of association represents a very relevant finding because, to the best of our knowledge, our present study is the first in which the association of these SNPs was analyzed with the same treatment outcomes used for their identification. In addition, the combined analysis with the three previous studies included in our meta-analysis (8-10) showed only a suggestive association of one of the four polymorphisms (even weaker than that reported in the study by Plant et al. (8)). These findings seem to exclude effects of sufficient magnitude to be useful in predicting response to treatment.

The lack of replication in our study could be ascribed to multiple differences between studies. It is commonly impossible to identify one of them as being more relevant than the others. Genetic differences between populations are an unlikely explanation of the results, given that the allele frequencies of the four tested polymorphisms were very similar between studies. Clinical differences between the patients with RA included in the different reports are possible and difficult to exclude. In this regard, it has already been mentioned that Plant et al. (8) evaluated the response to TNFi at 6 months, whereas the two subsequent studies used the response at 14 weeks. However, this difference does not apply to our study in which evaluation at 6 months evidenced negative results. A common cause of discrepant results is the overestimation of the true effect in the discovery study. This phenomenon has been characterized as the "winner's curse," and it has been very prevalent in genetic association studies (21). It should be noted that the four SNPs studied by Plant et al. (8) showed the highest effects in the discovery cohort (which was the only one with a clear association between these four polymorphisms and the clinical response), whereas the three replication studies

showed lower effect sizes (β values less different from zero), thus supporting this possibility. Indeed, significant heterogeneity between studies was observed in the meta-analysis of three of the four analyzed genetic variants. Interestingly, this heterogeneity disappeared when the discovery cohort of Plant et al. was removed (8). Therefore, variables other than the presence of the four SNPs considered herein could have influenced the efficacy of TNFi in this cohort, accounting for its singularity.

Other GWASs of responses to TNFi treatment in RA have been published (7, 9-11). This approach represents an important step forward in the understanding of the influence of genetic variability on the efficacy of this therapy. Only one of the observed associations has been found to reach the GWAS statistical significance level, however, and only after combination with data derived from replication studies (12). This highlights the important role of validation studies in determining the status of the remaining GWAS findings. It is to be expected that these combined efforts will produce useful insights.

Conclusions

The association of four polymorphisms (rs12081765, rs1532269, rs17301249 and rs7305646) previously identified as being associated with TNFi treatment response was not confirmed in the present study. Our results indicate that these four genetic variants are not useful predictors of response to TNFi in patients with RA.

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Additional Table S3.1: Association analysis of the four analyzed genetic variants with the EULAR response at 6 months in the Spanish RA patients

		6 months							
		Collection 1 (n = 438)				Collection 2 (n = 196)			
SNP	1/2	Subgroup (N)	MAF	P-value[a,b]	OR [95% CI][b]	Subgroup (N)	MAF	P-value[a,b]	OR [95% CI][b]
rs12081765	A/G	Responders (n = 336)	0.412			Responders (n = 162)	0.444		
		Non-responders (n = 82)	0.427	0.851	1.04 [0.71–1.52]	Non-responders (n = 29)	0.534	0.153	1.52 [0.85–2.71]
rs1532269	C/G	Responders (n = 337)	0.378			Responders (n = 156)	0.420		
		Non-responders (n = 82)	0.360	0.901	0.98 [0.67–1.43]	Non-responders (n = 28)	0.411	0.952	1.02 [0.57–1.82]
rs17301249	C/G	Responders (n = 334)	0.121			Responders (n = 161)	0.130		
		Non-responders (n = 82)	0.122	0.902	1.03 [0.59–1.82]	Non-responders (n = 28)	0.143	0.848	1.09 [0.46–2.60]
rs7305646	T/C	Responders (n = 336)	0.488			Responders (n = 160)	0.478		
		Non-responders (n = 82)	0.415	0.150	0.75 [0.50–1.11]	Non-responders (n = 28)	0.536	0.148	1.61 [0.84–3.05]

[a] All P-values have been calculated for the allelic model. [b] Adjusted for DAS28 at baseline, gender and anti-TNF treatment.
1 = minor allele, 2 = major allele.

Additional Table S3.2: Association analysis of the four analyzed genetic variants with the EULAR response at 12 months in the Spanish RA patients

		12 months							
		Collection 1 (n = 314)				Collection 2 (n = 131)			
SNP	1/2	Subgroup (N)	MAF	P-value[a,b]	OR [95% CI][b]	Subgroup (N)	MAF	P-value[a,b]	OR [95% CI][b]
rs12081765	A/G	Responders (n = 259)	0.398			Responders (n = 114)	0.395		
		Non-responders (n = 55)	0.473	0.226	1.31 [0.84–2.04]	Non-responders (n = 16)	0.500	0.255	1.54 [0.73–3.22]
rs1532269	C/G	Responders (n = 258)	0.396			Responders (n = 114)	0.399		
		Non-responders (n = 55)	0.418	0.593	1.12 [0.73–1.71]	Non-responders (n = 16)	0.563	0.114	1.78 [0.87–3.64]
rs17301249	C/G	Responders (n = 259)	0.134			Responders (n = 114)	0.162		
		Non-responders (n = 55)	0.118	0.536	0.81 [0.42–1.58]	Non-responders (n = 16)	0.094	0.309	0.52 [0.14–1.84]
rs7305646	T/C	Responders (n = 259)	0.494			Responders (n = 113)	0.469		
		Non-responders (n = 55)	0.445	0.43	0.82 [0.52–1.33]	Non-responders (n = 16)	0.500	0.76	1.13 [0.52–2.46]

[a] All P-values have been calculated for the allelic model. [b] Adjusted for DAS28 at baseline, gender and anti-TNF treatment.
1 = minor allele, 2 = major allele.

Additional Table S3.3: Meta-analysis of the four tested genetic variants in non-responder and responder RA patients from the two Spanish collections

			6 months				12 months			
SNP	Locus	1/2	Subgroup (N)	MAF	P-value[a]	OR [95% CI][b]	Subgroup (N)	MAF	P-value[a]	OR [95% CI][b]
rs12081765	*Intergenic*	A/G	Responders (n = 498)	0.423			Responders (n = 373)	0.397		
			Non-responders (n = 111)	0.455	0.379	1.15 [0.86–1.55]	Non-responders (n = 71)	0.479	0.084	1.40 [0.97–2.00]
rs1532269	*PDZ2D*	C/G	Responders (n = 493)	0.392			Responders (n = 372)	0.397		
			Non-responders (n = 110)	0.373	0.715	0.93 [0.68–1.28]	Non-responders (n = 71)	0.451	0.255	1.26 [0.86–1.83]
rs17301249	*EYA4*	C/G	Responders (n = 495)	0.124			Responders (n = 372)	0.143		
			Non-responders (n = 110)	0.127	0.967	1.04 [0.67–1.61]	Non-responders (n = 71)	0.113	0.449	0.78 [0.44–1.36]
rs7305646	*Intergenic*	T/C	Responders (n = 496)	0.485			Responders (n = 372)	0.487		
			Non-responders (n = 110)	0.446	0.336	0.86 [0.64–1.15]	Non-responders (n = 71)	0.458	0.575	0.89 [0.62–1.27]

[a] All P-values have been calculated for the allelic model. [b] Adjusted for DAS28 at baseline, gender and anti-TNF treatment.
1 = minor allele, 2 = major allele.

Chapter 4

Confirmation of *-174G/C interleukin-6* gene promoter polymorphism as a genetic marker predicting anti-TNF treatment outcome

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Abstract

Background

The *IL-6* -174G/C genetic variant has been recently associated with the clinical response to etanercept therapy in rheumatoid arthritis (RA) patients. Considering previous results, the aim of our study was to validate the role of this polymorphism as a predictor of the anti-TNF treatment outcome in RA.

Methods

Our study population was composed of 199 Spanish patients with RA receiving anti-TNF therapy. The *IL6* -174G/C (rs1800795) genetic variant was genotyped using the TaqMan® allelic discrimination technology. Patients were classified, according to the European League Against Rheumatism (EULAR) criteria, as responders (good and moderate response) and non-responders at 6, 12, 18 y 24 months after the first infusion.

Results

The -174*G allele was significantly associated with a good or moderated EULAR response at 12 (p = 0.015, OR = 2.93, 95% CI 1.29–6.70), 18 (p = 4.54E-03, OR = 5.17, 95% CI 1.80–14.85) and 24 months (p = 4.54E-03, OR = 14.86, 95% CI 2.91–75.91). A meta-analysis combining these data with the results from a previous study confirmed this association (p = 1.89^{E-02}, OR = 1.80, 95% CI 1.13–2.87, at 12 months).

Conclusion

Our results support the role of the-174G/C *IL-6* polymorphism as a genetic marker of responsiveness to anti-TNF therapy.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease characterized by polyarthritis, joint damage and functional disability (1). The cutaneous and systemic over-expression of several proinflammatory cytokines such as IL-2, IL-6, IL-8 and tumor necrosis factor-alpha (TNF-α), has been suggested to be responsible for the initiation, maintenance and recurrence of skin lesions and joint inflammation and destruction in RA (2, 3).

Research on the complex biology of TNF has uncovered many mechanisms and pathways by which TNF may be involved in the pathogenesis of RA (3, 4). The introduction of TNF-blocking agents, such as infliximab, etanercept and adalimumab revolutionized the treatment of RA, most notably because of the excellent clinical efficacy and ability of these agents to prevent further structural damage in patients who failed to respond to treatment with conventional disease-modifying antirheumatic drugs (DMARDs) (3, 5). However, the response to treatment with anti-TNF agents is variable and a substantial proportion of patients (20–50%) do not display any significant clinical improvement or lose an initially favorable response over time (5-8). The identification of pharmacogenetic markers of treatment response may be useful in predicting clinical response to anti-TNF therapies and would facilitate the development of individualized treatment (6, 9).

IL-6, produced by a variety of cell types, including monocytes, macrophages, fibroblasts, T-helper 2 cells and vascular endothelial cells, is a multifunctional cytokine that plays important roles in host defense, acute-phase reactions, immune responses and haematopoiesis (10-14). The *-174G/C* polymorphism (rs1800795), which is located in the negative regulative domain of the *IL-6* gene promoter, has been found to affect transcriptional regulation (15, 16). The *IL-6* -174*C allele has been associated in vivo with increased levels of IL-6 (17, 18), and C-reactive protein (CRP) (19) in the general population and in RA patients (20).

A recent study (21) has described a significant association of -174G/C with the clinical response to etanercept therapy at 12 months in Serbian patients with RA. This article showed that a higher number of responders were present among patients with the -174*GG genotype compared with patients carrying the -174*C allele, suggesting that this polymorphism may be a genetic marker of responsiveness to etanercept in RA. Replication of these results in independent and larger data sets is required in order to confirm the role of this genetic variant as predictor of anti-TNF outcome.

The aim of this study was to validate the reported association of the *IL-6* -174G/C polymorphism with the anti-TNF response in an independent cohort of Spanish RA patients.

MATERIAL AND METHODS

Patients and treatment

A total of 199 anti-TNF treated RA patients were recruited from five Spanish university medical centres (Hospital San Cecilio, Granada; Hospital Virgen de la Arrixaca, Murcia; Hospital La Paz, Madrid, Hospital Doctor Peset, Valencia; Hospital Virgen de la Victoria, Málaga). All patients were diagnosed with RA according to the 1987 American College of Rheumatology (ACR) criteria. Informed written consent from all participants and approval from the local ethical committees were obtained in accordance with the tenets of the Declaration of Helsinki. The characteristics of the patients enrolled in this study are shown in Table 4.1.

Table 4.1: Baseline characteristics of the rheumatoid arthritis

Parameters	N = 199	GG (N = 98)	GC (N = 83)	CC (N = 18)	p-value
Age (years) (mean ± SD)	53.08 ± 14.28	53.25 ± 13.84	51.64 ± 14.76	59 ± 13.52	0.608
Sex (female) [N (%)]	163 (81.9%)	83 (50.92%)	65 (39.88%)	15 (9.20%)	0.608
Swollen joints (mean ± SD)	6.10 ± 4.08	6.04 ± 4.18	5.79 ± 3.53	7.94 ± 5.45	0.138
Tenders joints (mean ± SD)	4.5 ± 3.14	4.11 ± 2.91	4.81 ± 3.20	5.64 ± 3.79	0.100
DAS28 (mean ± SD)	5.21 ± 1.12	5.10 ± 1.14	5.25 ± 1.11	5.62 ± 0.93	0.185
NSAID [N (%)]	132 (73.74%)	63 (47.73%)	59(44.7%)	10(7.58%)	0.179
Corticosteroids [N (%)]	130 (65.33%)	66 (50.77%)	54(41.54%)	10(7.69%)	0.833
MTX/DMARDS [N (%)]	157 (78.89%)	76 (48.41%)	68(43.31%)	13(8.28%)	0.471
ESR (> 8) [N (%)]	139 (69.85%)	69 (49.64%)	58(41.73%)	12(8.63%)	0.972
CRP ≥ 5 [N (%)]	25 (12.56%)	12 (12.44%)	10(12.04%)	3 (16%)	0.210
Positive RF ≥ 20 [N (%)]	70 (35.17%)	31 (31.63%)	32 (38.5%)	7 (38%)	0.110
Infliximab [N (%)]	61 (30.65%)	31(50.82%)	25(40.98%)	5(8.20%)	0.940
Etanercept [N (%)]	21(10.5%)	12(57.14%)	7(33.33%)	2(9.52%)	0.941
Adalimumab [N (%)]	117(58.8)	57(48.72%)	50(42.72%)	10(8.55%)	0.942

SD, standard deviation; DAS28, disease activity score 28; NSAID, non-steroidal anti-inflammatory drugs; MTX, methotrexate; ESR, erythrocyte sedimentation rate; CRP, c-reactive protein, RF, rheumatoid factor.

Infliximab was given intravenously and continuously at a dose of 5 mg/kg at weeks 0, 2 and 6 and every 8 weeks thereafter. Etanercept was given at a dose of 50 mg once per week subcutaneously. Adalimumab was subcutaneously administered at dose of 40 mg every two weeks. The choice between infliximab, etanercept and adalimumab was made according to the typology of patients and disease features, the onset of action in terms of clinical response.

Disease severity was evaluated using the disease activity score 28 (DAS28). DAS28 was measured at baseline and at four time points after the first infusion: 6, 12, 18 and 24 months. According to the European League Against Rheumatism (EULAR) response criteria (22, 23) patients were classified as good responders (good and moderate) or non-responders, using the individual amount of change in DAS28 (ΔDAS28) and DAS28 values at 6, 12, 18 and 24 months. Briefly, a good responder was classified if ΔDAS28 > 1.2, moderate responders were patients with ΔDAS28 ≤ 1.2 and > 0.6. Patients were classified as non-responders if they do not fall into any of these categories.

Following this criteria, most patients were responders to anti-TNF therapy at 6 (84.8%), 12 (87.6%), 18 (83.5%) and 24 months (88.5%).

-174G/C *IL-6* genotyping

For genotyping, cellular DNA was isolated from saliva using standard procedures. The *IL-6* -174G/C (rs1800795) gene promoter single-nucleotide polymorphism (SNP) was genotyped using the TaqMan® allelic discrimination assay technology from Applied Biosystems (Foster City, California, USA) on a LightCycler® 480 Real-time PCR system (Roche Applied Science).

Statistical analysis

Plink (v1.07) (http://pngu.mgh.harvard.edu/purcell/plink/) and StatsDirect v.2.6.6 (StatsDirect Ltd, Cheshire, UK) were used to perform 2x2 contingency tables and χ^2 test and/or Fisher's exact test. Odds ratios (OR) and 95% confidence intervals (CI) were obtained according to Woolf's method (24). The Benjamini & Hochberg (25) step-up false discovery rate (FDR) control correction for multiple testing was applied to the P-values. After correction, P-values lower than 0.05 were considered statistically significant. The analysis of the combined data from our study and the previous report was performed using Plink and StatsDirect. Breslow–Day (BD) test method (26) was used to estimate the homogeneity among populations. Pooled analyses were performed by Mantel-Haenszel test under fixed effects.

Clinical variables previously identified as being independent predictors of efficacy of anti-TNF agents, including age, gender, baseline DAS28, smoking status, rheumatoid factor status, previous and concomitant treatments, and, anticyclic citrullinated protein antibodies (anti-CCP) status, were assessed for association with treatment response. In the multivariate analysis using Plink, only baseline DAS28 was strongly associated with the efficacy of the therapy. Accordingly, analyses were adjusted for DAS28 at baseline.

RESULTS

Demographic and clinical features

A total of 199 RA patients receiving anti-TNF therapy were included. RA patients were aged (mean ± SD) 53.08 ± 14.28 years, 81.9% were female, 48.24% were rheumatoid factor (RF) positive and 78.89% had taken methotrexate/DMARDS. 30.65% were treated with infliximab, 10.5% with etanercept and 58.8% adalimumab. Demographic and clinical features at baseline according to genotype distribution are showed in Table 4.1. There were not significant differences in baseline features.

EULAR response in RA patients

The response to anti-TNF therapy was evaluated at months 6, 12, 18 and 24 after first infusion, according to the EULAR criteria. We consider good and moderate as responders. The EULAR responses were: 84.8% responders (162 out of 191 patients) and 15.18% non-responders (29 out of 191 patients) at 6 months, 87.6% responders (113 out of 129 patients) and 12.4% non-responders (16 out of 129 patients) at 12 months, 83.5% responders (66 out of 79 patients) and 16.45% non-responders (13 out of 79 patients) at 18 months and 88.4% responders (69 out of 78 patients) and 11.53% non-responders (9 out of 78 patients) at 24 months.

Association of *IL-6* polymorphism with response to anti-TNF-therapy

As shown in Table 4.2, when allelic frequencies were compared between responder and non-responder patients, the presence of the *IL-6* -174*G allele was associated with a good or moderated EULAR response at 12 (p_{FDR} = 0.015, OR = 2.93, 95% CI 1.29–6.70), 18 (p_{FDR} = 4.54^{E-03}, OR = 5.17, 95% CI 1.80–14.85) and 24 months (p_{FDR} = 4.54E-03, OR = 14.86, 95% CI 2.91–75.91). At 6 months, the number of patients carrying the -174*G allele was slightly increased in the group of responder patients compared with non-responders, however this association did not reach statistically significance (p = 0.456).

The administered anti-TNF agent did not affect the responder/non-responder status since none of them was associated independently with the response in any of the time points evaluated (data not shown).

Table 4.2: Genotype and allele distribution of the *IL-6 –174G/C* genetic variant in responder and non-responder rheumatoid arthritis patients treated with antitumor necrosis factor therapy

		Genotype, N (%)				Allele test		
	Subgroup (N)	CC	GC	GG	G allele frequency (%)	P-value*†	P_{FDR}**	OR (95% CI)***
6 months	Non-responders (n = 29)	3 (10.34)	13 (44.83)	13 (44.83)	67.24			
	Responders (n = 162)	15 (9.26)	65 (40.12)	82 (50.62)	70.68	0.456	0.456	1.27 (0.68–2.35)
12 months	Non-responders (n = 16)	6 (37.50)	4 (25.00)	6 (37.50)	50			
	Responders (n = 113)	7 (6.19)	48 (42.48)	58 (51.33)	72.57	**0.011**	**0.015**	2.93 (1.29–6.70)
18 months	Non-responders (n = 13)	7 (53.85)	2 (15.38)	4 (30.77)	38.46			
	Responders (n = 66)	4 (6.06)	31 (46.97)	31 (46.97)	70.45	**2.27^{E-03}**	**4.54^{E-03}**	5.17 (1.80–14.85)
24 months	Non-responders (n = 9)	6 (66.67)	2 (22.22)	1 (11.11)	22.22			
	Responders (n = 69)	5 (7.25)	32 (46.38)	32 (46.38)	69.57	**1.18^{E-03}**	**4.54^{E-03}**	14.86 (2.91–75.91)

* All p-values have been calculated for the allelic model.

† Adjusted for DAS28 at baseline; ** Benjamini and Hochberg step-up false discovery rate control; *** Odds ratio for the comparison of the G allele frequency between responder and non-responder patients.

Table 4.3: Meta-analysis of the *IL-6* -174 G/C genetic variant in non-responder and responder RA patients from Spain and Serbia at 6 and 12 months considering responder as patients with DAS28 improvement > 1.2

			Genotype, N (%)				Allele test	
6 months	Population	Subgroup (N)	CC	GC	GG	G allele frequency (%)	P-value*	OR (95% CI)**
	Spain	Non-responders (n = 61)	8 (13.11)	25 (40.98)	28 (45.90)	66,39		
		Responders (n = 130)	10 (7.69)	53 (40.77)	67 (51.54)	71,92	0.127	1.48 (0.89–2.45)
	Serbia (21)	Non-responders (n = 13)	2 (15.38)	8 (61.54)	3 (23.08)	53,85		
		Responders (n = 64)	7 (10.94)	37 (57.81)	20 (31.25)	60,16	0.551	1.30 (0.56–2.99)
	Overall meta-analysis	Non-responders (n = 74)	10 (13.51)	33 (44.59)	31 (41.89)	64,19		
		Responders (n = 194)	17 (8.76)	90 (46.39)	87 (44.85)	68,04	0.252	1.30 (0.86–1.95)
12 months	Population	Subgroup (N)	CC	GC	GG	G allele frequency (%)	P-value*	OR (95% CI)**
	Spain	Non-responders (n = 33)	6 (18.18)	11 (33.33)	16 (48.48)	65,15		
		Responders (n = 96)	7 (7.29)	41 (42.71)	48 (50.00)	71,35	0.069	1.83 (0.95–3.52)
	Serbia (21)	Non-responders (n = 17)	5 (29.41)	11 (64.71)	1 (5.88)	38,24		
		Responders (n = 60)	4 (6.67)	34 (56.67)	22 (36.66)	65,00	**5.10E-03**	3.00 (1.28–7.18)
	Overall meta-analysis	Non-responders (n = 50)	11 (22.00)	22 (44.00)	17 (34.00)	56,00		
		Responders (n = 156)	11 (7.05)	75 (48.08)	70 (44.87)	68,91	**1.89E-02**	1.80 (1.13–2.87)

* All p-values have been calculated for the allelic model.

** Odds ratio for the comparison of the G allele frequency between responder and non-responder patients.

Meta-analysis

Subsequently, as no heterogeneity between the ORs from our study and the previously published report (21) was evident by BD test ($p > 0.05$), a pooled analysis was performed (Table 4.3). Since in Jancic et al. (21) only the patients who had DAS28 improvement > 1.2 were defined as responders, the meta-analysis was performed following this criteria. Overall meta-analysis showed a consistent association between the *IL-6* -174*G allele and anti-TNF treatment response at 12 months ($p_{MH} = 1.89^{E-02}$, OR = 1.80, 95% CI 1.13–2.87) (Table 4.3). Again, no significant differences were found when responder and non-responder patients at 6 months were compared ($p_{MH} = 0.252$).

DISCUSSION

Treatment with anti-TNF agents results in a reduction of disease activity in most RA patients; however, a percentage of patients do not respond to this therapy for unknown reasons. Due to the extremely high costs of anti-TNF therapy and the risk of event adverse, it would be beneficial to predict whether an individual patient will respond to treatment in advance.

Our results confirm the role of the *IL-6* -174G/C polymorphism as a genetic predictor of the response to anti-TNF therapy in RA patients. A similar study (21) was achieved to address the potential influence of the -174G/C *IL-6* gene promoter polymorphism on disease activity and clinical response to etanercept therapy in patients with RA following 6 and 12 months after the initial treatment. According with our results, the authors of this article showed that a higher number of responders were present among patients with the -174GG genotype compared with the patients with the -174GC or CC genotypes (C alleles carriers), suggesting that the -174G/C *IL-6* gene polymorphism may be a genetic marker of responsiveness to etanercept in RA.

The combined analysis of our data and those previously published showed an association between this genetic variant and the clinical response to TNF-α blockers (Table 4.3).

IL-6 has the ability to induce an acute inflammatory reaction and, in the chronic phase, to support the activation of lymphocytes and myeloid cells, which may elevate the serum levels of *IL-6*, leading to increased inflammation. It may, therefore, be responsible for many of the systemic manifestations of RA (27). It has been shown that the neutralisation of the TNF-α results in the suppression of various proinflammatory cytokines, including *IL-6* (28, 29). Functional studies have reported that the -174*C allele is associated with higher serum levels of *IL-6* (15, 16) thus suggesting that increased expression of this cytokine in patients carrying the -174*C allele would result in a poorer response to anti-TNF treatment. In fact,

it has been shown that although both TNF-α and *IL-6* are major targets of therapeutic intervention in RA, baseline serum *IL-6* but not baseline TNF-α level is a potential biomarker reflecting disease activity (30).

According to our data, *-174G/C* was significantly associated with a good or moderated EULAR response at 12, 18 and 24 months, but not at 6 months. Moreover, the larger the treatment period the stronger the observed association signal. This highlights the importance of assessing the response to long-term anti-TNF treatment. This may be the reason that an association between this polymorphism and the clinical efficacy of anti-TNF therapy has not been reported in previous pharmacogenomic studies, most of which did not evaluate the clinical response beyond 6 months of treatment (8, 31-33).

CONCLUSION

The original effect on anti-TNF treatment response caused by the change *IL-6* -174G/C was successfully replicated in an independent population, supporting the role of this polymorphism as a genetic marker predicting anti-TNF treatment outcome.

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Chapter 5

FcGR genetic polymorphisms and the response to adalimumab in patients with rheumatoid arthritis

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Abstract

Aims

The aim of our study was to explore the potential of *FcGR* genetic polymorphisms as a predictor of adalimumab efficacy in rheumatoid arthritis patients.

Materials and methods

The study population was composed of 302 Dutch RA patients receiving adalimumab therapy. The *FcGR2A* (R131>H) (rs1801274) and *FcGR3A* (F158>V) (rs396991) genetic variants were genotyped using the TaqMan® allelic discrimination technology. Treatment outcome was evaluated with the use of the 28-joint disease activity score criteria (DAS28) and good response and remission were classified according to EULAR criteria.

Results

Comparing allelic frequencies between responders and non-responders, the presence of the *FcGR*2A*R allele was associated with EULAR good response at 14 weeks ($p = 0.017$, OR = 1.53, 95% CI 1.08–2.17). No significant association was found for *FcGR3A*, with good response or remission. The combined effect of both *FcGR2A* and *FcGR3A* SNPs showed a trend for association with EULAR good response ($p = 0.041$, OR = 1.38, 95% CI 1.01–1.89).

Conclusions

Our results indicate that *FcGR* polymorphisms could be a determinant of adalimumab efficacy in RA patients.

INTRODUCTION

The treatment with anti-TNF biological therapy has revolutionized the management of rheumatoid arthritis (RA). Anti-TNF treatment has demonstrated to be effective in suppressing inflammation and reducing the amount of long-term joint and tissue damage (1). However, despite the proven therapeutic value of TNFα antagonists, about 25% of patients show insufficient or no response (1-3).

At present, five TNF inhibitors are available for the treatment of RA, three of which are full-length monoclonal antibodies: infliximab, adalimumab and golimumab. The fourth agent, etanercept, is a fusion protein of two TNFR2 receptor extracellular domains and the Fc fragment of human immunoglobulin 1 (IgG1). Certolizumab is a humanized Fab fragment conjugated to polyethylene glycol (PEG) without IgG1 region (4).

Biological agents exert their pharmacological effects through their variable portion (designed to block the target molecule) and their constant portion (the Fc fragment of IgG1), which specifically binds the human FcG receptors (FcGRs) (5-8). FcGRs are expressed on the surface of most immune cells. Engagement of FcGRs by TNF antagonists could affect a number of cellular functions, including phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), induction of apoptosis, cytokine release and macrophage-mediated clearance of immune-complexes (8, 9).

Six types of human FcGR have been described: FcGR1A, FcGR2A, FcGR2B, FcGR2C, FcGR3A and FcGR3B (10).

Several candidate gene studies have suggested that the response to anti-TNF treatment is dependent on heterogeneity of the FcGR (11-16). Indeed, two FcGR subclasses, *FcGR*2A and *FcGR*3A, are known to be subject to genetic polymorphisms resulting in differential ligand binding. Each of these polymorphisms is located in the extracellular Fc-binding portion of the FcGR and hence affects the affinity with which the FcGR interacts with the various IgG subclasses (17) and thus may affect the clearance of immune complexes (18).

The *FcGR2A* polymorphism displays a single nucleotide polymorphism (A>G, Arg131His rs1801274) in the region specifying its ligand binding domain, causing an Arginine (R) to Histidine (H) amino acid substitution at position 131 (19). FcGR2A-H131 has higher affinity for human IgG1 and is the only FcGR that interacts with IgG2 (5). The functional consequence of this polymorphism was shown using IgG2-opsonized particles which were poorly internalized by phagocytes from FcGR2A-R131 homozygous donors, however, IgG2-opsonized particles were efficiently phagocytosed by FcGR*2A*-H131 expressing cells (20, 21). It has been suggested that FcGR*2A*-RR patients may clear anti-TNF drugs less efficiently

compared to patients carrying high affinity variants (HH or RH) and thus experience an increased beneficial clinical effect of anti-TNF drugs (11).

The *FcGR3A* (Phe158Val rs396991) displays an A>C substitution resulting in a Phenylalanine (F) to Valine (V) substitution at amino acid position 158. The FcGR*3A*-V158 allelic variant of FcGR3A protein has higher affinity for IgG1, IgG2 and IgG3 compared to the F158 allelic variant, and is also able to interact with IgG4 (17). It has been suggested that patients with FcGR*3A*-FF clear anti-TNF drug less efficiently from the circulation, thus increasing its beneficial clinical effect (11).

Several studies (Table 5.1) have evaluated the hypothesis of a decreased clearance due to *FcGR2A* and *FcGR3A* genetic polymorphisms by analyzing the effect of these SNPs on the response to different TNFα antagonists including infliximab, etanercept, and adalimumab in RA albeit with conflicting results (11-16). These disconcordant results may be explained by the small sample size, heterogeneity in the design (different anti-TNF agents), the use of different definitions of response and importantly also the use of different methods for genotyping. Indeed, genotyping of *FCGR3A* polymorphisms has shown to be difficult with some methods due to co-amplification of the homologous gene *FcGR3B*. As a result of the latter, reported allele frequencies of *FcGR3A* differ between studies and several studies report deviations from Hardy Weinberg equilibrium (HWE) (22). The aim of the current study was to explore *FcGR2A* and *FcGR3A* genetic polymorphisms for association with adalimumab efficacy in RA patients.

MATERIAL AND METHODS

Patients and treatment

Clinical data of 325 adalimumab treated patients were obtained from a database of AptheekZorg which facilitated the Dutch distribution of adalimumab. All patients were diagnosed with RA according to the 1987 revised American College of Rheumatology (ACR) criteria (26, 27). At that time of the study, adalimumab was reimbursed in the Netherlands only if prescribed according to the following protocol: 1) patients have used 2 DMARDs including MTX and 2) patients have a Disease Activity Score based on a 28-joint count (DAS28) of at least 3.2. Aditional inclusion criteria to the use of adalimumab for RA treatment were 18 years of age or older, an erythrocyte sedimentation rate (ESR) of at least 28 mm/hour, patient´s global assessment of their general well-being measured on a 100 mm horizontal visual analogue scale (VAS), the left end representing as good as can be and the right end representing as worse as possible, of at least 20 mm. Adalimumab

Table 5.1: Overview of pharmacogenetic studies involving *FcGR* polymorphisms and anti-TNF treatment in RA

Ref	Anti-TNF	n	Genotype	Endpoints	OR (95% CI), p-value (Low affinitiy genotype vs No-low affinity genotypes) (RR vs no RR for 2A, FF vs no FF for 3A)**	Genotyping methods
Cañete 2009 (11)	Infliximab	91	*FcGR2A*	ACR 20 at 30 weeks	2.89 (1.04–8.01), p = 0.035	PCT-SBT method
			FcGR3A	ACR 50 at 6 weeks	7.83 (1.26–48.54), p = 0.003	
				EULAR at 6 weeks	2.61 (0.98–6.92), p = 0.044	
Montes 2014 (16)	Infliximab	246	*FcGR2A*	EULAR at 3 months*	0.51 (0.32–0.81), p = 0.005	SNaPshot
	Adalimumab/ etanercept	164	*FcGR2A*	EULAR at 3 months*	Frequencies are not showed, p > 0.05	Multiplex Kit
Tutuncu 2005 (12)	Etanercept/ adalimumab/ infliximab	30	*FcGR3A*	No EULAR no ACR criteria 3 months	12.0 (0.63–226.5), p < 0.01	PCR-based specific method
Morales-Lara 2010 (13)	Infliximab	41	*FcGR3A*	ACR ≥ 20 at 12 months	4.27 (0.82–22.07), p = 0.0392	PCR and RFLP
				EULAR at 3 months*	2.84 (0.43–18.73), p = 0.130	
				ACR at 3 months	0.48 (0.12–1.89), p = 0.239	
Kastbom 2006 (14)	Infliximab/etanercept	282	*FcGR3A*	ACR 20 at 3 months ACR50 at 3 months ACR70 at 3 months EULAR at 3 months	Frequencies are not showed p = 0.8 p = 0.6 p = 0.9 data not showed	PCR and HPLC

Table 5.1 continues on next page.

Table 5.1: ***Continued***

Ref	Anti-TNF	n	Genotype	Endpoints	OR (95% CI), p-value (Low affinitiy genotype vs No-low affinity genotypes) (RR vs no RR for 2A, FF vs no FF for 3A)**	Genotyping methods
Criswell 2004 (15)	Etanercept	457	*HLA-DRB1* *TNF* *LTA* *TNFRSF1A* *TNFRSF1B* *FcGR2A* *FcGR3A* *FcGR3B*	ACR50 at 12 months	Frequencies are not showed	PCR and HPLC
Rooryck (23)	Infliximab	78	*FcGR3A* *TNFRSF1B* 	 ACR20 ACR50 ACR70	Frequencies are not showed p = 1 p = 0.924 p = 0.813	Primers designed and ABI-PRISM®
Sarsour (24)	Anti-TNF	390	*FcGR3A*	Changes in CDAI	Frequencies are not showed p > 0.05	Taqman®
Tsukahara (25)	Infliximab	33	*FcGR3A*	EULAR at 22 weeks	7.88 (0.34–183), p = 0.02	Taqman®

ACR, American College of rheumatology criteria; EULAR, European league against rheumatism.
* Response were considered good and moderate response.
** (RR vs no RR for 2A and FF vs no FF for 3A).

was subcutaneously administered at a dose of 40 mg every two weeks. The study protocol was approved by the ethics committee of the Leiden University Medical Center and all patients provided written informed consent.

Clinical evaluation

Clinical response was evaluated at 14 weeks and categorized in good response and remission according to EULAR criteria (26). Primary endpoint in our study was EULAR good response defined as a change of DAS28 > 1.2 and DAS28 at 14 weeks ≤ 3.2. EULAR remission was an exploratory endpoint defined as achieving DAS28 at 14 weeks ≤ 2.6.

FcGR2A and *FcGR3A* genotyping

After inclusion and with patients' written consent, 2 ml saliva samples were obtained for DNA extraction. Specifically, saliva samples were collected using OrageneTM DNA self-collection kit (DNA Genoteke Inc., Ottawa, Ontario, Canada) according to standard procedures. Isolation of DNA was performed according to manufacturer's prescription, quantified using nanodrop (Isogen, Maarsssen, The Netherlands) and diluted to 10 (ng/ul). *FcGR2A* rs1801274 and *FcGR3A* rs396991 were genotyped using pre-designed TaqMan® genotyping assays technology from Life Technologies and analyzed on a ViiA7® Real-time PCR system (Life Technologies, Bleiswijk, The Netherlands). Recently it was shown that standard genotyping methods are not always specific for *FcGR3A* but may co-amplify *FcGR3B* as well. Therefore, *FcGR3A* was also genotyped using a validated pyrosequencing method as described previously (22). Results for Taqman and pyrosequencing were in 100% concordance.

Statistical analysis

The statistical analysis was performed using SPSS v.20 (SPSS, Chicago, Illinois, USA). Initially SNPs were explored for associations under allelic and genotypic model using chi-square tests. The model that best described the data was selected and used for further analysis. Odds ratios (OR) and 95% confidence intervals (CI) were obtained according to Woolf's method (28). Hereafter, the potential influence of clinical and epidemiological factors including age, gender, concomitant MTX therapy and DAS28 at baseline on the clinical outcome was evaluated by logistic regression models. Since the study from which our data originate was not primarily designed to investigate the effect of *FcGR* genetic polymorphisms we performed a post-hoc power calculation.

Our study had a power of 97.4% (α = 0.025) to detect a 50% difference in response rate for carriers of the *FcGR2A*-RR compared to carriers of the *FcGR2A*-HR and *FcGR2A*-HH genotype.

Results were adjusted for age, gender, concomitant MTX and DAS28 at baseline. To correct for testing the effect of two independent genes (*FcGR2A* and *FcGR3A*) p-values lower than 0.025 were considered statistically significant.

RESULTS

Demographic and clinical features

For a total of 302 RA patients receiving adalimumab therapy a DNA sample was available. Clinical and demographic data and the distribution of *FcGR2A* and *FcGR3A* genotypes are shown in Table 5.2 and are similar to that reported for other Caucasian populations (14, 29, 30).

RA patients were aged (mean ± SD) 58.5 ± 11.5 years, 71.5% were female. The mean disease activity (DAS28) at baseline was 5.8 ± 0.97. The 82.1% of patients received concomitant MTX with an average dose of 22.36 ± 5.61 mg. In this cohort, 53 patients (17.9%) used adalimumab as monotherapy during evaluation period. Demographic, genetic and clinical characteristics are presented in Table 5.2.

EULAR response in RA patients

After 14 weeks of treatment with adalimumab 53.6% (162 out of 302 patients) and 31.1% (94 out of 302 patients) of the patients showed good response and remission response according to the EULAR criteria, respectively.

Genotype frequencies of *FcGR2A* and *FcGR3A*

Patients were genotyped for *FcGR2A*:p.Arg131His with a success rate of 94%. Genotype distribution was RR 29.8%, RH 47.4% and HH 22.80%. The call rate for *FcGR3A* p.Phe158Val was 94%, and genotype distribution was FF 38.54%, FV 45.18% and VV 16.28%. Genotype frequencies of both genes were in Hardy-Weinberg equilibrium (*FCGR2A* p = 0.38 and *FCGR3A* p = 0.32) and were similar to previously reported frequencies (6, 22).

Table 5.2: Epidemiological, clinical and genetic features of the study cohort (baseline and at 14 weeks)

Characteristics	Value
Number of RA patients	302
Age start-years (mean, SD)	58.5 (11.56)
Gender-female (%)	216 (71.5)
Concurrent MTX (%)	248 (82.1)
MTX dose/week in mg (mean, SD)	22.36 (5.61)
Previous biological agent (%)	12 (4.0)
SJC	
Baseline (mean, SD)	9.89 (4.93)
14 weeks (mean, SD)	2.56 (2.48)
TJC	
Baseline (mean, SD)	11.51 (7.63)
14 weeks (mean, SD)	2.35 (2.74)
ESR	
Baseline (mean, SD)	31 (23.5)
14 weeks (mean, SD)	16.38 (14.04)
VAS	
Baseline (mean, SD)	70.32 (17.17)
14 weeks (mean, SD)	25.83 (14.94)
DAS28	
Baseline (mean, SD)	5.80 (0.97)
14 weeks (mean, SD)	3.12 (1.10)
ΔDAS (mean, sd)	2.67 (1.02)
%change in DAS28 at 14 weeks (mean, SD)	47.71 (16.40)
Genotypes, n (%)	
*FcGR*2A	
GG (HH)	69 (22.8)
GA (HR)	143 (47.4)
AA (RR)	90 (29.8)
*FcGR*3A	
CC (VV)	49 (16.28)
CA (VF)	137 (45.51)
AA (FF)	115 (38.21)

SJC, swollen joint count; TJC, tender joint count; VAS, visual analogue scale; ESR, erythrocyte sedimentation rate.

Association of *FcGR2A* and *FcGR3A* polymorphisms with response to anti-TNF-therapy

When comparing allelic frequencies between responders and non-responders, the presence of the *FcGR2A**R allele was associated with EULAR good response at 14 weeks (p = 0.017, OR = 1.53, 95% CI 1.08–2.17) (Table 5.3).

Table 5.3: Genotype distributions and allele frequencies of the *FcGR2A* (R>H) and *FcGR3A* (F>V) genetic variants in responder and non-responder RA patients treated with adalimumab at 14 weeks

		Genotype, N (%)			Allele test				
	Subgroup (N)	RR	RH	HH	R allele frequency (%)	p-value	OR (95% CI)	p-value*	OR (95% CI)*
FcGR2A	Good response (n = 162)	56 (35)	75 (46)	31 (19)	57.7	**0.026**	**1.43 (1.04–1.98)**	**0.017**	**1.53 (1.08–2.17)**
	No good response (n = 140)	34 (24)	68 (49)	38 (27)	48.6				
	Remission (n = 94)	33 (35.1)	38 (40.4)	23 (24.5)	55.3	0.54	1.11 (0.78–1.57)	0.56	1.18 (0.76–1.63)
	No remission (n = 208)	57 (27.4)	105 (50.5)	46 (22.1)	52.6				
	Subgroup (N)	FF	FV	VV	F allele frequency (%)	P-value	OR (95% CI)	P-value*	OR (95% CI)*
FcGR3A	Good response (n = 160)	61 (38.1)	70 (43.8)	29 (18.1)	60	0.61	0.92 (0.66–1.27)	0.87	1.03 (0.72–1.47)
	No good response (n = 141)	54 (38.3)	67 (47.5)	20 (14.2)	62.1				
	Remission (n = 93)	40 (43)	38 (40.9)	15 (16.1)	63.4	0.40	0.86 (0.61–1.23)	0.17	0.76 (0.51–1.13)
	No remission (n = 208)	75 (36.1)	99 (47.6)	34 (16.4)	36.6				

Odds ratio for the comparison between responder and non-responder patients.
* Adjusted by age, gender, concomitant MTX and DAS28 at baseline.

These differences were not observed for remission response. No significant associations were found for the *FcGR3A* polymorphism and good response or remission (Table 5.3).

To analyze the potential combined effect of the 2 SNPs, we also performed a combined analysis. The number of low-affinity alleles (FcGR*2A*-R and FcGR*3A*-F) per patient was calculated, ranging from 0 to 4, where 0 indicates the absence of low affinity alleles (HHVV genotype) and 4 indicates the presence of 4 low-affinity alleles (RRFF). The number of low-affinity alleles in a regression model showed a trend towards association with good response ($p = 0.041$, OR = 1.38, 95% CI 1.01–1.89, $R^2 = 0.19$) at 14 weeks (adjusted for age, gender, concomitant MTX and DAS28 baseline).

DISCUSSION

To the best of our knowledge this is the first study to investigate the influence of *FcGR2A* and *FcGR3A* genes on treatment response in a cohort of RA patients using adalimumab as the anti-TNF drug. Our results indicate that the *FcGR2A* genotype shows a trend towards association with clinical efficacy of adalimumab defined as EULAR good response at 14 weeks. Low-affinity *FcGR2A*-R* allele shows a better EULAR good response at 14 weeks. However, we did not find an association with good response or remission response for the *FcGR3A* genotype. Recently, Montes et al. (16) reported a significant association between the *FcGR2*A polymorphism and response to treatment with infliximab at three months, but they could not find such an association combining etanercept and adalimumab treated patients. Unfortunately, no analysis of patients treated with adalimumab or treated with etanercept could be performed separately because these two groups consisted of too small numbers of patients. In our study we were able to include 302 patients treated with adalimumab, the largest sample size for a pharmacogenetic study of adalimumab treated patients published to date.

Previously, three papers studying the association of *FcGR3A* polymorphisms and response to anti-TNF drugs have been published (12-14). In a small study consisting of 30 RA patients, Tutuncu et al. (12) found that patients with *FcGR3A*-FF genotype had a better response to several anti-TNF drugs after 12 weeks than those carrying at least one *FcGR3A*-V allele. However, the response to therapy was not evaluated according to accepted standards such as the EULAR criteria. In contrast, Morales-Lara et al. (13) found no significant association between the *FcGR3A*-FF and good response-EULAR or ACR20 criteria at 3 months in their small cohort of 41 RA patients treated with infliximab, but the genotype was associated with ACR20 response at 12 months using ACR criteria. Kastbom et al. (14) did not find

an association between *FcGR3*A genotype and efficacy in 282 RA patients treated with infliximab or etanercept using ACR criteria. We also analyzed the combined influence of low-affinity alleles (*FcGR2A*-R and *FcGR3A*-F) since anti-TNF drugs are affected by both of these FcGR. A linear regression model showed a trend towards association between the number of low-affinity alleles and EULAR good response but not for remission. Low-affinity alleles may additively result in decreased FcGR-mediated drug clearance of adalimumab. Indeed, in a pharmacokinetic study it was shown that RA patients with low-affinity *FcGR2A* and *3A* alleles showed a decreased clearance of infliximab (18). In our study we did not collect plasma for adalimumab drug level measurement and therefore we cannot associate our genetic findings with pharmacokinetic endpoints. The applied additive genetic model for *FcGR2*A and *FcGR3*A is one of the possible interactions between these two gene variants. However, a comprehensive test of all potential models is not feasible given the sample size of our study. In addition, other epistatic interactions and copy number variation of *FcGR* genes may also affect ADCC, but this is not taken into account in this study.

A recent meta-analysis (31) demonstrated that *FcGR*3A polymorphism is not associated with anti-TNF therapy but was associated with rituximab. Despite showing similar results to ours in terms of anti-TNF therapy, heterogeneity, confounding factors and different criteria used for evaluating the response, may affect the meta-analysis.

Interestingly, Morales-Lara et al. (13) studied the role of *FcGR3A* in the response to infliximab in patients with psoriatic arthritis and ankylosing spondylitis and unexpectedly found that the high-affinity-V158 allele was associated with a better response to infliximab in patients with ankylosing spondylitis. In addition, in a recent publication (6) the presence of high-affinity alleles of *FcGR2A* and *FcGR3A* was significantly associated with a better response in the intermediated point of treatment but not at the end of the treatment in 70 PsA patients treated with different anti-TNF drugs suggesting that ADCC-mediated apoptosis of TNF-bearing cells by natural killer cells and macrophages might induce a faster clearance of milder lesions than those with higher score disease. These results suggest that the role of *FcGR* polymorphisms in response to anti-TNF drugs may be dependent on the disease as well.

The limitations of our study include the lack of analysis of drug blood levels and the presence of anti-drugs antibodies. Also, the period studied could also have influence on the results. It was shown that adalimumab levels varied widely among ankylosing spondylitis patients, however, some of them improved based on clinical measurements despite low adalimumab levels (32). Recently it has been shown that genetic variants in other genes including *NLRP3* (rs4612666) and *INFG* (rs2430561) are also associated with

anti-TNF response (33). Further studies taking these factors into account are needed in an independent cohort to establish a robust pharmacogenetic marker. However, this is the largest study of RA patients treated with adalimumab published to date.

In conclusion, the presence of the low affinity *FcGR2A** R-allele is associated with EULAR good response at 14 weeks in adalimumab treated of RA patients. The combined effect of both *FcGR*2A and *FcGR3A* SNPs showed a trend for association with EULAR good response. These results indicate that *FcGR* polymorphisms could be a determinant of adalimumab efficacy in RA patients.

SUMMARY POINTS

- This is the largest study investigating the relation between *FcGR* polymorphisms and treatment response in a cohort of RA patients receiving adalimumab.
- By comparing allelic frequencies between responders and non-responders, the presence of the *FcGR2A**R allele was associated with EULAR good response at 14 weeks.
- No significant associations were found for the *FcGR3A* polymorphism and good response or remission.
- The combined number of low affinity *FcGR2A* and *FcGR3A* alleles tends to be associated with good response in adalimumab treatment of RA patients.
- Further studies taking these factors into account are needed to establish a robust pharmacogenetic marker.

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ERRATUM

The article "FcGR genetic polymorphisms and the response to adalimumab in patients with rheumatoid arthritis" by Cristina Lucía Dávila-Fajardo et al. (Pharmacogenomics (2015) 16(4), 373–381) contained an error.

For the SNP in *FcGR2A* (A131>G; rs1801274) the A (H) and G (R) alleles were incorrectly assigned. As a result, the conclusion of the article changes. The high affinity allele (*FcGR2A-H*) instead of the low affinity allele (*FcGR2A-R*) is associated with good response at 14 weeks (p = 0.017, OR: 1.53, 95% CI: 1.08–2.17, adjusted by age, gender, concomitant methotrexate and DAS28 at baseline). Similarly, the potential combined effect of the two SNPs changes. The number of high-affinity alleles (*FcGR2A*-H and *FcGR3A*-V) per patient was calculated, ranging from 0 to 4, where 0 indicates the absence of high-affinity alleles (RRFF genotype) and 4 indicates the presence of four high-affinity alleles (HHVV). After regression analysis with the correct allele assignments, the number of high-affinity alleles no longer shows a trend for association with good response at 14 weeks (p-value = 0.095, OR: 1.19, 95% CI: 0.97–1.48, R^2 = 0.19, instead of p-value = 0.041, OR: 1.38, 95% CI: 1.01–1.89, R^2 = 0.19, adjusted for age, gender, concomitant MTX and DAS28 baseline).

A potential explanation for the association of *FcGR2A-H* with good response is that the action of high affinity alleles of *FcGR2A-H131* might lead to enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) of pathogenetically relevant cells expressing TNF on their membranes, producing a more rapid clinical response. In fact, ADCC-mediated apoptosis of TNF-bearing cells by natural killer cells and macrophages has been pointed out as a relevant mechanism of action of TNF blockers in RA and psoriasis.

We would like to acknowledge dr. Gilles Thibault for bringing this erroneous allele assignment to our attention and for the fruitful and constructive discussion afterwards in preparing this erratum.

Table 5.2 (corrected): Epidemiological, clinical and genetic features of the study cohort (baseline and at 14 weeks)

Characteristics	Value
Number of RA patients	302
Age start-years (mean, SD)	58.5 (11.56)
Gender-female (%)	216 (71.5)
Concurrent MTX (%)	248 (82.1)
MTX dose/week in mg (mean, SD)	22.36 (5.61)
Previous biological agent (%)	12 (4.0)
SJC	
Baseline (mean, SD)	9.89 (4.93)
14 weeks (mean, SD)	2.56 (2.48)
TJC	
Baseline (mean, SD)	11.51 (7.63)
14 weeks (mean, SD)	2.35 (2.74)
ESR	
Baseline (mean, SD)	31 (23.5)
14 weeks (mean, SD)	16.38 (14.04)
VAS	
Baseline (mean, SD)	70.32 (17.17)
14 weeks (mean, SD)	25.83 (14.94)
DAS28	
Baseline (mean, SD)	5.80 (0.97)
14 weeks (mean, SD)	3.12 (1.10)
ΔDAS (mean, sd)	2.67 (1.02)
%change in DAS28 at 14 weeks (mean, SD)	47.71 (16.40)
Genotypes, n (%)	
*FcGR*2A	
GG (RR)	69 (22.8)
GA (HR)	143 (47.4)
AA (HH)	90 (29.8)
*FcGR*3A	
CC (VV)	49 (16.28)
CA (VF)	137 (45.51)
AA (FF)	115 (38.21)

SJC, swollen joint count; TJC, tender joint count; VAS, visual analogue scale; ESR, erythrocyte sedimentation rate.

Table 5.3 (corrected): Genotype distributions and allele frequencies of the *FcGR2A* (R>H) and *FcGR3A* (F>V) genetic variants in responder and non-responder RA patients treated with adalimumab at 14 weeks

		Genotype, N (%)			Allele test				
	Subgroup (N)	HH	RH	RR	H allele frequency (%)	p-value	OR (95% CI)	p-value*	OR (95% CI)*
FcGR2A	Good response (n = 162)	56 (35)	75 (46)	31 (19)	57.7	**0.026**	**1.43 (1.04–1.98)**	**0.017**	**1.53 (1.08–2.17)**
	No good response (n = 140)	34 (24)	68 (49)	38 (27)	42.3				
	Remission (n = 94)	33 (35.1)	38 (40.4)	23 (24.5)	55.3	0.54	1.11 (0.78–1.57)	0.56	1.18 (0.76–1.63)
	No remission (n = 208)	57 (27.4)	105 (50.5)	46 (22.1)	52.6				
	Subgroup (N)	VV	FV	FF	V allele frequency (%)	P-value	OR (95% CI)	P-value*	OR (95% CI)*
FcGR3A	Good response (n = 160)	29 (18.1)	70 (43.8)	61 (38.1)	40	0.61	1.09 (0.78–1.52)	0.86	1.04 (0.71–1.50)
	No good response (n = 141)	20 (14.2)	67 (47.5)	54 (38.3)	60				
	Remission (n = 93)	15 (16.1)	38 (40.9)	40 (43)	36.6	0.40	0.86 (0.61–1.23)	0.17	0.76 (0.52–1.13)
	No remission (n = 208)	34 (16.4)	99 (47.6)	75 (36.1)	63.4				

Odds ratio for the comparison between responder and non-responder patients.
* Adjusted by age, gender, concomitant MTX and DAS28 at baseline.

Chapter 6

Association between *-174 Interleukin-6* gene polymorphism and biological response to rituximab in several systemic autoimmune diseases

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Abstract

Rituximab has become a pivotal treatment for systemic autoimmune diseases. The aim of this study was to determine whether the genetic variant *-174 IL-6* contributes to differences in the response to rituximab in patients with systemic autoimmune diseases, including systemic lupus erythematosus, inflammatory myopathies, anti-neutrophil cytoplasmic antibody-mediated vasculitis, systemic sclerosis, Sjöegren's syndrome, rheumatoid arthritis, and autoimmune hemolytic anemia. DNA samples from 144 Spanish patients with different systemic autoimmune diseases receiving rituximab were genotyped for *-174 IL-6* (rs1800795) gene polymorphism using the TaqMan allelic discrimination technology. Six months after the first infusion with rituximab, we evaluated the response to the drug: 60.4% of the patients showed a complete response, partial 27.8%, and 11.8% did not respond to the treatment. The CC genotype frequency was significantly increased in non-responders with respect to responders (23.5% vs. 7.1%, respectively; p = 0.049; odds ratio (OR) = 4.03, 95% confidence intervals (CI) 0.78–16.97). According to the genotype distribution, rituximab was effective in 69.2% of the CC carriers, 91.9% of the CG carriers, and 88.4% of the GG carriers. A similar trend was observed when SLE patients were analyzed separately (27.3% carried CC homozygosis in non-responders and 6.9% in responders; p = 0.066; OR = 5.10, 95% CI 0.65–31.73). Rituximab was effective in 62.5% of the CC carriers, 88.9% of the GC carriers, and 90% of the GG carriers. These results suggest that *-174 IL-6* (rs1800795) gene polymorphism plays a role in the response to rituximab in systemic autoimmune diseases. Validation of these findings in independent cohorts is warranted.

INTRODUCTION

Rituximab is a chimeric monoclonal immunoglobulin G1 antibody against the CD20 protein of B-lymphocytes promoting B cell depletion (1, 2). It has become a crucial therapy against systemic autoimmune diseases, since an aberrant B cell regulation is among the common pathogenic mechanisms of these diseases (3). The Food and Drug Administration has approved the use of rituximab in Non-Hodgkin's lymphoma, Chronic lymphocytic leukemia, and Rheumatoid arthritis (RA) in combination with methotrexate in adult patients with moderately to severely active RA who have an inadequate response to one or more tumor necrosis factor (TNF) antagonist therapies, Wegener's granulomatosis (WG), and microscopic polyangiitis (MPA) in adult patients in combination with glucocorticoids. Recent studies in other systemic autoimmune diseases show the importance of this therapy in refractory patients (4-8). Certain clinical and genetic characteristics, including the presence of positive rheumatoid factor (9), the presence of Epstein-Barr virus (10), low levels of type I interferons (11), and low levels of B lymphocyte stimulator (12), have been associated with a positive response to the drug. Pharmacogenetic studies have been suggested to explain variations in efficiency of biological treatments and predisposition of patients to a nonresponse to rituximab (13). Interleukin-6 (IL-6) is a cytokine expressed by lymphocytes, monocytes, and fibroblasts that plays a key role in B cell maturation and autoantibodies production (14). IL-6 actions are mainly controlled through a complex, including the membrane-bound IL-6 receptor (IL-6R) and two gp130 subunits. However, IL-6 can also signal via a soluble receptor (sIL-6R), which binds to IL-6 and then interacts with gp130 subunits (15). IL-6 acts as a proinflammatory mediator in response to inflammatory stimuli (16, 17). In autoimmunity, IL-6 inhibits the function of T-reg cells and induces the generation of pathogenic Th17 cells, essential in an inflammatory autoimmune response leading to tissue inflammation and destruction (18). During the acute inflammation phase in RA, monocytes and macrophages release IL-6 to serum and can be used as a biomarker of inflammation or disease activity (19). The *-174 G/C* genetic variant (rs1800795), located in the *IL-6* gene promoter region, has been seen associated to autoimmune diseases and involved in increased levels of IL-6 protein in serum in diverse inflammatory diseases, although it is unclear which allele or genotype is involved in these findings (20-22). Recently, Fabris et al. (23) reported a lower response to rituximab in RA patients that presented CC homozygosis in the *-174 IL-6* variation. The aim of our study was to assess the possible involvement of the *-174 IL-6* polymorphism in the clinical response to rituximab in different systemic autoimmune diseases.

MATERIALS AND METHODS

Study population

This study was performed using a Spanish Caucasian cohort comprising a total of 144 patients with systemic autoimmune diseases treated with rituximab, recruited from three university medical centers (Hospital Universitario San Cecilio, Granada; Hospital Carlos Haya, Málaga; Hospital Virgen del Rocío, Sevilla). Table 6.1 shows the main characteristics of the patients enrolled in this study. Systemic autoimmune diseases patients included 83 (57.6%) systemic lupus erythematosus (SLE) patients, 16 (11.1%) with different inflammatory myopathies such as polymyositis and dermatomyositis, 16 (11.1%) anti-neutrophil cytoplasmic antibody associated vasculitis patients, including WG, Churg-Strauss Syndrome, and MPA, and other systemic autoimmune diseases such as Sjögren syndrome, systemic sclerosis, or autoimmune hemolytic anemia in the remaining 29 patients. The most-frequently administered rituximab dose was 375 mg/m^2 of rituximab weekly for 4 weeks in most cases, although some patients received 1000 mg twice at an interval of 15 days. Six months after the first infusion, a clinical response to the drug was evaluated according to the ACR and EULAR recommendations (24-26). The criteria used to evaluate the response to rituximab in different autoimmune diseases have already been described in detail elsewhere (6). The response to rituximab was assessed on the basis of clinical evolution. Responders included complete responders and partial responders depending on improvement of initial disease activity, total or at least 50%, but not reaching complete remission, respectively. Non-responders were defined as patients with no significant improvement or a worsening of the disease. Previous and concomitant treatments are shown in Table 6.1. All patients gave written, informed consent before participation and an ethics committee approved the study protocol.

IL-6 genotyping

DNA was isolated from whole peripheral blood, using standard procedures. *IL-6-174* single nucleotide polymorphism (SNP) (rs1800795) genotyping was performed using the TaqMan allelic discrimination assay technology in a 7500 Real-Time PCR System, both from Applied Biosystems (Foster City, CA). The genotype call rate was 100% for the tested *IL-6* genetic variant. The probes were labeled with the fluorescent dyes 2-chloro-7-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) and 6-carboxyfluorescein (FAM).

Table 6.1: Main characteristics of systemic autoimmune diseases patients treated with rituximab Included in this study

Characteristics	n (%)
Female	114 (79.2%)
Male	30 (20.8%)
Systemic autoimmune diseases	
Systemic lupus erythematosus	83 (57.6%)
Inflammatory myopathies	16 (11.1%)
ANCA-mediated vasculitis	16 (11.1%)
Sjögren's syndrome	4 (2.77%)
Systemic sclerosis	5 (3.47%)
Hemolytic autoimmune anemia	3 (2.08%)
Pemphigus vulgaris	3 (2.08%)
Mixed connective tissue disease	2 (1.38%)
Idiopathic thrombocytopenic purpura	4 (2.77%)
Cryoglobulinemia	2 (1.38%)
Rheumatoid arthritis	3 (2.08%)
Axonal polyneuropathy associated with HCV	1 (0.69%)
Autoimmune trombocytopenia	1 (0.69%)
Sarcoidosis + RA	1 (0.69%)
Previous therapies	
Corticosteroids	137 (95.1%)
Cyclophosphamide	89 (61.8%)
Methotrexate	52 (36.1%)
Mycophenolate	50 (34.7%)
Intravenous immunoglobulins	49 (34.0%)
Antimalarials	38 (26.4%)
Azathioprine	38 (26.4%)
Cyclosporine A	11 (7.6%)
Leflunomide	5 (3.4%)
Other biologic therapies	5 (3.4%)
Plasma exchange	3 (2%)
Thalinomide	2 (1.38%)
Other therapies	11 (7.63%)
Concomitant therapies	
Corticosteroids	136 (94.4%)
Antimalarials	13 (9%)
Methotrexate	8 (5.5%)
Azathioprine	8 (5.5%)
Mycophenolate	7 (4.86%)
Cyclophosphamide	8 (5.5%)
Cyclosporine A	2 (1.38%)
Intravenous immunoglobulins	3 (2.08%)
Other therapies	2 (1.38%)
Response (n = 132)	
Complete	87 (60.4%)
Partial	40 (27.8%)
No response	17 (11.8%)

Statistical analysis

A statistical analysis for allelic and genotypic distributions was calculated by the chi-squared test or the Fisher's exact test, when necessary, using the Statcalc software packages (Epi Info 2002; centers for Disease Control and Prevention, Atlanta, GA); p-values, odds ratio (OR), and 95% confidence intervals (CI) were calculated according to this software. p-values lower than 0.05 were considered as statistically significant. The presence of heterogeneity between SLE and the remaining autoimmune diseases patients was tested on the basis of the Breslow-Day test using a significance level of 0.05 (StatsDirect, v. 2,6,6). A multivariate logistic regression analysis was performed using STATA v. 10. Statistical power of our study is 80% to detect an association of -174 G/C with the OR reported in previous studies (OR = 3) (23, 27).

RESULTS

According to the EULAR and ACR criteria (6, 24-26), clinical evaluation of the response to rituximab was carried out at month 6 after first infusion with the therapy. There were 87 (60.4%) good responders (complete remission of the symptoms and clinical characteristics that recommended the use of the drug), 40 (27.8%) partial responders (reduction in at least 50% of the disease activity), and 17 (11.8%) non-responders (reduction in less than 50% of the disease activity). Interestingly, when we stratified by sex, 10.6% of the women (12/113) were non-responders, whereas 17.2% of the men (5/29) did not respond, although this difference did not become significant. Main characteristics of systemic autoimmune diseases patients including in this study are shown in Table 6.1. Genotype frequencies of the IL-6 polymorphism in Spanish systemic autoimmune diseases patients were not significantly different from those previously described in RA and SLE studies in Caucasian populations (23, 28). Of the 144 patients analyzed, 69 (47.9%) were homozygous for GG, 62 (43.1%) were heterozygous GC, and 13 (9%) were homozygous for C allele. Different systemic autoimmune diseases patients were pooled, and homogeneity of odds ratios between SLE and the remaining autoimmune diseases patients was verified by the Breslow-Day test ($p > 0.05$). Table 6.2 shows genotypic and allelic frequencies in patients stratified into two groups, according to the response to rituximab. In the subgroup of patients presenting total or a partial response to rituximab, 61 (48%) were GG, 57 (44.9%) were GC, and 9 patients (7.1%) were CC. In non-responders, the CC genotype frequency was significantly increased with respect to responders (four patients = 23.5%, CC in non-responders vs. nine patients = 7.1%, CC in responders: $p = 0.049$; OR = 4.03, 95% CI 0.78–16.97), whereas GC and GG genotypes frequencies were diminished with respect to responders (five patients = 29.4% and eight patients = 47.1%, respectively), although these differences did not reach statistical significance. No significant differences in allelic frequencies

for the *-174 IL-6* gene promoter polymorphism were observed when patients that responded to the treatment were compared against non-responders (p = 0.301). A logistic regression analysis was performed to evaluate if the concomitant therapies could be confounding the observed association. This analysis found no confounding factors (data not shown).

In correlation with these results, only 69.2% (9/13) of the patients carrying the CC genotype were responders to the treatment with rituximab, whereas this drug was effective in 89.9% (118/131) of the patients carrying the GC or GG genotype (p = 0.049; OR = 4.03, 95% CI 0.78–16.97) (Table 6.2). On the other hand, when we analyzed separately SLE patients (84/144, 58.3% of the patients), we found a trend in the same direction, although it did not reach statistical significance (Table 6.3). The efficiency level of rituximab was lower in

Table 6.2: Distribution of *-174 IL-6* rs1800795 SNP and efficiency in systemic autoimmune diseases patients treated with rituximab

	Non-responders n = 17 n (%)	Responders n = 127 n (%)	Efficiency rituximab %	p-value	OR (95% CI)
Genotype					
GG	8 (47.1)	61 (48.0)	88.4	0.940	0.96 (0.31–2.94)
GC	5 (29.4)	57 (44.9)	91.9	0.226	0.51 (0.13–1.68)
CC	4 (23.5)	9 (7.1)	69.2	0.049[a]	4.03 (0.78–16.97)
Allele					
G	21 (61.8)	179 (70.5)	89.5	0.301	0.68 (0.30–1.52)
C	13 (38.2)	75 (29.5)	85.2	0.301	1.48 (0.66–3.28)

P-value comparing frequency and efficiency in non-responders versus responders. Significant p-values are in bold. [a] Fisher's exact test. OR, odds ratio; 95% CI, 95% confidence interval; SNP, single nucleotide polymorphism.

Table 6.3: Distribution of *-174 IL-6* rs1800795 SNP and efficiency in systemic lupus erythematosus patients treated with rituximab

	Non-responders n = 11 n (%)	Responders n = 73 n (%)	Efficiency rituximab %	p-value	OR (95% CI)
Genotype					
GG	4 (36.4)	36 (49.3)	90.0	0.423	0.59 (0.12–2.56)
GC	4 (36.4)	32 (43.8)	88.9	0.449[a]	0.73 (0.14–3.19)
CC	3 (27.3)	5 (6.9)	62.5	0.066[a]	5.10 (0.65–31.73)
Allele					
G	12 (54.6)	104 (71.2)	89.7	0.114	0.48 (0.18–1.32)
C	10 (45.5)	42 (28.8)	80.8	0.114	2.06 (0.76–5.61)

P-value comparing frequency and efficiency in non-responders versus responders. Significant p-values are in bold. [a] Fisher's exact test.

CC carriers (5/8 = 62.5% responded to the treatment) than in GC or GG carriers (68/76 = 89.5% did respond); p = 0.066, OR = 5.10; 95% CI 0.65–31.73.

DISCUSSION

Rituximab has become a pivotal therapy in the treatment of several autoimmune diseases, and the study of the genetic predisposition to a positive or a negative response to this drug has been suggested to be a first step to improve its efficacy.

In the present study, we have analyzed the association of the *-174 IL-6* promoter variation with the response to rituximab in a group of patients that presented diverse systemic autoimmune diseases. Frequencies for this SNP were similar to those previously reported in Caucasian populations (23, 28). Genotypic frequencies for CC were increased in non-responders, which correlates with the fact that patients carrying this homozygosis responded worse to the treatment with rituximab than those carrying GC or GG genotypes (69.2% vs. 90.2%). Fabris et al. (23) found a lower response to rituximab in RA patients that were homozygous for CC. Their findings agree with our results, both in the group of diverse systemic autoimmune diseases patients and in SLE patients analyzed separately, although, in SLE patients, the observed differences are not statistically significant, probably due to the lower statistical power of this stratified analysis. Pathogenesis of systemic autoimmune diseases involves inflammation cytokines IL-1, TNF alpha, and IL-6. Murine models in inflammatory diseases indicate that IL-6 deficiency reduces the severity of an inflammatory response (29). Recent studies have clarified evidence that antagonizing the action of proinflammatory cytokines, including IL-6, may exert a therapeutic effect in patients nonresponsive to other therapies. Tocilizumab, a humanized antibody to the IL-6 receptor, blocks IL-6 signaling and activity and decreases levels of inflammatory markers in RA (30, 31). Previous studies reported that B cell depletion induced by rituximab resulted in a downregulation of proinflammatory cytokines, including IL-6 and, consequently, a decrease of the autoimmune response and re-establishment of the immunotolerance (32). The lower efficiency of rituximab in systemic autoimmune diseases patients carrying the CC genotype, suggests an increase in the number of refractory patients to rituximab in this group. Biological therapies different to rituximab might be had under consideration to get an adequate and more effective response in these patients. According to our data, *-174 IL-6* SNP suggests a pharmacogenetic association with the clinical response to rituximab in systemic autoimmune diseases, and the hypothesis that this variation could be a predictive value, independently of other clinical or environmental factors.

Anyway, as the observed significant associations could be due to a casual finding resulting from multiple comparisons, larger replication studies are needed and still planned by our group to confirm present results.

Currently, there are very few data about genetic markers of prognosis that may be used in the future to facilitate treatment decisions. We herein provide preliminary evidence of a possible new genetic marker, the CC homozygosis of the *-174 IL-6* promoter polymorphism, as a predictor of non-response to rituximab in autoimmune diseases.

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Chapter 7

Association of the *FCGR3A-158F/V* gene polymorphism with the response to rituximab treatment in Spanish systemic autoimmune disease patients

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Abstract

Rituximab is being used as treatment for systemic autoimmune diseases. The objective of this study was to determine whether the genetic variant in the *Fc gamma-receptor III a* (*FCGR3A*) gene, 158F/V, contributes to the observed variation in response to rituximab in patients with systemic autoimmune diseases. DNA samples from 132 Spanish patients with different systemic autoimmune diseases receiving rituximab were genotyped for *FCGR3A-158F/V* (rs396991) gene polymorphism using the TaqMan allelic discrimination technology. Six months after infusion with rituximab we evaluated the response to the drug: 61% of the patients showed a complete response, partial 27% and 12% did not respond to the treatment. A statistically significant difference was observed in V allele frequency between responder (38%) and non-responder (16%) patients (p = 0.01; odds ratio [OR] = 3.24, 95% confidence interval [CI] 1.17–11.1). Rituximab was also more effective in V allele carriers (94%) than in homozygous FF patients (81%): p = 0.02; OR = 3.96, 95% CI 1.10–17.68. These results suggest that *FCGR3A*-158F/V (rs396991) gene polymorphism play a role in the response to rituximab in autoimmune diseases. Validation of these findings in independent cohorts is warranted.

INTRODUCTION

Systemic autoimmune diseases are a heterogeneous group of diseases with different pathogenesis and clinical manifestations but with common pathogenic mechanisms, including an aberrant B-cell regulation. The crucial role of B cells in autoimmune disorders has evidenced the importance of biological treatments that blockade these cells in refractory patients (1). Rituximab is a chimeric mouse-human monoclonal immunoglobulin G1 (IgG1) antibody that specifically targets the human B-lymphocyte CD20 surface protein (2), resulting in peripheral B-cell depletion (3). Rituximab use has been approved by the FDA for B-cell non-Hodgkin's lymphomas (4), for non-responders to patients with first-line antitumor necrosis factor-a rheumatoid arthritis (RA) (5), and recently, for vasculitis (6, 7) or lupus nephritis (8, 9), but there is growing evidence from observational studies and registries of patients that their usefulness can be extended to other autoimmune diseases (10-12). Studies have shown several different mechanisms by which rituximab can selectively deplete B cells, such as antibody-dependent cell-mediated cytotoxicity (ADCC), complement-mediated cytotoxicity, and direct induction of B-cell apoptosis (13).

Several factors have been associated with a better response to rituximab, for example, in patients with RA, the presence of positive rheumatoid factor (14), positive Epstein-Barr virus (15), low levels of B lymphocyte stimulator (16), and low levels of type I interferons (17).

The Fc portion of rituximab binds specifically to cell surface Fc-g receptors (FcGR), and this may affect certain immune responses such as removal of antigen–antibody complexes from the circulation, ADCC, or phagocytosis. There are three major classes of human FCGR that are encoded by 8 genes (*FCGR1A*, *B*, and *C*; *FcGR2A*, *B*, and *C*; *FcGR3A* and *B*), all located on chromosome 1. Three polymorphisms, two in positions 48 and 158 of *FcR3A* and one at codon 131 of *FcGR2A*, have been reported to affect receptor affinity for IgG (18-20). Functional studies have described a correlation between the *FcGR3A*-158 genotype and rituximab efficacy, but no correlation has been found with *FcGR2A*-131 (21). Moreover, genetic linkage of FcGR-48 and *FcGR2A*-131 with *FcGR3A*-158 has been demonstrated and points to the primacy of *FcGR3A*-158 in predicting rituximab response (22-25).

FcGR3A, also known as CD16, is expressed on macrophages, monocytes, and natural killer (NK) cells, all of which are involved in B-cell depletion. The nonsynonymous *FcGR3A*-158 polymorphism results in either a phenylalanine (F158) or a valine (V158) at this position in the membrane proximal domain of the molecule. The *FcGR3A*-158V single nucleotide polymorphism (SNP) exhibits a higher affinity for IgG subtypes than the *FcGR3A*-158F SNP (22, 26). This SNP has been associated to different autoimmune diseases such as type 1 diabetes, celiac disease, RA (27), and systemic lupus erythematosus (SLE) (28). In patients

with giant cell arteritis, an association was observed with the *FcGR2A FcGR3A* 131R-158F haplotype (29). However, no association between *FcGR3A*-158 and systemic sclerosis was described (30). Homozygosity for the higher-affinity V allele has also been shown to be associated with susceptibility to antibody-positive RA (31, 32).

An important pharmacogenetic association with biological response to rituximab has been shown in this polymorphism. Patients carrying the V/V isoform with non-Hodgkin lymphoma (NHL) and SLE showed a better biological response to rituximab (21, 33). Later, two studies conducted in healthy donors determined that this improved response observed in individuals expressing at least one valine at *FcGR3A*-158 seems to be due to an increased CD16 expression, rituximab binding, and ADCC activity mediated by NK cells (26, 34). In other diseases such as Sjögren's Syndrome (35) or chronic lymphocytic leukemia (36), this association was not observed, which may indicate that mechanisms of action of rituximab other than ADCC may be more important in these pathologies.

Only one study has examined the influence of *FcGR3A*-158F/V in the clinical response to rituximab in autoimmunity. This study conducted in patients with RA found that the V allele carriage was significantly associated with a higher response rate (37). It is possible however that the relative importance of ADCC as a mechanism for the activity of rituximab may differ between autoimmune disorders. The aim of our work was to investigate the possible involvement of the *FcGR3A*-158F/V polymorphism in the clinical response to rituximab in Spanish patients with different systemic autoimmune diseases.

MATERIALS AND METHODS

Patients and treatment

In total, 132 unselected patients with systemic autoimmune diseases treated with rituximab were recruited from three university medical centers (Hospital Universitario San Cecilio, Granada; Hospital Carlos Haya, Málaga; Hospital Virgen del Rocío, Sevilla). The characteristics of the patients enrolled in this study are shown in Table 7.1. Of the 132 patients, 81 (61.4%) were patients with SLE; 16 (12.1%) presented different inflammatory myopathies such as polymyositis and dermatomyositis; 13 (9.8%) were patients with ANCA mediated vasculitis, including Wegener's granulomatosis, Churg-Strauss Syndrome, and microscopic polyangiitis; and the remaining 22 patients presented other systemic autoimmune diseases such as Sjögren syndrome, systemic sclerosis, or autoimmune hemolytic anemia. The majority of patients received rituximab when conventional treatment had failed caused side effects or was contraindicated. Four 375 mg/m^2 doses

Table 7.1: Main characteristics of patients with systemic autoimmune diseases treated with rituximab included in this study

Characteristics	n (%)
Female	108 (82%)
Male	24 (18%)
Systemic autoimmune diseases	
Systemic lupus erythematosus	81 (61%)
Inflammatory myopathies	16 (12%)
ANCA-mediated vasculitis	13 (10%)
Sjögren's syndrome	4 (3%)
Systemic sclerosis	4 (3%)
Hemolytic autoimmune anemia	3 (2%)
Pemphigus vulgaris	3 (2%)
Mixed connective tissue disease	2 (1.5%)
Idiopathic thrombocytopenic purpura	2 (1.5%)
Rheumatoid arthritis	1 (1%)
Axonal polyneuropathy associated with HCV	1 (1%)
Autoimmune thrombocytopenia	1 (1%)
Sarcoidosis + RA	1 (1%)
Previous therapies	
Corticosteroids	127 (96%)
Cyclophosphamide	85 (64%)
Methotrexate	48 (36%)
Mycophenolate	47 (36%)
Intravenous immunoglobulins	46 (35%)
Antimalarials	38 (29%)
Azathioprine	35 (26%)
Cyclosporine A	11 (8%)
Leflunomide	5 (4%)
Other biologic therapies	5 (4%)
Plasma exchange	3 (2%)
Thalinomide	2 (1.5%)
Other therapies	9 (7%)
Concomitant therapies	
Corticosteroids	127 (96%)
Antimalarials	13 (10%)
Methotrexate	8 (6%)
Azathioprine	8 (6%)
Mycophenolate	7 (5%)
Cyclophosphamide	5 (4%)
Cyclosporine A	2 (1.5%)
Intravenous immunoglobulins	1 (0.5%)
Other therapies	2 (1.5%)
Response (n = 132)	
Complete	80 (61%)
Partial	36 (27%)
No response	16 (12%)

of rituximab (the recommended for treatment of lymphoma) were administered by intravenous infusion on days 1, 8, 15, and 22, in most cases, although some patients received 1000 mg twice at an interval of 15 days. Clinical response was evaluated according to the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) recommendations at 6th month after the first infusion (12, 38-40). Complete response was defined as disappearance of all symptoms and signs that led to the use of rituximab, while concomitant immunosuppressive therapy remained stable and in acceptable levels in clinical practice; partial response was defined as a significant improvement (at least 50%) of initial disease activity, based on clinical judgment, but not reaching complete remission; no response was defined as no significant improvement or a worsening of the disease. Previous and concomitant treatments are shown in Table 7.1. The study protocol was approved by an ethics committee, and all patients gave written, informed consent before participation.

FcGR3A genotyping

For genotyping, cellular DNA was isolated from peripheral blood, using QIAamp DNA blood midi/maxi extraction kit (Qiagen GmbH, Germany). *FcGR3A*-158F/V SNP (rs396991) was genotyped using a TaqMan allelic discrimination Assay-By-Design method (Applied Biosystems, Foster City, CA). The genotype call rate was 100% for the tested *FcGR3A* genetic variants. The probes were labeled with the fluorescent dyes 2-chloro-7-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) and 6-carboxyfluorescein (FAM), and a polymerase chain reaction was carried. Endpoint fluorescent readings were performed on an ABI PRISM 7500 Sequence Detection Systems using SDS 2.3 software for allelic discrimination (Applied Biosystems).

Statistical analysis

Statistical analysis for allelic and genotypic distributions was calculated by chi-squared test or Fisher's exact test, when necessary, using the Statcalc software packages (EpiInfo 2002; Centers for Disease Control and Prevention, Atlanta, GA); p-values, odds ratio (OR), and 95% confidence intervals (95% CIs) were calculated. The results were considered to be statistically significant when $p < 0.05$. The presence of heterogeneity between SLE and the remaining patients with autoimmune disease was tested on the basis of the Breslow-Day test using a significance level of 0.05 (StatsDirect, v. 2,6,6).

RESULTS

The response to rituximab was evaluated at month 6 after first infusion, according to the EULAR and ACR criteria (12, 38-40). Eighty of the patients (61%) were considered to be good responders (complete remission of the symptoms and clinical characteristics that recommended the use of the drug); 36 patients (27%) were partial responders (reduction in at least 50% of the disease activity); and sixteen (12%) were classified as non-responders (reduction in < 50% of the disease activity). Of the 132 patients analyzed, 62 (47%) were homozygous for F allele, 48 (36%) were heterozygous FV, and 22 (17%) were homozygous for V allele, similarly to previous studies in SLE in Caucasian populations (41).

When all patients were pooled, after checking for homogeneity of odds ratios between patients with SLE and the remaining patients with autoimmune diseases by Breslow-Day test ($p > 0.05$), and stratified into two groups according to the response to rituximab, genotypic frequencies in patients presenting total or partial response to rituximab were as follows: 50 patients (43%) were FF; 45 patients (39%) were FV; and 21 patients (18%) were VV, whereas in the subgroup of patients presenting non-response to rituximab, 12 (75%) were FF; 3 (19%) were FV; and 1 patient (6%) was VV. In responders, the frequency of V allele carriers (FV +VV) was significantly increased with respect to non-responders (66 patients = 57% vs. 4 patients = 25%; $p = 0.02$; OR = 3.96, 95% CI 1.10–17.68). A significant association was also found when comparing the FCGR3A-158V allele frequency between responders (87 patients = 38%) and non-responders (5 patients = 16%); $p = 0.01$; OR = 3.24, 95% CI 1.17–11.13 (Table 7.2).

Table 7.2: Distribution of *FCGR3A-158F/V* (rs396991) single-nucleotide polymorphism and efficiency in patients with systemic autoimmune diseases treated with rituximab

	Responders n = 116 n (%)	Non-responders n = 16 n (%)	Efficiency rituximab %	p-value	OR (95% CI)
Genotype					
FF	50 (43)	12 (75)	81	0.02	0.25 (0.06–0.91)
FV	45 (39)	3 (19)	94	0.12	2.75 (0.70–15.7)
VV	21 (18)	1 (6)	95	0.21	3.32 (0.46–146.1)
Carriers					
FV + VV	66 (57)	4 (25)	94	0.02	3.96 (1.10–17.68)
FF	50 (43)	12 (75)	81	-	-
Allele					
F	145 (62)	27 (84)	84	0.01	0.31 (0.09–0.86)
V	87 (38)	5 (16)	95	0.01	3.24 (1.17–11.13)

OR, odds ratio; 95% CI, 95% confidence interval; p-value compares the frequency and efficiency in responders versus non-responders.

In correlation with these results, rituximab was effective in 94% of the patients carrying V allele (66 responders carrying the V allele/70 patients carrying the V allele) and 81% of the homozygous FF patients (50 responders FF homozygotes/62 patients FF homozygotes) (p = 0.02) (Table 7.2).

Finally, we analyzed separately patients with SLE (81/132, 61.4% of the patients) and patients with other autoimmune diseases (51/132, 38.6% of the patients). In both groups, we found a similar trend to those observed in the global analysis. In patients with SLE, 49% of the

Table 7.3: Distribution of *FCGR3A-158F/V* (rs396991) single-nucleotide polymorphism and efficiency in patients with systemic lupus erythematosus treated with rituximab

	Responders n = 71 n (%)	Non-responders n = 10 n (%)	Efficiency rituximab %	p-value	OR (95% CI)
Genotype					
FF	36 (51)	8 (80)	82	0.08	0.26 (0.02–1.44)
FV	23 (32)	1 (10)	96	0.14	4.31 (0.53–197.10)
VV	12 (17)	1 (10)	92	0.5	1.83 (0.21–86.83)
Carriers					
FV + VV	35 (49)	2 (20)	95	0.08	3.89 (0.70–39.51)
FF	36 (51)	8 (80)	82	-	-
Allele					
F	95 (68)	17 (85)	85	0.10	0.36 (0.06–1.33)
V	47 (32)	3 (15)	94	0.10	2.80 (0.75–15.58)

P-value compares the frequency and efficiency in responders versus non-responders.

Table 7.4: Distribution of *FCGR3A-158F/V* (rs396991) single-nucleotide polymorphism and efficiency in patients with no systemic lupus erythematosus treated with rituximab

	Responders n = 45 n (%)	Non-responders n = 6 n (%)	Efficiency rituximab %	p-value	OR (95% CI)
Genotype					
FF	15 (33)	4 (67)	79	0.13	0.25 (0.02–2.03)
FV	22 (49)	2 (33)	92	0.39	1.91 (0.24–22.88)
VV	8 (18)	0 (0)	100	0.34	Undefined
Carriers					
FV + VV	30 (67)	2 (33)	94	0.13	4.00 (0.49–47.55)
FF	15 (33)	4 (67)	79	-	-
Allele					
F	52 (58)	10 (83)	85	0.08	0.27 (0.03–1.41)
V	38 (42)	2 (17)	94	0.08	3.65 (0.71–35.81)

P-value compares the frequency and efficiency in responders versus non-responders.

responders carried the V allele while it was present in 20% of non-responders (p = 0.08, OR = 3.89, 95% CI 0.70–39.51). Rituximab was effective in 95% of the V carriers and 82% of the homozygousFF (p = 0.08) (Table 7.3). Likewise, in patients with no SLE, the frequency of the V allele was increased in responder versus non-responder patients (42% vs. 17%; p = 0.08, OR = 3.65, 95% CI 0.71–35.81). Rituximab was effective in 94% of the V carriers and 79% of the patients with FF homozygotes (p = 0.13) (Table 7.4).

DISCUSSION

The establishment of pharmacogenetic markers to predict the response to rituximab therapy becomes a pivotal requirement, given the expanding clinical use of this drug in the treatment of several autoimmune diseases.

Rituximab is recognized and bound to the surface of NK cells and macrophages through the FCGR, triggering ADCC immune system mechanism, essential for the activity of rituximab to deplete B cells. FCGR3A is expressed by immune effector cells and shows specific affinity for IgG monoclonal antibodies, such as rituximab. The importance of FCGR3A in the response to rituximab has been shown in studies where mice lacking FCGR3 presented a decrease in the response to this drug (42).

In the present study, we have analyzed the association of the *FCGR3A-158F/V* polymorphism with the response to rituximab in patients with autoimmune diseases. Genotypic frequencies for this SNP were similar to those described previously for several patients with autoimmune diseases in Caucasian populations (43, 44, 27). It is remarkable that frequencies were elevated for V carriers in responders, which correlates with the fact that patients carrying the V allele at this position presented a better response to the treatment with the drug than those with homozygous FF genotype.

Functional studies have demonstrated that the 158V allele is correlated with a better biological response to rituximab in autoimmunity. Anolik et al. (21) showed that in patients with SLE carrying the high-affinity V allele (FV or VV), rituximab was more effective in depleting peripheral B cells than in those homozygous for the low-affinity FF. Recently, the *FCGR3A-158F/V* SNP has been associated with the clinical response to rituximab in RA. This study conducted in 111 patients found that the V allele carriage was significantly associated with a higher response rate (91% of responder vs. 70%; p = 0.006, OR = 4.6, 95% CI 1.5–13.6) (37).

The findings in SLE and RA are in line with our results that showed a better response to rituximab in patients with autoimmune diseases that carried the V allele (FV or VV) than in

patients with homozygous FF. Additionally, based on the previous association observed in patients with SLE and on the fact that this was the largest group, we analyzed separately patients with SLE. We found a similar pattern, and patients carrying the V allele showed a better response to rituximab treatment, although it did not reach statistical significance ($p = 0.08$). Finally, we examined the group of patients with no SLE to establish whether this association is shared by different autoimmune disorders. As in the case of patients with SLE, we observed a similar effect, but this association did not reach statistical significance either ($p = 0.08$). This suggests that the influence of the 158F/V polymorphism in the therapeutic response to rituximab is common to various autoimmune diseases; however, the reduced numbers involved in these stratified analysis leads to poor statistical power, and therefore the conclusions are provisional.

It should be noted that copy number variation (CNV) has been shown to be present in the *FCGR3A* gene (45-47). The presence of common CNVs can cause false SNP genotyping results that can lead to fail the Hardy–Weinberg equilibrium (HWE) and may blur the association of the studied SNPs with disease susceptibility. In our study, the genotypic frequencies were significantly different from those predicted by HWE, but only in the group of patients with SLE. This may be due to existence of an association between the *158F/V* polymorphism and this disease (48). In fact, in our cohort of healthy controls (previously published genotypic data), genotype frequencies for this SNP were in the HWE (49). Moreover, the frequency of CNV has been reported to vary significantly in different ethnic populations, which can result in contradictory findings, but in this case, frequencies observed in patients were similar to those previously described, and the results reported to date are fairly consistent.

Previous findings showed that patients carrying the V allele in *FCGR3A-158F/V* increased expression of CD16 in NK cells (34). A correlation between the number of cell surface CD16 receptors and the enhancing of the ADCC activity mediated by NK cells was found. These observations would explain the better response to rituximab observed in patients with systemic autoimmune diseases carrying the V allele and would highlight the importance of the ADCC mechanism for clearance of B cells by rituximab in autoimmune diseases.

In summary, our results together with previous findings (21, 50, 37) suggest that FCGR3A plays an important role in response to rituximab in patients with systemic autoimmune diseases and support the hypothesis that the 158F/V variant could be used as a potential predictor of those patients who will respond better to treatment with rituximab.

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Chapter 8

IL2/IL21 region polymorphism influences response to rituximab in systemic lupus erythematosus patients

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Abstract

To determine whether the *IL2/IL21* region, a general autoimmunity locus, contributes to the observed variation in response to rituximab in patients with systemic lupus erythematosus as well as to analyze its influence in a cohort including other autoimmune diseases. rs6822844 G/T polymorphism at the *IL2–IL21* region was analyzed by TaqMan assay in 84 systemic lupus erythematosus (SLE) and 60 different systemic autoimmune diseases Spanish patients receiving rituximab. Six months after the first infusion patients were classified, according to the EULAR criteria, as good responders, partial responders and non-responders. A statistically significant difference was observed in GG genotype frequency between responder (total and partial response) (83.56%) and non-responder (45.45%) SLE patients ($p = 0.010$, odds ratio (OR) = 6.10 [1.28–29.06]). No association with the response was evident in the group of patients with autoimmune diseases other than lupus. Furthermore, when both groups of patients were pooled in a meta-analysis, a reduced statistical significance of the association was observed ($p = 0.024$, OR = 3.53 [1.06–11.64]). Our results show for a first time that *IL2–IL21* region seems to play a role in the response to rituximab in SLE patients but not in other autoimmune diseases.

INTRODUCTION

Rituximab is an anti-CD20 monoclonal antibody that suppresses inflammation effectively in multiple autoimmune diseases (AD) (1). It was initially approved by FDA for the treatment of B cell lymphomas and later for rheumatoid arthritis (RA) refractory to anti-tumor necrosis factor therapies (2, 3). The precise mechanisms by which rituximab exerts its effects are not fully understood. Different mechanisms have been proposed for explaining the therapeutic action of this drug in AD. On the one hand, rituximab is hypothesized to suppress disease injury by promoting rapid and long-term elimination of circulating and possibly lymphoid-tissue-associated B cells (4-6). On the other hand, rituximab-opsonized B cells may act as decoy immune complexes that effectively divert monocytes or macrophages from interactions with tissue associated immune complexes (7).

Recently, studies in the research field of pharmacogenetics have reported potential markers associated with clinical response on treatment with rituximab. In this way, polymorphisms located in *FcGR3A*, *IL6* and *TGFB1* genes seem to act as predictors of response in patients with RA (8-10).

Certain clinical factors have also been associated with a better response to rituximab, including the presence of positive rheumatoid factor in RA patients, positive Epstein-Barr virus in bone marrow, depletion of B cells after first infusion (11, 12) low levels of B lymphocyte stimulator (BLyS) and low levels of type I interferons (13, 14).

Several early clinical investigations of the combination of interleukin-2 (IL2) and rituximab have reported an increased efficacy of this drug by expansion of circulating NK cells, leading to an increased antibody-dependent cellular cytotoxicity (ADCC) (15, 16), therefore evidencing the key role played by this cytokine in rituximab response.

The *IL2/IL21* region at 4q27 is a susceptibility locus for multiple autoimmune diseases (17). Both genes, *IL2* and *IL21*, are plausible functional candidates as genetic modifiers of autoimmunity (18, 19). IL2 stimulates T cell proliferation and activation and regulates the adaptive immune response by stimulating both T-regulatory cells and activation-induced cell death in antigen-activated T cells. Although different polymorphisms in this region have been associated with autoimmunity, rs6822844 has been the most consistently replicated in independent studies and different populations (17, 20-25).

Polymorphisms in susceptibility genes for RA have been shown to be associated with treatment response (26, 27); we hypothesized that rs6822844, known to have a role in several autoimmune diseases, may also influence the response to rituximab therapy. Our main aim was to analyze the role of this genetic variant in the rituximab response in a

cohort of SLE patients and, additionally, to check whether this polymorphism is a common factor influencing the response in different autoimmune disorders.

MATERIALS AND METHODS

Patients and treatment

In the present study were included 84 SLE patients and 60 patients with other systemic autoimmune diseases (16 patients (26.7%) presented different inflammatory myopathies including polymyositis and dermatomyositis, 16 (26.7%) were ANCA-mediated vasculitis patients including Wegener's granulomatosis, Churg–Strauss syndrome and microscopic polyangiitis and the remaining 29 (48.3%) patients presented other systemic autoimmune diseases, such as systemic sclerosis, Sjögren's syndrome, rheumatoid arthritis and autoimmune haemolytic anemia), all of them Spanish Caucasian patients treated with rituximab. Patients were recruited from three university medical centers (Hospital Universitario San Cecilio, Granada; Hospital Carlos Haya, Málaga; Hospital Virgen del Rocío, Sevilla). The main characteristics of the patients enrolled in this study are shown in Table 8.1.

The administered intravenous dose of rituximab was 375 mg/m^2 weekly for 4 weeks in most cases, although some patients received 1,000 mg twice at an interval of fifteen days. Clinical response was evaluated 6 months after the first infusion of rituximab, according to the ACR and EULAR recommendations (28-30). The criteria used to evaluate the response to rituximab in different autoimmune diseases have already been described in detail elsewhere (1). Complete response was defined as disappearance of all symptoms and signs of the systemic disease that recommended the use of rituximab. Partial response was defined as a significant improvement (at least 50%) of initial disease activity, based on clinical judgment. Responders included complete responders and partial responders; no response was defined as no significant improvement or worsening of the disease. Concomitant and previous treatments are shown in Table 8.1. The study was approved by an ethic committee, and all patients gave written informed consent before participation.

Genotyping

DNA was isolated from whole peripheral blood, using standard procedures. *IL2–IL21* SNP (rs6822844) genotyping was performed using the Taqman allelic discrimination assay technology in a 7,500 real-time PCR system, from applied biosystems (Foster City, California, USA). The genotype call rate was 100% for the tested genetic variant. The probes were labeled with the fluorescent dyes VIC and FAM and PCR reaction was carried.

Table 8.1: Main characteristics of systemic autoimmune diseases patients treated with rituximab included in this study

Characteristics	n (%)
Female	114 (79.16)
Male	30 (20.83)
Systemic autoimmune diseases	
Systemic lupus erythematosus	84 (57.64)
Inflammatory myopathies	16 (11.11)
ANCA-mediated vasculitis	16 (11.11)
Sjo¨gren's syndrome	4 (2.77)
Systemic sclerosis	5 (3.47)
Hemolytic autoimmune anemia	3 (2.08)
Pemphigus vulgaris	3 (2.08)
Mixed connective tissue disease	2 (1.38)
Idiopathic thrombocytopenic purpura	4 (2.77)
Cryoglobulinemia	2 (1.38)
Rheumatoid arthritis	3 (2.08)
Axonal polyneuropathy associated with HCV	1 (0.69)
Autoimmune trombocytopenia	1 (0.69)
Sarcoidosis + RA	1 (0.69)
Previous therapies	
Corticosteroids	137 (95.14)
Cyclophosphamide	89 (61.80)
Methotrexate	52 (36.11)
Mycophenolate	50 (34.72)
Intravenous immunoglobulins	49 (34.02)
Antimalarials	38 (26.38)
Azathioprine	38 (26.38)
Cyclosporine A	11 (7.6)
Leflunomide	5 (3.4)
Other biologic therapies	5 (3.4)
Plasma exchange	3 (2)
Thalinomide	2 (1.38)
Other therapies	11 (7.63)
Concomitant therapies	
Corticosteroids	136 (94.4)
Antimalarials	13 (9)
Methotrexate	8 (5.5)
Azathioprine	8 (5.5)
Mycophenolate	7 (4.86)
Cyclophosphamide	8 (5.5)
Cyclosporine A	2 (1.38)
Intravenous immunoglobulins	3 (2.08)
Other therapies	2 (1.38)
Response (n = 144)	
Complete	87 (60.42)
Partial	40 (27.77)
No response	17 (11.81)

Statistical analysis

Statistical analysis for allelic and genotypic distributions was calculated by Chi squared test or Fisher's exact test, when necessary, using the StatCalc software packages (Epi Info 2002; Centers for Disease Control and Prevention, Atlanta, GA); p-values, odds ratio (OR) and 95% confidence intervals (CI) were calculated according to this software. p-value lower than 0.05 was considered as statistically significant.

The presence of heterogeneity between SLE and the remaining autoimmune diseases patients was tested on the basis of the Breslow–Day test using a significance level of 0.05 (StatsDirect, v. 2,6,6). Multivariate logistic regression analysis was performed using STATA v. 10.

RESULTS

The clinical response of the treatment with rituximab was evaluated at month 6 after the first infusion, according to the EULAR and ACR criteria (1, 28-30). In the group of SLE patients, 49 patients (58.3%) responded well to the treatment, 24 (28.6%) were considered as partial responders and 11 (13.1%) were considered non responders. In patients with other autoimmune disorders, 38 patients (63.3%) showed a good response, 16 patients (26.7%) were considered as partial responders and 6 patients (10%) did not respond to the treatment. The response rate observed in patients including in our study is similar to those described in other studies (31, 32).

Genotypic and allele frequencies of the *IL2/IL21* polymorphism observed in responder and non-responder autoimmune diseases patients are summarized in Table 8.2. These frequencies were not significantly different from those previously described in Caucasian populations (22).

In SLE patients, both GG genotype and G allele frequencies were increased in responders compared with non-responders (83.6% vs 45.5%; p = 0.010, OR = 6.10 [1.28–29.06] and 91.8% vs 72.7%; p = 0.016, OR = 4.19 [1.12–14.06], respectively) (Table 8.2). Nevertheless, no differences between responder and non-responder patients were observed in the group of non-SLE patients (Table 8.2).

Subsequently, different systemic autoimmune diseases patients were pooled and homogeneity of odds ratio between SLE and the remaining autoimmune diseases patients was verified by Breslow-Day test (p > 0.05). In responders, GG genotype frequency was significantly increased with respect to non-responders (83.5% vs 58.82%; p = 0.024, OR = 3.53 [1.06–11.64]). Significant differences were also observed in the allelic frequencies between responder and non-responder patients (91.7% vs 79.4%; p = 0.032, OR = 2.88 [1.00–8.01]) (Table 8.2).

Table 8.2: Distribution of rs6822844 *IL2/IL21* genetic variant in systemic lupus erythematosus, non-systemic lupus erythematosus and autoimmune diseases patients

		Genotype, n (%)			Genotype test		G allele	Allele test	
Disease	Subgroup (n)	GG	GT	TT	p-value[a]	OR (95% CI)	frequency (%)	p-value	OR (95% CI)
SLE	Non-responders (n = 11)	5 (45.45)	6 (54.55)	0			72.70		
	Responders (n = 73)	61 (83.56)	12 (16.44)	0	**0.010**	6.10 (1.28–29.060	91.78	**0.016**	4.19 (1.12–14.06)
Non-SLE	Non-responders (n = 6)	5 (83.33)	1 (16.67)	0			91.67		
	Responders (n = 54)	45 (83.33)	9 (16.67)	0	0.683	1.00 (0.02–10.67)	91.69	0.666	1.00 (0.02–8.55)
Autoimmune diseases pooled	Non-responders (n = 17)	10 (58.82)	7 (41.18)	0			79.41		
	Responders (n = 127)	106 (83.46)	21 (16.54)	0	**0.024**	3.53 (1.06–11.64)	91.73	**0.032**	2.88 (1.00–8.01)

[a] P-values have been calculated comparing responders carrying GG versus non-responders carrying GG.
SLE, systemic lupus erythematosus.
Bold text denotes significant p-values.

Finally, to evaluate if socio-demographic variables and concomitant therapies could be confounding the observed association in SLE patients, a multivariate logistic regression analysis, considering the effect of the GG genotype on the rituximab response as the dependent variable and gender, age and concomitant therapies as independent variables, was performed. As shown in Table 8.3, this analysis observed no confounding factors.

Table 8.3: Multivariable model of *IL2/IL21* rs6822844 GG genotype carriage adjusting for potential confounding factors in systemic lupus erythematosus

	p-value	OR (95% CI)
rs6822844 GG genotype unadjusted	0.008[a]	6.1 (1.60–23.26)[a]
Adjusted for individual covariates		
Gender	0.007[b]	6.37 (1.64–24.67)[b]
Age	0.012[b]	6.63 (1.51–29.11)[b]
Corticosteroids	0.005[b]	7.38 (1.80–30.18)[b]
DMARDs	0.006[b]	7.43 (1.78–31.03)[b]
Other therapies	0.006[b]	7.13 (1.74–29.18)[b]
Adjusted for all the covariates	0.016[b]	6.43 (1.42–21.07)[b]

[a] p-value and OR correspond to the effect of the rs6822844 GG genotype in rituximab response.
[b] p-values and OR correspond to the effect of the rs6822844 GG genotype in rituximab response adjusted for different covariates considered individually and altogether.
DMARDS, disease-modifying antirheumatic drugs.

DISCUSSION

Treatment with rituximab results in a reduction of disease activity in most autoimmune diseases patients. However, a percentage of patients do not respond to this therapy and/or experience toxicity (33). The reason for this non-response is unknown, but genetic and environmental factors are thought to be implicated. Given the potential toxicities and the high cost of rituximab therapy, it would be beneficial to predict whether an individual patient will benefit from this treatment, beforehand.

Knowledge about related genetic variants, mostly SNPs, may help to predict drug response or optimal dose in the individual patient. Classically, explorative pharmacogenetic association studies are aimed at finding polymorphisms potentially predictive (34).

Our results indicate that SLE patients homozygous for rs6822844 G allele show a better clinical response to rituximab at month 6 than patients with GT genotype. On the contrary, no association was evident in the group of non-SLE patients. It could be speculated that this lack of association was a consequence of a lower statistical power in the latter analysis;

however, it should be noted that no effect size was suggested in this case (i.e. OR = 1) and, in addition, a reduction of the statistical significance of the association was observed when the non-SLE patients were meta-analyzed with those showing SLE (which increases the statistical power). Taken together, our data suggest that the influence of the *IL2/IL21* rs6822844 polymorphism in the therapeutic response to rituximab is specific of the SLE condition.

Although the mechanism of action of rituximab remains unclear, accumulating data suggest that antibody-dependent cellular cytotoxicity (ADCC) may play a dominant role (35). ADCC is mediated through immune effector cells, mainly NK cells, via expression of an activating receptor for the Fc portion of IgG antibodies (FcGR). The majority of human NK cells are CD16 positive (FcGRIII) and express the intermediate affinity interleukin-2 receptor. It has been described that intermediate doses of IL2 are capable of expanding CD16 positive NK cells and activating cytotoxic effector functions, including ADCC activity (36-40).

Several studies have demonstrated that this ability of IL2 to promote NK cell expansion and cytotoxicity influences the efficiency of rituximab treatment and correlates with the clinical response (15, 16, 41-44). Furthermore, the relationship between IL2 and the efficacy of rituximab is supported by the fact that soluble interleukin-2 receptor is used as a prognostic factor in patients with lymphoma receiving rituximab (45-49).

An alteration of the function of B cells is a key factor contributing to SLE athophysiology; however, some clinical trials with rituximab in this disease have failed to show efficacy. Murine models of SLE based on antibody mediated cellular depletion evidenced that this lack of efficacy can be explained by a defect in macrophage and neutrophil IgG-dependent phagocytosis induced by serum IgG (50). In this context, the role of IL2 promoting rituximab-mediated ADCC could become more critical in the efficacy of rituximab in lupus than in other autoimmune diseases in which this drug acts through all its mechanisms.

The *FCGR3A-158* polymorphism is currently shown to enhance rituximab mediated ADCC and improve clinical response to this drug (51). Similarly, rs6822844 variant could affect the cytotoxic activity of NK cells and, therefore, the efficacy of rituximab in SLE condition; although to date no functional studies analyzing this issue have been published.

In conclusion, we show for a first time that *IL2–IL21* rs6822844 G/T polymorphism influences the clinical efficacy of rituximab in SLE patients. The replication of this association in independent studies could enable the potential use of this variant as a pharmacogenetic marker.

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Chapter 9

Summary

Rheumatoid arthritis (RA) is a common autoimmune disease associated with progressive disability and systemic complications. The etiology of this inflammatory disease remains largely unclear due to complexity of interacting factors including genetic and environmental determinants. Disease-modifying antirheumatic drugs (DMARDs) (including methotrexate, MTX), anti-TNF drugs and rituximab in RA have the capacity of reducing progression or preventing damage to the joints and preserving their integrity and function by modulating the immune response.

Systemic lupus erythematosus (SLE) is a chronic inflammatory disease of unknown cause that can affect virtually any organ of the body. Patients present with variable clinical features ranging from mild joint and skin involvement to life-threatening renal, hematologic, or central nervous system involvement. A general approach to treating SLE includes the use of immunosuppressive agents targeting B-cell pathways as belimumab and rituximab.

However, it is widely recognized that interindividual responses both with regard to efficacy and toxicity vary for all drugs used in RA and SLE, including MTX, anti-TNF acting drugs and other agents targeting B-cell pathways as rituximab. A possible explanation is that the genetic profile of an individual influences drug efficacy or drug toxicity. Indeed, genes encoding drug metabolizing enzymes and drug transporters but also pharmacodynamic proteins are found highly variable between individuals. Early identification of subjects who respond or do not respond to these drugs may be of help when establishing the most efficacious and safe treatment with these drugs.

RHEUMATOID ARTHRITIS

In the first part of the thesis studies were presented concerning genetic variability contributing to differences in response to anti-TNF and toxicity to MTX in RA patients. In **chapter 2**, we reviewed the scientific literature for evidence for markers for MTX-induced hepatic injury in RA treatment. These genetic and nongenetic determinants may be useful to predict the individual patients´ risk for MTX-induced hepatotoxicity and could help to reduce the incidence and morbidity of MTX-induced liver injury. The possible nongenetic risk factors include the cumulative MTX dose and duration of treatment, the use of other hepatotoxic drugs or chemicals such as alcohol, impaired renal function and the concomitant use of drugs that decrease the elimination of MTX and history of liver disease. In addition, genetic susceptibility plays an important role in the occurrence of hepatotoxicity and increased risk of developing drug-induced liver injury (DILI). In general, from the published studies, *MTHFR* C677T appears to be the most promising genetic marker

predicting low-dose MTX-induced hepatotoxicity, although because the limited power of studies to identify genetic biomarkers for hepatotoxicity, conflicting results exist limiting its clinical application.

Recently, researchers in pharmacogenetic studies have reported several genetic variants associated with clinical response to anti-TNF treatment. In **chapter 3**, the association of four polymorphisms (rs1532269 and rs17301249, intronic polymorphisms mapped within *PDZD2* and *EYA4*, respectively, and rs12081765 and rs7305646 located at intergenic regions on chromosomes 1 and 12, respectively) previously identified in a genome-wide association study (GWAS) as being associated with anti-TNF treatment response in patients with RA was performed in our study.

These 4 polymorphisms were genotyped in a total of 634 Spanish RA patients treated with anti-TNF drugs. Four results were evaluated: changes in the Disease Activity Score in 28 joints (DAS28) after 6 and 12 months of treatments and classification according to the European League Against Rheumatism (EULAR) response criteria at the same time points. In addition, we combined our data with those of previously reported studies in a meta-analysis including 2,998 RA patients. None of the four genetic variants showed an association with response to anti-TNF drugs in any of the four outcomes analyzed in our Spanish patients. However, rs1532269, mapped within *PDZD2* gene, yielded a suggestive association with the response to anti-TNF when available data from previous studies were combined in the meta-analysis.

This heterogeneity between studies was also seen in the results of **chapter 5**, where we present the results of the first large study on the influence of *FcGR2A* and *FcGR3A* genes on treatment response in a cohort of 302 Dutch RA patients using adalimumab as the anti-TNF therapy. Similarly to chapter 2, treatment outcome was evaluated with the use of the DAS28 criteria and responses were classified according to EULAR criteria. The presence of *FcGR2A*-H allele was associated with EULAR good response at 14 weeks. No significant association was found for *FcGR3A* with good response or remission. The combined effect of both SNPs showed no association with EULAR good response.

In **chapter 4** it was shown that the response to anti-TNF therapy is also influenced by a polymorphism affecting the disease activity, suggesting that increased expression of IL-6 in patients carrying the *-174*C* allele would result in a poorer response to anti-TNF treatment. The original effect on anti-TNF treatment response caused by the change in *IL-6 -174G/C* was replicated in an independent population of 199 Spanish RA patients receiving anti-TNF therapy. Patients were classified according to EULAR criteria as responders and non-responders at 6, 12, 18 and 24 months after the first infusion. The *-174*G* allele was

significantly associated with good or moderate EULAR response at 12, 18 and 24 months. The combined analysis of our data and those previously published showed an association between this genetic variant and the clinical response to anti-TNF.

SLE AND OTHER AUTOIMMUNE DISEASES

The second part of the thesis was focused on studies concerning genetic variability contributing to differences in response to rituximab in several autoimmune diseases, mainly SLE.

Recent studies have provided evidence that antagonizing the action of proinflammatory cytokines, including IL-6 and IL-2, may exert a therapeutic effect in autoimmune disease patients nonresponsive to other therapies. The *-174G/C* genetic variant (rs1800795), located in the *IL-6* gene promoter region, has been found to be associated with autoimmune diseases and involved in increased levels of IL-6 protein in serum in diverse inflammatory diseases; the GG homozygotes have circulating IL-6 concentrations approximately twice higher than those homozygous for the C allele. In **chapter 6** we analyzed the association of the *-174 IL-6* promoter variation with the response to rituximab in a group of 144 Spanish patients that presented diverse systemic autoimmune diseases, including SLE. Six months after the first infusion with rituximab, we evaluated the response to the drug. The CC genotype was more frequent in non-responders as compared to those carrying GC or GG genotypes. A similar trend but not statistically significant was observed when SLE patients were analyzed separately.

In healthy subjects, stratification according to the *IL2–IL21* region polymorphism (rs6822844) revealed significant differences in circulating interleukin-2 with the lowest levels in GG genotype carriers (12). This is in agreement with our results in **chapter 8** where the role of this genetic variant on the rituximab's response was studied in 144 Spanish patients with different systemic autoimmune diseases. The response was evaluated according to EULAR criteria at six months after the first infusion. In the group of SLE patients, both GG genotype and G allele frequency were increased in responders compared with non-responders. No association with response was evident in non-SLE patients. Interestingly, these findings show conflicting results with the results obtained in **chapter 6** where the allele associated previously with lower levels of IL6 were associated with worse response to rituximab.

Rituximab is recognized and bound to the surface of NK cells and macrophages through the FcGR, triggering ADCC immune system mechanism, essential for the activity of rituximab

to deplete B cells. The importance of FcGR3A in the response to rituximab has been shown in studies where mice lacking FcGR3 presented a decrease in the response to this drug (13). In **chapter 7**, a genetic variant in *FcGR3A* gene in the response to rituximab was studied in 132 Spanish patients with different systemic autoimmune diseases. Rituximab was more effective in V allele carriers than in homozygous FF in patients with different autoimmune diseases. We analyzed separately SLE patients and we found a similar trend to those observed in the global analysis but it was not statistically significant.

Chapter 10

Samenvatting

Reumatoïde artritis (RA) is een veel voorkomende auto-immuunziekte geassocieerd met progressieve invaliditeit en systemische complicaties. De etiologie van deze ontstekingsziekte is grotendeels onduidelijk en complex vanwege interacterende factoren, waaronder genetische en omgevingsfactoren. Disease-modifying antirheumatic drugs (DMARD's; inclusief methotrexaat, MTX), anti-TNF-geneesmiddelen en rituximab bij RA kunnen de schade aan de gewrichten verminderen door hun immuun-modulerende werking.

SLE is een chronische inflammatoire ziekte van onbekende oorzaak die vrijwel elk orgaan van het lichaam kan treffen. Patiënten vertonen verschillende klinische kenmerken, variërend van een lichte gewrichts- en huidaandoening tot levensbedreigende nier-, hematologische of centrale zenuwstelselschade. De hoeksteen van de behandeling van SLE is het gebruik van immunosuppressiva waaronder belimumab and rituximab.

Echter, de individuele respons op de geneesmiddelen die worden gebruikt bij RA en SLE, waaronder MTX, anti-TNF en andere middelen gericht tegen B-cellen zoals rituximab, is sterk variabel zowel met betrekking tot de werkzaamheid als de toxiciteit. Een mogelijke verklaring hiervoor is dat het genetische profiel van een individu de werkzaamheid van een geneesmiddel of de toxiciteit van geneesmiddelen beïnvloedt. Het is bekend dat genen die coderen voor geneesmiddel-metaboliserende enzymen en geneesmiddeltransporters, maar ook farmacodynamische eiwitten, zoals receptoren, verschillen tussen individuen. Vroegtijdige herkenning van patiënten die al dan niet positief reageren op deze geneesmiddelen, kan van pas komen bij het vaststellen van de meest werkzame en veilige behandeling van RA- en SLE-patiënten.

REUMATOÏDE ARTRITIS

In het eerste deel van het proefschrift worden studies gepresenteerd over de genetische variabiliteit tussen patiënten die bijdraagt aan verschillen in respons op anti-TNF en toxiciteit voor MTX bij RA-patiënten.

In **hoofdstuk 2** hebben we de wetenschappelijke literatuur onderzocht op aanwijzingen voor markers ter voorspelling van MTX-geïnduceerde leverbeschadiging bij RA-behandeling. Deze genetische en niet-genetische determinanten kunnen behulpzaam zijn om het individuele patiëntenrisico voor MTX-geïnduceerde hepatotoxiciteit te voorspellen en kunnen mogelijk de incidentie en morbiditeit van leverschade verminderen. Als niet-genetische risicofactoren komen naar voren: de cumulatieve MTX-dosis, de duur van de behandeling, het gebruik van andere hepatotoxische geneesmiddelen of chemicaliën zoals

alcohol, een verminderde nierfunctie en het gelijktijdig gebruik van geneesmiddelen die de eliminatie van MTX vertragen, en eerder doorgemaakte leveraandoeningen. Als genetische factor voor het optreden van hepatotoxiciteit en het ontwikkelen van geneesmiddel-geïnduceerde leverbeschadiging (DILI) komt *MTHFR* C677T uit de gepubliceerde studies naar voren. Echter, het bewijs is nog te beperkt om deze biomarker in de klinsiche praktijk toe te gaan passen.

Recentelijk hebben onderzoekers verschillende genetische varianten gerapporteerd die verband houden met de klinische respons op de ant-TNF-behandeling. In **hoofdstuk 3** hebben we de associatie onderzocht van vier polymorfismen rs1532269 en rs17301249, gelegen in resp. het gen *PDZD2* en *EYA4*, en rs12081765 en rs7305646 gelegen op resp. chromosoom 1 en 12, die eerder werden geïdentificeerd in een genoom-brede associatiestudie, met anti-TNF-behandelingsrespons bij patiënten met RA. Deze 4 polymorfismen werden genotypeerd in totaal 634 Spaanse RA-patiënten die werden behandeld met anti-TNF-geneesmiddelen. Er is gekeken naar veranderingen in de ziekte-activiteitsscore (DAS28) na 6 en 12 maanden behandeling en de EULAR-classificatie op dezelfde tijdstippen. Tevens hebben we onze gegevens gecombineerd met die van eerder gepubliceerde studies in een meta-analyse met 2.998 RA-patiënten. Geen van de vier genetische varianten vertoonde een associatie met respons op anti-TNF-geneesmiddelen in ons cohort. Echter, in de meta-analyse liet de variant rs1532269 gelegen in het *PDZD2*-gen een trend voor associatie met de anti-TNF-respons zien.

Deze heterogeniteit tussen studies werd ook gezien bij **hoofdstuk 5**, waarin we de resultaten presenteren van de eerste grote studie naar de invloed van *FcGR2A*- en *FcGR3A*-genen op de behandelrespons in een cohort van 302 Nederlandse RA-patiënten die adalimumab als de anti-TNF-therapie gebruiken. Net als in hoofdstuk 2 werd het behandelresultaat geëvalueerd met behulp van de DAS28-criteria en werden de responsen geclassificeerd volgens EULAR-criteria. De aanwezigheid van het *FcGR2A*-H allel was geassocieerd met een EULAR goede respons na 14 weken. Er werd geen significante associatie gevonden voor FcGR3A met een EULAR goede respons of remissie. Het gecombineerde effect van beide 'SNP's toonde geen associatie met EULAR goede respons.

In **hoofdstuk 4** wordt aangetoond dat de respons op anti-TNF-therapie ook wordt beïnvloed door een polymorfisme dat de RA-ziekteactiviteit beïnvloedt: een verhoogde IL-6-expressie bij patiënten die het *-174*C*-allel dragen resulteert in een slechtere reactie op anti-TNF-behandeling. We voerden een replicatiestudie uit naar de rol van *IL-6 -174G/C* op de effectiviteit van de anti-TNF-behandeling in een onafhankelijke populatie van 199 Spaanse RA-patiënten. Patiënten werden ingedeeld op basis van EULAR-criteria als responders en

non-responders op 6, 12, 18 en 24 maanden na de eerste infusie. Het *-174*G*-allel was significant geassocieerd met goede of matige EULAR-respons na 12, 18 en 24 maanden. De gecombineerde analyse van onze gegevens en de eerder gepubliceerde data liet ook een significant verband zien tussen deze genetische variant en de klinische respons.

SLE EN ANDERE AUTO-IMMUUNZIEKTEN

Het tweede deel van het proefschrift richt zich op studies met betrekking tot genetische variabiliteit die bijdragen aan verschillen in respons op rituximab bij verschillende auto-immuunziekten, voornamelijk SLE. Recente onderzoeken hebben aangetoond dat het antagoneren van de werking van pro-inflammatoire cytokines, waaronder IL-6 en IL-2, een therapeutisch effect kan hebben bij patiënten met auto-immuunziekten die niet reageren op andere therapieën. De *IL6 -174G/C* variant (rs1800795), gelokaliseerd in het IL-6 genpromotorgebied, blijkt geassocieerd te zijn met auto-immuunziekten en leidt tot verhoogde concentraties van IL-6-eiwit in serum bij diverse ontstekingsziekten: de GG-homozygoten hebben een ongeveer tweemaal hogere concentratie IL-6 in vergelijking met personen die CC-homozygoot zijn. In **hoofdstuk 6** onderzochten we de associatie van de *IL-6 -174* variant met de respons op rituximab bij een groep van 144 Spaanse patiënten met verschillende systemische auto-immuunziekten, waaronder SLE. Zes maanden na de eerste infusie met rituximab evalueerden we de reactie op het geneesmiddel: het CC-genotype kwam vaker voor bij non-responders in vergelijking met degenen die GC- of GG-genotypen hadden. Een vergelijkbare maar niet statistisch significante trend werd waargenomen in de subgroep van SLE-patiënten.

Studies in gezonde personen lieten zien dat er significante verschillen in circulerende interleukine-2-concentraties bestaan afhankelijk van het *IL2-IL21*-polymorfisme (rs6822844): de laagste IL-2-concentraties werden gevonden in personen met het GG-genotype. Deze bevindingen zijn in lijn met onze resultaten in **hoofdstuk 8**, waarin de rol van deze genetische variant op de respons van rituximab werd bestudeerd bij 144 Spaanse patiënten met verschillende systemische auto-immuunziekten. De respons werd beoordeeld volgens de EULAR-criteria zes maanden na de eerste infusie. In de groep van SLE-patiënten was zowel het GG-genotype als de G-allelfrequentie verhoogd in responders vergeleken met niet-responders. Er werd geen associatie gevonden bij niet-SLE-patiënten. Interessant is dat deze bevindingen niet in lijn zijn met de resultaten die zijn verkregen in **hoofdstuk 6**, waar het allel dat eerder met een lager niveau van IL6 was geassocieerd, geassocieerd was met een slechtere respons op rituximab.

Rituximab wordt herkend door en gebonden aan het oppervlak van NK-cellen en macrofagen via de FCGR, waardoor het ADCC-immuunsysteem wordt geactiveerd. Dit is essentieel voor de activiteit van rituximab om B-cellen te depleteren. Het belang van FCGR3A in de respons op rituximab is aangetoond in onderzoeken waarbij muizen zonder FCGR3 een afname van de respons op dit geneesmiddel vertoonden. In **hoofdstuk 7** werd de rol van een genetische variant in het *FcGR3A*-gen op de respons op rituximab bestudeerd bij 132 Spaanse patiënten met verschillende systemische auto-immuunziekten. Bij patiënten met diverse auto-immuunziekten bleek rituximab effectiever bij V-alleldragers dan bij patiënten die het homozygote FF-genotype hadden. We analyseerden ook de subgroep van SLE-patiënten en we vonden daarin een vergelijkbare trend, hoewel niet statistisch significant.

Chapter 11

General discussion and future perspectives

RA is an autoimmune disease characterized by chronic inflammation of the synovial joints resulting in joint destruction, polyarthritis and functional disability. SLE is a chronic inflammatory disease of unknown cause that can affect virtually any organ of the body. In recent years, the use of DMARDs, anti-TNF drugs and rituximab has resulted in an improvement in the treatment of RA patients by reducing both inflammation and joint damage, and their clinical use has become widespread (1, 2). Similarly, rituximab has become a pivotal therapy in the treatment of SLE, since an aberrant B cell regulation is among the common pathogenic mechanism of these diseases (3, 4).

However, despite the use of the above-mentioned drugs, it is known that there are considerable differences in individual responses to MTX, anti-TNF and rituximab both regarding efficacy and toxicity.

The reason for this variable response is unknown, but genetic and environmental factors are thought to be implicated. Given the potential toxicities and the high cost of therapies, it would be a great improvement to be able to predict whether an individual patient will benefit from this treatment, beforehand. Knowledge about related genetic variants, mostly SNPs, may help to predict drug response or the optimal dose in the individual patient. Classically, explorative pharmacogenetic association studies are aimed at finding polymorphisms potentially useful as predictive biomarkers of drug response.

RHEUMATOID ARTHRITIS

In **chapter 2**, we reviewed the scientific literature for evidence for genetic markers for MTX-induced hepatic injury in RA treatment. Overall, we found limited evidence and a low number of studies. Such studies may be difficult due to the relative low incidence of MTX-induced hepatotoxicity. In addition, the use of different definitions of hepatotoxicity, differences in MTX dose and folic acid supplementation and the lack of replication studies hampers solid conclusions. Nevertheless, the identification of genetic predictors for MTX-induced hepatotoxicity presents an important opportunity to identify individual patients at risk for this debilitating adverse event. In general, from the published studies, *MTHFR* C677T appears to be the most promising genetic marker predicting low-dose MTX-induced hepatotoxicity (5), although because the limited power of studies to identify genetic biomarkers for hepatotoxicity, conflicting results exist limiting its clinical application.

In **chapter 3**, the association of four polymorphisms (rs1532269 and rs17301249, intronic polymorphisms mapped within *PDZD2* and *EYA4*, respectively, and rs12081765 and rs7305646 located at intergenic regions on chromosomes 1 and 12, respectively) previously

identified as being associated with anti-TNF treatment response in patients with RA was not confirmed. In addition, the combined analysis with the three previous studies included in our meta-analysis (6-8) showed only a suggestive association of one of the four polymorphisms (rs1532269) (even weaker than that reported in the study by Plant et al. (6). These findings seem to exclude effects of sufficient magnitude to be useful in predicting response to treatment. The lack of replication provided in pharmacogenetic studies could be ascribed to multiple differences between studies including ethnic background, phenotype definition or exposure to other risk factors. It is commonly impossible to identify one of them as being more relevant than the others. Genetic differences between populations are an unlikely explanation of the results, given that the allele frequencies of the four tested polymorphisms were very similar between studies. Clinical differences between the patients with RA included in the different reports are possible and difficult to exclude. In this regard, it has already been mentioned that Plant et al. (6) evaluated the response to TNFi at 6 months, whereas the two subsequent studies used the response at 14 weeks. However, this difference does not apply to our study in which evaluation at 6 months evidenced negative results.

It has been shown repeatedly that in the first study of an association, the effect is overestimated, and that there is only a modest correlation between effects in first and in subsequent studies on the same association (9-12). There is a phenomenon known as 'winner's curse' (13) or 'Jackpot effect' (14) originating in the fact that the associations with the strongest effects are inflated (10). This occurs primarily because with a small sample, a weak effect becomes significant only if the effect is overestimated. This phenomenon is aggravated by a selective reporting of the analyses, possibly biased interpretation of results and publication and other forms of bias (10, 11, 15).

It should be noted that the four SNPs studied by Plant et al. (6) showed the highest effects in the discovery cohort (which was the only one with a clear association between these four polymorphisms and the clinical response), whereas the three replication studies showed lower effect sizes (β-values less different from zero), thus supporting this possibility. Indeed, significant heterogeneity between studies was observed in the meta-analysis of three of the four analyzed genetic variants. Interestingly, this heterogeneity disappeared when the discovery cohort of Plant et al. was removed (6). Therefore, variables other than the presence of the four SNPs considered herein could have influenced the efficacy of TNFi in this cohort, accounting for its singularity. Other GWASs of responses to TNFi treatment in RA have been published (15-18). This GWAS approach represents an important step forward in the understanding of the influence of genetic variability on the efficacy of this therapy. Only one of the observed associations has been found to reach the GWAS statistical

significance level, however, and only after combination with data derived from replication studies (19). This highlights the important role of validation studies in determining the status of the remaining GWAS findings. It is to be expected that these combined efforts will produce useful insights.

This heterogeneity between studies was also seen when preparing for **chapter 5**. Several studies evaluated the hypothesis of a decreased clearance of anti-TNF drugs in RA due to *FcGR2A* and *FcGR3A* genetic polymorphisms by analyzing the effect of these SNPs on the response to different TNFα antagonists in RA with conflicting results. These disconcordant results could be explained by the small sample size, heterogeneity in the design (different anti-TNF agents), the use of different definitions of response, the different observational period and the use of different methods for genotyping. In **chapter 5**, we present the results of the first large study on the influence of *FcGR2A* and *FcGR3A* genes on treatment response in a cohort of RA patients using adalimumab as the anti-TNF drug being investigated. Our results indicate that the *FcGR2A* genotype shows a trend toward association with clinical efficacy of adalimumab defined as EULAR good response at 14 weeks. However, we did not find an association with good response or remission response for the *FcGR3A* genotype. Recently, Montes et al. (20) reported a significant association between the *FcGR2A* polymorphism and response to treatment with infliximab at 3 months, but they could not find such an association combining etanercept and adalimumab treated patients. Unfortunately, no analysis of patients treated with adalimumab or treated with etanercept could be performed separately because these two groups consisted of too small numbers of patients. In our study we were able to include 302 patients treated with adalimumab, the largest sample size for a pharmacogenetic study of adalimumab-treated patients published to date.

Previously, three papers studying the association of *FcGR3A* polymorphisms and response to anti-TNF drugs have been published (21-23). In a small study consisting of 30 RA patients, Tutuncu et al. (21) found that patients with *FcGR3A*-FF genotype had a better response to several anti-TNF drugs after 12 weeks than those carrying at least one *FcGR3A*-V allele. However, the response to therapy was not evaluated according to accepted standards such as the EULAR criteria. In contrast, Morales-Lara et al. (22) found no significant association between the *FcGR3A*-FF and good response EULAR or ACR20 criteria at 3 months in their small cohort of 41 RA patients treated with infliximab, but the genotype was associated with ACR20 response at 12 months using ACR.

Similarly, different articles have shown that the role of *FcGR* polymorphisms in response to anti-TNF drugs may be dependent on the disease as well. Several articles have studied the

association between *FcGR3A* in the response to infliximab in patients with psoriatic arthritis and ankylosing spondylitis and unexpectedly found that the high-affinity-V158 allele was associated with a better response to infliximab in patients with ankylosing spondylitis. In addition, in a recent publication (24) the presence of high-affinity alleles of *FcGR2A* and *FcGR3A* was significantly associated with a better response in the intermediated point of treatment but not at the end of the treatment in 70 PsA patients treated with different anti-TNF drugs suggesting that ADCC-mediated apoptosis of TNF-bearing cells by natural killer cells and macrophages might induce a faster clearance of milder lesions than those with higher score disease. These results suggest that the role of *FcGR* polymorphisms in response to anti-TNF drugs may be dependent on the disease as well.

In **chapter 4** it is shown that the response to anti-TNF therapy is also influenced by a polymorphism affecting the disease activity. Increased expression of IL-6 in patients carrying the *-174*C* allele would result in a poorer response to anti-TNF treatment (16, 17). The original effect on anti-TNF treatment response caused by the change in *IL-6 -174G/C* was successfully replicated in an independent population, supporting the role of this polymorphism as a genetic marker predicting anti-TNF treatment outcome. The combined analysis of our data and those previously published showed an association between this genetic variant and the clinical response to anti-TNF. IL-6 has the ability to induce an acute inflammatory reaction and, in the chronic phase, to support the activation of lymphocytes and myeloid cells, which may elevate the serum levels of IL-6, leading to increased inflammation. It may therefore be responsible for many of the systemic manifestations of RA (25). It has been shown that the neutralization of the TNF-a results in the suppression of various proinflammatory cytokines, including IL-6 (26, 27). Functional studies have reported that the *-174*C* allele is associated with higher serum levels of IL-6 (16, 17), thus suggesting that increased expression of this cytokine in patients carrying the *-174*C* allele would result in a poorer response to anti-TNF treatment. In fact, it has been shown that although both TNF-a and IL-6 are major targets of therapeutic intervention in RA, baseline serum IL-6 but not baseline TNF-a level is a potential biomarker reflecting disease activity (28). According to our data, *-174G/C* was significantly associated with a good or moderate EULAR response at 12, 18, and 24 months, but not at 6 months. Moreover, the longer the treatment period, the stronger the observed association signal was. This highlights the importance of assessing the response to long-term anti-TNF treatment. This may be the reason that an association between this polymorphism and the clinical efficacy of anti-TNF therapy has not been reported in previous pharmacogenetic studies, most of which did not evaluate the clinical response beyond 6 months of treatment (6-8, 29).

The possible interactions between two gene variants could not explain the response to anti-TNF treatment. In **chapter 5**, the high affinity *FcGR2A**H allele was associated with EULAR good response at 14 weeks in adalimumab treated of RA patients, but not with high affinity *FcGR3A*-V allele. The applied additive genetic model for *FcGR2A* and *FcGR3A* didn't show an association with EULAR good response.

SLE AND OTHER AUTOIMMUNE DISEASES

Recent studies have provided evidence that antagonizing the action of proinflammatory cytokines, including IL-6 and IL-2, may exert a therapeutic effect in autoimmune disease patients nonresponsive to other therapies. B cell depletion induced by rituximab resulted in a downregulation of proinflammatory cytokines and consequently, a decrease of the autoimmune response and re-establishment of the immunotolerance (18). The establishment of pharmacogenetic markers to predict the response to rituximab therapy becomes a pivotal requirement, given the expanding clinical use of this drug in the treatment of several autoimmune diseases. The *-174G/C* genetic variant (rs1800795), located in the *IL-6* gene promoter region, has been found associated to autoimmune diseases and involved in increased levels of IL-6 protein in serum in diverse inflammatory diseases, the GG homozygotes have circulating IL-6 concentrations approximately twice higher than those homozygous for the C allele (16). In **chapter 6** we have analyzed the association of the *-174 IL-6* promoter variation with the response to rituximab in a group of patients that presented diverse systemic autoimmune diseases. The CC genotype was borderline more frequent in non-responders as compared to those carrying GC or GG genotypes (p-value = 0.049). However, these differences were not statistically significant in SLE patients. Genotypic frequencies for CC were increased in non-responders, which correlates with the fact that patients carrying this homozygous genotype responded worse to the treatment with rituximab than those carrying GC or GG genotypes (69.2% vs. 90.2%). Fabris et al. (30) found a lower response to rituximab in RA patients that were homozygous for CC.

Their findings are in agreement with our results, both in the group of diverse systemic autoimmune diseases patients and in SLE patients analyzed separately, although, in SLE patients, the observed differences were not statistically significant, probably due to the lower statistical power of this stratified analysis. Pathogenesis of systemic autoimmune diseases involves inflammation cytokines IL-1, TNF alpha, and IL-6. Murine models in inflammatory diseases indicate that IL-6 deficiency reduces the severity of an inflammatory response (31). Recent studies have clarified evidence that antagonizing the action of

proinflammatory cytokines, including IL-6, may exert a therapeutic effect in patients nonresponsive to other therapies. Tocilizumab, a humanized antibody to the IL-6 receptor, blocks IL-6 signaling and activity and decreases levels of inflammatory markers in RA (32, 33). Previous studies reported that B cell depletion induced by rituximab resulted in a downregulation of proinflammatory cytokines, including IL-6 and, consequently, a decrease of the autoimmune response and re-establishment of the immunotolerance (18). The lower efficiency of rituximab in systemic autoimmune diseases patients carrying the CC genotype, suggests an increase in the number of refractory patients to rituximab in this group. Biological therapies different to rituximab might be had under consideration to get an adequate and more effective response in these patients. According to our data, *-174 IL-6* SNP suggests a pharmacogenetic association with the clinical response to rituximab in systemic autoimmune diseases, and the hypothesis that this variation could be a predictive value, independently of other clinical or environmental factors. Anyway, as the observed significant associations could be due to a casual finding resulting from multiple comparisons, larger replication studies are needed and still planned by our group to confirm present results. Currently, there are very few data about genetic markers of prognosis that may be used in the future to facilitate treatment decisions. We herein provide preliminary evidence of a possible new genetic marker, the CC homozygosis of the *-174 IL-6* promoter polymorphism, as a predictor of nonresponse to rituximab in autoimmune diseases.

In **chapter 4**, the *-174*G* allele was significantly associated with a good or moderate EULAR response at 12, 18 and 24 months in an independent cohort of Spanish RA patients treated with anti-TNF therapy. A meta-analysis combining these data with the results from a previous study (34) confirmed this association. In **chapter 6**, The *-174 IL-6 CC* genotype was significantly increased in non-responders with respect to responders in several autoimmune disease patients treated with rituximab. Therefore, in some way, the *-174 IL-6*G* allele could be a genetic marker of response to rituximab in different autoimmune diseases.

Rituximab is recognized and bound to the surface of NK cells and macrophages through the FcGR, triggering ADCC immune system mechanism, essential for the activity of rituximab to deplete B cells. *FcGR3A* is expressed by immune effector cells and shows specific affinity for IgG monoclonal antibodies, such as rituximab. The importance of *FcGR3A* in the response to rituximab has been shown in studies where mice lacking *FcGR3* presented a decrease in the response to this drug (35). In **chapter 7**, we have analyzed the association of the *FcGR3A*-158F/V polymorphism with the response to rituximab in patients with autoimmune diseases. Genotypic frequencies for this SNP were similar to those described previously for several patients with autoimmune diseases in Caucasian populations (36-38). It is remarkable that frequencies were elevated for V carriers in responders, which correlates

with the fact that patients carrying the V allele at this position presented a better response to the treatment with the drug than those with homozygous FF genotype. Functional studies have demonstrated that the 158V allele is correlated with a better biological response to rituximab in autoimmunity. Anolik et al. (39) showed that in patients with SLE carrying the high-affinity V allele (FV or VV), rituximab was more effective in depleting peripheral B cells than in those homozygous for the low-affinity FF. Recently, the *FcGR3A*-158F/V SNP has been associated with the clinical response to rituximab in RA. This study conducted in 111 patients found that the V allele carriage was significantly associated with a higher response rate (91% of responder vs. 70%; p = 0.006, OR = 4.6, 95% CI 1.5–13.6) (40). The findings in SLE and RA are in line with our results that showed a better response to rituximab in patients with autoimmune diseases that carried the V allele (FV or VV) than in patients with homozygous FF. Additionally, based on the previous association observed in patients with SLE and on the fact that this was the largest group, we analyzed separately patients with SLE. We found a similar pattern, and patients carrying the V allele showed a better response to rituximab treatment, although it did not reach statistical significance (p = 0.08). Finally, we examined the group of patients with no SLE to establish whether this association is shared by different autoimmune disorders. As in the case of patients with SLE, we observed a similar effect, but this association did not reach statistical significance either (p = 0.08). This suggests that the influence of the *158F/V* polymorphism in the therapeutic response to rituximab is common to various autoimmune diseases; however, the reduced numbers involved in these stratified analysis leads to poor statistical power, and therefore the conclusions are provisional.

It should be noted that copy number variation (CNV) has been shown to be present in the *FcGR3A* gene (42-44). The presence of common CNVs can cause false SNP genotyping results that can lead to fail the Hardy–Weinberg equilibrium (HWE) and may blur the association of the studied SNPs with disease susceptibility. In our study, the genotypic frequencies were significantly different from those predicted by HWE, but only in the group of patients with SLE. This may be due to existence of an association between the *158F/V* polymorphism and this disease (41). In fact, in our cohort of healthy controls (previously published genotypic data), genotype frequencies for this SNP were in the HWE (38). Moreover, the frequency of CNV has been reported to vary significantly in different ethnic populations, which can result in contradictory findings, but in this case, frequencies observed in patients were similar to those previously described, and the results reported to date are fairly consistent. Previous findings showed that patients carrying the V allele in *FcGR3A*-158F/V increased expression of CD16 in NK cells (45). A correlation between the number of cell surface CD16 receptors and the enhancing of the ADCC activity mediated by NK cells was found. These

observations would explain the better response to rituximab observed in patients with systemic autoimmune diseases carrying the V allele and would highlight the importance of the ADCC mechanism for clearance of B cells by rituximab in autoimmune diseases. In summary, our results together with previous findings (39, 40) suggest that *FcGR3A* plays an important role in response to rituximab in patients with systemic autoimmune diseases and support the hypothesis that the *158F/V* variant could be used as a potential predictor of those patients who will respond better to treatment with rituximab.

In **chapter 7**, rituximab was more effective in V allele carriers than in homozygous FF in RA patients. However, in **chapter 5**, no significant associations were found for the *FcGR3A* polymorphism and response to adalimumab in RA patients, and the combined influence of high-affinity alleles (*FcGR2A*-H and *FcGR3A*-V) showed no association between the number of high-affinity alleles and EULAR good response neither for remission. These conflicting results regarding the role of *FcGR3A* on the response to different drugs (adalimumab and rituximab) in RA patients could have a biological explanation. On the one hand, patients with high affinity allele (V) are more effective in depleting peripheral B cells and have better response to rituximab (47). On the other hand, patients with low affinity allele (F) may have decreased FcGR-mediated drug clearance of adalimumab and then a better response to this drug. This could mean a first step toward personalized medicine in RA and to choose the drug by the *FcGR3A* genotype.

In healthy subjects, stratification according to the *IL2–IL21* region polymorphism (rs6822844) revealed significant differences in circulating interleukin-2 with the lowest levels in GG genotype (19). In **chapter 8**, SLE patients homozygous for rs6822844 G allele show a better clinical response to rituximab at month 6 than patients with GT genotype. On the contrary, no association was evident in the group of non-SLE patients. It could be speculated that this lack of association was a consequence of a lower statistical power in the latter analysis. However, it should be noted that no effect size was suggested in this case (i.e. OR = 1) and, in addition, a reduction of the statistical significance of the association was observed when the non-SLE patients were meta-analyzed with those showing SLE (which increases the statistical power). Taken together, our data suggest that the influence of the *IL2/IL21* rs6822844 polymorphism in the therapeutic response to rituximab is specific of the SLE condition. Although the mechanism of action of rituximab remains unclear, accumulating data suggest that ADCC may play a dominant role (48). ADCC is mediated through immune effector cells, mainly NK cells, via expression of an activating receptor for the Fc portion of IgG antibodies (FcGR). The majority of human NK cells are CD16 positive (FCcRIII) and express the intermediate affinity interleukin-2 receptor. It has been described that intermediate doses of IL2 are capable of expanding CD16 positive NK

cells and activating cytotoxic effector functions, including ADCC activity (49-53). Several studies have demonstrated that this ability of IL2 to promote NK cell expansion and cytotoxicity influences the efficiency of rituximab treatment and correlates with the clinical response (54-59). Furthermore, the relationship between IL2 and the efficacy of rituximab is supported by the fact that soluble interleukin-2 receptor is used as a prognostic factor in patients with lymphoma receiving rituximab (60-64). An alteration of the function of B cells is a key factor contributing to SLE pathophysiology; however, some clinical trials with rituximab in this disease have failed to show efficacy. Murine models of SLE based on antibody mediated cellular depletion evidenced that this lack of efficacy can be explained by a defect in macrophage and neutrophil IgG-dependent phagocytosis induced by serum IgG (65). In this context, the role of IL2 promoting rituximab-mediated ADCC could become more critical in the efficacy of rituximab in lupus than in other autoimmune diseases in which this drug acts through all its mechanisms. The *FCGR3A-158* polymorphism is currently shown to enhance rituximab mediated ADCC and improve clinical response to this drug (45). Similarly, rs6822844 variant could affect the cytotoxic activity of NK cells and, therefore, the efficacy of rituximab in SLE condition; although to date no functional studies analyzing this issue have been published. In conclusion, we show for a first time that *IL2–IL21* rs6822844 G/T polymorphism influences the clinical efficacy of rituximab in SLE patients. The replication of this association in independent studies could enable the potential use of this variant as a pharmacogenetic marker.

In **chapter 8** the results indicate that SLE patients homozygous for rs6822844 G allele at the *IL2–IL21* region show a better clinical response to rituximab at month 6 than patients with GT genotype. On the contrary, no association was evident in the group of non-SLE patients. Interestingly, these findings show conflicting conclusions with the results obtained in **chapter 6** where the allele associated previously with lower level of IL6 were associated with worse response to rituximab.

FUTURE PERSPECTIVES

It is well known that personalized medicine is a tool that allows predicting the response or toxicity to drugs before the administration. This approach is very well accepted in some clinical areas, such as oncology, psychiatry, and is also starting in cardiology. Probably this is due to the high level of evidence of the association between genetic polymorphisms and the clinical outcome which led to the development of PGx guidelines in these areas. However, in other areas such as autoimmune diseases, among which we highlight AR and LES, at present it has not been possible to find validated genetic markers that predict

the response to drugs and thus can be used in daily clinical practice. In this case, it has been difficult to transfer knowledge of the effect of genetic polymorphisms into specific recommendations because the low evidence of the association or even disagreement between different studies.

Regulators are often confronted with challenges involved in translating data from pharmacogenomic studies into clinically relevant and meaningful product information, starting with the level of scientific evidence required to justify the inclusion of PGx data in the product information (66). For developing new drugs there is a guideline published by European Medicines Agency (EMA) which provides a framework on where it is recommended that pharmacogenetics should be implemented in the drug development process (66, 67). For authorized drugs, such as MTX, anti-TNF drugs and RTX used in RA and SLE, the guideline for the use of pharmacogenomic methodologies in the pharmacovigilance evaluation of medicinal products should be followed by researchers in order to find biomarkers associated with the response or toxicity of the drugs (68). With both guidelines, EMA intends further to enable the potential of PGx during drug development and surveillance and to gain insight into the associated scientific challenges and discusses potential solutions. The guidelines are expected to improve genomic data-informed drug development and clinical experience, thereby promoting understanding of interindividual drug response variations and, consequently, provide guidance towards more personalized treatments in the interest of the patient and public.

This thesis reflects the need to do more studies to find genetic markers that are associated with the response to drugs used in RA and SLE. The steps to follow would be the following.

Some limitations of these research studies in autoimmune diseases are that most of them use measures, including DAS-28, American College of Rheumatology, or EULAR response criteria, which include subjective measures of disease and are known to have a placebo effect (69) and they have not taken into account one of the reason that explain that patients with RA which continue with the active disease, or relapses, even during current biological therapy is the immunogenicity associated to these drugs (70).

There are different exploratory approaches providing different levels of evidence. On one side of the spectrum non-randomized (cohort, case-control or single arm) studies are performed and on the other side of the spectrum randomised controlled studies (RCTs -prospective or retrospective evaluation) are executed. The search for genetic biomarkers can be done without a hypothesis using GWAS approaches. Typically, GWAS is a search strategy rather than specific developmental design. GWAS have revolutionized genetic research as they allow the discovery of multiple gene variants with individually small effects.

The advantage of GWAS is that they eliminate the need to choose, a priori, candidate genes or variants. GWAS are highly suitable to identify genetic variants contributing to complex phenotypes such as drug response or drug- induced toxicity. The GWAS approach enables novel and less obvious genetic markers to be identified, particularly for genetic variation affecting drug pharmacodynamics, which is more complex and often less well understood than pharmacokinetics. While very interesting and affordable, GWAS also suffer from limitations. In this thesis we have shown that biomarkers found using non-RCT, including cohort studies and GWAS could not be replicated and validated in other independent studies, probably because the different definition of outcomes, the low sample size, and lack sufficient rigor to establish the predictive value of the biomarkers and to quantify its sensitivity and specificity.

Novel studies that overcome these limitations are necessary to find biomarkers which predict the response or toxicity to drugs used in RA and LES. And, after that, probably it´s necessary to conduct a clinical trial where preliminary information regarding the value of a predictive biomarker is based on published literature or from early studies within a development programme.

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Appendix

List of publications
Curriculum vitae
Acknowledgements

LIST OF PUBLICATIONS

Dávila-Fajardo CL*, Swen JJ*, Cabeza Barrera J, Guchelaar HJ. Genetic risk factors for drug-induced liver injury in rheumatoid arthritis patients using low-dose methotrexate. Pharmacogenomics 2013;14(1):63-73.

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CURRICULUM VITAE

Cristina Lucía Dávila Fajardo was born in Granada, Spain on August 7th 1977. After finishing her secondary school at Regina Mundi College in Granada in 1995, she started her Pharmacy study at Granada University. In 2000 she obtained her pharmacy Degree and then she moved to Madrid for doing her thesis in Centro de Investigaciones Biológicas (CIB-CSIC) after getting a national grant from Spanish Ministry of Science and Technology. The defense of her thesis was completed in 9th February 2006. This Thesis was related to the molecular basis of antibiotic resistance plasmid and structure of proteins. In May 2016 she went to Imperial College of London for doing a short internship related to resolution protein structure by X-ray crystallography. In January 2007 she did the national exam for specialized medical training and in May 2007 she started her residency at the Clinical Pharmacy department from San Cecilio University hospital, Granada and it was completed in May 2011. During the 3rd and 4th years, she did an internship at the Clinical Pharmacy and Toxicology Department from Leiden University Medical Center, The Netherlands and she started working on the studies presented in this PhD thesis. After her residency, she received a grant from the Institute of Health Carlos III, the main Public Research Entity funding, dedicated to managing and carrying out biomedical research in Spain, and she could continue with the project of this thesis. Actually, Cristina lives in Granada and works as a clinical pharmacist and researcher in the field of pharmacogenetics at San Cecilio University Hospital at Granada.

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In 2007 I started my residence in the Pharmacy department at San Cecilio University Hospital. I always thought that something could be done for improving health outcomes through research. Then Pharmacogenetics field was presented as a very relevant proposal for a clinical pharmacist as it promised to bring the patient closer to personalized medicine. In that search I found Prof. Henk-Jan Guchelaar who had done numerous studies in this area with very good results. I decided to write him to request a stay in his Pharmacy and Toxicology department at the LUMC and what was my surprise when I received a kind response accepting me as a rotating resident. For me, Prof. Henk-Jan Guchelaar has been a very important person in my professional career. Not only because of the knowledge that I have learned over the years thanks to him, but also because of the great person who is: very enthusiastic, concerned about his teammates and also knows how to achieve the expected results due to the great communication capacity he has. Thanks to him, and to all the companions of the LUMC Pharmacy service, this thesis, which in principle was not intended, has finally been able to defended. Among those colleagues I would like to highlight Jesse for all the help he has always given me with articles, designs, revisions, results... and René and Tahar for helping me in the laboratory with samples and devices. In recent years I have also been fortunate to be able to work with Cathelijne that has shown me the hardworking person and good companion she is. I would like to thank Wendy for all the help with the administrative part of the thesis. I would also like to thank Renate for all the help provided for the editing of the thesis that for me has been essential to defend it. And in general, thanks to all the people that I have been able to meet in the LUMC Pharmacy department.

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