Universidade de Lisboa

Faculdade de Medicina de Lisboa

THE CLINICAL RELEVANCE OF DRUG IMMUNOGENICITY

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Doutoramento Medicina

Reumatologia

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À memória da minha mãe Rita e do meu mano Vitor

ABSTRACT

The introduction of biologic therapies into clinical practice has greatly improved the treatment of chronic disabling inflammatory diseases, such as Rheumatoid Arthritis, Spondylarthritis or Inflammatory Bowel Diseases, among others. However, a sizeable fraction of patients never achieve therapeutic response or, more often, cannot maintain therapeutic response over time. Among the pitfalls of biologicals is their potential immunogenicity and the associated anti-drug antibodies (ADAb) produced by the patients, which promote faster clearance/neutralization of the drug in circulation and thus interfere with drug efficacy. Moreover, ADAb have also been associated with adverse events.

In this work we aimed at formally document the impact of ADAb in clinical outcomes, and use this analysis to construct and test an algorithm for therapeutic decisions based on explicit biomarkers of immunogenicity.

To evaluate the clinical relevance of biological drug's immunogenicity, we first performed a systematic review of the literature coupled with a meta-analysis. We evidenced that in the presence of detectable ADAb therapeutic response may be reduced by as much as 80%. Such impact is attenuated, although not abrogated, by concomitant immunosuppression, particularly with methotrexate that associates with reduced ADAb production. Differences in the immunogenic profile of specific biologics were also verified, with monoclonal antibodies exhibiting higher immunogenicity than fusion proteins. We next assessed the impact of immunogenicity on drug's safety profile by following a cohort of patients receiving intravenous infliximab, a TNF-inhibitor. Infusion-related adverse events occurred exclusively in ADAb-positive patients and nearly half of the ADAb-positive patients

developed an acute reaction during or immediately after the infusion, requiring medical intervention.

To evaluate the relevance of drug immunogenicity assessment for therapeutic decisions, we first defined a convenient method to assess immunogenicity on a routine basis. We verified that a newly developed Bridging ELISA performed as well as antigen-binding radio-immuno assay, currently considered by many as the "gold-standard" to assess ADAb. Next, we designed an algorithm for the management of patients receiving biologic therapies, which combines the usual clinical evaluation with immunogenicity assessment at every three months. This algorithm was tested in a cohort of RA patients treated with one of the three most commonly used biologics. We evidenced that patients who followed therapeutic strategies concordant with the proposed algorithm had close to 10-times higher probability of achieving low disease activity, when compared to those who followed other strategies commonly adopted in current clinical practice.

Our work demonstrates that a personalized, evidence-based approach for the management of patients receiving biologic therapies will lead to safer and most cost-effective strategies. These findings have important clinical, societal and economic consequences.

RESUMO

As terapêuticas biológicas revolucionaram o prognóstico de doenças inflamatórias crónicas e incapacitantes como a Artrite Reumatóide, as Espondilartrites, as Doenças Inflamatórias do Intestino, entre outras. Ainda assim, um número significativo de doentes não responde a esses fármacos ou, mais frequentemente, perde a resposta inicial ao longo do tempo de tratamento. Uma das principais limitações destas terapêuticas é o seu potencial imunogénico e a consequente formação de anticorpos anti-fármaco (AAF) por parte dos doentes a quem são administrados. Os AAF promovem uma rápida eliminação/neutralização do fármaco em circulação e podem também interferir com o seu perfil de segurança, associando-se a um maior risco de efeitos adversos.

Este trabalho pretende documentar formalmente o impacto clínico dos AAF, utilizando essa informação na construção e validação de um novo algoritmo de apoio à decisão terapêutica baseado em biomarcadores de imunogenicidade.

Para avaliar a relevância clínica da imunogenicidade foi inicialmente realizada uma revisão sistemática da literatura e uma meta-análise. Evidenciámos que na presença de AAF detetáveis há uma redução de até 80% da resposta à terapêutica. Este impacto é atenuado, mas não eliminado, pelo tratamento concomitante com imunossupressores, particularmente com metotrexato, que se associa a uma redução da produção de AAF. Foram também verificadas diferenças importantes no perfil imunogénico entre os fármacos, com os anticorpos monoclonais a exibirem maior imunogenicidade que as proteinas de fusão. Seguidamente foi avaliado o impacto da imunogenicidade no perfil de segurança destes fármacos, avaliando no tempo uma cohort de doentes tratados com infliximab, um inibidor do TNF-alfa. Reações adversas agudas associadas à infusão do fármaco ocorreram exclusivamente em doentes com AAF detetáveis, sendo que cerca de metade dos doentes

com AAF tiveram uma reação aguda durante ou imediatamente após a infusão do biológico, requerendo intervenção médica imediata.

No sentido de avaliar a relevância da avaliação da imunogenicidade para a melhoria das decisões terapêuticas, começámos por definir o método laboratorial mais conveniente para monitorização da imunogenicidade na prática clínica de rotina. Constatámos uma boa concordância entre um novo método de ELISA desenvolvido ("Bridging ELISA") e o método de radioimmunoensaio ("RIA-ABT"), considerado por muitos como *"gold standard"* na deteção dos indivíduos AAF-positivos. Seguidamente, desenhámos um algoritmo de apoio à decisão terapêutica para doentes medicados com terapêuticas biológicas, que combina a atual avaliação clínica com a monitorização da imunogenicidade a cada três meses. Este algoritmo foi testado numa cohort de doentes com Artrite Reumatóide inicialmente medicados com um dos três agentes biológicos mais utilizados no tratamento desta doença. Evidenciámos que os doentes que tinham seguido estratégias terapêuticas concordantes com o algoritmo proposto tiveram cerca de 10 vezes maior probabilidade de alcançarem uma baixa atividade de doença, quando comparados com os doentes que seguiram outras estratégias, frequentemente adotadas na atual prática clínica.

O nosso trabalho demonstra que uma abordagem personalizada e cientificamente orientada aos doentes medicados com terapêuticas biológicas permite desenhar estratégias mais seguras e custo-efetivas. Os nossos resultados têm não só uma elevada relevância clínica, como também económica e social.

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TABLE OF CONTENTS

Abstract .... **vii**

Resumo .... **ix**

Agradecimentos .... **xi**

Table of Contents .... **xv**

List of Figures .... **xix**

List of Tables .... **xxi**

Acronyms .... **xxiii**

1 General Introduction **1**

1.1 Biologic Therapies **3**

1.2 Imunoglobulins Structure and Function **3**

1.3 Antibody Production: from hybridoma technique to advanced biothechnology **6**

1.4 Drug Immunogenicity **9**

1.4.1 Drug-related Factors Influencing Immunogenicity 11

1.4.2 Patient-related Factors 13

1.4.3 Immunogenicity Assessment 16

1.5 Rheumatoid Arthritis **23**

1.6 Tumor Necrosis Factor Alpha **26**

1.7 TNF Inhibitors in RA **28**

1.8 Non-TNFi Biologic Therapies in RA **31**

1.9 The Current Management of RA And The Treat-To-Target Strategy **33**

2 Objectives/Scope of the Thesis **39**

3 Results **41**

3.1 The Immunogenicity of TNFi Therapies in Immune-mediated Inflammatory Diseases – a systematic review of the literature with a meta-analysis **41**

3.1.1 Introduction 41

3.1.2 Methods ... 42
3.1.3 Results ... 43
3.1.4 Discussion ... 46
3.2 The impact of Immunogenicity on Drug Safety Profile ... 74
3.2.1 Introduction ... 74
3.2.2 Objectives ... 75
3.2.3 Methods ... 75
3.2.4 Results ... 76
3.2.5 Discussion ... 78
3.3 Immunogenicity Assessment in Routine Clinical Practice ... 84
3.3.1 Introduction ... 84
3.3.2 Objectives: ... 85
3.3.3 Methods: ... 85
3.3.4 Results: ... 89
3.3.5 Discussion ... 90
3.4 A Preliminary Algorithm Introducing Immunogenicity Assessment in the Management of RA Patients Receiving Tumor Necrosis Factor Inhibitors Therapies ... 97
3.4.1 Introduction ... 97
3.4.2 Objectives ... 98
3.4.3 Methods ... 99
3.4.4 Results ... 102
3.4.5 Discussion ... 105

4 General Discussion ... 115
4.1 The Impact of ADAb on Therapeutic Responses ... 116
4.2 The Influence of Concomitant Immunosuppression and Other Possible Modulator Factors on ADAb Detection ... 117
4.3 Etanercept (fusion protein): Biologic Free of Significant Immunogenicity ... 119
4.4 The Impact of ADAb on Drug's Safety Profile ... 121
4.5 Suitable Assays to Monitor Immunogenicity in Routine Clinical Practice ... 123
4.6 Integrating Immunogenicity Information in the Management of Patients Receiving Biologic Therapies ... 125
4.7 Drug Mechanism of Action and ADAb Production ... 132

4.8 Biologic Dose Reduction Programs Based on Serum Drug Levels 135

5 Conclusions 139

5.1 Relevance (and perspective) of This Work for Clinical Practice 139

5.2 Relevance for Economic and Societal Values 140

6 References 143

Appendix 209

LIST OF FIGURES

Figure 1 – Immunoglobulins Structure ... 4

Figure 2 – Complementary-Determining Regions (CDR1, CDR2 and CDR3) ... 5

Figure 3 – Bridging ELISA ... 21

Figure 4 – Radioimmunoassay (RIA) – Antigen Binding Test (ABT) ... 21

Figure 5 – Flow of studies through the systematic review (SR) process ... 56

Figure 6 – Effect of ADAb positivity on TNFi response (excluding the study “Bartelds 2011”) ... 57

Figure 7 – Effect of ADAb positivity on TNF responsei (excluding the study “Bartelds 2007”) ... 58

Figure 8 – Effect of ADAb positivity on TNFi response (including “Pascual-Salcedo 2011” and “Plasencia 2012” at 6 months of follow-up time) ... 59

Figure 9 – Effect of ADAb positivity on TNFi response (including “Pascual-Salcedo 2011” and “Plasencia 2012” at 12 months of follow-up time) ... 60

Figure 10 – Effect of ADA positivity on TNFi response (including “Pascual-Salcedo 2011” and “Plasencia 2012” at > 48 M follow-up time) ... 61

Figure 11 – Effect of ADAb positivity on TNFi response ... 62

Figure 12 – Effect of ADAb positivity on TNFi response (IS<67% and IS≥67%) ... 63

Figure 13 – Effect of ADAb positivity on TNFi response (MTX<74% and MTX≥74%) ... 64

Figure 14 – Effect of ADAb positivity on TNFi response (by diagnosis) ... 65

Figure 15 – Effect of ADAb positivity on TNFi response (by diagnosis) ... 66

Figure 16 – Effect of ADAb positivity on TNFi response according to initial biologic dose ... 67

Figure 17 – Egger’s publication bias plot for the effect of ADAb positivity on TNFi response ... 68

Figure 18 – Effect of immunosuppression on ADAb production (by excluding “Bartelds 2010” and “Bartelds 2011”) ... 68

Figure 19 – Effect of immunosuppression on ADAb production (by excluding “Bartelds 2007” and “Bartelds 2011”) ... 69

Figure 20 – Effect of immunosuppression on ADAb production (by excluding “Bartelds 2007” and “Bartelds 2010”) 70

Figure 21 – Effect of immunosuppression on ADAb production 71

Figure 22 – Effect of immunosuppression on ADAb production (by assay) 72

Figure 23 – Egger’s publication bias plot for the effect of IS on ADAb production 73

Figure 24 – Patients with infusion-related adverse events (IrAE) 82

Figure 25 – Patients with detectable ADAb 82

Figure 26 – Proportion of patients with IrAE among ADAb-positives and ADAb-negatives 83

Figure 27 – Serum drug levels at every week between two infliximab infusions in 4 IBD patients who had IrAE 83

Figure 28 – ELISA to assess serum drug levels 95

Figure 29 – Assays used to measure ADAb 95

Figure 30 – Proportion of ADAb-positive patients detected in each assay. 96

Figure 31 – ADAb titres assessed by IgG-RIA-ABT and specific IgG4-RIA-ABT 96

Figure 32 – Preliminary Algorithm to Approach RA Patients receiving TNF Inhibitor Therapies, introducing immunogenicity assessment 113

Figure 33 – C-Reactive Protein (CRP) concentration among ADAb-negative and ADAb-positive patients, at study beginning 114

LIST OF TABLES

Table 1 – Eligibility criteria for studies included in the systematic review 50

Table 2– Study and baseline patient characteristics // 1. Quantitative Evidence 51

Table 3 – Meta-Regression stratified by clinical characteristics to address the effect of ADAb on drug response and the effect of IS on ADAb detection 55

Table 4 – Patient's Baseline Characteristics.. 110

Table 5 – Response and Low Disease Activity in patients from Group A and Group B, over one year after therapeutic decision 111

Table 6 – Therapeutic Response and Low Disease Activity among ADAb-negative and ADAb-positive patients, at study beginning 111

Table 7 – Patient's Baseline Characteristics among ADAb+ e ADAb- 112

ACRONYMS

Abs – Antibodies

ACPA – Anticitrullinated Protein Antibodies

ACR – American College of Rheumatology

ADAb – Anti-drug Antibodies

ADCC – Cell-mediated Cytotosicity

ADCP – Antibody-dependent Cellular Phagocytosis

AE – Adverse Events

AE – Ankylosing Spondylitis

AIRE – Autoimmune Regulator Protein

APC – Antigen Presenting Cell

ASAS – Assessment of Spondyloarthritis International Society

ASDAS - Ankylosing Spondylitis Disease Activity Score

AZA – Azathioprine

BCR – B Cell Receptor

C – Constant

CD – Crohn's Disease

cDNA – Complementary Deoxyribonucleic Acid

CDR – Complementary-Determining Regions

CI – Confidence Intervale

CNS – Central Nervous System

CRP – C-Reactive Protein

CTLA-4 – Cytotoxic T-Lymphocyte-associated Antigen 4

DAS – Disease Activity Score

DC – Dendritic Cells

DMARDs – Disease-modifying Antirheumatic Drugs

DNA – Deoxyribonucleic Acid

EAE – Experimental autoimmune Encephalomyelitis

ELISA – Enzyme-linked Immunosorbent Assay

EMA – European Medicines Agency

ESR – Erythrocyte Sedimentation Rate

EULAR – European League Against Rheumatism

FADD – Fas-Associated Death Domain

FDA – Food & Drug Administration

GEE – Gneralized Estimation Equation

GM-CSF – Granulocyte-Macrophage Colony Stimulating Factor

HCQ – Hydroxycloroquine

HLA – Human Leucocyte Antigen

IBD – Inflammatory Bowel Diseases

IFN – Interferon

Igs – Immunoglobulins

IL – Interleukin

IQR – Interquartile Range

IrAE – Infusion-Related Adverse Events

IS – Immunosuppression

IVIG – Intravenous Immunoglobulin

LFN – Leflunomide

LPS – Lypopolysaccharide

LTa – Lymphotoxin Alpha

mAb – Monoclonal Antibody

MCP – 6-Mercaptopurine

MHC – Major Histocompatibility Complex

MS – Multiple Sclerosis

mTEC – Medullary Thymic Epithelial Cells

MTX – Metothrexate

Nab – Neutralizing Antibody

NF-kB – Nuclear Factor kB

NK – Natural Killer

NSG – NOD scid gamma

OMERACT – Outcomes Measures in Rheumatology Initiative

OR – Odds Ratio

PAD – Peptidyl Arginine Deaminase

PCR – Polymerase Chain Reaction

PD – Pharmacodynamics

PK – Pharmacokinetics

Ps – Psoriasis

PsA – Psoriatic Arthritis

RA – Rheumatoid Arthritis

RCTs – Randomized Clinical Trials

REM – Random Effect Models

RF – Rheumatoid Factor

RIA – Radioimmunoassay

RR – Risk Ratio

SD – Standard Deviation

SDAI – Simplified Disease Activity Index

SLZ – Salazopirine

SpA – Spondylarthritis

TACE – TNF Alpha Converting Enzyme

TCR – T Cell Receptor

TEC – Thymic Epithelial Cells

TLR – Toll-like Receptor

TNFa – Tumor Necrosis Factor alpha

TNFi – Tumor Necrosis Factor inhibitors

TRAF – TNF Receptor-Associated Factor

Tregs – T Regulatory Cells

TSA – Tissue-Specific Antigens

UC – Ulcerative Colitis

V – Variable

1 GENERAL INTRODUCTION

Chronic inflammatory immune-mediated diseases are a heterogeneous group of poorly understood disorders, which aetiology remains largely unknown. They have been conceived as a result of a combination of genetic variants, acquired environmental triggers and stochastic events.

Rheumatoid Arthritis (RA), Spondylarthritis (SpA), Psoriasis (Ps) and Inflammatory Bowel Diseases (IBD) are among the most prevalent chronic inflammatory immune-mediated diseases that affect predominantly young people at productive age of life, inducing significant morbidity and mortality. Therefore, their social and economic impact on society is extremely high.

Despite the poorly understood aetiology of those diseases, their physiopathology ends in a chronic inflammatory response against self-tissues with the release of inflammatory mediators, production of autoantibodies and activation of leukocytes that will perpetuate immune response, leading to an extensive tissue damage and malfunction of the corresponding target organs.

Cytokines are potent-rate limiting extracellular molecules that specifically regulate the inflammatory response, the tissue damage and the repair mechanisms. An increasing body of evidence has revealed the critical role that cytokines play in the initiation and perpetuation of autoimmunity [1, 2]. The important role of cytokines in immune-mediated inflammatory mechanisms had lead to the idea that cytokine-based manipulation could offer a possibility to interfere with autoimmune process.

Such idea has revolutionized the treatment approach of the above-mentioned diseases, which the most well known example has been RA.

RA is a chronic disabling disease that affects primarily the joints, inducing an irreversible joint damage with significant loss of functionality. In the beginning of 90's a paradigm shift occurred in the treatment approach of RA. In addition to small chemical molecules, such as methotrexate (MTX) or corticosteroids, large therapeutic proteins, also known as biologics, were introduced in the arsenal of therapeutic options for RA treatment. Biologics represent a distinct therapeutic class, which are produced through living organisms using

biotechnology and genetic engineering instead of being simply chemically synthesized. There are today a wide variety of biotechnologic-derived therapeutic proteins. In the context of chronic inflammatory immune-mediated diseases, biologics are mainly represented by monoclonal antibodies, and to a less extend by fusion proteins, which target several cytokines or cells that play critical role on immune-mediated inflammation. The first biologics used in the treatment of RA targeted the Tumor Necrosis Factor (TNF). Given the successful results other targets emerged, such as CD-20, co-stimulatory molecules B7.1/B7.2 and interleukin (IL)-6. These therapies provide better control of inflammation, increasing patient's quality of life and, most importantly, their functionality.

The clinical benefit that biologics have brought to RA patients has been extensively demonstrated in randomized clinical trials (RCTs). However, biologics have now been used in clinical practice for more than a decade and a general pattern seemed to emerge, best analysed for Tumor Necrosis Factor inhibitors (TNFi) in RA: there is evidence that i) about one third of the patients would not respond to the biological therapy (primary non-responders); ii) one third would show clinical response, but the beneficial effect would fade away within the first 6-12 months of continuous therapy (secondary non-responders); and iii) the remaining third would maintain a clinical response beyond a year.

Over the last years increasing evidence has revealed the production of anti-drug antibodies (ADAb), naturally produced by the patients upon administration of biologics, as one of the main factors interfering with drug efficacy and safety profile. Although there were good indications that immunogenicity of TNFi is one of the main mechanisms behind treatment failure, this notion did not permeate the clinical practice.

The lack of suitable assays to assess immunogenicity in clinical practice has also prevented the expansion of the field. Immunogenicity assessment is technically challenging and only recently optimized assays, specifically tailored to detect ADAb, have emerged though little experience still exists.

Despite the great improvement in overall clinical responses afforded by biologicals, therapeutic failures to these drugs are frequent. Therapeutic decisions in these cases, such as whether to increase the dose or to switch to another biological of similar or different mechanism of action, would benefit to be guided by reliable biomarkers. Moreover, in responding patients, the same lack of guiding biomarkers prevents an educated and desirable

dose reduction program, as these therapies are supposed to be maintained for life. Monitoring drug levels and potential immunogenicity should help optimize the use of biological therapies. However, the way such information might be integrated in clinical practice towards more cost-effective strategies remains to be defined.

The high costs of biologic therapies represent a big concern for societies. Monitoring drug levels and ADAb might represent a very promising tool for an optimized and personalized use of biological therapies, as it allows revision of the costs engaged in these therapies while keeping as a priority the welfare of patients.

1.1 BIOLOGIC THERAPIES

The critical role of cytokines in all stages of the immune-mediated inflammatory process, lead to the idea that the manipulations of cytokine network could modulate immune responses and autoimmune diseases [1, 2]. The easiest and more efficient way to block cytokines is through monoclonal antibodies (mAbs), although other type of molecules such as decoy receptors can also bind cytokines with specificity and high affinity. The therapeutic potential of mAbs has revolutionized the pharmaceutical industry over the last years, which has lead to important refinements in the antibody manufacturing techniques.

Biotechnology-derived therapeutic proteins represent a group of medicines that are produced through live organisms and not simply chemically synthetized. Hence, they are often mentioned as biologics. There are a wide variety of biotechnology-derived therapeutic proteins available today for the treatment of several diseases. Beyond mAb and soluble receptors, enzymes, clotting factors, hormones, or cellular growth factors also exist, namely for the treatment of some genetic diseases where there is an absence or pathologic modification of the endogenous protein. Nonetheless, mAbs represent the great bulk of biologics today produced with therapeutic purposes.

1.2 IMUNOGLOBULINS STRUCTURE AND FUNCTION

Antibodies (Abs) are a family of structurally related glycoproteins, also known as Immunoglobulins (Igs). Antibodies represent the secreted form of Igs that can also be produced in a membrane form (B cell receptor, BCR). All Abs have a common symmetric core structure of two identical covalently linked heavy chains and two identical light chains, each linked to one of the heavy chains – Figure 1.

Abs are classified into different isotypes and subtypes on the basis of amino-acid differences in the heavy chain constant (C) regions, which consist of three or four C domains [3]. The antibody classes or isotypes are called IgM, IgD, IgE, IgA and IgG. IgG isotype consist of four subtypes, numbered according to their frequency in peripheral blood: IgG1, IgG2, IgG3 and IgG4. Each Ig molecule contains 2 light chains of the same isotype, kappa (κ) or lambda (λ), which differ in their single C domain. Even within the same isotype, slight differences in the amino-acid sequences of the constant heavy or light chain also exist among different individuals, designated as allotypes [4].

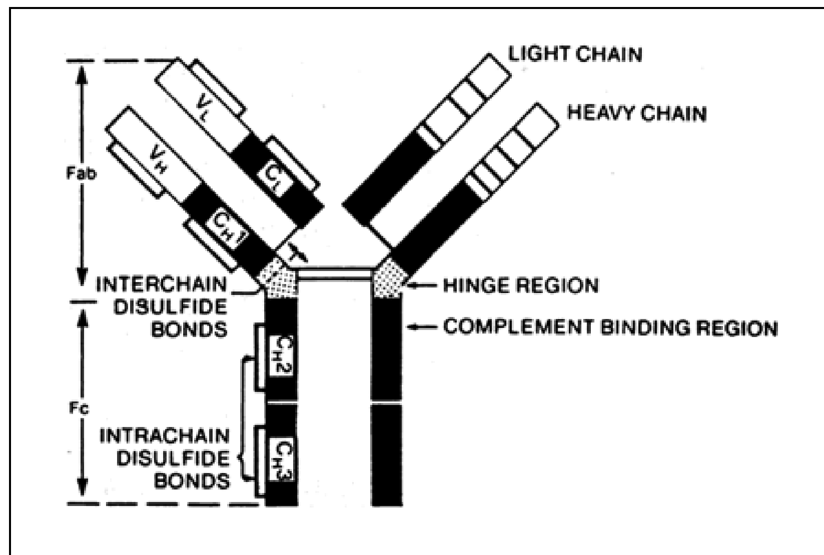

Figure 1 – Immunoglobulins Structure

Allotypes expressed on the constant region of IgG heavy chain are referred as Gm (genetic markers) together with the isotype. With the exception of IgG4, different allotypes have been described for IgG1 (G1m), IgG2 (G2m) and IgG3 (G3m). Allotypes expressed on the constant region of κ light chain are referred as Km. No allotypes have been described for λ light chains. Specific Gm haplotypes exist in different populations. In a Caucasian population the G1m1,17 allotype is much less frequent than G1m3 [5]. Even within the same population group, inter-individual variations may also occur [4].

The N-terminal domains of heavy and light chains form the variable (V) regions of Ab molecules, which differ among antibodies of different specificities. The V regions of heavy and light chains each contain three separate hypervariable regions of about 10 a.a that are spatially assembled to form the antigen-combining site of the antibody molecule, known as complementary-determining regions (CDR) – Figure 2.

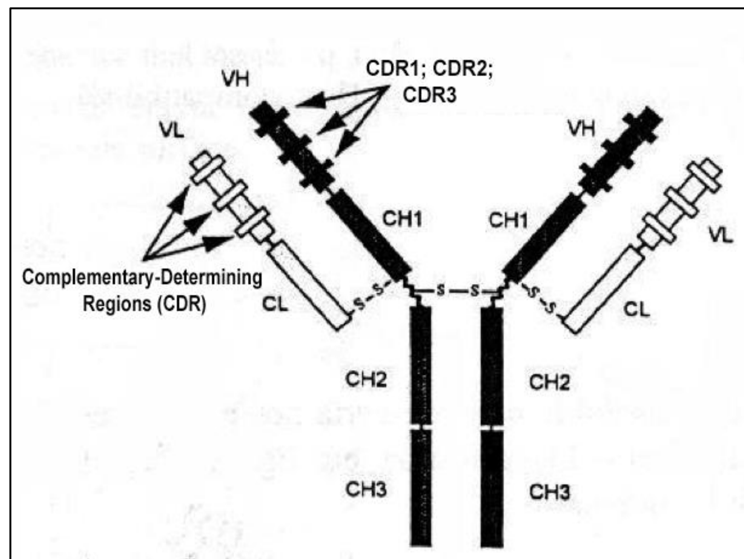

Figure 2 – Complementary-Determining Regions (CDR1, CDR2 and CDR3)

Antibody binding to antigen can be highly specific, but cross-reactions may also occur and the same antibody may bind different antigens. Antibodies can bind to two, or in the case of IgM, up to ten identical epitopes simultaneously, leading to enhanced avidity of the antibody-antigen interaction. The relative concentrations of antigens and antibodies may favour the formation of immune complexes.

The antibodies' role does not extinguish in the antigen recognition. Antibodies also have effector functions, which are mainly mediated by the C regions of the heavy chains [6-8]. IgG1, IgG3 and to a lesser extend IgG2 are able to activate the complement system, by binding to the C1 complex via their Fc terminals [9]. Activation of the complement system may lead to target cell lysis, phagocytosis of pathogens, release of anaphylatoxins, among other pro-inflammatory effects [10]. Antibodies may also interact with Fc receptors, displayed at surface of immune cells such as macrophages, monocytes, dendritic cells or platelets, leading to their activation. This interaction may result in antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cell-mediated cytotoxicity (ADCC) and cytokine release [11]. The interaction of Fc regions with neonatal Fc receptor protects IgG from degradation, increasing their half-live and mediates IgG transfer from mother-to-fetus [12]. In contrast, IgG4 has limited effector functions due to its low affinity for complement and Fc receptors [13, 14]. IgG4 represents a small fraction of total IgG content and it has been described in cases of prolonged or repeated antigenic stimulation [15]. It has been thought that IgG4 production may have regulatory or protective effect against chronic inflammatory reactions [16, 17]. Human IgG4 has the particular ability to exchange half of

the molecule with another IgG4 molecule of different specificity, resulting in a bispecific immunoglobulin molecule unable to cross-link identical antigens [17-21]. This dynamic Fab arm exchange introduces unpredictability on the binding ability of IgG4, which may be unwanted in case of human immunotherapy.

1.3 ANTIBODY PRODUCTION: FROM HYBRIDOMA TECHNIQUE TO ADVANCED BIOTHECHNOLOGY

The production of antibodies at a large scale, in a reliable manner, started in 1975, with the development of mouse hybridoma technology by Cesar Milstein and Georges Köhler [22]. The contribution of such invention for science and medicine conceived to its inventors the Nobel Prize of 1984 for Medicine and Physiology, shared with Niels Jerne, who also made critical contributions to immunology.

The hybridoma technique consists in the fusion of a mouse myeloma and mouse spleen cells from an immunized donor, able to secrete antibodies of a single specificity against a predetermined antigen. Such hybrid cells can be grown *in vitro* in massive cultures to provide specific antibodies. Mouse hybridomas were developed for a number of research and therapeutic applications. However, clinical studies with murine mAbs had been disappointed due to their weak effector functions, short half-life and ability to evoke immune reactions in the human organism [23-25]. An intensive work was subsequently conducted in order to refine the production and the clinical use of therapeutic antibodies. To that, much had certainly contributed the easy access to hybridoma technology, which was never patented by its inventors, deliberatively.

In 1986, the US Food and Drug Administration (FDA) approved OKT3 (muromonab), the first therapeutic monoclonal antibody for human use. OKT3 is a murine monoclonal antibody of the IgG2a isotype, which targets CD3, a molecule that is part of a multimolecular complex found in association of T-cell receptor (TCR) for antigen. OKT3 acts by blocking T-cell function and it was shown to effectively treat acute allograft rejection [26, 27]. Subsequently, many other monoclonal antibodies emerged, providing great advantages for the treatment of several disabling diseases. However, the use of mouse monoclonal antibodies in humans was also accompanied by important immune reactions, limiting its clinical use [28-30]. This fact has propelled the pharmaceutical companies to refine the production techniques of biologics, creating products which are increasingly identical to

human constituents. Even though, immune reactions to biologics have been difficult to overcome [31, 32].

One of the major challenges in applying biopharmaceuticals for medical purposes has been to ensure that they circumvent recognition by the immune system, being accepted as self-molecules and do not elicit a specific or non-specific immune response against themselves.

Improved techniques have been developed to reduce non-human sequences in biologics. One of the first techniques developed with that aim was the antibody chimerization, a process in which mouse variable domain regions are fused to human constant regions [33]. This process generates chimeric antibodies, which have reduced immunogenicity when compared to fully mouse antibodies [34-37].

Later on, humanized antibodies were developed by grafting mouse CDRs into human antibody backbone, restricting even more the non-human sequences [38, 39]. The exclusive replacement of the CDRs without framework regions may affect the antibody affinity to the antigen, which led to the replacement of human amino acids in the framework region by their corresponding mouse sequences. This replacement is then followed by "resurfacing" approach, where the exposed mouse residues at surface are replaced by human amino acids [40, 41].

The clinical success obtained with chimeric and humanized antibodies have motivated continuous innovation at the production level, and currently an increasing number of fully human antibodies exist [42]. These human antibodies are typically derived from large phage display libraries expressing human antibodies fragments or from transgenic mice engineered with human immunoglobulin genes [43].

Phage display platforms started in 1985 with the discovery that foreign deoxyribonucleic acid (DNA) sequences could be cloned into filamentous bacteriophages, being displayed at the surface of phage particles as fusion proteins [44]. This discovery was combined with polymerase chain reaction (PCR) amplification methods developed in the late 1980s for cloning expressed immunoglobulin variable region complementary DNA (cDNA) repertoires derived from B cells, to create diverse libraries of antibody variable regions that reflect the natural B cell repertoire [45]. The library is then used to select against the target protein to capture the phage that binds specifically to the protein. These libraries allow a

rapid screen of large number of antibodies against the target protein, in contrast to the laborious generation of hybridomas. Libraries started to be built from immune fragments isolated from immunized animals or infected humans, resulting in biased libraries toward certain specificities [46, 47]. Then *naïve* libraries emerged, built from a pool of healthy donors, followed by a synthetic repertoire rearranged *in vitro*, which is not biased and therefore can be used for selecting specificities against a wide range of targets [48, 49]. Large and diverse semi-synthetic libraries represent a valuable source of antibodies against a large number of target proteins.

In 1994, by genetic engineering, it was possible to generate mice expressing fully human antibody repertoires [50, 51]. These engineered animals comprised target disruptions of the endogenous mouse heavy and κ light chain genes together with introduced transgenes of unrearranged human heavy and κ light chain gene segments. The observation that a limited germline repertoire can be used by the mouse immune system to construct high specificity and high affinity antibodies to a wide variety of antigens reflects the relevance of somatic recombination and affinity maturation processes in generating diversity [52]. Subsequent progress has included the expression of more V segments by the transgenic mice, thereby expanding the potential repertoire of recovered antibodies [53].

There are no major advantages for the use of one technique over another. Transgenic mice-derived antibodies undergo affinity maturation *in vivo*, which obviates the requirement for subsequent *in vitro* affinity maturation, thereby reducing timelines. However, the reported affinities for antibodies derived from the two types of platforms fall into the same range, with no overall significant difference between affinity-optimized phage displays derived mAbs and un-optimized transgenic derived mAbs [43]. It is often desirable to obtain species-crossreactive antibodies, which allow the evaluation of their biologic function in animal models, which are often mice. Typically self-reactive mice-derived antibodies are not selected, due to processes of immune tolerance induction. This constitutes a particular strength of phage display libraries, which in contrast to transgenic mice platforms and also hybridoma technology, allows direct selection for exquisitely specific binding properties, such as species cross-reactivity [54].

Despite all the above mentioned advances in mAbs manufacturing, drug immunogenicity still represents one of the major drawbacks in the usage of these biologics in clinical practice.

1.4 DRUG IMMUNOGENICITY

Immunogenicity is the ability that virtually all therapeutic proteins have to elicit and unwanted immune response against themselves, with the production of anti-drug antibodies (ADAb).

The theoretical basis for biologic's immunogenicity relies on two main principles: on the foreign nature of biopharmaceutical (neo-antigens or non-self antigens) or on their similarity to self-molecules in cases of protein replacement, where little or no endogenous protein is expressed. In the later case, immune tolerance was never developed for the defective or absent protein and the biologic will be recognized as a foreign molecule by the recipient's immune system.

To elicit an antibody response, therapeutic protein must interact with three major types of cells: professional antigen presenting cells (APCs), T helper (Th) cells and B cells.

The first step involves the uptake of therapeutic protein by APCs, through pinocytosis, receptor-mediated endocytosis or phagocytosis. The efficiency of this process varies according several factors, such as the aggregation state of the therapeutic protein, its receptor-binding affinity, the route of administration, the capacity of binding membrane receptors that will lead to internalization and effective protein processing [55-58]. Receptor-mediated endocytosis provides a more efficient antigen uptake than non-specific endocytosis, enabling presentation of antigens that are present at very small concentrations [59]. Inside the APCs, therapeutic protein is cleaved in a mixture of small peptides. Each peptide will bind human leukocyte antigen class II (HLAII) molecules, and those who bind with "strongly enough" affinity will be displayed at the cell surface. As such, the concentration of a particular epitope that is being presented is a function of the amount loaded and, crucially, of its affinity for HLAII receptors. HLA polymorphism and its impact on the binding of specific peptides (HLA restriction) are primary mechanisms by which patients genetics contributes to immune responses to particular protein therapeutics [60-63]. Antigen-specific T cells, through its T cell receptor (TCR), will recognize a specific complex peptide-HLAII. This interaction, together with co-stimulatory signals provided by the APC through CD80 and CD86 molecules, fully activates the specific Th cell. In the absence of this co-stimulation, T cells become inactive. Once fully activated, T cells divide and produce an array of pro-inflammatory cytokines. Naive B cells that will recognize, through IgM and

IgD surface receptors, a cognate antigen bound via a specific T cell epitope-HLA/TCR will be activated, proliferate and mature toward a plasma cell. This interaction results in the engagement of CD40 and CD40L between T and B cell, which will provide a further signal to B cells that will lead to B cell clonal expansion and differentiation into antibody-secreting plasma cells and memory B cells. In the absence of activated Th cells, *naïve* B cells do not fully mature, and are rendered anergic or undergo apoptosis.

T-cell independent activation of B cells may also occur, usually induced by highly repetitive structures, where the co-stimulatory signal can be mediated via alternative signalling pathways, such as Toll-like receptors (TLR) engagement [64]. Antibodies produced by direct B cell activation are mostly IgM or low affinity IgG isotypes. However, the great bulk of ADAb are high affinity IgG antibodies, denoting T cell help to antigen-specific B cells [65].

The great dynamic diversity of TCRs and BCRs ensures the recognition of a wide variety of sequences and structures, which is important for the defence role of the immune system. On the other hand, such diversity implies the generation of new hypervariable sequences to which immune system is not tolerant. This explains, in part, why even fully human therapeutic mAbs can be immunogenic.

Tolerance is ensured by complex mechanisms with origin at the thymus. Promiscuous gene expression by thymic epithelial cells (TEC) of tissue-specific antigens (TSA) is highly relevant to ensure tolerance, as demonstrated in autoimmune regulator protein (AIRE)-deficient mice [66-68]. It has been argued that T cells carrying TCRs with “too much high” affinity for self HLA-peptide complex will be subjected to negative selection mediated by medullary TECs (mTECs) [69, 70]. However, clonal deletion at the polyclonal level is incomplete, and autoreactive T cells often escapes into periphery [71-76]. Those autoreactive T cells are controlled at the periphery by a particular subset of lymphocytes, which although less frequent than effector cells, are able to regulate auto-reactive T cells, as elegantly demonstrated by Coutinho et al in the beginning of 90’s [77]. Those cells are known today as T regulatory cells (Tregs), which are phenotypically characterized as CD4 cells that express the high affinity receptor for IL-2 (CD25) and also Foxp3, a transcriptional factor codified by X chromosome that is essential for these cells’ development and function [78-81]. It has been suggested that Foxp3+ Tregs are selected on high-affinity self-reactive TCRs, having higher resistance to negative selection than conventional T cells [82-87].

Therefore, Tregs cells will be selected in an antigen-specific manner, according to the antigens presented in the thymus during T cell development. It is thus conceivable to postulate that newly variable sequences as those continuously generated through V(D)J recombination might represent foreign sequences, not represented in the thymus, thereby able to elicit an immune response. Recent evidence has, however, demonstrated that the antigens may also reach the thymus via the blood stream or immigrating antigen-loaded dendritic cells [88-90]. This, together with the possibility of antigen co-presentation by thymic APCs and the existence of inducible Tregs at periphery, may explain tolerance for antigens not expressed by TEC [91-96].

Taken together, this highlights the complexity of immune response to biologics, which represents a highly complex phenomenon resulting from the interaction of drug- and patient-related factors not fully elucidated.

1.4.1 Drug-related Factors Influencing Immunogenicity

Perhaps the most understandable factor responsible for an immune response to biopharmaceuticals is the proportion of non-self sequences in the biopharmaceutical. Non-human sequences represent a primary target of human immune system. However, even fully human sequences may elicit the production of ADAb. T cell epitope content represents one of the major aspects influencing immunogenicity. Fab regions represent the highly immunogenic part of mAbs [60]. As mentioned before, the hypervariable CDR regions may represent new determinants unlikely to be present in the thymus during T cell development.

That notion has propelled the development of immunoinformatic tools, able to easily identify T-cell epitopes present in a biologic [97-100]. Despite the good correlation that has been found between the *in silico* prediction and *in vivo* immunogenicity, often *in silico* assays leads to an overestimation of the potential immunogenic T cell epitopes, as not all peptides that fit into the HLAII groove are generated by protein processing *in vivo*. Therefore, a wide array of *in vitro* and *in vivo* methodologies exist to further validate the ability of those peptides to elicit immune responses. Those methodologies include HLA binding assays, antigen and presentation assays, T cell proliferation or cytokine assays, T cell phenotyping (effector *versus* regulatory T cells), *naïve* blood assays, or humanized mouse models [101].

In the course of searching for T effector epitopes, regulatory T-cell epitopes (Tregitopes) were also identified in the structure of biologic proteins [102]. Tregitopes are specific, highly conserved and promiscuous epitopes from conserved regions of human immunoglobulins, able to activate Tregs, with the phenotypic properties of "natural" Tregs that supress immune responses *in vitro* and *in vivo* [102, 103]. In contrast to T effector epitopes, which mainly resides in the CDR regions of immunoglobulins, Tregitopes have been found mainly at the Fc portion of the human immunoglobulins [102]. There is a close correlation between the presence of highly promiscuous HLAII Tregitopes and the absence of HLA-binding T effector epitopes, with lack of immunogenicity in published clinical studies [104]. Therefore, validated Tregitopes have been integrated in the immunogenicity prediction, greatly improving the accuracy of the *in silico* analysis [104]. Hence, tolerization represents today an emerging approach to reduce unwanted immune responses to therapeutic mAb and biologic proteins. With the introduction of tolerogenic sequences in the biologic, which are thought to induce T regulatory cell expansion, a tolerogenic immune response to biologic is expected [103, 105-108]. Preliminary studies have demonstrated that co-administration of antigens with Tregitopes *in vivo* and *in vitro* leads to the induction of antigen-specific tolerance and suppression of both humoral and cellular immune responses to co-administered antigens [102, 105, 106, 109, 110]. Other strategy that has been adopted to induce tolerance has been the design of 'stealth' antibodies. These 'stealth' antibodies are composed of a peptide "mimotope" linked to the antibody via a flexible linker and can enable the host to develop tolerance to a non-binding conformation of the therapeutic protein. This approach has been achieved for alemtuzumab in which tolerance was induced to a soluble non-binding (single mutation) alemtuzumab variant [58].

One of the highest risk factor for drug immunogenicity is still protein aggregation despite all the advances in drug formulation over the last years. Aggregates are one of the major concerns of regulatory agencies, as their presence may results in quantitative and qualitative changes in T cell epitope presentation and cellular activation [64, 111, 112]. Certain aggregates, such as fibrils, which are large and highly ordered proteins aggregates can be presented in an "array" format, such is the case of viral capsids and bacterial cell walls. Such arrays may act as potent immunostimulators of innate defence system, which in turn leads to an adaptive, more specific immune response [64, 113, 114]. Multiple sources of protein aggregation, particles and leachates exist, namely at the stages of product manufacture, storage, shipping and drug infusion [115-119]. Patients' serum characteristics (e.g.

hyperlipidemia) may also influence protein aggregation, an issue that needs to be further explored. Product mishandling by patients or health care professionals can also contribute to protein aggregation, although the extent of this problem remains unknown [120]. Methods for predicting aggregation are not currently available.

Glycosylation pattern is also a key aspect in the biologic's structure. Non-human carbohydrate residues might be highly immunogenic [121-123]. The challenge of engineering and analysing glycosylation is a cornerstone of biopharmaceutical drug-design in nowadays [124]. Therapeutic proteins are produced in cell lines that arederived from a variety of sources, including mamals (human and non-human), bacteria, plants, yeast and viruses. Small differences in the protein sequence and/or post-translational modifications, such as glycosylation, oxidation, damination, acylation and alkylation, may influence the immunogenicity profile of the therapeutic protein.

Other important sources of immunogenicity in a biopharmaceutical are the so-called product-related factors. These factors represent the additional compounds other than the active ingredient found in the final product, such as degradation products, process- or product-related impurities and additives. These impurities, even in small quantities, have the potential to stimulate an unwanted immune response [125].

Even though, despite all the advances in protein engineering technologies, chemistry and manufacturing techniques, which have lead to the production of highly pure fully human or humanized mAbs, immunogenicity is still a limitation of biologic therapies. However, the fact that the same biological may be highly immunogenic in some patients, but not in all, strongly suggests that also patient-related factors are determinant to immunogenicity.

1.4.2 Patient-related Factors

Genetic differences among individuals might certainly influence the ability to produce and maintain a significant immune response against a biopharmaceutical.

Allelic differences in HLAII molecules are known to account to the inter-individual susceptibility to autoimmune diseases, specific infectious diseases and efficacy of some vaccines [126-129]. Similarly, those individual allelic differences may also affect drug immunogenicity, as demonstrated for factor VIII or Interferon-alpha (IFNα) [130, 131].

Detailed *in silico* studies have also confirmed the link between HLA and immunogenicity [132].

Interindividual variation of T cell repertoire may also contribute to differences in immunogenicity between individuals. T-cell repertoire is though to be the result of previous exposures to related epitopes, vaccination and gut microbiome [133, 134]. Such individual specificity, in addition to antigen processing may explain why some HLA binding peptide sequences do not induce T cell activation in some individuals [135].

Recently, a correlation between Il-10 gene polymorphisms and antibody production against adalimumab (fully human TNFi) has been described, but a causal relation has not been investigated [136].

It has been recently proposed that drug immunogenicity may possibly be affected by pre-existing antibodies, namely by the natural antibody repertoire. Pre-existing antibody responses can regulate immune responses following subsequent antigen challenge [137]. It was demonstrated that the presence of antibodies specific for Galα1-3Galβ1-4GlcNAc-R (αGal), encoded in the natural antibody repertoire, increases T and B cell responses to poorly immunogenic antigens that have been modified to express αGal epitopes [121]. Although the exact mechanism is not fully elucidated, it has been shown that the presence of αGal-specific antibodies may allow immune complex formation or efficient antigen presentation by B cells, which increases the efficiency of priming immune response to the antigen [121, 138, 139]. Anti-hapten responses have shown to be either increased or suppressed depending on the titre of pre-existing antibodies [140, 141]. Similarly, in IBD patients, pre-treatment infliximab-Fab reactive IgGs were significantly higher in patients developing infusion-reaction to infliximab than in remaining patients, while were significantly lower among those who were in remission one year after infliximab treatment [142]. The role of antibodies as immune regulators has first described more than one century ago [143], and may explain the effectiveness of intravenous immunoglobulin (IVIG) therapy in many autoimmune conditions. IVIG exerts its effect through a wide array of mechanisms, including immune complexes formation and immuno-modulation via anti-idiotypic interactions [144, 145].

The evidence that our immune system contains a large variety of antigenic structures in the form of the V-regions of immunoglobulins and TCR, able to interact even in the absence of nominal antigens, lead Niels Jerne, in early 70’s, to postulate that idiotypic interactions

would be on the basis of the selection of "pre-immune repertoires" and in the establishment of natural tolerance [146]. Natural antibodies are critical in providing early protection against pathogens, although they may also participate in T-cell dependent immune responses [147-150]. A huge fraction of natural antibodies are conserved among different individuals [151]. Computer simulations of the immune network show that the greater the degree of connectivity of a clone, the greater is its degree of tolerance to chronic antigenic stimulation [152]. The study of those specificities might integrate further models of drug design, aiming to select V-regions of therapeutic antibodies that show the wider connectivity profile with pre-existing clones.

The type of the disease and the immune status of the patient might also influence immunogenicity. We might expect to verify higher immune responses in patients with autoimmune diseases that are more prone to antibody production. High disease activity and baseline inflammatory markers (C-reactive protein) have been detected among RA patients who become ADAb-positives, maybe reflecting a highly active immune system [153]. Neutralizing ADAb to a Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) product was described in 95% of immune-competent cancer patients but only in 10% of immune-compromised cancer patients [154]. Similarly, rituximab, a chimeric anti-CD20 mAb, elicited no immune response from B cell chronic lymphocytic leukemia patients, but was immunogenic in 27% of Sjögren syndrome and in 65% of Systemic Lupus Erythematous [155-158].

Drug immunogenicity may also be age related, as protein turnover is different in children compared to adults. However, there is a lack of studies specifically comparing the immunogenicity of biologics in these two populations.

The use of concomitant immunosuppressive therapies has also been associated with decreased immunogenicity. Co-treatment with methotrexate, azathioprine, 6-mercaptopurine and hydrocortisone has been associated with lower antibody responses to TNFi in RA or Crohn's Disease patients [159, 160]. Similarly, pre-treatment with methotrexate plus rituximab ± IVIG in children receiving alglucosidase for Pompe disease or pre-treatment with IVIG in hemophilic patients receiving recombinant human FVIII, was associated with reduced immunogenicity and better clinical outcomes [161, 162].

Factors such as administration route, drug regimen and dosage may also affect drug immunogenicity. Administration of a biologic into sites with a high prevalence of dendritic cells (DCs), such as subcutaneous tissue, seems to increase the risk of immunogenicity due to prolonged exposure to and uptake by DCs, as compared with intravenous administration [163-165]. In a recent study, intravenous abatacept (a fusion protein consisting of CTLA4-Fcγ1) was revealed to be less immunogenic than subcutaneous abatacept [166]. Moreover, the immune complexes formation may lead to local inflammation at injection site, and may also reduce the absorption of the biopharmaceutical, preventing them to reach the target sites.

Intuitively, we would say that frequent drug administration increase the potential drug immunogenicity. However, regular, scheduled administrations have been associated with lower incidence of ADAb, in comparison with episodic or on-demand regimens [160, 167, 168]. Similarly, it is conceivable to assume that higher doses of biologic would increase immunogenicity, as higher amounts of drug (antigen) and product-related factors would be administered. However, it has been shown that in contrast to initial lower-doses of antigen, initial higher doses lead to small numbers of memory T cells and less efficient immune responses upon re-challenge with the same antigen [169, 170]. This point is of major relevance, since induction dosages are preconized only for some biologics in the treatment of specific diseases [171, 172].

In comparison to the extensive knowledge that has emerged about the drug-related factors, very few are still known about the patient-related factors implicated in drug immunogenicity. Further research is warranted to better understand the inter-individual variability on immune responses, aiming to predict the patients at risk to develop significant immune responses to a given biologic and to modulate immunogenicity below to its clinical significance.

1.4.3 Immunogenicity Assessment

As drug immunogenicity may have dramatic impact on product safety and efficacy, the assessment of immunogenicity starts at the very early phases of drug development. This notion have led the regulatory agencies to develop risk-based guidelines for immunogenicity screening, which is mandatory for the approval of biopharmaceuticals [173, 174].

Currently, combined in silico, *in vitro* and *in vivo* strategies are adopted by drug developers in order to rapidly screen therapeutic proteins for potential immunogenicity. Different bioinformatics tools are today available to screen T cell epitopes that bind HLAII with high affinity [97-101]. However, those *in silico* methods cannot be used alone to evaluate immunogenicity, as they cannot evaluate aspects such as processing and peptide presentation, affinity or peptide-HLA stability, TCR affinity or post-translational modifications that may influence the immune response against therapeutic proteins. Therefore, further *in vitro* and *in vivo* validation is warranted to confirm the potential of predicted epitopes to induce an immune response.

A wide array of *in vitro* assays can be used: 1) HLA binding assays to evaluate the ability of peptides to bind HLA class II proteins; 2) Dendritic Cell (DC) assays to detect potential endogenous and exogenous DC stimuli, either related to the drug (aggregates, misfolding, denaturation) or related to the formulation (impurities and excipients); 3) T cell assays to evaluate cellular proliferation, phenotype and cytokine release, which also provides the information about the nature of the T cell response. However, *in vitro* testing may not reflect differences in how the protein is processed and presented *in vivo* or the inter-individual variability in DC responses or T cell repertoire, which may lead to some discrepancies in the results.

In vivo methods represent another mean of evaluating the potential immunogenicity of a protein or peptide. Most commonly, NOD *scid* gamma (NSG) mice transplanted with human immune systems and/or transgenic 'immune tolerant' mice that express the protein therapeutic have been used to predict immunogenicity in humans [175]. They also allow us to modulate factors such as dosing, drug concentration and route of administration. Transgenic animal models can be used to study the immune response as a consequence of a break in tolerance [176-178]. One example of such an approach is the use of a transgenic mouse model expressing human IFNβ, which was used to model the development of ADAs against various recombinant IFNβ products. These transgenic mice were more sensitive than previous 'hybrid' transgenic mice models, in that they were not only suitable for studying factors that break immunological tolerance but could also be used to dissect the effects of protein structure, formulation and aggregation on the induction of ADAs [175, 179, 180]. However, important limitations exist in each model because the mechanisms underlying

immunogenicity are still unknown and there may be important differences between the human and animal responses.

Such combined approach for immunogenicity prediction at very early phases of drug development allows the rapid identification of potentially high immunogenic drugs before entering in clinical phase, saving time, costs and efforts. Aditionally, the identification of T effector epitopes in therapeutic proteins also allow the identification of the best candidates for de-immunization with the aim to eliminate or camouflage those epitopes. Such strategy reduces the interaction of therapeutic proteins with immune effector cells, thereby reducing their immunogenicity [29, 181-186]. Point mutations, pegylation or glycosylation are examples of techniques that have been used to mask the immunogenic epitopes [187-189]. However, de-imunization techniques have also some limitations, as those changes may alter the structural and functional properties of therapeutic proteins, which may render them non-functional or even more immunogenic.

Still, it should be also stressed that despite the good correlation that has been verified between the T cell epitope content predicted by *in silico* tools and the development of significant immunogenicity in further clinical trials, immunogenicity at population level can be quite different from immunogenicity at individual level [61, 132, 190]. Inter-individual variability, such as HLA haplotype, TCR repertoire, immune status or concomitant therapies may influence immunogenicity at individual level. Therefore, there is a need to monitor immunogenicity beyond the approval phase, as recently recommended by the European Medicines Agency (EMA) [191].

Because ADAb production is the end-result of immune response to therapeutic proteins, circulating ADAb has been the chief criterion for defining an immune response to biologics. However, the detection of ADAb is technically challenging.

Different types of ADAb may be defined according to the target epitope region in the therapeutic antibodies: anti- idiotypic, anti-isotypic and anti-allotypic ADAb.

Anti-idiotypic antibodies, represent the bulk of antibody response against therapeutic mAbs [60, 192-194]. Those ADAb will compete with the endogenous ligand for the binding to the drug and are designated as neutralizing antibodies because they can immediately inhibit the working mechanism of the drug [153, 195]. Antibodies targeting different regions of the

drug have also been described, although they represent a minor part of ADAb [196]. It would be expected that allotypic mismatch would induce ADAb formation [4]. However, the association between the patients' allotypes and the presence or concentration of ADAb have not been verified so far [196-198]. Nonetheless, ADAb that target different regions of biologic molecule than idiotypic regions are designated as non-neutralizing antibodies as they did not prevent the binding between the drug and the endogenous ligand.

Several methods are available to detect antibodies, including binding assays based on immunochemical procedures such as solid or liquid phase immunoassays, radioimmunoprecipitation assays and biophysical methods such as surface plasmon resonance [199, 200].

Immunoassays have been the most commonly used methods to measure ADAb. Immunoassays use the specific interaction of antibody with antigen to provide quantitative information about antibody (or antigen) concentration. They can be conducted using a variety of formats and/or detection systems, including direct, indirect, bridging and competitive platforms using radioligand, enzymatic, fluorescent, chemi-luminescent or electrochemical luminescence detection systems [201]. However, the detection of antibodies against antibodies offers particular concerns and all the methods currently available have their own limitations that should be taken into account for a proper interpretation.

The most common methods employed to assess ADAb are enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays (RIAs). Different assays exist of each method, with different sensitivity and specificity [202]. The initial assays used to assess ADAb were mainly ELISA methods. However, several limitations were found in those initial methods, which may had contributed to an underestimation of true immunogenicity induced by the long-term use of biologics in humans. Often, in those initial ELISAs, an anti-immunoglobulin antibody was used as secondary reagent, which is not appropriate to detect ADAb, as it can also bind to the therapeutic antibody used in the coating. To circumvent this problem several strategies were developed, such as coating the microtiter plates with drug-F(ab')2 or drug-Fab fragments. However, it has been verified that serum of healthy individuals contains high titers of IgG antibodies against coated F(ab')2 or Fab fragments, which lead to very high cut-off levels in normal serum and, thus, high false-negative rates in positive samples [203-205]. Furthermore, the immobilization of Fab/F(ab')2 fragments on a plastic surface may alter the antigen conformation, masking some epitopes or revealing

new ones, such that antibodies specific to the original epitope of interest may not be recognized [206]. In such instances, it may be necessary to preform an “indirect assay” by immobilizing a capturing agent (e.g. monoclonal antibody specific to the antigen or streptavidin to capture antigen conjugated to biotin), which can then be used to anchor the therapeutic protein [206].

The majority of therapeutic antibodies are of κ light chain in contrast to the majority of antibodies produced by human immune system, which are mainly of λ light chain. Anti-λ antibodies have been used in an attempt to more specifically assess ADAb. However, it has been verified that anti-λ light chain antibodies may also target other antibodies present in the sera, which are able to bind to the coated therapeutic antibody through Fc-Fc interactions. These antibodies have been detected even in healthy individuals or untreated patients [207]. Once more, the high cut-off levels in normal sera lead to a significant underestimation of the presence of ADAb. On the other hand, although being a minority, ADAb of λ light chain-type cannot be revealed by this strategy.

Several optimizations of ELISA method have been conducted, improving its performance in the detection of ADAb. Adoption of a “bridging antibody” format in which ADAb is captured by immobilized antigen and detected using the labelled antigen can provide ELISAs with high specificity because ADAb must be recognized twice for detection. The bridging capacity that is required for ADAb detection, might prevent the detection of he majority of IgG4, as human IgG4 tend to exchange half of the molecule with other IgG4 molecule that can be of different specificity, creating a monovalent Ig that loose bridging ability [17, 208] – Figure 3.

Nonetheless, compared with the previous formats, Bridging ELISA has high sensitivity and relatively low background. Cross-linking by C1q and rheumatoid factors (RF) may still occur, although both can be eliminated by pre-treatment of serum with RF-neutralization reagent, which consists of IgG coated latex particles. This type of assay has been increasingly used to detect antibodies against therapeutic antibodies [209-212].

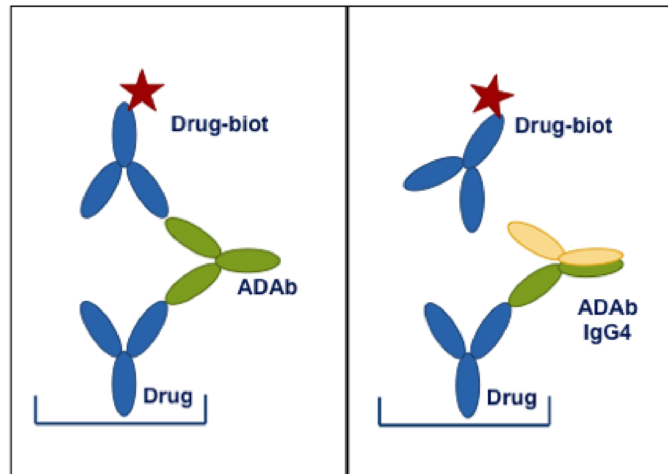

Figure 3 – Bridging ELISA

Radioimmunoassays have been also designed specifically to quantify ADAb, and are considered by many as the current "*gold-standard*" to assess ADAb [195, 202, 213]. RIA-ABA offers some advantages over ELISAs: it provides a fluid-phase platform, where therapeutic antibodies are in solution, which prevents the denaturation effect of coating; in contrast to bridging ELISAs, RIAs can detect monovalent IgG4 ADAb and discriminate them from other isotypes by the use of isotype-specific sepharose-coupled antibodies – Figure 4.

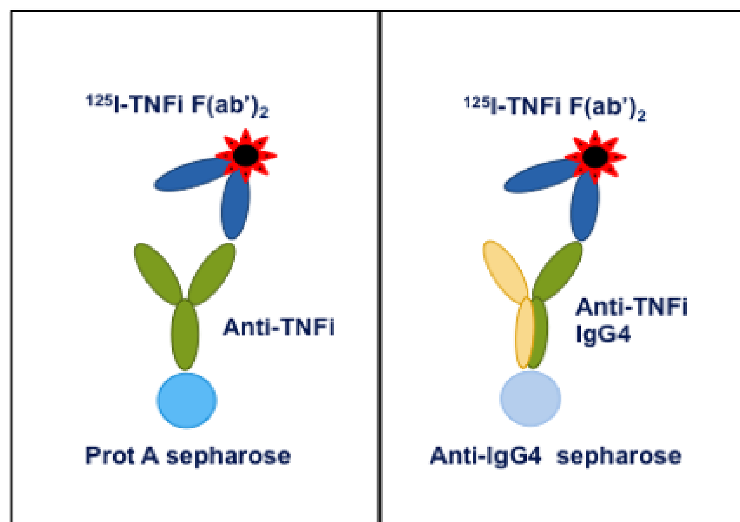

Figure 4 – Radioimmunoassay (RIA) – Antigen Binding Test (ABT)

RIAs are also less sensitive to drug interference, when compared with ELISAs [209]. On the other hand, RIAs are more difficult to automate than ELISAs and samples throughput is normally slower. RIAs can also be prone to artefacts, as the radiolabelling processes can mask/denaturate epitopes recognized by antibodies. However, the major disadvantage of RIAs is the requirement of high amounts of radioactivity and special laboratory conditions

for its implementation, which limits its use at a large-scale basis on the routine clinical practice.

The newly developed RIAs and Bridging ELISAs have been developed toward the detection of anti-idiotypic antibodies, as they use as secondary reagents the drug or the drug-Fab2. However, in rigor the neutralizing capacity of ADAb can only be confirmed through bioassays, which are vitro assays based on a functional aspect of the protein or mechanism of action. Different type of bioassays can be used. Both cell based and ligand-binding assays can be pursued for the detection of neutralizing antibodies (NAb) [199, 214-218]. While cell-based assays are believed to be the preferred NAb assay format, they do have disadvantages including high variability, low throughput and low sensitivity [214] . However, new generation reporter gene assays using growth-arrested cells are able to provide the desired sensitivity and limited variability. It is important to remember that irrespective of the NAb assay format it will only determine the potential of *in vitro* neutralization, whereas for *in vivo* translatability additional factors need to be taken into account, including clearance of ADA-drug immune complexes, pharmacokinetics in relation to ADA levels and efficacy or the equilibrium/affinity *in vivo* and *in vitro* between the drug/antibody/target. Due to the constraints associated with NAb assays, it could be considered whether it is helpful to monitor the pharmacokinetic/pharmacodynamic (PK/PD) profile of the biologic as indirect indicator for the presence of neutralizing factors. Furthermore, recent data including that of different TNFi have demonstrated that virtually all ADA detected in patients through antigen-binding radioimmunoassay are neutralizing antibodies since they are directed against the CDR of the therapeutic mAb [219]. This evidence calls into question the usefulness of evaluating a second method for the measurement of the neutralizing capacity of the ADA against mAb in routine clinical practice.

EMA has recently recommended the routine immunogenicity assessment beyond the approval phase of biologics [191]. However, the proper way to do this assessment is not yet formally established and some controversies have emerged regarding the type of assays and strategies that should be implemented. Irrespectively of the strategy that will be adopted, most important than to simply assess ADAb will be to correlate their presence with parameters such as PK and/or PD, together with clinical consequences, for an appropriate evaluation of their clinical significance.

1.5 RHEUMATOID ARTHRITIS

The paradigmatic example of the clinical success of anti-cytokine biologic approach is Rheumatoid Arthritis, starting by TNFi therapies that soon had extended to other cytokines, as the complexity of cytokine network in the disease became increasingly known.

Today, RA represents one of the diseases with more different biologics approved for its treatment, which makes it a good disease model to evaluate the impact of drug immunogenicity and how its routine assessment might improve the use of these therapies towards a higher benefit for the patients and for the society.

RA is chronic disabling inflammatory disease that primarily affects the synovium, cartilage and bone. It affects 1% of the population and is associated with significant increase in morbidity and mortality [220].

Despite RA has been generally accepted as an autoimmune disease, it remains somehow controversial as the identification of a dominant arthritogenic autoantigen is still missing and the reproducibility of rheumatoid arthritis in animal models has been difficult to demonstrate, despite some similarities with human disease [221, 222].

The idea that autoimmune mechanisms might underlie RA pathogenesis arises with the discovery of rheumatoid factors (RFs) in the blood of affected patients [223, 224]. RFs are autoantibodies targeting the Fc-part of human IgG. They are mainly of IgM isotype, although other isotypes, such as IgG, have also been described in some subgroups of RA patients [225]. RFs are thought to form immune complexes with IgGs activating complement in the joint, which in turn leads to increased vascular permeability and the release of chemotactic factors recruiting immune-competent effector cells to the joint [226]. Even though, RF are not sufficient neither necessary for arthritis development, as RF are present in up to 15% of healthy individuals and many other medical conditions, whilst around 20% of RA patients test negative for the presence of RF [227-229]. Therefore, the role of RFs in disease aetiology has not been established.

More recently, citrulline-specific reactivities against a number of citrullinated proteins, such as fibrinogen or vimentin, have been identified in RA patients [230]. Citrullination is a process by which arginine residues in a given protein are post-translationally modified (“deaminated”) in the presence of high calcium concentrations by an enzyme called PAD

(peptidyl arginine deaminase). Citrullination is a physiological process, which is believed to be important for degradation of intracellular proteins during apoptosis. Anti-citrullinated protein antibodies (ACPA) are found in 60-70% of RA-patients, but have been rarely detected in other diseases or healthy subjects [231]. Despite their unique (>95%) specificity for RA, the extent to which ACPA are directly involved in RA pathogenesis continues to be the subject of intense debate and investigation.

Genetic factors are known to be critical in the disease pathogenesis. The estimated heritability of RA, i.e., to which extent a condition in a population can be explained by genetic variation, has been calculated as up to 60% [232-234]. Due to technical advances in genotyping, over the last few years, an increasing number of genetic variations that show an association with RA have been identified.

The strongest and most relevant genetic risk factor for the development of RA, contributing around 30% to the total genetic effect, is found in the HLAII-encoding locus (chromosomal position 6p21.3). Similarities in HLA-DRB1 have been found among RA patients, exhibiting a common amino acid sequence at position 70-74 in the third hypervariable region of the DRβ1-chain. This sequence, consisting of glutamine/arginine-leucine/arginine-arginine-alanine-alanine (Q(R)K(R)RAA), is associated with RA and has been termed the "shared epitope" [235-237]. This conserved amino acid sequence was thought initially to be situated in the antigen binding cleft of HLA-DRB1 and, thus implicated in binding of a putative arthritogenic peptide ("shared epitope hypothesis")[235]. More recently, by crystallography techniques, it was verified that the shared amino acids actually face away from the antigen-binding cleft. Interestingly, this shared epitope despite being a primary risk factor for RA *per se*, it also represents a risk factor for ACPA positivity [236], with these autoantibodies developing preferentially (but not exclusively) in patients that harbour one or two shared-epitope alleles. Residues at the position 70-71 of the shared epitope have found to be critical for the affinitiy between HLA II molecules and the citrullinated peptides, and may influence risk of ACPA-positivity by modulating T-cells responses. In contrast to the sequence Q(R)K(R)RAA at position 70-74 in the third hypervariable region of the DRβ1-chain, the amino acid sequence DERAA at the same position have demonstrated an independent protective role in its carriers from the development of RA, even in the presence of coexisting shared epitope alleles. The mechanism behind this protection conferred by DERAA

sequence remains to be elucidated. DERAA-encoding alleles are found in approximately 30% of healthy caucasian individuals, but only in 16% of RA-patients [238, 239].

Non-HLA genetic association with RA has also been described. With the increasing number of genome-wide association studies, several polymorphisms at several genes, namely PTPN22, STAT4, CTLA-4, IL2RA, among many others, have been identified as risk factors for RA susceptibility [240-249]. However, the functionality of these polymorphisms remains to be formally established. Moreover, these non-HLA genetic variants invariably confer only very modest independent disease risk, often displaying individual odds ratios of little more than 1. The additive effects of such minor genetic determinants are unlikely to ever describe all of the unaccounted heritability of RA, and it seems likely that distinct genetic risk factors may provide multiplicative, rather than merely additive, combined risk, with gene environment interactions further completing the picture.

Environmental factors are also key players in the induction, magnitude and rate of the disease progression, in individuals who are genetically susceptible. Among environmental factors, cigarette smoking has been described as the most important risk factor for RA. A correlation between cigarette smoking and an increased risk of severe disease, has been described, in proportion to the number of pack-years [249]. Moreover, smoking is seen to represent a risk factor for ACPA-positivity, particularly in the presence of shared epitope alleles (up to an estimated 21-fold as compared to shared-epitope negative non-smokers), illustrating the multiplicative effect of combined risk factors [248, 250]. Citrullinated proteins have been detected in bronchoalveolar lavage fluid from smokers but not from non-smokers, suggesting an induction of protein-citrullination by smoking, through a mechanism not fully elucidated [250].

Other environmental factors, such as infectious microorganisms or sex hormones have also been proposed as risk factors for the disease. According to the available evidence, no compelling evidence has yet implicated a single microorganism as the cause for RA. The higher prevalence of RA in women and a tendency for disease to improve in pregnancy, suggests some influence of sex hormones in disease pathogenesis, although the precise relationship is not known [251].

The main affected tissue in RA is the synovium, which can be easily obtained by tissue biopsy. Largely driven by this factor, the histologic and cytokine pattern in the inflamed tissue has been extensively scrutinized.

Rheumatoid synovium is classically hyperplastic, exhibiting a marked cellular infiltrate, which includes CD4+ T cells, CD8+ cells, B cells, plasma cells, neutrophils, natural killer (NK) cells and also NKT cells. The interaction of these cells with other residential synovial cells, including macrophages, fibroblasts, mast cells and dendritic cells, allows the release of a multitude of cytokines, chemokynes and other molecules that play an essential role in the continuation of the inflammatory process [252-254]. The inflamed synovium invades adjacent cartilage and promotes articular destruction, which is mediated by the activation of osteoclasts, chondrocytes and synovial fibroblasts [255].

The first analysis of the cytokine pattern of the rheumatoid synovium started in the 1980s, when cytokine cDNAs were cloned and tools to measure cytokine expression became available. It was observed that the rheumatoid synovium is enriched with almost every cytokine known, particularly key pro-inflammatory cytokines such as Tumor Necrosis Factor Alpha (TNFa), IL1 and IL6 [256-258]. By using short-term cultures of rheumatoid synovial cells and neutralizing antibodies against specific cytokines, it was possible to demonstrate the dominance of TNFa within the complex cytokine network existed in rheumatoid synovium [258]. A linear model was proposed at the time, in which TNFa would drive downstream cytokines, such as IL1 and IL6 sequentially [259]. These studies represented the first evidence revealing TNFa as a good target in RA.

1.6 TUMOR NECROSIS FACTOR ALPHA

TNFa, named for its ability to cause rapid necrotic tumor regression, was the first described member of the TNF ligand superfamily, which is known to have pleiotropic functions including cell proliferation, differentiation, activation and apoptosis [260]. TNFa is primarily produced by monocytes and macrophages, although T- and B-lymphocytes also produces significant amounts of TNFa [261, 262]. Other cells, such as neutrophils, endothelial cells, keratinocytes and fibroblasts may also be sources of TNFa during acute inflammatory responses [263]. Its synthesis is initiated by the transcriptional activation of the TNFa gene, which is largely driven by several nuclear factor-κB (NF-κB) responsive elements in the 5’ promoter region [264]. TNF gene lies in the class III region of the Major

Histocompability Complex (MHC), approximately 250 kilobases centromeric of the HLA-B locus and 850 kilobases telomeric of HLA-DR [265]. TNFa expression is kept under tight control and the bulk of TNFa expression is regulated post-transcriptionally [266-269].

TNFa exists in two bioactive forms, a transmembrane and a soluble form. It is initially expressed as a transmembrane protein of 26 KDa, which is proteolytically cleaved off the membrane by TNFa converting enzyme (TACE) to form a secreted protein of 17 KDa [270, 271]. The 17KDa form of TNFa then aggregates into trimolecular complexes, which bind and activates its receptors.

TNFa exert their functions via two different cell surface receptors: p55-TNFR (TNFR-I, CD120α) and p75-TNFR (TNFR-II, CD120b). p55-TNFR is virtually expressed in all cell types, whereas p75-TNFR exhibits more restrictive expression, confined to certain lymphocytes subpopulations, including CD4+ and CD8+ cells [272], B cells [273], thymocytes [274, 275], oligodendrocyte progenitors [276] and few other cell types [277, 278]. Each of the cell types bearing p75-TNFR also expresses p55-TNFR. The ratio of their expression fluctuates according the cell type, its function and its activation state [279, 280].

TNF receptors are initially synthesized as membrane-anchored proteins, but they can be released form the cell surface by proteolysis and are constitutively released in the circulation [281]. TNF soluble receptor levels increases in response to several stimuli, such as TNF, lipopolysaccharide (LPS), T cell and neutrophil activation [282-285]. The release of TNFR into the circulation in response to all of those stimuli leads to an acute decrease in the number of TNFR on the cell surface, which probably serves to transiently desensitize cells to the TNF action, while the soluble receptors might act as a physiological attenuators of the TNF activity, by competing for the ligand with the cell surface receptors.

Both receptors display structurally similar extracellular domains but signal through distinct intracellular regions, with p55-TNFR containing a death domain that is not present in the p75-TNFR [286]. In general, TNFa largely relies on p55-TNFR for apoptosis and on p75-TNFR for any function related to T-cell survival [279, 287-289]. Overall, TNFa binding to p55-TNFR activates apoptosis through a pathway involving the adaptor proteins p55-TNFR-associated death domain (TRADD) and Fas-associated death domain (FADD), to coordinate downstream signalling by the caspase cascade [290]. By contrast, p75-TNFR signalling begins with the recruitment of the adaptor proteins TNF receptor-associated factor 1 (TRAF

1) and TRAF 2, ending with the mobilization and nuclear entry of the pro-survival transcription factor NF-κB to promote transcription of pro-survival genes [291, 292]. However, some degree of crosstalk and overlap in functions between the two receptors has been described, by mechanisms that are not fully known [293, 294].

The contribution of TNFa to joint inflammation and bone erosions is quite extensive. TNF is able to induce fibroblast-like synoviocytes and collagenases, inhibiting proteoglycan synthesis by articular chondrocytes, leading to cartilage destruction [295-299]. Additionally, TNFa also stimulate osteoclastogenesis and bone resorption, responsible for the erosive bone disease [300, 301].

However, the role of TNFa in immune pathophysiology is highly complex. Despite all the well-known pro-inflammatory properties of TNFa, an increasing body of evidence has emerged over the last years highlighting the immunoregulatory properties of TNFa [302, 303].

While TNFa overexpression in newborn NOD mice accelerates disease progression, in adult mice it is able to prevent autoimmune diabetes [304-308]. Despite the participation of TNFa in inflammatory demyelination of the central nervous system (CNS), demonstrated in several animal models, the blockade of TNFa in Multiple Sclerosis (MS) patients had completely unsuccessful results [309-318]. The same was verified by using TNFi in SLE patients and animal models of the disease, leading to the exacerbation of autoimmune phenotype. Similarly, prolonged TNF blockade in patients with RA or IBD have been associated with autoimmune phenomena [319-322].

The level, timing and duration of TNFa exposure, as the differential distribution of TNF receptors in different cell types and the genetic background of the organism may account for such pleiotrophism [303, 323].

1.7 TNF INHIBITORS IN RA

The critical role of TNFa in rheumatoid arthritis pathogenesis was successfully tested in animal models, such as collagen-induced arthritis. These studies provided the rationale for clinical trials of anti-TNFα therapy in patients with long-standing RA.

The first TNFi tested in RA occurred in 1992, using Infliximab: a chimeric mouse Fv-human IgG1 monoclonal antibody that binds soluble and membrane-bound TNFa with high neutralizing capacity [324]. The results were highly encouraging, as infliximab treatment in comparison to placebo, lead to a 60–70% reduction in the measures of disease activity, such as swollen or tender joint counts and inflammatory markers such as C-reactive protein (CRP) [325]. However, the duration of this study was very short in order to be able to have patients in placebo not dropping out. Longer follow up studies were only possible by enrolling patients with active disease despite therapy with methotrexate (MTX), one of the most potent therapies in RA. Consequently, infliximab trials have always been conducted in combination therapy with MTX, with treatment with infliximab plus MTX revealing significant better clinical and radiographic outcomes than MTX alone [326]. Long-term efficacy and safety had also been demonstrated. At 52-weeks of follow-up, infliximab treatment demonstrated improvement in patient's quality of life and functionality [327]. The efficacy of infliximab has been demonstrated either in established as in early RA, ie, ≤ 3 years' disease duration [327, 328]. Infliximab was approved for RA treatment in combination with MTX, with a recommended regimen of an i.v. infusion of 3mg/Kg body weight at 0, 2, 6 and every 8 weeks thereafter, although higher doses (6 and 10 mg/Kg) had revealed slightly better outcomes, with no significant increase in adverse events [327, 328]. Dose escalation is recommended in case of partial or non-response, in an attempt to (re)gain efficacy.

With the successful results obtained with infliximab, other clinical trials started to be conducted with other TNFi that were being developed at the time.

The second TNFi to be tested in RA was Etanercept, a dimeric fusion protein comprised of two extracellular portions of p75-TNFR (75-kDa TNF receptors) linked to the Fc portion of a human IgG1. Only the junction between these two domains, the hinge region, is composed by few non-human a.a. residues. Etanercept inhibits TNFa activity by binding soluble and cell bound TNFa with high affinity and by competing with natural TNFa receptors. However, the affinity of etanercept to membrane TNFa is lower when compared with infliximab or adalimumab, two monoclonal antibodies targeting TNFa [329]. Etanercept can also neutralize lymphotoxin alpha (LTa). The efficacy and safety of etanercept have been demonstrated either in patients with persistently active RA despite MTX treatment, as well in MTX-*naïve* patients, with or without concomitant MTX [330-335]. However, combination therapy has been always associated with better clinical outcomes. Short and

long-term efficacy and safety of etanercept has been demonstrated, both in established and in early RA [332, 336, 337]. The recommended regimen is 25 mg s.c. twice a week or 50 mg s.c. weekly, with equivalent efficacy and, with or without concomitant MTX [338].

Few years later, adalimumab emerged as the first fully human monoclonal antibody against TNFa. Adalimumab is an IgG1κ monoclonal antibody, made by phage display technique. Adalimumab has also proven efficacy and safety in both longstanding and early RA, MTX resistant or not [339-343]. Again, a synergistic effect was verified with the concomitant MTX in comparison with MTX alone or adalimumab alone [340, 342]. Therefore, although approved in monotherapy for the treatment of RA, adalimumab is recommended in association with MTX, in a dose of 40 mg subcutaneously every other week.

Recently, two other TNFi were introduced in the market: golimumab and certolizumab, for RA treatment.

Golimumab is an IgG1κ monoclonal antibody produced through genetically engineered transgenic mice immunized with human TNF, resulting in an antibody with human-derived antibody variable and constant regions. It has high specificity and affinity for soluble and transmembrane TNFa [344]. Similar to the previous TNFi, short and long-term efficacy and safety have been demonstrated in patients with either early or longstanding RA, MTX-resistant or MTX-*naïves* patients, in monotherapy or in combination with MTX [345-348]. Because golimumab was the fourth TNFi entering in the market, it was important to assess its safety and efficacy in patients who had been previously submitted to one or more TNFi (switcher patients). This study, conducted up to 14 weeks, revealed better therapeutic outcomes in golimumab-group than in placebo-group. The type of previous TNFi was not considered and, although it was described that 58% of patients had switched due to lack of effectiveness and 53% due to other reasons (intolerance and accessibility issues), the outcomes were not compared between these two groups [349]. Nevertheless, golimumab was considered efficient and safe for both biologic *naïve* and switcher patients, at a recommended dosage of 50 mg s.c. once a month [344].

Certolizumab is a PEGylated recombinant, humanized antibody Fab' fragment specific for human TNFa. Several clinical trials have demonstrated superior efficacy of certolizumab plus MTX versus MTX alone at 24 weeks of treatment. The combined therapy significantly improved disease activity scores, inflammatory

parameters, radiographic progression, patients' functionality and quality of life [350, 351]. When compared with placebo, certolizumab-treated patients also exhibited better clinical outcomes at week 24 [352]. Certolizumab has also shown efficacy in RA patients with secondary inadequate response or intolerance to previous TNFi (switchers) [353, 354]. Certolizumab is approved for RA treatment at a recommended initial dosage of 400 mg at weeks 0, 2 and 4, followed by maintenance dose of 200 mg every other week. A more convenient dosing regimen of 400 mg every four weeks can be, however, considered.

The therapeutic success of TNFi has motivated the emergence of other anti-cytokine drugs for RA treatment. However, TNF blockade still represent the most widely used strategy adopted in RA patients.

1.8 NON-TNFI BIOLOGIC THERAPIES IN RA

Although TNFa has been shown to play a central role in RA physiopathology, it has also been demonstrated that TNFa is not absolutely required for osteoclastogenesis, osteolysis and erosive arthritis, since all of those events may occur in the absence of TNFa [355-357]. Additionally, there is still a subgroup of RA patients that never achieve response to TNFi agents, being described in the literature as the "TNF-independent" RA. Taken together, this evidence propelled the introduction of biologic therapies with different targets than TNFa for the treatment of RA, with successful results.

Anakinra is a recombinant, non-glycosylated version of the human interleukin-1 receptor antagonist (IL-1Ra) that competitively inibiths the binding of IL1 to IL1 receptor. IL1 is a pivotal pro-inflammatory cytokine mediating many cellular responses including those important in synovial inflammation and subsequently joint destruction in RA [358]. A correlation has been reported between IL1concentration in the plasma and the activity of the disease [359]. Clinical trials of anakinra have shown that it reduces the signs and symptoms of active disease and slows the rate of radiographic destruction in adults with RA [360, 361]. Anakinra was approved by EMA in 2002 for the treatment of moderately to severely active RA patients who have failed one or more synthetic disease-modifying antirheumatic drugs (DMARDs) [362]. Anakinra can be used alone or in combination with DMARDs other than TNFi, in a daily subcutaneous injection of 100 mg. Although no direct head-to-head studies

have been performed between anakinra and other biologics, such as TNFi, indirect comparisons with adalimumab, etanercept and infliximab, showed a trend towards greater efficacy for the TNFi [363]. Anakinra also seems to be associated with comparably high rates of injection reactions [363]. Such evidence has lead to an underuse of anakinra in RA and in nowadays its use is merely occasional [364].

Rituximab is a chimeric human-murine anti-human antigen CD20 monoclonal antibody [365]. Initially developed for the treatment of certain lymphomas, the efficacy and safety of rituximab for the treatment of RA have been extensively demonstrated in both clinical trials and observational studies [366-372]. Although their efficacy was verified in methotrexate-naive patients and as first line biologic therapy for RA treatment, a favourable risk-to-benefit ratio has not been established in these populations. Therefore, in RA, rituximab is only approved in combination with methotrexate for treatment of moderately to severely active RA, in adults with disease that has shown inadequate response to ≥1 TNFi [365]. The recommended regimen is 1 g administered i.v. 2 weeks apart, on days 1 and 15 (for a total of 2 doses). A similar course of rituximab might be repeated every 24 weeks or based on clinical response, although no sooner than every 16 weeks between courses [365].

Abatacept is a recombinant fusion protein with an extracellular domain of human cytotoxic T-lymphocyte-associated antigen (CTLA-4) and modified Fc domain of human immunoglobulin G1. CTLA4Ig binds to CD80 and CD86 on antigen-presenting cells, blocking the engagement of CD28 on T cells, thus preventing T-cell activation [373]. The efficacy and safety of abatacept were demonstrated for early or longstanding active RA, in MTX-resistant or MTX-*naïve* population, in combination or not with MTX [374-376]. Moreover, treatment with abatacept has been also efficacious in patients who had had an inadequate response to TNFi therapy, with clinical improvements observed at 6 months and maintained throughout the next 2 years [377]. Therefore, abatacept can be used alone or with other DMARDs for the treatment of active RA despite synthetic DMARD therapy. Abatacept was recently approved in Europe as first-line biologic therapy in RA. The recommended dosage varies according to the patient's weight [373], as following: <60 kg weight: 500 mg i.v. at 0, 2, and 4 weeks, then every 4 weeks; 60–100 kg weight: 750 mg i.v. at 0, 2, and 4 weeks, then every 4 weeks; >100 kg weight: 1 g i.v. at 0, 2, and 4 weeks, then every 4 weeks.

Tocilizumab is a recombinant humanized IgG1monoclonal antibody specific for IL6 receptor [378]. The efficacy and safety of tocilizumab have been demonstrated by several RCTs, in different RA subpopulations: in patients with early or longstanding resistant RA, despite conventional synthetic DMARDS (MTX, chloroquine or hydroxychloroquine, sulfasalazine, leflunomide, azathioprine, or parenteral gold); in MTX-*naïve* patients; either in combination with MTX or as monotherapy [379-385]. Tocilizumab has also been tested in patients with moderate to severe RA who had a previously inadequate response to TNFi related to safety or inadequate efficacy [381]. Notably, high remission rates have been observed with tocilizumab treatment. Studies enrolling MTX- or other synthetic DMARDs-inadequate responders have revealed remission rates at 6 months that rage from 27%-30.2% in patients receiving tocilizumb, in comparison to 0.8%-3.4% in the placebo-DMARDs group [379, 380]. Even greater results have been described in observational studies, where 50 to 60% of tocilizumab-treated patients achieved remission after 6-12 months of therapy [386-389]. Additionally, tocilizumab has been, so far, the only agent showing no inferiority (or even slight superiority) in monotherapy when compared with combination therapy with MTX. A significant higher remission rate was verified among patients treated with tocilizumab monotherapy (34%) when compared to MTX (12%) [382, 383]. Tocilizumab is also approved in Europe as first-line therapy for RA treatment, either in monotherapy or in combination with synthetic DMARDS. The recommended dosage is 4 mg/kg i.v. once every 4 weeks, which may be increased to 8 mg/kg once every 4 weeks based on clinical response. Doses higher than 800 mg are not recommended [378].

Many other cytokines are currently under investigation for its role in RA. Recent research has revealed that the complexity of cytokine network is far more complex than the linear model initially proposed with TNFa at the apex controlling all downstream mechanisms.

1.9 THE CURRENT MANAGEMENT OF RA AND THE TREAT-TO-TARGET STRATEGY

The general approach to RA treatment has changed remarkably in recent years. The key message from many recent studies is the requirement for early recognition of disease and early "aggressive" intervention with traditional synthetic DMARDs and if necessary with biologic DMARDs, to that remission or at least low disease activity can be achieved [364, 390]. Examples of traditional synthetic DMARDs are Methotrexate (MTX), Salazopirine (SLZ), hydroxicloroquine (HCQ) or Leflunomide (LFN).

Disease activity is commonly assessed in RA by a composite index, named DAS28 (Modifyied Disease Activity Score). This index evaluates: the number of tender and swollen joints in 28 predefined joints (including small joints of the hands and feet, elbows, shoulders and knees); the erythrocyte sedimentation rate (ESR, mm/1st h) or C-reactive protein (CRP, mg/dL); and patient global assessment, through a visual analogue (0-10) scale. The DAS28 can be calculated using the following formula: DAS28 = 0.56 * sqrt(tender28) + 0.28 * sqrt(swollen28) + 0.70 * ln(ESR) + 0.014 * GH [391].

Clinical remission has been until very recently defined as DAS28<2.6 [392]. However, such definition has not been regarded as sufficiently stringent today to define remission, since multiple joints can remain swollen or tender at that score [393-396]. This led the Ammerican College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR), together with the Outocome Measures in Rheumatology Initiative (OMERACT) to form a committee to redefine remission in RA. It was agreed that remission should be defined as: 1) when scores on tender joint count, swollen joint count, CRP (mg/dL) and patient global assessment (0-10 scale) are all ≤ 1 or 2) when the score on the Simplified disease Activity Index (SDAI) is ≤ 3.3 [390]. SDAI is an index similar to DAS28 (CRP, mg/dL) that additionally includes the physician global assessment of disease activity through a visual analogue (0–10) scale [397].

Despite remission is today the treatment target in RA, low disease activity defined by composite measures, such as DAS28<3.2, might be an alternative goal for many patients who cannot attain remission, such as those with long-standing disease [398, 399].

The importance of an early diagnosis in RA has recently lead the EULAR Task Force to reformulate the RA classification criteria, such that it would be possible to identify more patients at very early stages of the disease. The early institution of DMARDs lead to better structural and functional outcomes [400-403].

The therapeutic target should ideally be achieved within the first 3 months of diagnosis and definitively attained by a maximum of 6 months [404], as there is evidence that disease activity states at 3–6 months after treatment initiation predict outcome at later time points [405]. If there is no improvement by at most 3 months after treatment start or the target have not been reached by at 6 months, therapy need to be adjusted. Such strategy requires fast and assertive therapeutic decisions, with a tight control of disease activity [406-408]. However,

the abundant therapeutic options available and insufficient information on differential efficacy and safety, make treatment decisions in clinical practice remains highly challenging.

The EULAR Task Force is continuously updating recommendations for the management of RA with synthetic and biologic DMARDs, based on systematic literature reviews and expert opinion committee. Several countries, including Portugal, have based their own national recommendations on such EULAR guidelines.

According to the last published recommendations, MTX remains the anchor drug on RA treatment and should be part of the first treatment strategy in patients with active RA [364]. Despite MTX has revealed to be effective as monotherapy in some RA patients, in the majority of the cases a combination therapy with glucocorticoids and other synthetic or biologic DMARDs are required [328, 409, 410]. Low dose glucocorticoids should also be part of the initial treatment strategy (in combination with MTX or other synthetic DMARDs) for up to period of 6 months, after which its risk- benefit profile is not clear yet [411, 412].

If the treatment target is not achieved within 3-6 months of the first DMARD strategy, and in the absence of poor prognostic factors, switch to, or the addition of, other synthetic DMARDs might be an option. If poor prognostic factors are present, such as high disease activity, autoantibody positivity (rheumatoid factor and/or antibodies to citrullinated proteins) and the early presence of joint damage, addition of a biologic DMARD should be considered [413, 414].

Currently, there are several biologics approved for RA treatment: five TNFi (one chimeric mAb, Infliximab; two fully-human mAbs, Adalimumab and Golimumab; one construct p75-TNFR-Fcγ soluble receptor, Etanercept; and one F(ab)2' fragment, Certolizumab, which has very recently approved in Portugal); one IL6-R antagonist, Tocilizumab; one CTLA4-Fcγ construct, Abatacept and one anti-CD20 agent, Rituximab.

Overall, similar efficacy and safety profile have been found in the different Randomized Clinical Trials (RCTs) but there are very few comparative head-to-head RCTs involving the different biologics approved for RA treatment [415, 416]. Indirect inferences have been drawn from systematic reviews of the literature and meta-analysis of RCTs. Those pooled analysis have revealed no significant differences in efficacy between the different TNFi

[417-422]. However, the majority of those results were associated with very large confidence intervals and significant heterogeneity between studies, which may have clinical relevance.

Both TNFi, Il6-R antagonist and abatacept can be now used as first-line biologic therapies in patients responding insufficiently to MTX and/or other synthetic DMARD strategies, with or without glucocorticoids [415, 416, 423-425].

Rituximab, despite effective in MTX-*naïve* patients and as a first-line biologic agent, did not show a favorable risk-to-benefit ration in these populations. Therefore, it may be used as first-line therapy in cases where contra-indications exist for other agents, such as recent history of lymphoma, latent tuberculosis with contraindications to the use of chemoprophylaxis, living in a tuberculosis-endemic region, previous history of demyelinating disease or recent history of malignancies [426, 427].

Significant disparities between data obtained from randomized versus observational studies in RA patients treated with biological have been observed by several investigators [420, 428, 429]. Emerging evidence from observational registries suggests differences in the effectiveness of different biologics, although with some inconsistencies. From the analysis of Dutch Rheumatoid Arthritis Monitoring (DREAM) registry, by monitoring RA patients who start on TNFi agents for the first time, it was verified that over one-year of follow-up, patients treated with etanercept or adalimumab had a significant decrease in DAS28 than patients receiving infliximab. No significant differences in the one-year effectiveness were verified between etanercept and adalimumab [430]. In contrast, the results from the Danish DANUBIO registry revealed clinical superiority from adalimumab over etanercept [431, 432]. Consistent results across several observational studies have revealed higher drug survival for etanercept in comparison with infliximab or adalimumab [422, 432-435].

Given such heterogeneity among the published studies, the EULAR Task Force decided that no preference of one over another biological agent should be expressed in the EULAR recommendations for the management of biologic-*naïves* RA patients. Thus, the choice between different biologics remains an empirical rather than a scientific-based decision.

Despite the great overall clinical benefit that biologics have brought to the RA patients, some are never able to achieve therapeutic response to those therapies or, more often, they loose an initial good response over time, usually within the first year of therapy. In case of non-

response to the current biologic therapy, assessed at every 3 months, patients should switch to another biologic agent, of similar or different mechanism of action. Similarly of what was described for biologic-*naïve* patients, the Task Force has considered that current evidence does not suggest superiority of any one agent over others in biologic-experienced patients. Therefore, the choice of the next therapy also represents an empirical clinical decision.

Several observational studies, including meta-analysis results, have shown the effectiveness and safety of switching between different TNFi [436-440]. However, the heterogeneity that is often observed in those pooled analysis, also suggests that therapeutic response to subsequent TNFi may vary according to different subgroups of switchers. Better outcomes have been achieved in switchers that had discontinued the previous TNFi by loss of efficacy (secondary non-response) or adverse events, when compared with patients that had never achieved response to the first TNFi [440-444]. Other analyses have suggested that patients who had already experienced failure to etanercept are unlikely to achieve response to another TNFi agent [443, 445, 446], although not consistently [447-449]. Some studies have reported that the likelihood of response to subsequent TNFi declines with the increasing number of previous TNFi, with some revealing an inefficiency of the third TNFi after the failure of two previous TNFi [446, 450]. Two independent prospective cohort studies, from the British Society for Rheumatology Biologics Register and from the Swiss Clinical Quality Management RA cohort, revealed that switching to Rituximab (B-cell depleting agent) is more effective than switching to another TNFi [372, 451]. Contradictory results exist, revealing no differences in efficacy and safety by switching from one TNFi to another TNFi versus to an agent with different mechanism of action [452-454].

In all the above mentioned studies, the reason for therapeutic failure is never taking into account and switchers have been always considered as a homogeneous group. However, such heterogeneity in the results is likely due to some confounder(s) factors that were not properly addressed. Over the last years, an increasing number of papers have revealed a clear association between the presence of ADAb and poor therapeutic responses [153, 160, 195, 210, 211, 213, 455, 456]. Differences in immunogenicity among individuals and among different therapies might well underlie such heterogeneity. Despite the biologic plausibility for the interference of ADAb on the therapeutic outcomes of patients receiving biologic therapies, this notion did not permeate the clinical practice and immunogenicity assessment is still not part of the current approach to biologic-treated patients.

2 OBJECTIVES/SCOPE OF THE THESIS

Accumulating evidence has suggested that drug immunogenicity might be one of the major factors underlying therapeutic inefficacy. Thus, its routine assessment might represent a powerful tool to optimize the use of biologic therapies. However, the clinical significance of drug immunogenicity is not formally established. Additionally, the way drug levels and anti-drug antibodies (ADAb) should be assessed and integrated in routine clinical practice remains to be defined.

The main objectives of this thesis work are:

1. To formally document the impact of ADAb in therapeutic responses

Given the clinical, scientific and societal/economic relevance of the topic, the clinical consequences of drug immunogenicity need to be clearly defined.

Systematic reviews of the literature with meta-analysis represent today the top of the evidence for clinical decision-making. Meta-analysis is a statistical technique for combining data from independent studies, having higher statistical power to detect an effect than individual studies. Moreover, it is less influenced by local biases than single studies will be, as it combines several studies. Therefore, we aim to determine the clinical impact of drug immunogenicity on therapeutic efficacy/effectiveness, by conducting a systematic review of the literature with a meta-analysis.

2. To evaluate the impact of drug immunogenicity on drug safety profile

Some studies have revealed an association between the presence of ADAb and acute adverse reactions to infusions of biologics, though other reasons may mediate such type of adverse reactions. We aim to evaluate the association between the presence of ADAb and infusion-related adverse events, in a cohort of patients receiving infliximab treatment (an intravenous TNFi).

3. To define a convenient assay to assess immunogenicity on routine clinical practice

After establishing the clinical relevance of drug immunogenicity, the way drug immunogenicity can be assessed in routine clinical practice might be defined. Radioimmunoassay-Antigen Binding Test (RIA-ABT) has been considered by many as the *"gold standard"* to quantify ADAb. However, RIA requires high radioactivity dose and special conditions to its implementation. We aim to compare the assay performance in the detection of ADAb between a newly developed Bridging ELISA with RIA-ABT, while a simple ELISA will be tested to assess serum drug levels.

4. To construct and test an algorithm for therapeutic decisions based on explicit biomarkers of immunogenicity

We aim to integrate immunogenicity information in the current clinical approach of patients receiving biopharmaceuticals. We intend to design a new treatment algorithm introducing immunogenicity data. The concordance between rheumatologist´s current clinical practice and our proposed algorithm will be assessed. Therapeutic response rates, over one year, between patients who followed the proposed algorithm and patients who followed other therapeutic strategies will be compared. Secondarily, we aim to evaluate the role of ADAb as a mediator of therapeutic response.

3 RESULTS

3.1 THE IMMUNOGENICITY OF TNFI THERAPIES IN IMMUNE-MEDIATED INFLAMMATORY DISEASES – A SYSTEMATIC REVIEW OF THE LITERATURE WITH A META-ANALYSIS

3.1.1 Introduction

Tumor Necrosis Factors Inhibitor (TNFi) therapies, such as infliximab, adalimumab and etanercept, are effective in treating Rheumatoid Arthritis (RA), Spondyloarthritis (SpA), Psoriasis (Ps) and Inflammatory Bowel Diseases (IBD), such as Crohn's Disease (CD) and Ulcerative Colitis (UC). Some patients maintain active disease and others show loss of efficacy after continued treatment [457]. Recent studies have highlighted drug immunogenicity as a mechanism behind treatment failure [153, 160, 458].

Immunogenicity is the ability that biotechnology-derived therapeutic proteins have in generating antibodies against themselves, since they contain unique sequences that can elicit an immune response [60].

The European Medicines Agency (EMA) and Food & Drug Administration (FDA) define the assessment of immunogenicity as mandatory for the approval of biopharmaceuticals; however, clinical trials may not reflect true immunogenicity induced by long-term treatment.

Quantification of such antibodies is challenging and undertaken by different assays [202, 205, 209, 459, 460]; assays have been optimized with improved sensitivity and specificity [196, 213, 461-463]. New evidence reveals a significant impact of immunogenicity on treatment response to biologics [153].

A systematic review (SR) and meta-analysis (MA) were undertaken to evaluate the impact of anti-drug antibodies (ADAb) on therapeutic response and the effect of immunosuppression (IS) on ADA detection.

3.1.2 Methods

We followed the PRISMA guidelines for reporting SRs and MAs and MOOSE recommendations for observational studies [464, 465].

3.1.2.1 Eligibility Criteria

To be included in this review, studies had to meet the following pre-defined eligibility criteria, defined according to the "PICOS" strategy [465]. The rationale for these criteria is also provided [466-469] – Table 1.

3.1.2.2 Data Sources

A comprehensive search strategy was designed to retrieve relevant clinical data from published literature. The following databases were examined up to August 19 2012: PubMed, EMBASE and Cochrane Library. Article reference lists were also scanned. The following search terms were used: (("Arthritis, Rheumatoid") OR ("Spondylitis, Ankylosing"[470]) OR ("Arthritis, Psoriatic"[Mesh]) OR ("Psoriasis"[Mesh]) OR ("Colitis, Ulcerative"[Mesh]) OR ("Crohn Disease"[Mesh])) AND "Treatment Outcome"[Mesh] AND (("TNFR-Fc fusion protein "[Substance Name]) OR ("infliximab "[Substance Name]) OR ("Adalimumab "[Substance Name])).

3.1.2.3 Study Selection

Two independent authors selected studies and extracted data. Disagreements were resolved by discussion between the two reviewers. To ascertain the validity of eligible studies and to minimize the risk of bias, two blinded reviewers determined the adequacy of study characteristics. We decided to use more broad inclusion criteria and then to perform metaregressions, subgroup analyses, and sensitivity analysis, rather than using highly restrictive study quality assessment scores [464].

3.1.2.4 Data Extraction

The following information was extracted from each study: 1) Population demographic characteristics: age, gender; 2) clinical data: diagnostic criteria and disease activity; 3) treatment: TNFi, duration, dose, schedule, route of administration, immunosuppressors;

4) exposure and outcome: therapeutic response, serum trough ADAb, time of assessment, assay; 5) publication: study design, follow-up period, author, year.

3.1.2.5 Statistical Analysis

Data were analyzed by calculating risk ratios (RR) for each study and performing forest plots; uncertainty was expressed by 95% confidence intervals (CI). Assuming clinical and methodological heterogeneity in the available body of evidence, random-effects models (REM), according to the Laird method, were used to cluster the results. Heterogeneity of study results was examined by calculating the $\chi2$ test for heterogeneity (when the $\chi2$ test had a $P< 0.1$) and the I2 measure of inconsistency [471]. Potential sources of heterogeneity were explored through analysis of the following predefined subgroups and confirmed through meta-regression: a) primary diagnosis b) proportion of patients co-treated with IS: methotrexate (MTX) or azathioprine/6-mercaptopurine (AZA/MCP); c) proportion of patients who underwent TNFi dose escalation; d) proportion of patients who started with higher initial doses of TNFi; d) scheduled treatment regimens; e) assay for ADAb detection; f) population characteristics and g) study characteristics. Publication bias was analyzed through the Egger's plot and test. STATA version SE 12 was used.

3.1.3 Results

Figure 5 shows the flow of studies through the SR process. Search of literature databases yielded 2082 references. Seventeen eligible studies were evaluated quantitatively, through MA. Five additional studies were exclusively qualitatively assessed because of significant missing data, but considered important evidence.

To address the impact of ADA on drug response, 865 patients (540 RA, 132 SpA, 58 Ps, 130 IBD) from 12 observational prospective cohort studies were analyzed [153, 211, 213, 455, 458, 463, 472-477]; and 5 additional studies were described qualitatively [212, 478-481] – Table 2.

Two publications used the same cohort of patients.[153, 455] We selected "Bartelds 2007" for the MA based on it being the original study and showing less heterogeneity in the pooled analysis – Figure 6 and Figure 7.

Two studies had multiple time points of assessment [211, 463]. The 12-month time point was selected for the MA, because of consistency with the included studies and less heterogeneity in the pooled analysis – Figure 8 to Figure 10.

Overall, detectable ADAb reduced the drug response rate by 68% (RR=0.32, 95%CI= 0.22-0.48) – Figure 11. Significant between-study heterogeneity was observed (I2=45.5%, p=0.037). Seeking for potential sources of heterogeneity, we observed a trend to an increased effect size in studies where the proportion of patients co-treated with IS (%IS) was <67% - Figure 12. However, univariate meta-regression did not detect %IS as a significant effect modifier of the effect of ADA on response rates - Table 3. A closer look at these subgroups showed that among studies where %IS <67%, detectable ADAb was associated with a reduction in therapeutic response by 78% (RR=0.22, 95%CI=0.12-0.39) and in studies where %IS ≥67%, the effect size reduction of drug response was attenuated to 59% (RR=0.41, 95%CI=0.27-0.62) – Figure 12.

Univariate meta-regression detects the proportion of patients co-treated with MTX (%MTX) as a significant effect modifier of ADAb on drug response – Table 3. In the subgroup analysis, we observed that in studies where %MTX<74%, the presence of ADAb reduced therapeutic response by 77% (RR=0.23, 95%CI=0.15-0.36), while in studies where that proportion was ≥74%, the effect size reduction was attenuated to 51% (RR=0.49, 95%CI=0.35-0.69) – Figure 13.

Univariate meta-regression also showed primary diagnosis (AR vs others) and initial higher doses of biologics to be significant effect modifiers of the effect of ADAb on drug response - Table 3. We verified a decreased effect size in studies evaluating RA patients and in studies where patients received initial lower doses of biologics – Figure 14 to Figure 16. These studies are also those with lower proportion of patients receiving IS and MTX. Significant heterogeneity in the pooled analysis was abrogated by dividing the studies according to the proportion of patients receiving IS, and particularly MTX, independently of the disease or the doses of biologics that were used.

The impact of ADAb on drug response was not significantly affected by the proportion of patients who underwent TNFi dose escalation – Table 3.

We could not test the effect size modification by scheduled treatment regimens or by the assay used for ADAb detection since most studies used schedule regimens and RIAs to assess ADAb.

The Egger's test provided evidence for no significant publication bias – Figure 17.

Studies with etanercept were not analyzed quantitatively because they did not fit the eligibility criteria and/or because no anti-etanercept antibodies were detected. Three studies are described qualitatively. Both described absence of anti-etanercept antibodies, by using Bridging ELISA and fluid-phase RIA, in a total of 332 RA patients [212, 480], and 53 AS patients [478], treated with 25 mg twice weekly or 50 mg weekly – Table 2. The absence of anti-etanercept antibodies persisted 3 months after therapy withdrawal [478].

We found two additional studies among Ps patients, which could not be included in the MA due to missing data. In a study enrolling 15 Ps patients receiving infliximab, those with detectable ADAb had higher PASI values than patients without such antibodies (PASI (mean, sd)= 10(4.9) vs. 5.3(2.4); p=0.02) [481]. Similarly, another study, among 22 PsA patients, revealed that ADAb-positive patients had a significantly worse DAS28 at 12 months of adalimumab therapy, when compared with patients without such antibodies (DAS28 (SEM)= 5.05 (0.84) vs. 2.58 (0.32); p=0.01) [479].

To address the influence of IS on the detection of ADAb 936 patients (376 RA, 94 SpA, 29 Ps, 437 IBD) from 12 studies were analyzed quantitatively [153, 159, 160, 211, 455, 473-476, 482-484] – Table 2.

MTX (dose range: 7.5-25 mg/wk) was the main immunosuppressor used in RA patients; AZA (2-2.5 mg/Kg/d) or MCP (1-1.25 mg/Kg/d) were the main immunosuppressors used in IBD patients.

ADAb were assessed through slightly different fluid-phase RIAs,[455, 474, 476, 484] and 3 different ELISA methods [159, 160, 211, 473, 474, 482, 483].

Two of the included studies used the same cohort of patients [153, 455, 484]. We selected "Bartelds 2007" for the MA based on the fact that it was the original study and

also the one that offered lower heterogeneity in the pooled analysis – Figure 18 to Figure 20.

Immunosuppressors reduced the proportion of patients with detectable ADAb by about 41% (RR=0.59, 95%CI=0.50-0.70) – Figure 21. Meta-regression confirmed the assay as a significant source of heterogeneity – Table 3. Concomitant IS reduced detectable ADA by 64% (RR=0.36; 95%CI=0.23-0.55) when RIA was used to detect ADAb, while when ELISA methods were used the effect size reduction of detectable ADA was attenuated to 37% (RR=0.63, 95%CI=0.42-0.67) – Figure 22.

It was not possible to evaluate potential differences in detectable ADAb between MTX and AZA/MCP, due to limited data.

The Egger's test provided evidence of significant publication bias for the studies evaluating the effect of immunosuppression on ADA production – Figure 23. Positive results may be more likely to be published. However, the presence of small and heterogeneous studies may also underlie that result.

3.1.4 Discussion

Immunogenicity reduces therapeutic response to TNFi, an effect that is attenuated by immunosuppressors. Detectable ADAb decreases TNFi response by as much as 80%. This contrasts with descriptions in most RCTs, considering immunogenicity as a minor problem [326, 340, 485, 486]. ADAb might reduce drug efficacy by competing with the endogenous ligand (neutralizing antibodies) and/or by forming immune complexes, which accelerates the clearance of the drug from the circulation, decreasing its bioavailability. Anti-idiotypic antibodies, which include neutralizing antibodies, represent the most significant part of the antibody response against infliximab and adalimumab [192, 197, 198]. The detection of ADAb is technically challenging and the assay may influence the results. The majority of studies in this paper used two independent but similar RIAs, with higher specificity than ELISAs [196, 202, 209, 461]. They are both fluid-phase RIAs, which avoids the artifacts induced by solid-phase adsorption of proteins and they both detect ADA against drug-F(ab')2, not favoring the detection of low-avidity antibodies. RIA is less susceptible than ELISAS to drug interference and is able to detect monovalent IgG4 ADA, which may represent a

significant proportion of ADAb [17, 209]. ELISAs have been the main method used in RCTs, perhaps explaining the contrasting descriptions in RCTs. We believe that the association remains underestimated: four of the included studies used ELISAs; the assays employed cannot detect ADAb that are in complex with the drug, not even RIA and we included EULAR moderate response within the responder group.

Despite few published studies reporting anti-etanercept antibodies [331, 333, 487, 488], all showed transient low titers, with no impact on drug response; this suggests the presence of "binding antibodies" or false positive results, since ELISAs of low specificity were used. Etanercept is administered more often than other biologics, possibly creating more drug interference in ADA detection. However, the absence of anti-etanercept antibodies has been confirmed months after therapy withdrawal [478]. Etanercept also blocks lymphotoxin-alpha (LTa), which among other functions is important for germinal center formation [489]. The involvement of LTa on drug immunogenicity remains unknown. The absence of clinically significant immunogenicity attributable to etanercept is consistent with the higher drug survival that has been reported for etanercept in comparison with infliximab or adalimumab [432, 490, 491].

Factors able to modulate the clinical impact of immunogenicity need to be identified. We verified that concomitant IS attenuated the impact of ADA on drug response, particularly MTX. The exact mechanism remains unknown. Concomitant MTX has been shown to be efficient in reducing immunogenicity in a dose-dependent manner, either by reducing the frequency of detectable ADA or by delaying its detection [195, 211, 492]. The time point of ADA assessment is also important. In our MA the majority of included studies assessed ADA at 12 months, showing a lower ADA frequency in patients receiving concomitant IS. That reduction was more apparent when RIA was used compared with ELISA methods (64% reduction vs. 34%), which may be explained by the increased specificity and less drug interference of RIAs over ELISAs.

These results are of high clinical interest, since they reveal a putative beneficial role of concomitant IS in diseases such as SpA, by modulating immunogenicity, increasing drug survival and treatment effectiveness. A recent study, in Ankylosing Spondylitis (AS) patients, failed to demonstrate the influence of MTX on infliximab

pharmacokinetics [493]. Infliximab is administered more frequently and at higher doses in AS, compared to RA, which may create drug interference and false-negative results if highly drug-sensitive assays are used to detect ADAb. That study was conducted up to 18 weeks of infliximab treatment, which for some patients may be too early for full development of immunogenicity. RCTs, with larger follow-up times should be conducted to show the beneficial effect of IS/MTX in these patients.

We could not assess differences between MTX and AZA/MCP regarding their impact on ADAb detection, due to the low number of studies using AZA/MCP and the fact that some studies in IBD also enrolled patients receiving MTX, who cannot be separated from the group receiving IS. However, univariate meta-regression detected %MTX as a significant source of heterogeneity, but not %IS (MTX + AZA/MCP). Subgroups based on %MTX resulted in homogeneous groups, whereas, the same did not happen for %IS subgroups. Further studies are warranted to verify whether this effect modifier is extended to other immunosuppressive agents and whether dose and scheduled regimens influence response by detectable ADA.

In some trials, initial higher doses of infliximab or adalimumab have revealed to be less immunogenic [159, 494]. It is not clear if that is a true effect or just an assay limitation of detecting ADAb in the presence of high drug concentration. We could not draw any conclusions in our MA because the studies that used higher biologic doses enrolled only patients with SpA, Ps and IBD, who also had lower proportion of patients co-treated with IS, confounding the results. Moreover, the limited number of studies and the clinical heterogeneity among them also prevent robust conclusions. Despite the high costs of biologic therapies, it would be of great clinical interest to know if higher induction doses of biologics would reduce immunogenicity, which could result in an improvement of its cost-effectiveness over the long-term.

Some reports show that dose escalation decreased ADAb detection, improving drug response [213, 455], but others [458], show that the procedure can indeed boost the immune response with serious consequences, such as infusion-related adverse events [463] or severe thromboembolic phenomena [495]. The absence of ADAb might be explained by drug interference, but it is striking why some ADAb-positive patients do not boost the ADA production. In our analysis, the proportion of patients who

underwent dose escalation did not interfere with the impact of ADAb on drug response. Very limited data are available and more studies are warranted to specifically address this question, since dose escalation is often adopted in clinical practice in case of inadequate response. We believe that increasing the dose may be risky since, so far, there is no way to identify which patients are at risk of boosting an anti-biologic response after dose escalation.

Scheduled regimens have revealed to be less immunogenic, by poorly defined mechanisms [160, 167, 168]. The limited number of studies prevented us to draw such conclusions in our analysis

A limitation to our study is that patient population, assessment of antibodies and therapeutic response definitions are not standardized across studies, although we addressed those sources of heterogeneity in the pooled analysis. The power of meta-regression analysis has limitations by the relatively small number of studies and the imprecision of the measurement techniques. The results obtained driven largely by findings in patients with RA. Although we excluded significant effect size modification by the disease itself, generalizations should be done cautiously.

This is the first SR and MA on TNFi immunogenicity. Our study strongly supports the notion that drug immunogenicity should be considered in clinical practice, during long-term use of therapeutic proteins. It is also an exploratory study, trying to identify factors able to influence the clinical impact of drug immunogenicity. It adds some evidence that unwanted immunogenicity may be modulated. Increasing evidence shows that monitoring immunogenicity will help us to better understand the clinical heterogeneity among patients, representing a very promising tool for an optimized and more personalized usage of biologic therapies.

Table 1 – Eligibility criteria for studies included in the systematic review

Criteria	Inclusion criteria	Rationale
Inclusion criteria	**Population** Age: Adults (≥18 years) Gender: Any Race: Any Disease: RA, SpA, Ps, CD or UC	The patient population has been restricted to adults, both genders, with diagnoses of RA, SpA, Ps, CD or IBD
	Intervention Infliximab Adalimumab Etanercept	The review included pharmacological interventions, which are used in clinical practice for RA, SpA, Ps, CD or IBD. Different formulations or route of administration of the same drug were included as supplement evidences
	Comparator Any of the included interventions Placebo	These comparators were selected to potentially enable the comparison of different drug responses
	Outcomes Drug response, assessed by: - the European League Against Rheumatism (EULAR) criteria for RA, [440]. Both EULAR good and moderate responders were included within the responder group. - the Assessment in Ankylosing Spondylitis 20% response criteria (ASAS-20) or Ankylosing Spondylitis Disease Activity Score (ASDAS) for SpA, [441, 442] - the Psoriasis Area and Severity Index (PASI) for Ps [443] - Empirically by an expert physician for IBD ADAb detection	Therapeutic responses were defined according to the internationally defined criteria for each disease. In IBD patients, therapeutic responses are often defined by an expert physician, in routine clinical practice. The review was not restrictive regarding the assay employed to assess ADA, if described.
	Study design Controlled Clinical Trials and Observational studies Published in peer-reviewed journals. Unpublished data, "recommendations", reviews and MAs were excluded from the analysis but used to hand-search additional bibliographic references.	RCTs are the gold standard of clinical evidence, minimizing the risk of confounding and allowing the comparison of the efficacy of interventions. To enhance the level of evidence, Observational Studies were included
	Phases of trial All phases	All phases of trials evaluating pharmacological interventions were included to avoid missing of any potential study
	Language restrictions No Language Restriction	The SR was meant to be as complete as possible.
	Publication timeframe No date or publication status restrictions were imposed	The SR was meant to be as complete as possible
Exclusion criteria	**Population** The study population was limited to a known type of drug response (ex. non-responders)	We are interested in the comparison between responders and non-responders.
	Exposure ADAb were assessed outside the trough period or in which ADAb were assessed before week 6 The assay was not specified Studies without a specified IS or administration schedule (to address the influence of IS on ADAb detection).	To avoid drug interference if outside the trough period; and to minimize false negative results before week 6 (for the majority of patients ADAb became detectable between 12 and 24 weeks of treatment) [2, 4, 12, 14]. Whenever ADAb were assessed at multiple time points, we selected the measurement closest to a) the last evaluation or b) the majority of the follow-up times of the included studies.

Table 2– Study and baseline patient characteristics // 1. Quantitative Evidence

	Study	Disease	Total Population No.	Women %	Age, mean (SD)/ median (IQR), y	Disease Duration, mean (SD)/ median (IQR), y	Disease Activitya, mean (SD)	MTX%	AZA/ MCP %	ST %	Other IS %	No IS %	Biologic	Posology
OBJECTIVE 1	Wolbink 2006 [204]	RA	51	82	56f (13)	12f (9)	6.0 (1.3)	86	na	na	8	6	INF	Indb+3mg/Kg q8w
	Radstake 2009 [446]	RA	34	79	56f (10)	na	5.7 (1.0)	41	na	26	0	59	ADL	40mg q2w
	Radstake 2009 [446]	RA	35	86	57f (10)	na	5.6 (1.2)	100	na	29	0	0	INF	Indb+3mg/Kg q8w
	Pascual-Salcedo 2011 [437]	RA	85	81	54f (14)	na	5.5 (1.3)	34	na	74	18	1	INF	Indb+3mg/Kg q8w
	de Vries 2007 [432]	AS	38	31	40f (10)	na	6.4 (1.2)	0	na	8	16	na	INF	Indc+5mg/Kg q6w
	Hoffman 2011 [451]	Ps	29	45	na	na	14.7(10)	24	na	na	7	na	INF	Indc+5mg/Kg q8w
OBJECTIVE 1 & 2	Bartelds 2007 [429]	RA	121	79	53f (13)	12f (10)	5.3 (1.1)	79	na	34	12	20	ADL	40mg q2w
	Bender 2007 [447]	RA	15	67	56f (8.1)	12f (8.2)	6.5 (1.2)	67	0	100	7	27	INF	40mg q2w
	Bartelds 2011 [160]	RA	272	81	54f (12)	8g (3-17)	5.2 (1.2)	74	na	91 (33)	19 (7)	9	ADL	40mg q2w
	Lecluse 2010 [450]	Ps	29	31	44 (11)	22 (na)	15.5(na)	10	na	na	na	0	ADL	Inde + 40mg q2w
	West 2008 [448]	CD	30	77	36h (21-73)	na	na	13	5 (17)	13	0	57	ADL	Indd + 40mg q2w

Table 2 (Cont.) – Study and baseline patient characteristics // 1. Quantitative Evidence (Cont.)

	Study	Disease	Total Population No.	Female %	Age, mean (sd)/ median (IQR), y	Disease Duration mean (sd)/ median (IQR), y	Disease Activitya, mean (sd)	MTX %	AZA/ MCP %	CST %	Other IS %	No IS %	Biologic	Scheme
OBJ 1 & 2	Steenholdt 2011 [449]	CD/UC	106	54	30-41j	3-10j	na	7	62	1	20 (19)	na	INF	Indc + 5mg/Kg q4-12w
	Plasencia 2012 [202]	SpA	94	44	50 (11)	na	3.1 (1.3)	22	na	42	27	23	INF	Indc + 5mg/Kg q6w
OBJECTIVE 2	Baert 2003 [167]	CD	125	66	35h (17-73)	na	260h (0-575)	2	45	42	na	14	INF	5mg/Kg SD or Indc + 5mg/Kg OD
	Vermiere 2007 [456]	CD	174	61	39h (18-73)	na	na	29	37	na	0	34	INF	5mg/Kg SD or Indc + 5mg/Kg OD
	Afif 2009 [457]	CD/UC	155	55	39h (26-50)	14g (na)	na	8	37	10	0	42	INF	5mg/Kg SD or Indc + 5mg/Kgq8w
	Maini 1998 [166]	RA	29	72	51f (na)	10f (na)	6.7	52	0	55	0	0	INF	Indb+3mg/Kg q4w
	Bartelds 2010 [458]	RA	235	79	53f (12)	9g (4-17)	5.2 (1.2)	82	na	34	na	na	ADL	40mg q2w

Table 2 (Cont.) – Study and baseline patient characteristics // 2. Qualitative Evidence

	Study	Disease	Total Population No.	Female %	Age, mean (SD)/ median (IQR), y	Disease Duration, mean (SD)/ median (IQR), y	Disease Activitya, mean (SD)	MTX %	AZA/ MCP %	CST %	Other IS %	No IS %	Biologic	Scheme
OBJECTIVE 1	Van Kuijk 2010 [453]	PsA	22	36	43h (21-61)	6g (1-18)	4.9i (0.3)	55	na	na	na	na	ADA	40mg q2w
	Adisen 2010 [455]	Ps	15	47	38-74j	30-40j	11-36j	na	na	27	na	na	INF	Indc+5mg/Kg q8w
	Jamnitski 2012 [203]	RA	292	82	53f (13)	8g (3-16)	5.2 (1.3)	76	na	28	36	na	ETA	25mg biw or 50mg qw
	Hoshino 2012 [454]	RA	40	88	60.5g (23-80)	6.5g (1-45)	5.4 (3.1-8.1)	58	0	85	18	0	ETA	25mg biw
	De Vries 2009 [452]	AS	53	25	41f (11)	na	6.4 (1.3)	na	na	na	na	na	ETA	25mg biw or 50mg qw

Legend: ADL= Adalimumab s.c.; AS= Ankylosing Spondylitis; AZA= Azathioprine; biw= twice a week; qw= every week; qXw= every X weeks; CD= Crohn's Disease; CST= Corticosteroids; ETA= Etanercept s.c; INF= Infliximab i.v.; IS= Immunosuppression; MCP= 6-Mercaptopurine; MTX= Methotrexate; na= not available; OD= on demand; Ps= Psoriasis; PsA= Psoriatic Arthritis; RA= Rheumatoid Arthritis; SD= single dose; sd= standard deviation; SpA= Spondyloarthritis; UC= Ulcerative Colitis; wk= weeks.

a= DAS28 for RA and PsA patients, ASAS 20 or ASDAS for SpA and AS patients, PASI for Ps patients; b= Induction dose of 3mg/Kg at 0, 2 and 6 weeks; c= Induction dose of 5mg/Kg at 0, 2 and 6 weeks; d= Induction dose of 160mg at wk 0 and 80mg at wk 2; e = Induction dose of 80mg at wk 0 and 40mg at wk 2; f= mean (SD); g= median (range/IQR); h= mean (range/IQR); i= mean (Standard Error Mean); j= range

Table 3 – Meta-Regression stratified by clinical characteristics to address the effect of ADAb on drug response and the effect of IS on ADAb detection

CLINICAL CHARACTERISTIC	RR (95% CI)	P for interaction
1. Effect of ADAb on Drug Response		
Imunosupressors, %		
Lower Proportion IS (<67%)	0.24 (0.11-0.53)	0.220
Higher Proportion IS (≥67%)	0.40 (0.27-0.61)	
Methotrexate, %		
Lower Proportion MTX (<74%)	0.24 (0.14-0.40)	0.028
Higher Proportion MTX (≥74%)	0.50 (0.35-0.71)	
Primary diagnosis		
RA	0.47 (0.33-0.65)	0.034
Other diseases	0.22 (0.12-0.40)	
Initiated higher biologic doses		
No	0.47 (0.33-0.65)	0.034
Yes	0.22 (0.12-0.40)	
Dose Escalation, %	0.31 (0.17-0.56)	0.57
2. Effect of IS on ADAb Detection		
Assay		
ELISAs	0.63 (0.50-0.79)	0.035
RIAs	0.36 (0.23-0.57)	

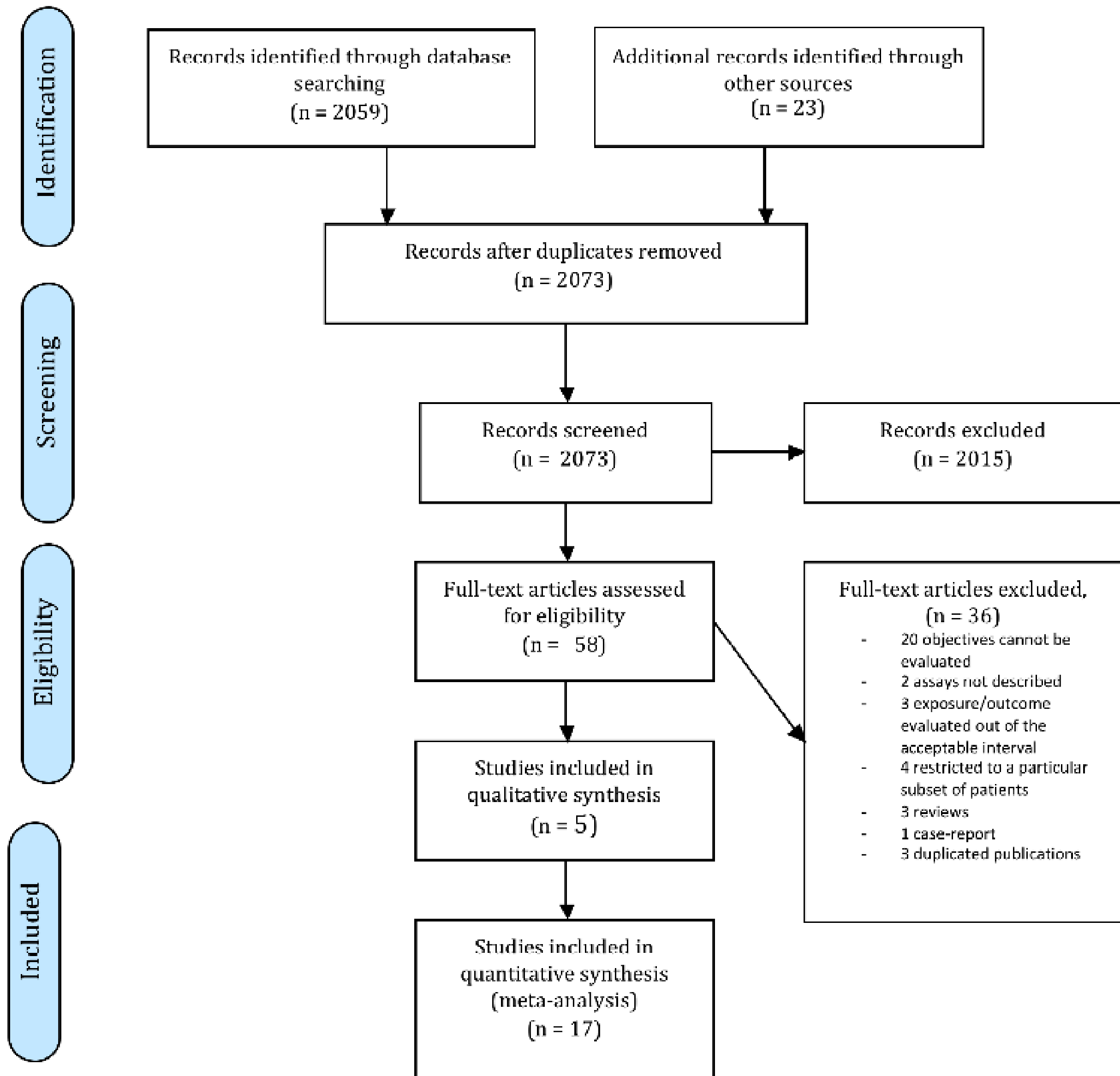

Figure 5 – Flow of studies through the systematic review (SR) process

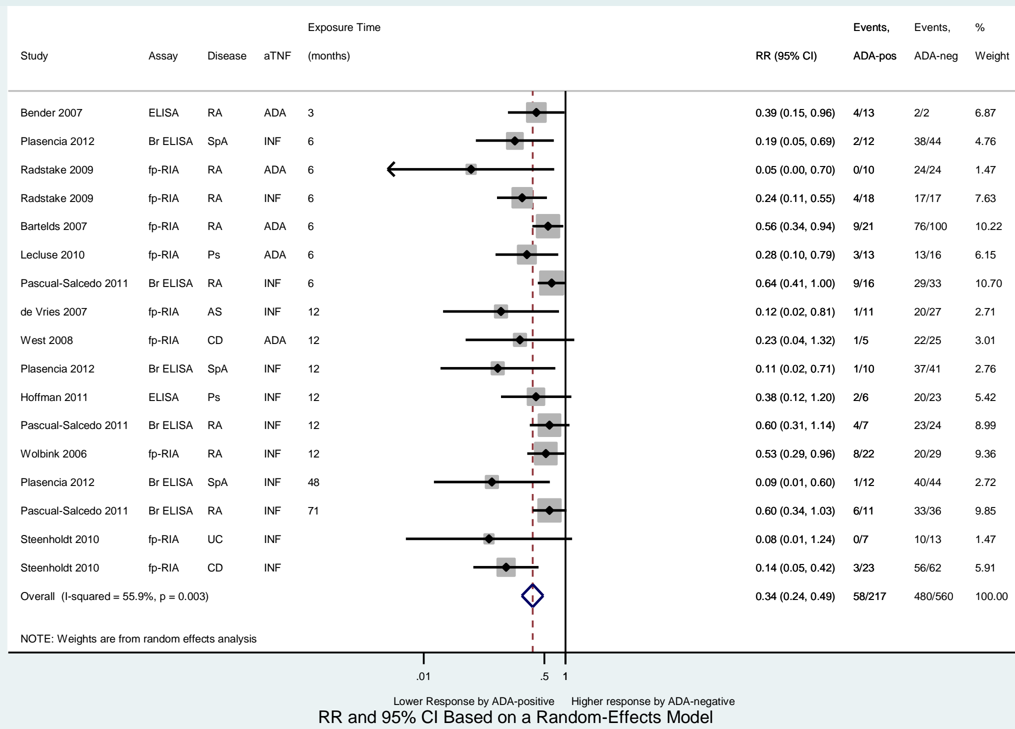

Figure 6 – Effect of ADAb positivity on TNFi response (excluding the study "Bartelds 2011")

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor, UC, Ulcerative Colitis.

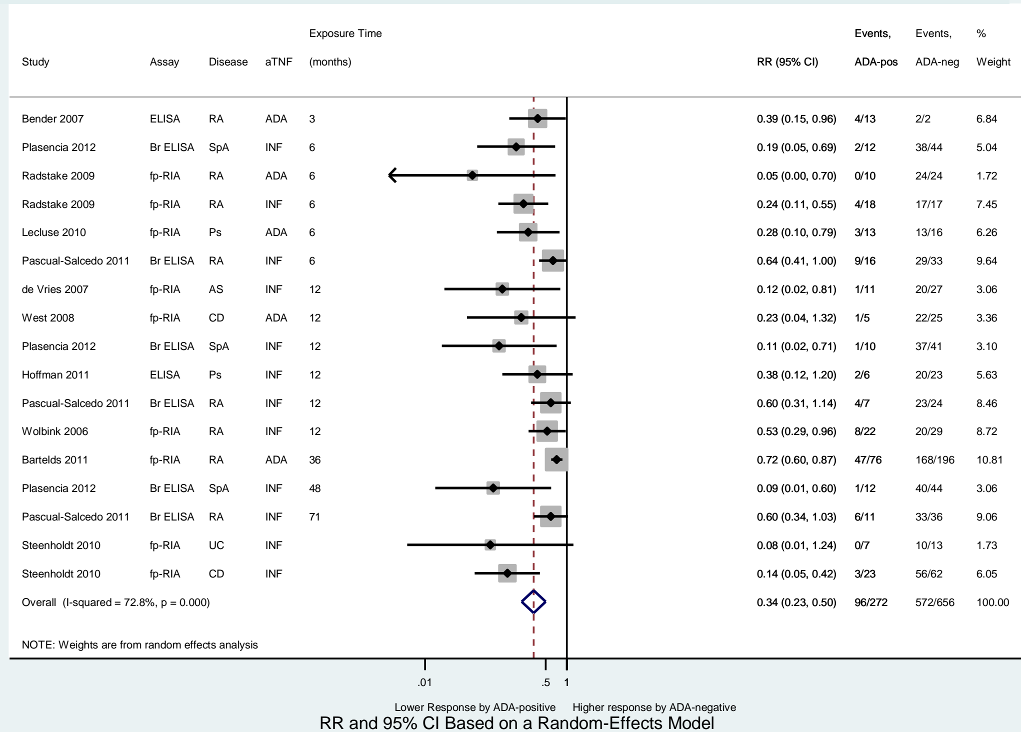

Figure 7 – Effect of ADAb positivity on TNF responsei (excluding the study “Bartelds 2007”)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn’s Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

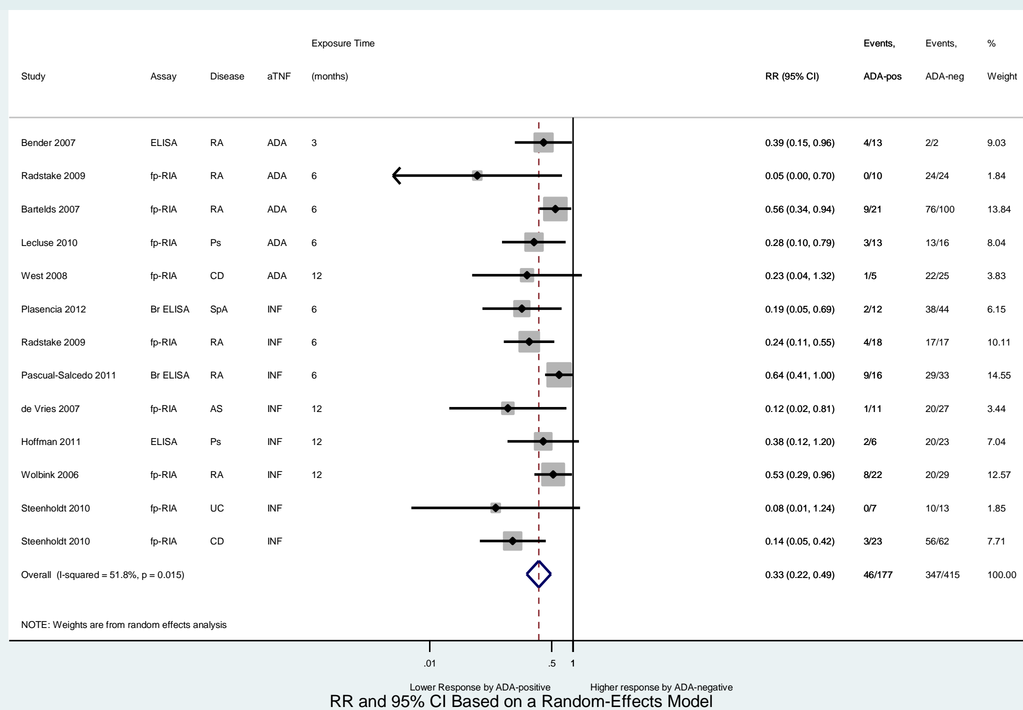

Figure 8 – Effect of ADAb positivity on TNFi response (including “Pascual-Salcedo 2011” and “Plasencia 2012” at 6 months of follow-up time)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn’s Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

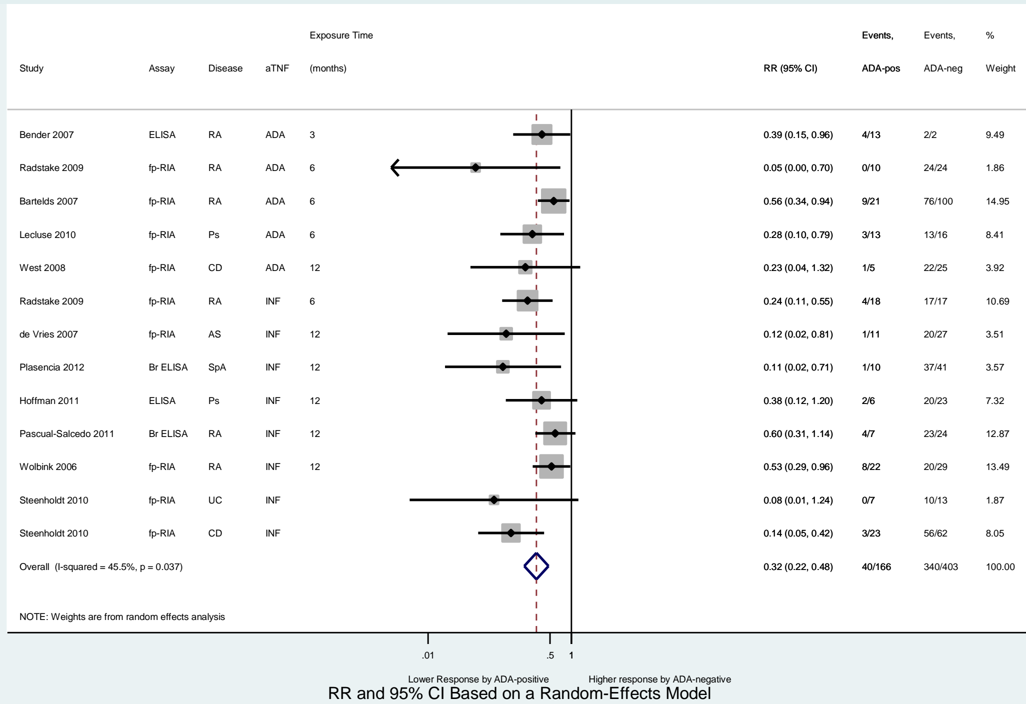

Figure 9 – Effect of ADAb positivity on TNFi response (including “Pascual-Salcedo 2011” and “Plasencia 2012” at 12 months of follow-up time)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn’s Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondylarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

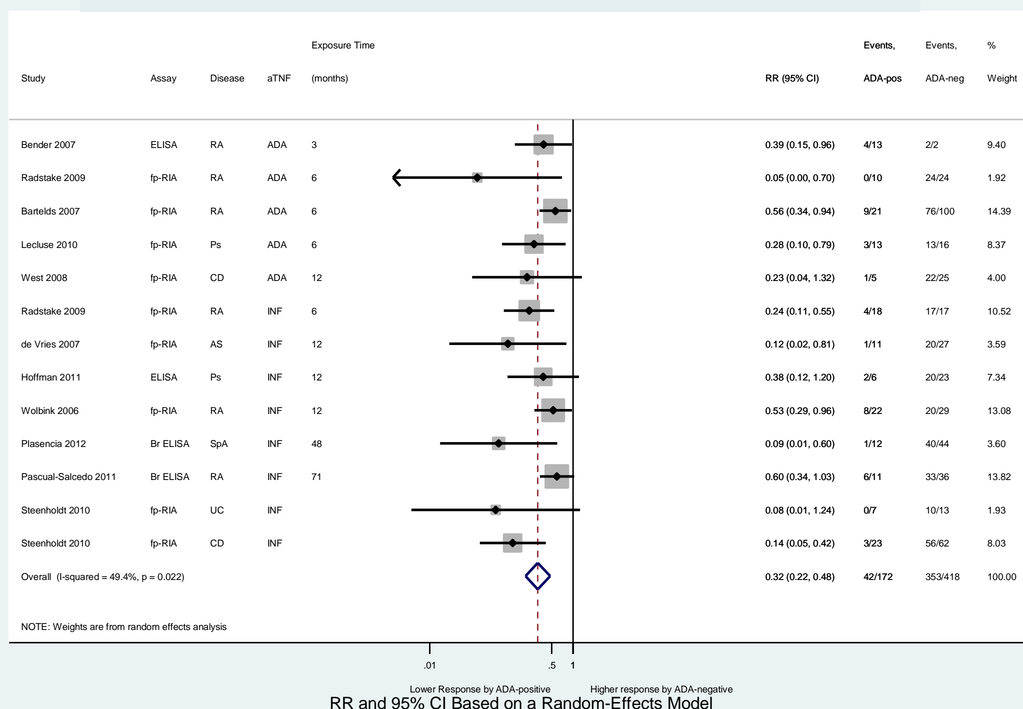

Figure 10 – Effect of ADA positivity on TNFi response (including “Pascual-Salcedo 2011” and “Plasencia 2012” at > 48 M follow-up time)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn’s Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondylarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

Note: The time point of 12 months offered less heterogeneity in the pooled analysis, when compared with the time point of 6 or >48 months (I2=45.5%, p=0.037 vs I2=51.8%, p=0.015 vs I2=49.4%, p=0.022).

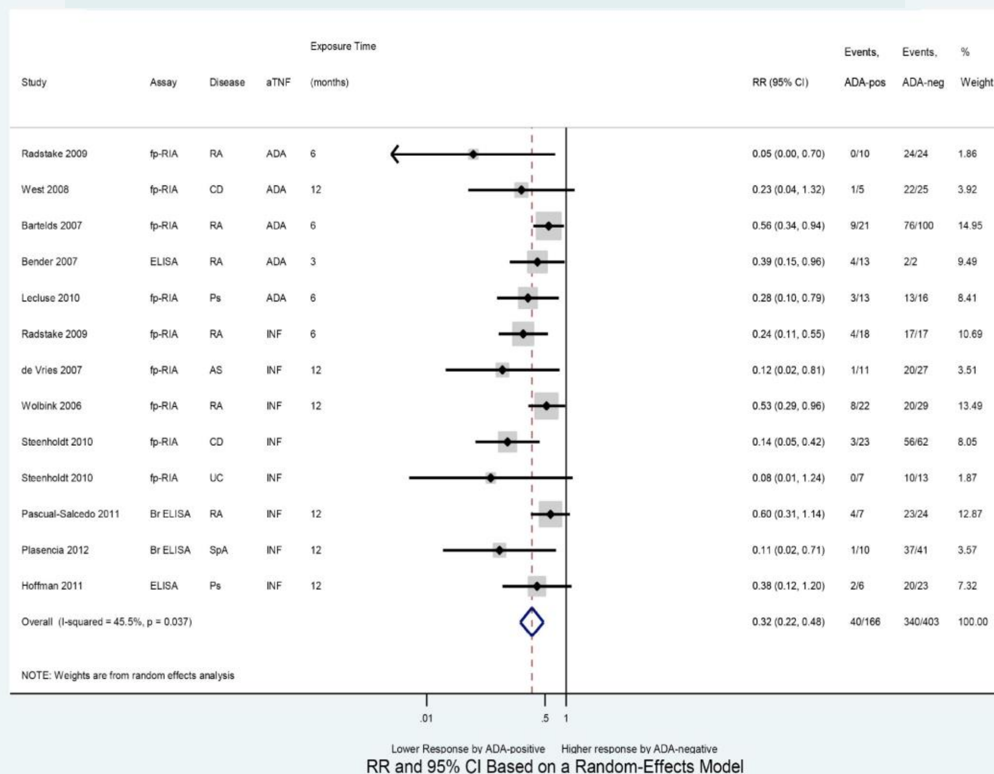

Figure 11 – Effect of ADAb positivity on TNFi response

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

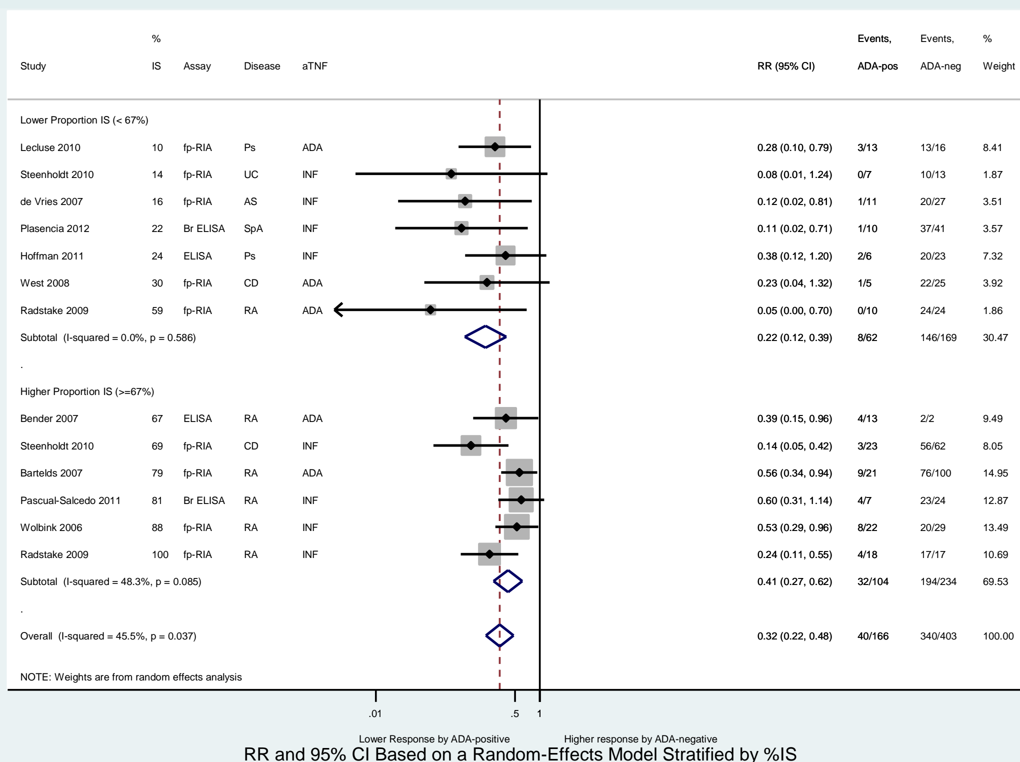

Figure 12 – Effect of ADAb positivity on TNFi response (IS<67% and IS≥67%)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; IS, Immunosuppression; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

Effect of ADAb-Positivity on TNFi Response (MTX<74% & MTX≥74%)

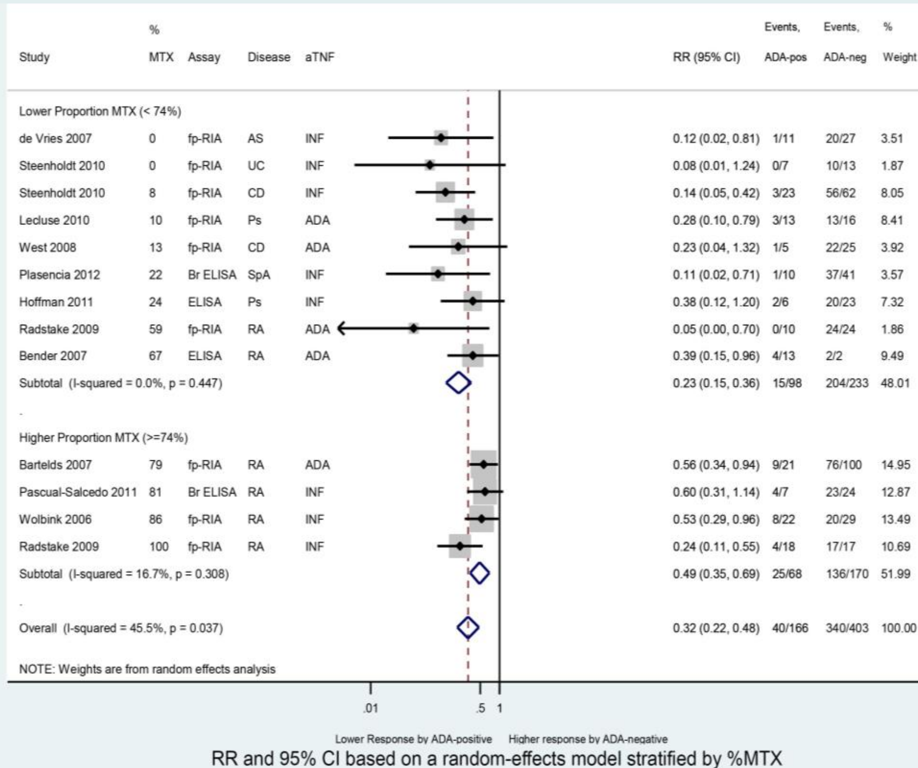

Figure 13 – Effect of ADAb positivity on TNFi response (MTX<74% and MTX≥74%)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn’s Disease; INF, Infliximab; MTX, Methotrexate; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

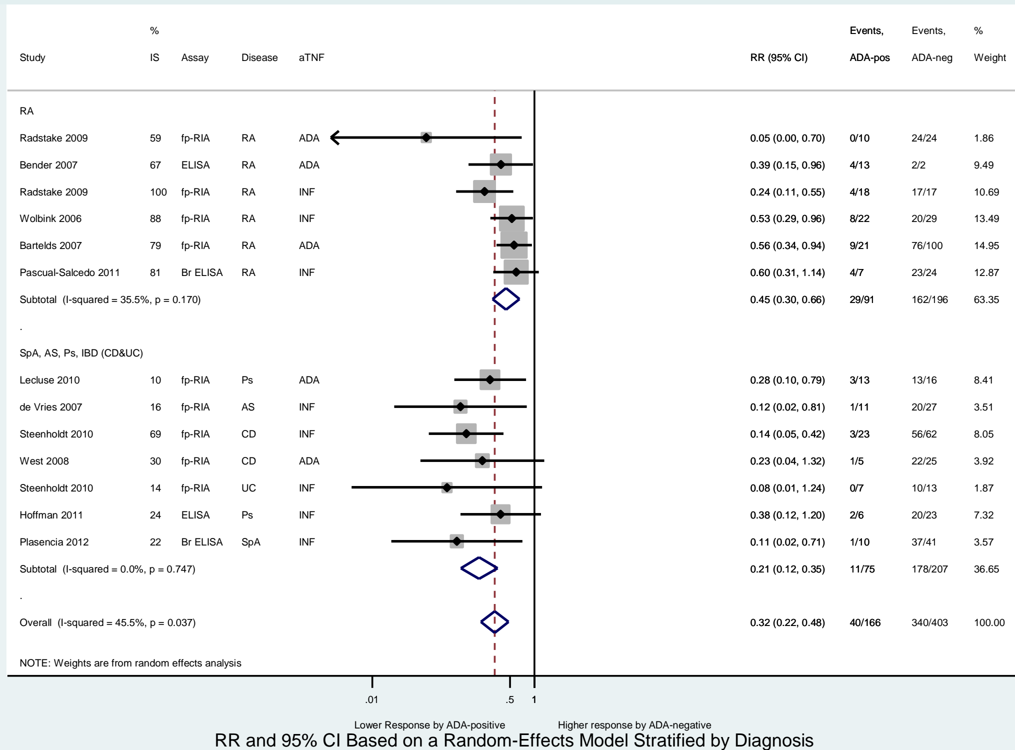

Figure 14 – Effect of ADAb positivity on TNFi response (by diagnosis)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

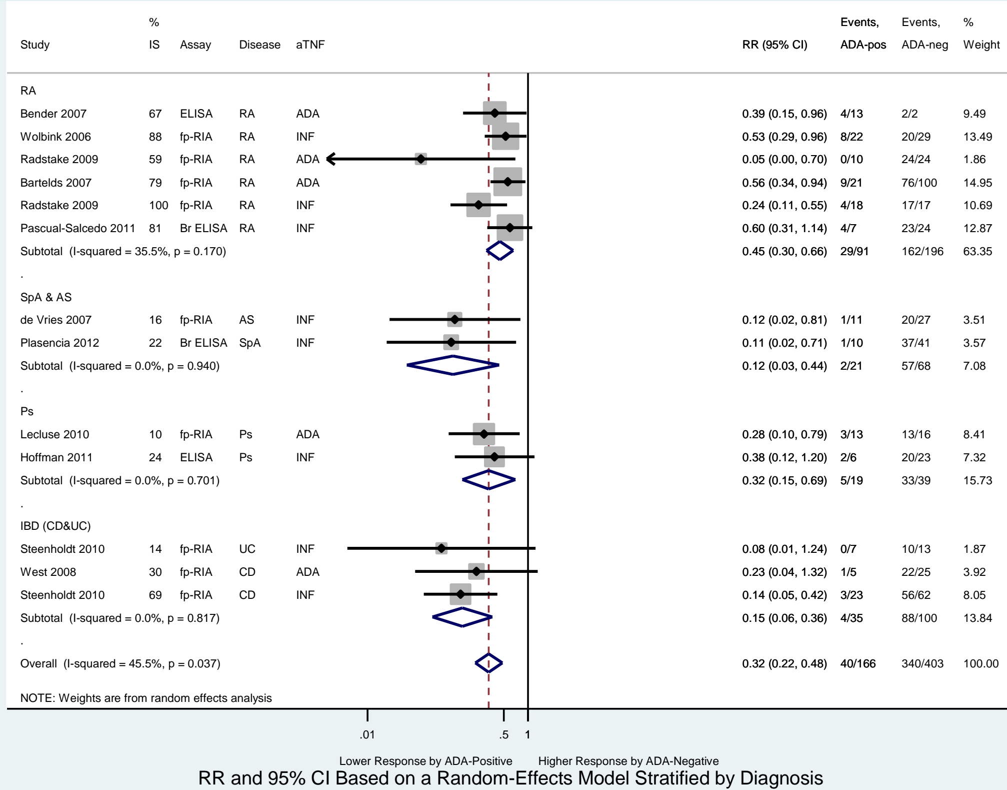

Figure 15 – Effect of ADAb positivity on TNFi response (by diagnosis)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

Note: The stratification by each diagnosis individually offers higher heterogeneity than the stratification by RA vs other diagnosis.

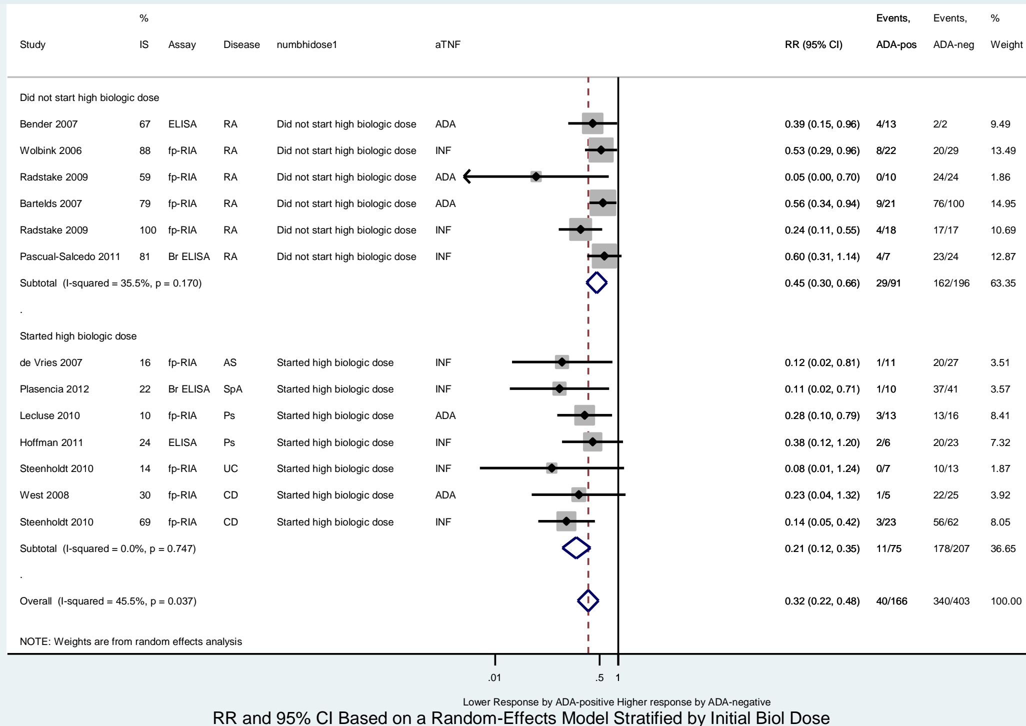

Figure 16 – Effect of ADAb positivity on TNFi response according to initial biologic dose

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn’s Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; TNFi, Tumor Necrosis Factor Inhibitor; UC, Ulcerative Colitis.

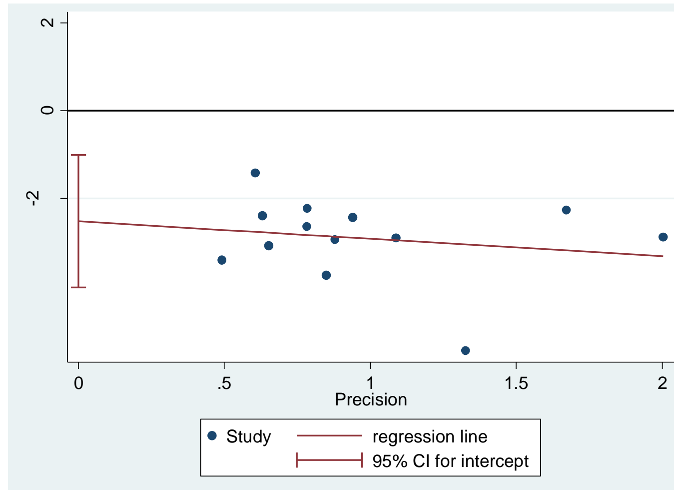

Figure 17 – Egger's publication bias plot for the effect of ADAb positivity on TNFi response

Legend: ADAb, anti-drug antibodies; TNFi, Tumor Necrosis Factor Inhibitor.

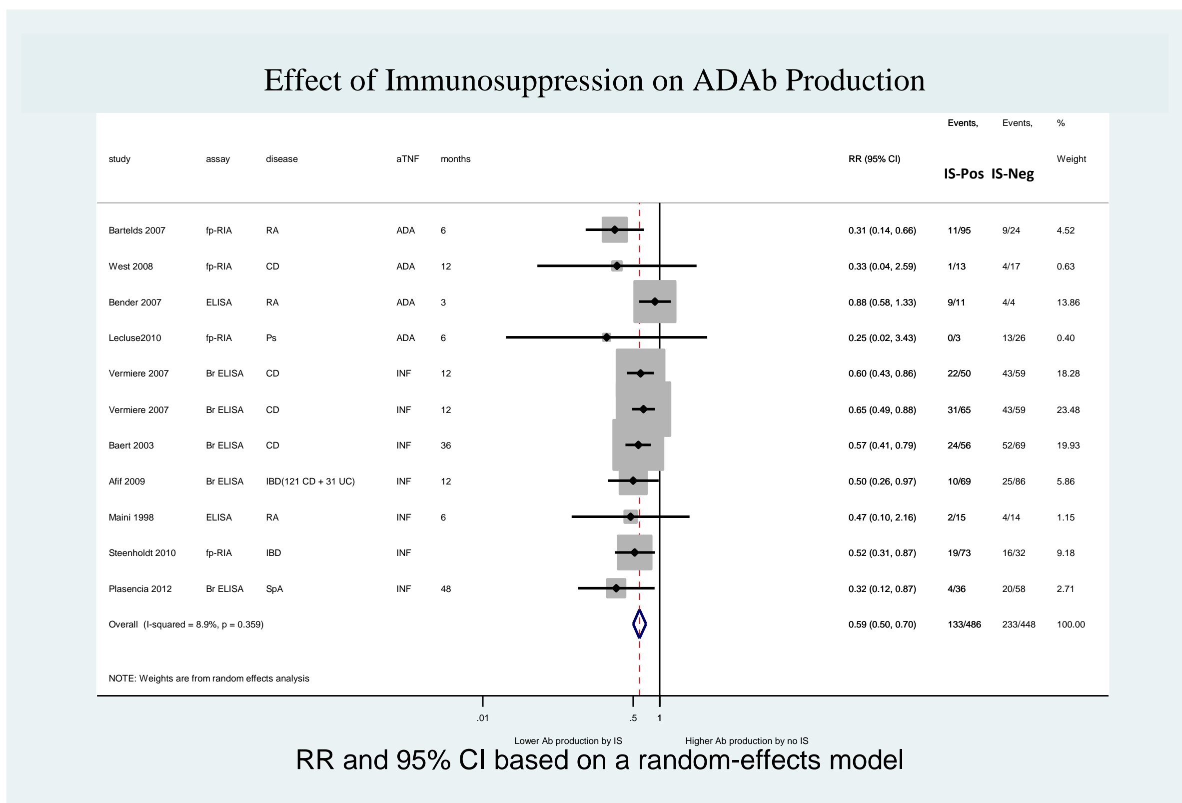

Figure 18 – Effect of immunosuppression on ADAb production (by excluding "Bartelds 2010" and "Bartelds 2011")

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; UC, Ulcerative Colitis.

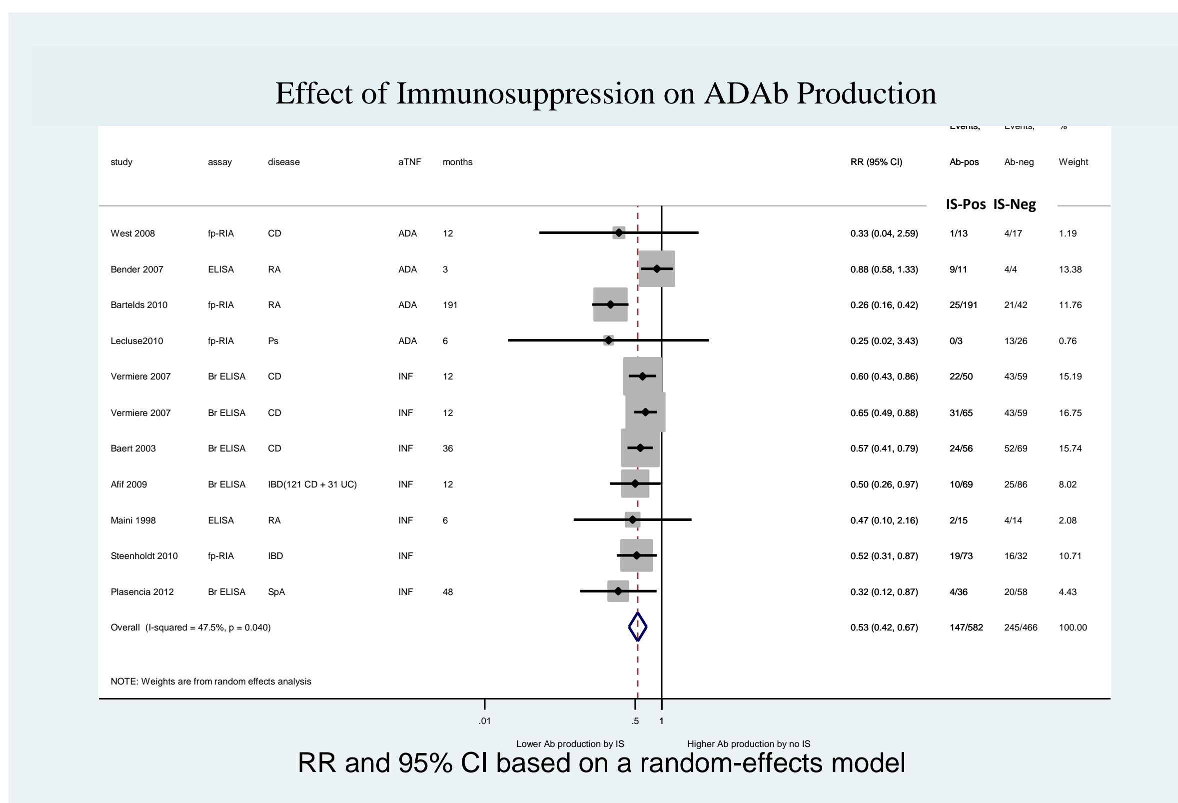

Figure 19 – Effect of immunosuppression on ADAb production (by excluding "Bartelds 2007" and "Bartelds 2011")

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; UC, Ulcerative Colitis.

Note: By leaving the study "Bartelds 2010" we introduced more heterogeneity in the pooled analysis, in comparison with Figure 18.

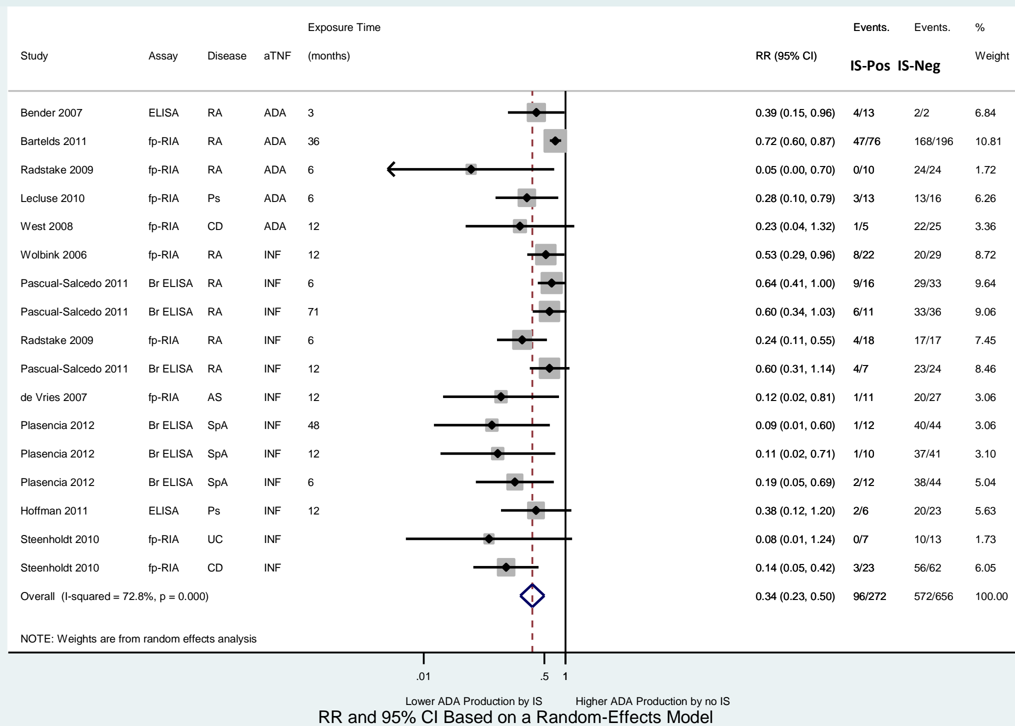

Figure 20 – Effect of immunosuppression on ADAb production (by excluding "Bartelds 2007" and "Bartelds 2010")

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; UC, Ulcerative Colitis.

Note: By leaving the study "Bartelds 2011" we introduced more heterogeneity in the pooled analysis, as compared with Figure 18.

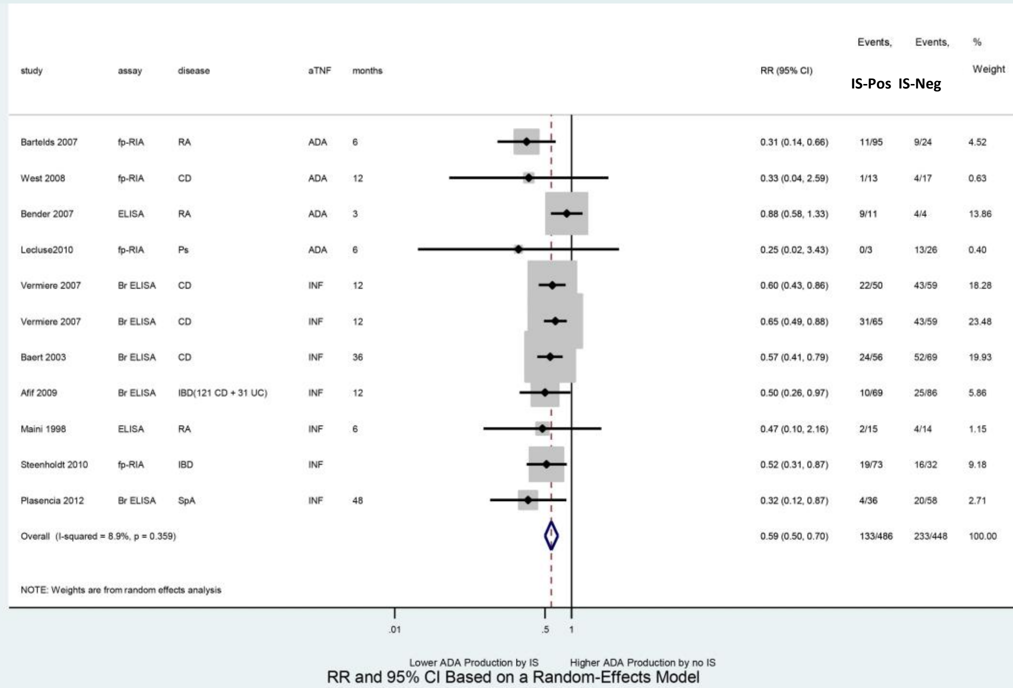

Figure 21 – Effect of immunosuppression on ADAb production

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn’s Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; UC, Ulcerative Colitis.

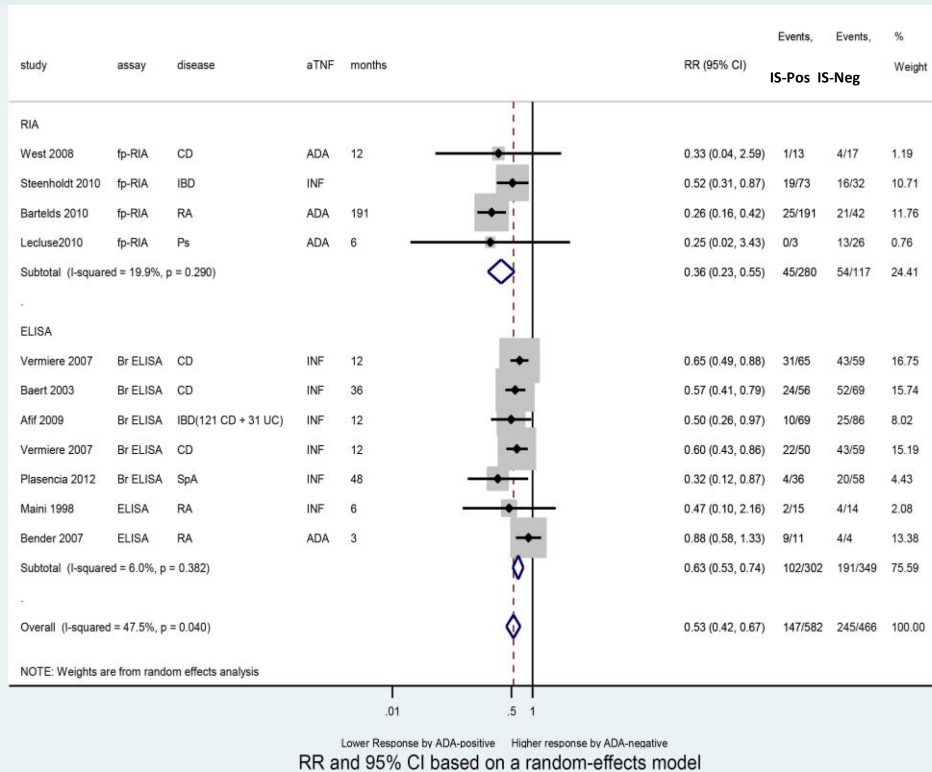

Figure 22 – Effect of immunosuppression on ADAb production (by assay)

Legend: ADAb, anti-drug antibodies; AS, Ankylosing Spondylitis; Br ELISA, Bridging enzyme-linked immunosorbent assay; ELISA, Enzyme-linked immunosorbent assay; fp-RIA, Fluid-phase radioimmuno assay; CD, Crohn's Disease; INF, Infliximab; Ps, Psoriasis; RA, Rheumatoid Arthritis; SpA, Spondyloarthritis; UC, Ulcerative Colitis.

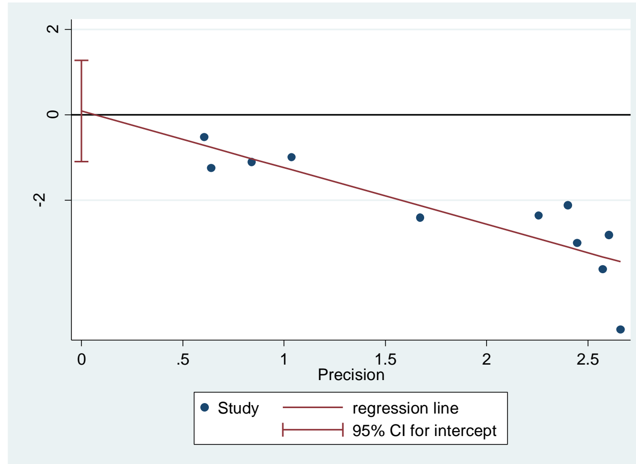

Figure 23 – Egger's publication bias plot for the effect of IS on ADAb production

Legend: ADAb, anti-drug antibodies; IS, immunosuppressors.

3.2 THE IMPACT OF IMMUNOGENICITY ON DRUG SAFETY PROFILE

3.2.1 Introduction

Biologics, and particularly monoclonal antibodies, are structurally immunogenic, as virtually all of them exhibit epitopes that can elicit an immune response.

Over the last years, an increasing body of evidence has highlighted the clinical significance of drug immunogenicity. Previously, we formally established the impact of anti-drug antibodies (ADAb) on therapeutic responses [496]. However, ADAb have also been associated with increased incidence of drug-related adverse events (AE) [160, 210, 211, 213, 458, 495, 497-501]. Acute infusion reactions occur during or within 1 h after infusion, while delayed reactions may occur from 1h to 14 days post-infusion [502, 503]. AE to biological are clinically very heterogeneous. The majority of those adverse reactions are mild (headache, flushing, nausea) but moderate or even severe reactions, with dyspnoea, angioedema, wheezing and stridor may also occur [502, 504-509]. Different mechanisms may underlie AE to biologics, and a specific classification based on those mechanisms have been proposed, aiming to guide clinicians in the management of such adverse reactions [503]. However, that classification is not easily applicable in clinical practice and the management of AE, as the decision to rechallange or discontinue treatment, has been based on the severity of the symptoms [505]. Severe reactions usually impose treatment discontinuation, while mild-to-moderate reactions are usually managed by temporary infusion interruption, reduction of the infusion rate and symptoms management, with re-treatment schemes. Those re-treatment schemes do not minimize adverse reactions in all patients and it medium-long term effectiveness has not been established [510]. Beyond the management of AE, the identification of markers able to predict the higher risk

patients to develop AE to biologics is warranted. The presence of ADAb might well represent a biomarker able to identify patients at higher risk to develop AE and those where re-treatment schemes might not be advisable, as rechallenge may boost the immune response to biologic. Therefore, immunogenicity assessment might represent a tool to help us preventing important adverse events, improving the biologics' management and their safety profile.

3.2.2 Objectives

We aim to evaluate the association between acute infusion-related adverse events (IrAE) and the presence of ADAb in patients with chronic immune-mediated inflammatory diseases receiving Infliximab. Secondarily, we assessed therapeutic response maintenance in patients who had IrAE.

3.2.3 Methods

During a period of 2 years (January 2010 - December 2011) we followed all adult patients (≥18 years) with Rheumatoid Arthritis (RA), Ankylosing Spondylitis (AS), Psoriatic Arthritis (PsA) and Inflammatory Bowel Disease (IBD), receiving infliximab 3-5mg/Kg i.v. at 0, 2, 6 and every 6 or 8 weeks thereafter, in monotherapy or with concomitant immunosuppressors, at the Department of Rheumatology and Department of Gastroenterology of Hospital Garcia de Orta, Portugal. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA, the Assessment of SpondyloArthritis international Society (ASAS) 2009 classification criteria for spondyloarthritis or clinical, endoscopic, histological and/or radiological criteria (ECCO criteria) for CD or UC [511-514]. All patients followed the Portuguese recommendations for the management of RA, SpA or IBD patients receiving biologic

therapies[515-518]. The study was approved by the hospital ethics committee. All patients gave written informed consent.

Drug immunogenicity was assessed every 3-6 months. Through serum samples were collected just before the next administration of a biologic. Serum drug trough levels were measured by ELISA, using a setup as described before [462, 519]. Limit of detection was determined by testing 100 sera of RA patients before treatment. The mean +/- 6 times standard deviation (SD) was chosen as a cut off. Limit of detection was about 2 ng/ml for all TNFi. ADAb were tested by Bridging ELISA as described before [209]. The sensitivity of these assays depends on the affinity of the ADAb. We used a series of patient-derived monoclonal antibodies to establish the sensitivity of the assay. A monoclonal antibody to adalimumab with the median affinity showed reached half-maximal extinction at 10 ng/ml and a detection limit <1 ng/ml. However non-specific binding of serum components such as rheumatoid factor or C1q sets the limit of detection for sera at about 20 ng/ml.

Acute infusion-related adverse event was defined as any abnormal sign or symptom occurring during or within two hours after the infliximab infusion.

Therapeutic response was defined according to the European League Against Rheumatism (EULAR) criteria for RA and RA-like PsA [466]; ASAS group guidelines for AS and AS-like PsA [513, 514]; and by an expert clinician for IBD patients.

3.2.4 Results

The study included 94 consecutive patients (22 AR, 33 AS, 9 PsA and 30 IBD), 76% females, with mean (SD) age of 48 (10.2) years, disease duration of 8 (6.4) years, and receiving biologic

therapy by 2.9 (2.0) years. Patients were receiving infliximab at 3-5 mg/Kg every 6 or 8 wks. All RA patients and 89% of PsA were receiving concomitant methotrexate (MTX), at a dose range of 10-20mg every week. IBD patients were receiving concomitant azathioprine, at a dose range of 2-2.5 mg/Kg/day and additionally 100 mg i.v. hydrocortisone, 2 mL i.v. clemastin and 500 mg i.v. acetaminophen prior to each Infliximab infusion.

During the follow-up period, twelve patients (13%) developed an overt IrAE (4 RA patients, 2 PsA, 2 AS and 4 IBD patients), between the fourth and tenth infusion – Figure 24. All the reactions were mild-moderate requiring hydrocortisone and anti-histaminic administration. Twenty-five patients (27%) had detectable ADAb (41% of RA, 33% of PsA, 18% of AS and 23% of IBD patients) – Figure 25.

Nearly half (48%) of ADAb-positive patients developed an IrAE, while none of ADAb-negative patients had acute adverse reactions to infliximab – Figure 26. Notably, in all cases, except two, the detection of ADAb occurred prior to the IrAE. In these two exceptional cases, we had no access to the patients' serum before the development of IrAE. All patients who developed acute IrAE were unable to maintain therapeutic response over time (one year) and re-treatment schemes did not prevent treatment failure. Furthermore, in 4 patients re-treatment increased the severity of adverse reaction (chest pain, severe hypotension).

Although not planned in the initial study protocol, we assessed serum drug levels every week between two infliximab administrations in the four IBD patients who had an IrAE. We intended to evaluate drug kinetics between two infliximab administrations in patients who had a mild IrAE and were kept in the same treatment. Notably, in three patients serum drug levels become undetectable already from week 2 after the infusion of 5mg/Kg of infliximab. In one patient the

drop in drug levels was slower, but still, no circulating drug was detected from week 5 after treatment – Figure 27.

3.2.5 Discussion

In the present study, acute IrAE to Infliximab occurred exclusively in patients with detectable ADAb and nearly half of ADAb-positive patients developed an adverse reaction. Notably, in all cases ADAb could be detected prior to the IrAE and none of those patients were able to maintain therapeutic response over time.

Despite the great benefit that biologic therapy brings to the majority of patients with chronic inflammatory diseases, they are not always effective. Moreover, they can cause a great variety of adverse side effects. Different type of IrAE may occur with the use of therapeutic proteins, requiring different approaches. There are several known mechanisms by which a therapeutic protein can induce an acute IrAE [32, 503]. Those adverse events may be related to the drug itself and to its molecular structure, or they may be related to the drug's target and biologic activity [520].

The great bulk of biologic therapies are represented by monoclonal antibodies. Antibodies are structurally immunogenic, even the fully human ones. The association between ADAb and acute IrAE have been extensively reported in the Literature, over the last years [502, 504-509]. As in our study, the vast majority of acute IrAE occurred in ADAb-positive patients [210, 211, 458, 501, 521]. Exception is a study published by Rutgeerts et al, where a similar incidence of acute IrAE to infliximab was found between ADAb-positive and –negatives [167]. In this study, an

ELISA with low specificity and sensitivity was used to assess ADAb, and false positive or negative results cannot be excluded.

The development of ADAb does not necessarily lead to drug adverse events, but in our study nearly half of ADAb-positive patients developed an acute IrAE, which is highly significant. Other groups have also reported extremely high incidence of IrAE among ADAb positives, ranging from 32% to 80% [210, 211, 458, 501, 521].

The presence of ADAb can be detected from the very beginning of infliximab treatment and before a clinically overt adverse reaction, as we also confirmed [210, 211, 458, 501, 521]. This fact is of high clinical relevance because it highlights the predictive value of ADAb for the development of IrAE. ADAb titres tend to increase with treatment continuation and IrAE have been associated mainly, although not exclusively, with the highest ADAb titers [210, 213, 521, 522]. This may also explains why all patients with IrAE were not able to sustain therapeutic response over time.

The majority of acute IrAE occur after the third infusion, which suggests a sensitization phase as occur in IgE-mediated hypersensitivity reactions. We did not assess IgE-ADAb in our study but previous studies have demonstrated that the great bulk of ADAb are of IgG isotype and not of IgE [461, 523]. Even though, IgE antibodies to infliximab and other biologics have been reported, often in association with a positive skin test [500, 501, 524-527]. Both IgE and non-IgE acute reactions are clinically undistinguishable, despite the former tend to induce more severe reactions and to occur at the very beginning of the infusion course [501]. Virtually all the acute IrAE are usually accompanied by symptoms suggestive of anaphylatic reaction [506-509, 521, 527, 528]. However, the mechanism behind might be an alternative pathway IgE-

independent, mediated by IgG, FcγRIII, macrophages, basophils and platelet activating factor (PAF) [529, 530]. Infliximab-specific IgG may also lead to another effector mechanism involving complement activation and the production of anaphylatoxins (complement-cleavage products), which may or may not activate mast cells unspecifically. A mast cell-independent pathway may explain the absence of increased serum tryptase levels reported in many cases of biologic-related anaphylaxis [531].

Although IgE-mediated IrAE to infliximab seem to represent a minor proportion of the acute adverse events, it would be clinically relevant to confirm a true IgE-mediated anaphylaxis, as those reactions are potentially more severe and cannot be avoided with pre-medication [524, 532]. These patients should not be kept in the same therapy. An *in vitro* assessment of IgE-ADAb and an *in vivo* skin test have been proposed to identify the IgE-mediated IrAE [501, 521]. However, IgE-ADAb assessment is technically challenging due to low serum concentration of IgE antibodies and interference of IgG-ADAb antibodies. Further assays' optimization need to be conducted in order to better assess different ADAb isotypes. Skin testing protocols have been proposed, which despite the high specificity (90%) still have low sensitivity (26%). Additionally, positive skin tests became negative over time, meaning that they need to be performed shortly after an immediate IrAE [501].

In our study, as in others in the literature, all patients with IrAE had detectable ADAb [210, 213, 458]. However, IrAE have also been reported in ADAb-negative patients, suggesting an immunogenicity-independent mechanism [160, 211, 500]. Supporting this notion is the fact that some patients develop acute IrAE at the first infusion and can be successfully retreated by reducing the rate of infusion [531, 533]. Most likely, a cytokine release might be on the basis of

such acute reactions, which may be clinically indistinguishable from type I hypersensitivity [499]. Although the precise mechanism underlying this syndrome is not fully understood, it has been hypothesized as being related to the cross-linking of therapeutic monoclonal antibodies bound to target cells with subsequent complement activation and apoptosis of the target cell, leading to cytokine and chemokines release. In contrast to type I hypersensitivity reactions, cytokine-release syndromes can be managed by short-term cessation of biologic infusion, administration of histamine blockers and corticosteroids, restarting the infusion at a slower rate [534-536]. A pre-existing sensitization towards some additives may be a further mechanism accounting for some acute IrAE during the first infusion of the biologic. This event has been described for polysorbate, used for rapid solubilisation of pharmaceuticals in aqueous solution [537, 538].

In our study all the IrAE occurred from the fourth infliximab infusion and were all associated with ADAb, which explains the recurrence of acute reactions despite pre-medication and infusion-rate reduction.

This study has also some limitations. It is a small, single-centre observational study, with possible bias. However, our studies are in clear agreement with other larger studies published in the literature. We did not access ADAb titers neither isotypes, as our primary intention was to simply verify the association between the presence of ADAb and acute IrAE.

Our study demonstrates a clear association between ADAb to infliximab and acute IrAE, highlighting the predictive value of ADAb for adverse reactions. Our results stress the high clinical value of routine immunogenicity assessment, which represents a powerful tool to optimize the management of biologics and their safety profile.

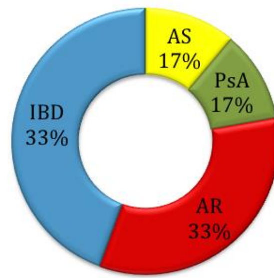

Figure 24 – Patients with infusion-related adverse events (IrAE)

Legend: During the follow-up period 12 patients had an IrAE, 4 (33%) of RA, 2 (17%) of PsA, 2 (17%) of AS and 4 (33%) of IBD patients

AS, Ankylosign Spondylitis; IBD, Inflammatory Bowel Diseases; PsA, Psoriatic Arthritis; RA, Rheumatoid Arthritis

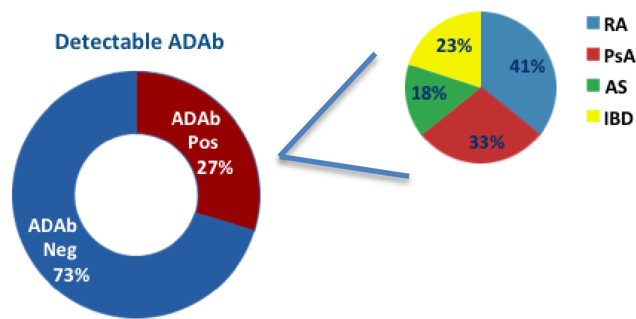

Figure 25 – Patients with detectable ADAb

Legend: During the follow-up period 25 (27%) patients developed ADAb, 41% of RA, 33% of PsA, 18% of AS and 23% of IBD patients

ADAb, Anti-Drug Antibodies; AS, Ankylosign Spondylitis; IBD, Inflammatory Bowel Diseases; PsA, Psoriatic Arthritis; RA, Rheumatoid Arthritis

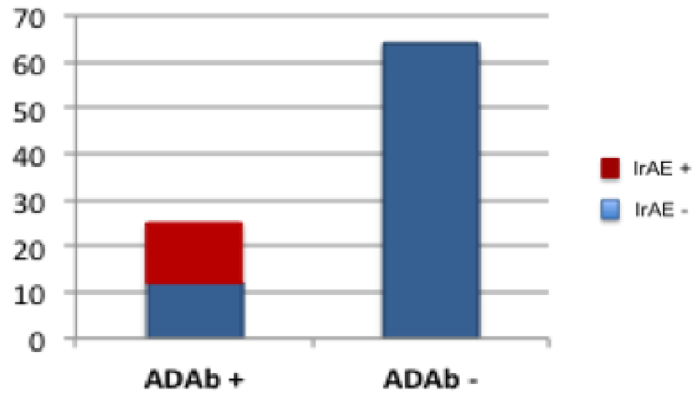

Figure 26 – Proportion of patients with IrAE among ADAb-positives and ADAb-negatives

All patients with IrAE had detectable ADAb. None IrAE were described among ADAb-negative patients whereas 48% of ADAb-positive patients developed an IrAE

ADAb, Anti-Drug Antibodies; IrAE, Infusion.Related Adverse Events

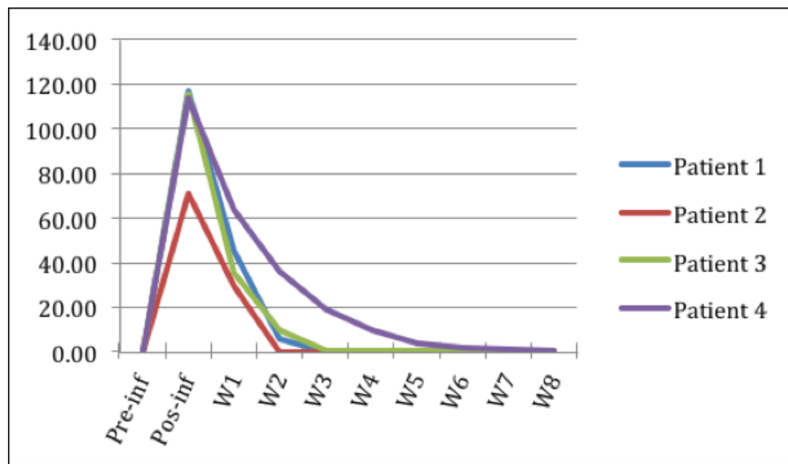

Figure 27 – Serum drug levels at every week between two infliximab infusions in 4 IBD patients who had IrAE

Legend: Four IBD patients who had an IrAE were selected to measure serum drug levels (ug/mL of infliximab) between two infliximab administrations. Three patients had undetectable serum drug levels from week 2 and one from week 5.

Pre-inf, Pre-infusion; Pos-inf, Pos-infusion; W, Week

3.3 IMMUNOGENICITY ASSESSMENT IN ROUTINE CLINICAL PRACTICE

3.3.1 Introduction

The immunogenicity of biologics has been associated with therapeutic failures and adverse events. ADAb have been detected before overt adverse reactions to biologics, which reveals an important predictive value in preventing those reactions. Moreover, immunogenicity assessment may help us to identify the reason for therapeutic failure in non-responder patients, which might be relevant for future therapeutic decisions [484, 539].

Despite all the advances in predicting immunogenicity at pre-clinical phases of drug development, it has become evident that the long-term use of biologics in clinical practice seems to induce a grade of immunogenicity different from that initially predicted. Such fact has attracted the attention of regulatory agencies, which have emitted guidelines on immunogenicity assessment beyond the approval phase [191].

Despite that, immunogenicity assessment is still not part of the routine clinical practice. This deficiency may be partially explained by technical issues of the available monitoring assays. The assessment of drug immunogenicity is technically challenging. Most commonly, ADAb are detected by radioimmunoassays (RIAs), specifically tailored to detect antibodies against therapeutic antibodies. Several formats of RIAs exist and they are considered by many as the "gold-standard" to assess ADAb. However, RIAs represent a method of high complexity and low throughput, which also imposes safety concerns related to the handling of radioactive material, limiting its use on a routine basis. To overcome these limitations a particular ELISA have been developed, using a bridging format, based on the principle that bivalent ADAb will crosslink the biologic (drug) coated on plate and the biotinylated biologic (drug) that will be

added as secondary reagent. This “bridging antibody” format implies that ADAb must be recognized twice for their detection, which increases the assay’s specificity. On the other hand, it may prevent the detection of ADAb of IgG4 isotype. Human IgG4 tend to exchange half of the molecule with other IgG4 molecule that can be of different specificity, creating a monovalent, bispecific antibody that loose bridging ability [17, 208]. Even though IgG4 represent a minor proportion of total IgG and ADAb of exclusively IgG4 isotype are unlikely to occur. Taken together, this indicates that ELISA format may represent a convenient method to use in the routine clinical practice. Additionally, it represents a simple and relatively cheap method that does not require special equipment and laboratory conditions to its implementation.

3.3.2 Objectives:

This study aims to compare the proportion of ADAb-positive patients assessed by RIA-ABT and by Bridging ELISA, in a cohort of 110 patients with chronic inflammatory diseases receiving TNFi. Secondarily we evaluated: a) the proportion of IgG4-ADAb among total IgG-ADAb; b) the presence of ADAb at least three months after therapy discontinuation; c) IgM-ADAb to etanercept.

3.3.3 Methods:

3.3.3.1 Patients

Consecutive patients with diagnosis of Rheumatoid Arthritis (RA), Ankylosing Spondylitis (AS), Psoriatic Arthritis (PsA) and Inflammatory Bowel Diseases (IBD) were included.

All patients were followed at the day hospital of Hospital Garcia de Orta, Almada, receiving infliximab i.v. at a dose ranging from 3-5mg/Kg every 6-8 weeks, adalimumab 40 mg eow,

etanercept 50 mg every week, according to national recommendations for the use of biologic therapy to each disease [515-518].

The study was approved by the hospital ethics committee. All patients gave written informed consent.

Serum samples were collected in trough, immediately before the next drug administration.

3.3.3.2 ELISA to assess serum drug levels

TNFi levels were measured by Elisa developed at Sanquin, Amsterdam, the Netherlands, using a setup as described before [212, 462, 519] – Figure 28. Briefly, a mouse monoclonal anti-TNF antibody was pre-incubated overnight onto microtitre plates. Thereafter, recombinant TNF (10ng/mL) in High Performance ELISA (HPE) buffer was added. After one hour plates were washed with phosphate buffered saline 0.02% Tween. Subsequently patient serum sample in different dilutions was added and incubated for 1 hour at 37°C. After washing the plates with phosphate buffered saline 0.02% Tween plates were incubated with biotinylated polyclonal rabbit antibodies against infliximab or adalimumab or etanercept in HPE buffer for 1 hour at 37°C. After washing the plates with phosphate buffered saline 0.02% Tween, poly-HRP streptavidin was added for 30 min at 30°C, followed by incubation with tetramethylbenzidine (TMB). Afterwards reaction was stopped and absorption at 450 nm was assessed. Test results were reading out of a titration curve infliximab or adalimumab or etanercept, which was present in each plate. Limit of detection was determined by testing 100 sera of RA patients before treatment. The mean +/- 6x SD was chosen as a cut off. Limit of detection was about 2 ng/ml.

Because relevant serum levels of these drugs are > 1 μg/ml, sera can be easily tested at 1:100 dilution.

3.3.3.3 Bridging ELISA to assess ADAb

ADAb against infliximab were measured by Bridging ELISA as described previously [209, 212] - Figure 29. In short, maxisorp ELISA plates (NUNC) were coated overnight with infliximab or adalimumab or etanercept in PBS at room temperature. After washing 5 times with PBS/0.02% Tween, plates were incubated with patient's serum, serially diluted in High Performance ELISA (HPE) buffer, from Sanquin, Amsterdam, the Netherlands) for 1 h. The highest serum concentration was 10%. All incubations were performed at room temperature in an assay volume of 100 uL. Subsequently the plates were washed in PBS/0.02% Tween and incubated for 1 hour with biotinylated infliximab or adalimumab or etanercept in HPE for one hour. After washing, streptavidin-poly-HRP (Sanquin) (1/10 000 in HPE) was added for 20 minutes for detection. After washing with PBS Tween, the ELISA was developed with 100ug/mL tetramethylbenzidine (TMB) in 0.11 M sodium acetate (pH 5.5) containing 0.003% (v/v) H2O2. The reaction was stopped with 2M H2SO4. Absorption at 450 nm was measured with an ELISA reader (Multiskan; Titertek, Elfab Oy, Filand). The sensitivity of these assays depends on the affinity of the ADAb. Results were related to a titration curve of infliximab- or adalimumab- or etanercept-specific rabbit serum containing anti-infliximab or anti-adalimumab or anti-etanercept antibodies in each plate. The lowest level of detection was 1ng/mL. However non-specific binding of serum components such as rheumatoid factor or C1q sets the limit of detection for sera at about 20 ng/ml, which when converted to arbitrary units set the limit of detection in 2AU/mL.

3.3.3.4 RIA-Antigen Binding Test (ABT) to assess total IgG-ADAb

ADAb of IgG isotype were measured by RIA-ABT as described previously [209, 462] – Figure 29. One uL of serum diluted in PBS/0.3% bovine serum albumin (BSA) (PA buffer) was incubated over night with 1 mg Sepharose-immobilized protein A (GE healthcare, Chalfont St. Giles, UK) in a final volume of 800 uL for total IgG detection. Subsequently, the samples were washed with PBS 0.005% Tween and specific ADAb binding was detected by over night incubation with 20 000 dpm (approximately 1 ng) 125I-labeled F(ab')2 infliximab or adalimumab or etanercept diluted in Freeze buffer (Sanquin). Unbound label was removed by washing, and protein A bound radioactivity was measured. When binding was more than 25% of the input, sera were further titrated. Antibody levels were compared to a standard rabbit serum containing anti-infliximab or anti-adalimumab or anti-etanercept antibodies and expressed in arbitrary units (AU). One AU corresponds to approximately 12 ng. In the etanercept assay, serum from an etanercept-vaccinated rabbit served as positive control. The lower limit of detection for ADAb was determined as 6 SD above the average signal measured for a panel of 100 RA patients that were not yet treated with TNFi and corresponds to 12AU/mL [455].

3.3.3.5 RIA-Antigen Binding Test (ABT) to assess specific IgG4 or IgM-ADAb

Antibodies to infliximab or adalimumab or etanercept of IgG4 subclass were measured as described before [15] – Figure 29. Briefly, 80 mg anti-human IgG4 monoclonal antibody (MH164-1, Sanquin) or anti-human IgM monoclonal antibody was coupled to 8g CNBR-activated sepharose (GE Healthcare). Per test 1 mg sepharose was incubated with serm in 750 uL. After washing, IgG4 ADAb was detected by incubation with 125I-labeled F(ab')2 of infliximab or adalimumab or etanercept in Freeze buffer. Antibody levels were compared to a

titration curve of infliximab- or adalimumab- or etanercept-specific rabbit serum containing anti-infliximab or anti-adalimumab or anti-etanercept antibodies, and expressed as arbitrary units (AU). The lower limit of detection was 19 AU/mL, determined as described above.

3.3.3.6 RIA-Antigen Binding Test (ABT) to assess specific IgM-antietanercept

IgM antibodies to etanercept were detected by using a RIA-ABT as above described, although instead of using anti-human IgG, an anti-human IgM was used.

3.3.3.7 Statistical Analysis:

Descriptive statistic was used and data is presented as values, proportions, mean (standard deviation) or median (interquartile range), as appropriate.

3.3.4 Results:

We evaluated 110 patients (12 PsA, 43 RA, 22 IBD and 33 AE), 63% female, with mean (SD) age of 44 (13). Eighty-two patients were receiving infliximab, 13 patients adalimumab and 15 patients were treated with etanercept, with a mean (SD) biologic duration of 3.2 (1.4) years.

A total of 26 patients (24%) tested positive for ADAb in RIA-ABT (IgG total): 21anti-infliximab and 5 anti-adalimumab. All those patients had also detectable ADAb measured by Bridging ELISA. No anti-etanercept antibodies were detected in any of the assays, either of IgG or IgM isotype. Among the 26 ADAb-positive patients, 16 (62%) also tested positive for IgG4-ADAb – Figure 30. Eight patients had similar ADAb titres assessed by both total IgG-RIA and specific IgG4-RIA, while the remaining 8 had IgG4-ADAb of significant lower magnitude than

total IgG-ADAb, suggesting the presence of other IgG isotype rather than IgG4 – Figure 31. No ADAb of exclusively IgG4 isotype were detected.

All ADAb-positive patients had undetectable serum trough drug levels and only one patient had undetectable etanercept levels in the absence of ADAb. Compliance was evaluated in that patient and nearly six months after, serum etanercept trough levels had normalized.

We tested ADAb in 16 patients who had discontinued TNFi (8 adalimumab and 8 etanercept) for more than 3 months. In 3 out of 8 adalimumab patients, anti-adalimumab remain detectable in both RIA-total IgG and Bridging ELISA. No anti-adalimumab of IgG4 isotype were detected in those patients. Among etanercept patients, anti-etanercept antibodies remain undetectable even three months after therapy discontinuation.

3.3.5 Discussion

In out study Bridging ELISA was able to detect the same ADAb-positive patients as RIA-ABT, even in cases where IgG4 isotype represented a significant proportion of total ADAb. ADAb exclusively of IgG4 isotype were not detected. Low amounts of circulating drug prevent ADAb detection by Bridging ELISA but also by RIA-ABT, denoting significant drug interference in both assays. Therefore, all ADAb-positive patients had undetectable drug levels. No anti-etanercept antibodies (IgG or IgM) could be detected by any of the assays. In some patients ADAb remain detectable several months after therapy discontinuation, which was not verified for etanercept.

The increasing evidence of the impact of drug immunogenicity in clinical practice has propelled the development of better and simpler assays able to monitor immunogenicity on a routine basis.

So far, on of the most optimized assays to specifically assess ADAb to biologic drugs have been the RIA-ABT. This method has proven to be a method with reasonable sensitivity and high specificity to assess ADAb. However, as stressed before, one of the major drawbacks of RIA is the requirement of high doses of radioactivity, special laboratory conditions and relatively low throughput. Several companies have done great efforts in the optimization of ELISA-based assays to assess immunogenicity, given it simplicity, relatively low price and high throughput. For this work we used two ELISA-based assays developed at Sanquin, Netherlands, with whom we established a partnership for the use and optimization of these assays, specially tailored to monitor drug levels (simple ELISA) and ADAb (Bridging ELISA).

Previous work has demonstrated that Bridging ELISA is more sensitive than RIA-ABT for ADAb detection, although more susceptible to drug interference [209]. Consequently, Bridging ELISA can only detect ADAb in the absence of circulating drug. RIA-ABT or IgG4-RIA are also highly affected by the presence of the drug, though less than Bridging ELISA. This explains why in some studies higher proportion of ADAb positive patients have been detected in RIA-ABT, when compared to Bridging ELISA [209]. However, in all those cases the ADAb levels are low and not able to fully neutralize the drug. The clinical significance of such low ADAb titres is not so clear and remains to be established [462]. In our study, we detected the same positive patients in both assays, Bridging ELISA and RIA-ABT. This might be due to the fact that, in contrast to the above mentioned studies, we used a cohort with relatively long treatment duration and relatively high ADAb levels.

Because the detection of ADAb by Bridging ELISA is only possible in the absence of drug levels, some authors have claimed that Bridging ELISA do not offer additional information to

the assessment of drug levels by using a simple ELISA. However, other reasons may underlie undetectable drug levels, namely patient's non-adherence to the therapy. Increasingly evidence has revealed that patient's non-adherence represents a major problem in our society, being even more dramatic in patients with chronic diseases [540]. Undetectable drug levels in the absence of detectable ADAb by Bridging ELISA may easily reveal those patients. In our small study we identify one patient in such conditions, to whom an educational reinforcement about the importance of compliance to those treatments was applied. Few months after, at the following evaluation, drug levels had returned to normal values.

A significant proportion of ADAb-positive patients also tested positive for IgG4 isotype, which is in agreement with previous studies [193, 209]. Repeated antigenic stimulation may lead to IgG4 class switch [16]. As mentioned before, IgG4 is able to exchange half of the molecule with another IgG4 molecule of different specificity, creating a monovalent antibody that cannot bridge [17, 208]. Even though, IgG4 represents a small fraction of total IgG and in presence of significant amount of IgG4-ADAb, it is likely that the Fab arm exchange occur with another IgG4 of the same specificity. This may explain why in our study all patients who tested positive for IgG4-ADAb had also tested positive in Bridging ELISA. Moreover, ADAb of exclusively IgG4 isotype is not common and often ADAb of IgG1 isotype co-exist. However, some patients have tested positive in IgG4-RIA and not in RIA-ABT, which can be simply explained by different drug interference with both assays [209].

No anti-etanercept antibodies could be detected either by RIA-ABT or Bridging ELISA, which is consistent with many other studies that have used more recent and optimized assays [496]. Etanercept is able to neutralize both TNFa and Lymphotoxin-alpha (LTa). Among other

important functions in the immune system, LTa is important for germinal centre formation [489]. Though hypogammaglobulinaemia have not been associated with etanercept treatment, still, we decided to assess anti-etanercept of IgM isotype, with negative results. As etanercept is administered to patients more often than infliximab or adalimumab (every week; e6-8w; eow, respectively), we wonder if residual circulating drug was interfering with ADAb detection. However, no anti-etanercept antibodies were found even several months after therapeutic withdrawal, which has also been reported by others [478]. On the other hand 3 out of 8 patients previously exposed to adalimumab, remain ADAb positive years after therapy discontinuation, assessed by Bridging ELISA and by RIA-ABT but not by IgG4-RIA. ADAb status in those patients, at the time of therapeutic failure, was not known. We cannot exclude that in some patients ADAb became undetectable over time after stopping therapy. However, our results highlight the notion that these ADAb are hypermutated, high affinity and class switch antibodies, resulting from a mature immune response that induces immunologic memory. Such fact is of high clinical relevance, as sometimes patients return to previous treatment long after its discontinuation. Re-challenging these patients with the same antigenic drug may boost the immune response against the drug with important adverse consequences.

Our study represents a very small-scale study, which was not designed to detailed comparissons between the above mentioned assays. However, our main purpose was to validate in our lab the performance and the easy implementation of this ELISA-based therapeutic monitoring (ELISA and Bridging ELISA), in order to evaluate to which extend these two assays can be proposed to monitor immunogenicity in routine clinical practice. We concluded that Bridging ELISA is a simple and effective method in detecting clinically significant immunogenicity, though not being able to detect ADAb of low levels and in the presence of residual circulating drug levels.

However, the clinical significance of those low levels is not entirely clear, as some patients are able to maintained such low titres with no apparent clinical consequences. Thus, only those patients with undetectable drug levels should be assessed for ADAb, by using Bridging ELISA. The assessment of ADAb in such conditions will help us to distinguish immunogenicity from non-adherence, which requires different approaches. This type of therapeutic monitoring represents a powerful tool to optimize the use of biologic therapies.

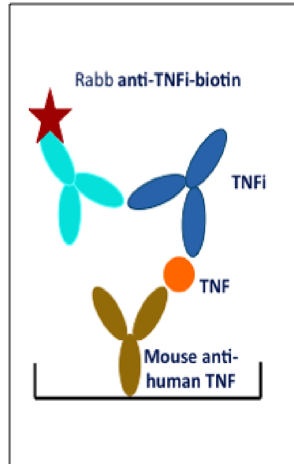

Figure 28 – ELISA to assess serum drug levels

Legend: TNF, Tumor Necrosis Factor; TNFi, Tumor Necrosis Factor Inhibitor

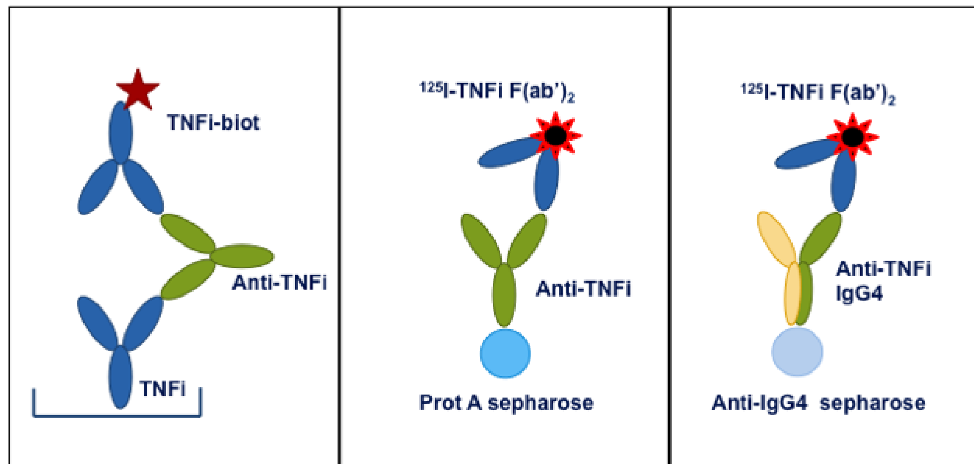

Figure 29 – Assays used to measure ADAb

Legend (from the left to the right): Bridging ELISA; RIA-ABT using Protein A sepharose able to detect total serum IgG; IgG4-RIA-ABT using anti-IgG4 sepharose to specifically assess serum IgG4.

TNF, Tumor Necrosis Factor; TNFi, Tumor Necrosis Factor Inhibitor

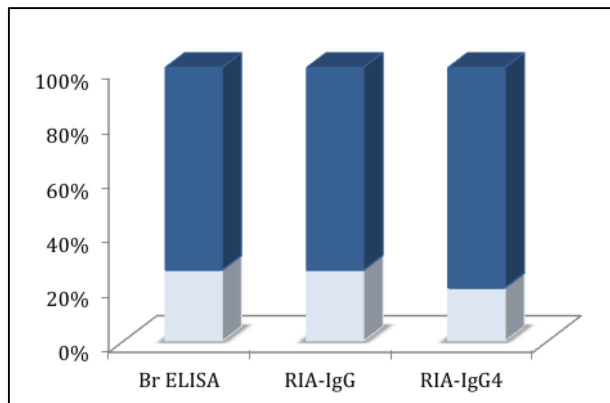

Figure 30 – Proportion of ADAb-positive patients detected in each assay.

Legend: Twenty-six out of 110 patients (24%) tested positive for ADAb in Bridging ELISA and in RIA-ABT (IgG total). Sixteen (62%) also tested positive for IgG4-ADAb in IgG4-RIA-ABT.

ABT- Antigen Binding Test; ADAb, Anti-drug antibodies; Br ELISA, Bridging Enzyme-Linked Immunosorbent Assay; RIA, Radioimmuno assay

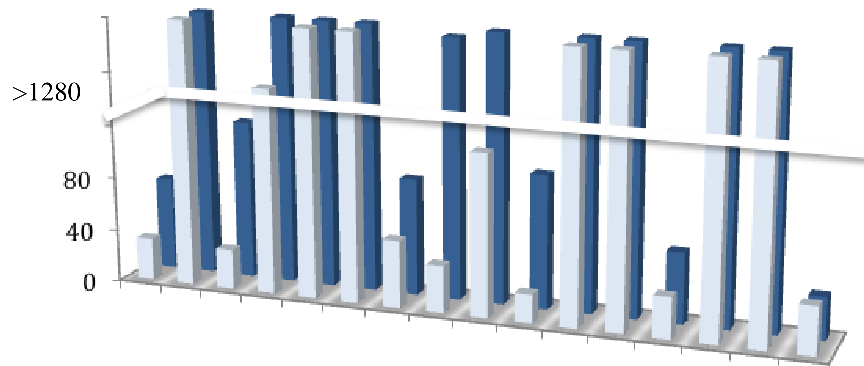

Figure 31 – ADAb titres assessed by IgG-RIA-ABT and specific IgG4-RIA-ABT

Legend: Eight out of 16 patients had similar ADAb titres (AU/mL) assessed by both total IgG-RIA and specific IgG4-RIA, while the remaining 8 had IgG4-ADAb of significant lower magnitude than total IgG-ADAb.

ABT- Antigen Binding Test; ADAb, Anti-drug antibodies; Br ELISA, Bridging Enzyme-Linked Immunosorbent Assay; RIA, Radioimmuno assay

3.4 A PRELIMINARY ALGORITHM INTRODUCING IMMUNOGENICITY ASSESSMENT IN THE MANAGEMENT OF RA PATIENTS RECEIVING TUMOR NECROSIS FACTOR INHIBITORS THERAPIES

3.4.1 Introduction

The "treat to target" strategy, now part of the European League Against Rheumatism (EULAR) recommendations, has revealed the importance of an early tight control of disease activity among Rheumatoid Arthritis (RA) patients [404, 541-543]. To reach clinical remission or at least low disease activity, fast and assertive therapeutic decisions are required. No formal recommendations exist to guide the order of the sequence of biologics, particularly after the failure of previous Tumor Necrosis Factor Inhibitors (TNFi), which represents a common situation in daily practice. The effectiveness of cycling between different TNFi is controversial and currently, the decision to switch mechanism of action (MOA) is largely empirical.

Drug immunogenicity has been proposed as one of the main mechanisms behind biologic therapeutic failure [153, 210, 213, 461]. We recently conducted a systematic review and meta-analysis concluding that anti-drug antibodies (ADAb) are clinically relevant and lead to significant decrease of therapeutic response rates. [496]

The presence of ADAb results in functional neutralization of the drug and formation of immune complexes that promotes a faster clearance of the drug from circulation [193, 544]. ADAb-positive patients exhibit very low or undetectable serum drug trough levels, in contrast to ADAb-negative patients, who often have normal or even high serum drug trough levels [210, 455, 472, 519].

Non-responder patients, who exhibit adequate serum drug levels and no detectable ADAb, have lower probability of response to another agent with the same MOA and may benefit in switching to a drug with a different MOA [484]. Non-responders, who have no detectable serum trough levels and detectable ADAb, may benefit in switching to a less immunogenic drug [539]. These patients may have a higher probability of developing ADAb against the new biopharmaceutical [484]. Neutralizing ADAb against etanercept or abatacept have not been detected [193, 496, 545].

The added value of assessing immunogenicity in current clinical practice has been questioned. Based on available evidence we designed a preliminary algorithm that introduces immunogenicity assessment in the current clinical approach to RA patients receiving biologic therapies – Figure 32. We propose to evaluate the concordance between the new algorithm and current clinical practice, comparing the effectiveness of "immunogenicity-based" *versus* "empirical-based" switches. The combination of clinical and immunogenicity data may provide a tool to optimize the use of biologic therapies.

3.4.2 Objectives

This study aims to evaluate how concordant rheumatologist´s current clinical practice was with our proposed treatment algorithm and to compare therapeutic response rates between patients who followed the proposed algorithm and those who followed other therapeutic strategies.

Therapeutic responses over one year in non-responders, who switched according to the following two main branches of our algorithm were compared to other strategies in both

occasions: 1) Switching to non-TNFi if serum drug levels were detectable; 2) Switching to a less immunogenic drug if serum drug levels were undetectable and ADAb testing positive.

Secondarily, we evaluated the role of ADAb as a mediator of therapeutic response.

3.4.3 Methods

3.4.3.1 Patients

During a period of 2 years (January 2010 - December 2011) we followed all adult patients (≥ 18 years) with established RA, receiving TNFi (infliximab 3mg/Kg i.v. at 0, 2, 6, 14 weeks and every 8 weeks thereafter, adalimumab 40 mg s.c. every other week, or etanercept 25 mg s.c. twice a week or 50 mg once a week) in monotherapy or with concomitant immunosuppressors, at the Department of Rheumatology, Hospital Garcia de Orta, Portugal. All patients fulfilled the American College of Rheumatology 1987 revised criteria for RA and followed the Portuguese recommendations for the management of RA patients receiving biologic therapies [512, 546]. The study was approved by the hospital ethics committee. All patients gave written informed consent.

For the concordance between rheumatologist´s current clinical practice and our proposed treatment algorithm, disease activity was evaluated in all patients every 3 months, using the Disease Activity Score in 28 joints (DAS28), according to the rheumatologist's standard of care. Therapeutic response was defined as EULAR good and moderate responses (improvement $DAS28>1.2$ and $DAS28\leq 3.2$; improvement $DAS28>1.2$ and $DAS28>3.2$ or improvement $0.6>DAS28\leq 1.2$ and $DAS28\leq 5.1$) and low disease activity as a $DAS28\leq 3.2$, according to national and international guidelines [466, 546].

Drug immunogenicity was assessed every 3 months, through serum samples collected just before the next administration of a biologic. Serum drug trough levels were measured by ELISA, using a setup as described before [462, 519]. Limit of detection was determined by testing 100 sera of RA patients before treatment. The mean +/- 6 times standard deviation (SD) was chosen as a cut off. Limit of detection was about 2 ng/ml for all TNFi. ADAb were tested by Bridging ELISA as described before [209]. The sensitivity of these assays depends on the affinity of the ADAb. We used a series of patient-derived monoclonal antibodies to establish the sensitivity of the assay. A monoclonal antibody to adalimumab with the median affinity showed reached half-maximal extinction at 10 ng/ml and a detection limit <1 ng/ml. However non-specific binding of serum components such as rheumatoid factor or C1q sets the limit of detection for sera at about 20 ng/ml.

Clinicians were blind to immunogenicity test results and therapeutic decisions were undertaken according to the Portuguese recommendations for the management of RA patients [546].

We classified patients into Group A if, during the entire study follow-up, they followed, empirically, any of the branches proposed in our algorithm, and Group B, those who followed different therapeutic strategies. Therapeutic responses were evaluated over one year after therapeutic decision, which may have included to switch or maintain therapy.

For comparison of therapeutic responses over one year in non-responder patients who switched according to the two main branches of our algorithm, therapeutic response was assessed before the switch and at 3, 6, 9 and 12 months thereafter. The proportion of patients with therapeutic response and the proportion of patients with low disease activity were compared, over one year after the switch, between: a) non-responders with detectable serum trough levels who switched

to another TNFi and non-responders with adequate serum trough levels who switched to a non-TNFi; b) non-responders with undetectable serum drug trough levels, ADAb-positives, who switched to therapeutic monoclonal antibodies and non-responders with undetectable serum drug trough levels, -positives, who switched to etanercept or abatacept.

To evaluate the role of ADAb as a mediator of therapeutic response, the proportion of patients with therapeutic response was assessed at study beginning, between ADAb-positive and ADAb-negative patients.

3.4.3.2 Confounders or effect modifiers:

To assess the impact of the proposed algorithm on therapeutic response and the influence of "immunogenicity-based" switches versus "empirical-based" switches on therapeutic response and low disease activity rates, we defined as potential confounders or effect modifiers: age, disease duration, disease duration before biologic DMARD introduction, exposure time to biologic, concomitant immunosuppressors, Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP), DAS28 and response status (responder vs. non-responder) at the study beginning or time of therapeutic decision.

3.4.3.3 Statistical analysis

Differences in patient characteristics between groups at study beginning were analyzed by chi-square test (binary variables), T-test or Mann-Whitney U test (continuous, variables), as appropriate. The threshold for significance was set at a P value (p) of less than 0.05, 2-sided.

To estimate the course of therapeutic response and low disease activity status over time in patients that followed specific therapeutic strategies, we used generalized estimation equation

(GEE) with binary response. All the variables previously defined as potential confounders or effect size modifiers were tested in our model. Statistical software SPSS 17.0 (Chicago, Illinois) and R 2.14.2 platform - package "GEE" were used [547].

3.4.4 Results

3.4.4.1 Characteristics of the cohort

The study included 105 RA patients, 45% switchers, with a median (interquartile range [IQR]) disease duration of 10.1 (4.6-12.6) years. At the study beginning, 22.9% patients were receiving infliximab, 31.4% adalimumab and 45.7% etanercept. During the follow-up period, 51.4% of patients (Group A) had therapeutic concordant decisions with the proposed algorithm, whereas 48.6% (Group B) had discordant. Therapeutic decisions concordant with the proposed algorithm were undertaken with a median delay of 249 days (IQR 116-388). Patient's baseline characteristics of both cohorts are listed in Table 4.

3.4.4.2 The Impact of the Proposed Algorithm on Clinical Response Over One Year after Therapeutic Decision

GEE demonstrated that patients from Group A had significantly higher probability of achieving therapeutic response (OR=7.91, $p<0.001$, 95% CI=3.27-19.13) and low disease activity over one year after the therapeutic decision (OR=9.77, $p<0.001$, 95%CI=4.69-20.37), in comparison with patients from Group B – Table 5.

By excluding ADAb-positives patients, the probability of achieving therapeutic response (OR=5.97, $p<0.001$, 95% CI=2.38-14.99) and low disease activity (OR=7.88, $p<0.001$,

95%CI=3.57-17.39) remained significantly higher in patients in Group A when compared to those in Group B.

Response status (responder vs. non-responder) at the time of therapeutic decision had a significant effect modification on both outcomes: therapeutic response (OR= 9.59, $p<0.001$, 95%CI=4.21-21.84) and low disease activity (OR=5.23, $p<0.001$, 95%CI=2.30-11.89). In Group A, higher DAS28 scores at the time of therapeutic decision were associated with a lower probability of achieving low disease activity over the following year (OR=0.40, $p<0.001$, 95%CI=0.27-0.61), whereas in group B the DAS 28 scores did not affect the probability of achieving low disease activity (OR=0.84, p=0.33, 95%CI=0.58-1.20). In both situations, either response status or DAS28 scores at the time of therapeutic decision did not abrogate the association between therapeutic decision and the studied outcomes, which remained highly significant and nearly unchanged (less than 10%). The same result was verified after controlling for all other considered confounders or effect modifiers, assessed at the time of therapeutic decision.

3.4.4.3 Therapeutic responses over one year in non-responder patients who followed the two main branches of our algorithm

Non-responders to a TNFi in the presence of detectable serum drug trough levels and no detectable ADAb had higher probability of achieving response by switching to a drug with different MOA, rather than another TNFi, even after adjusting for potential confounders, such as DAS28 at the time of switch (OR=6.76, p=0.004, 95%CI=1.82-25.04). Despite a trend toward higher probability of achieving low disease activity, statistical significance was not reached (regression coefficient=0.24, p=0.73).

By analysing the 18 non-responder patients with undetectable serum drug trough levels, ADAb-positives, we verified that: 1) only two patients switched to a less immunogenic drug, both achieving response and low disease activity after the switch; 2) 15 patients maintained immunogenic drugs (14 remained within the same MOA and one switched to a different MOA), none was able to achieve therapeutic response; 3) one patient, despite ADAb-positivity, was considered as a responder and maintained the same therapy, achieving low disease activity and even clinical remission over the following year.

3.4.4.4 ADAb as a mediator of therapeutic response

At the study beginning, ADAb was detected in 37.5% of infliximab-treated patients and in 27.3% of adalimumab-treated patients. No detectable ADAb were verified among patients receiving etanercept. All ADAb-positive patients had undetectable serum drug trough levels, which were not verified in patients without detectable ADAb. Patient's baseline characteristics among ADAb-positive and ADAb-negative are listed in Table 7.

Lower proportion of responders was observed among ADAb-positive patients than ADAb-negatives (22.2% vs. 62.1%, p=0.003). One patient with detectable ADAb had low disease activity, in comparison with 34.5% of patients without detectable ADAb (5.6% vs. 34.5%, p=0.02) - Table 5. ADAb-positive patients had significantly higher mean (SD) CRP values, compared with ADAb-negatives: 5 (3-10) vs. 2.15 (1-4) mg/L, p=0.001 – Figure 33 – C-Reactive Protein (CRP) concentration among ADAb-negative and ADAb-positive patients, at study beginning.

3.4.5 Discussion

Our results demonstrate that therapeutic decisions according to the proposed algorithm lead to better disease control with significantly better clinical outcomes in RA patients, receiving biologic therapies. These results were independent of ADAb status and DAS28 scores at the time of therapeutic decision. Less than half of patients empirically followed therapeutic strategies concordant with the proposed algorithm. Had our algorithm been followed, about 8 months would have been gained, with important clinical and economic impact. Serum drug trough levels predict therapeutic responses in switchers. Non-responders to etanercept, adalimumab or infliximab, in the presence of detectable serum drug trough levels, were most likely to respond when switched to a drug with different MOA, in comparison with those who switched to another TNFi. In this subset, TNF might not play a central role in disease pathogenesis. Our results are in agreement with two previous studies, where switchers who failed previous TNFi (infliximab or adalimumab) in the absence of ADAb had poor responses to a second TNFi [484, 539].

Non-responders to TNFi in the presence of undetectable serum drug trough levels, ADAb-positives, were poorly represented in our study and no robust conclusions should be drawn exclusively from our data. Our data suggest that those patients might benefit in switching to a less immunogenic drug, TNFi or non-TNFi. Previous studies have revealed that patients, who discontinued a TNFi due to immunogenicity, were able to achieve response to another TNFi, if free of significant immunogenicity [539]. In contrast, lower therapeutic responses were verified when the second TNFi was a monoclonal antibody [484, 539].

Previous studies have revealed that TNFi therapy is more efficient in patients who discontinued the first TNFi due to secondary failure or adverse events, rather than by primary failure [438, 548]. Therapeutic algorithms based on the clinical distinction between primary and secondary failures have been proposed by some authors, who have underestimated the added value of immunogenicity assessment in clinical practice [549]. Such a clinical distinction is not straightforward, as therapeutic response assessment is based on subjective criteria that can be strongly affected by certain biases. The placebo effect, “regression to the mean”, or optimization of concomitant therapies may be the cause of initial clinical response.

A decision tree algorithm based on immunogenicity monitoring was recently proposed by others [550]. In this work, contrarily to ours, the first branching implicitly concerns clinical response, as the study addresses specifically patients with primary and secondary failures to TNFi. We propose a first branching according to drug level that offers the possibility to readily identify patients that are over or uselessly treated. The previous algorithm also considers dose escalation in non-responders presenting low drug level and undetectable ADAb. In some of these cases ADAb might be hidden by the presence of drug and dose escalation may boost ADAb production with serious adverse events [209, 210, 462, 495]. In the previous algorithm, a switch to another TNFi is recommended for non-responder patients that present optimal serum drug levels and ADAb positive. Such ADAb titers, which have no significant impact in serum drug concentrations, are unlikely to fully neutralize drug’s bioactivity [193, 462]. No clinical response, despite TNF neutralization, may warrant switching to an agent with a different MOA. We did not include this category of patients in our own algorithm, as in presence of optimal drug levels, ADAb are unlikely to be revealed by using Bridging ELISA or RIA assays [195, 209, 210, 455].

The inclusion of responders in our algorithm should help identify patients for whom drug dose reduction or increased interval between drug administrations might increase treatment cost-effectiveness. Correlation between DAS28 improvement and serum drug trough levels has been verified up to a threshold of drug level, above which no significant DAS28 changes occur [551].

EULAR recommendations, according to expert opinion, suggest that tapering a biological DMARD should only be considered in patients in remission for at least 12 months [404]. Biologic withdrawal might be considered earlier among patients in remission despite undetectable drug levels and high titres of ADAb. Subclinical synovitis might lead to bone damage in patients who clinically seem to have controlled RA [552, 553]. Image techniques, including ultrasonography or magnetic resonance imaging (MRI), have revealed higher sensitivity and reproducibility than clinical evaluation in assessing active synovitis and may be used to confirm remission [554].

Our experience suggests that high ADAb titres in the trough are associated with undetectable drug levels during most of the interval between two drug administrations. It seems reasonable to postulate that in these cases, remission is not maintained by the therapy. Further double-blind, randomized controlled trials may better clarify this point.

At the study beginning, 37.5% of infliximab-treated patients and 27.3% of adalimumab-treated patients had detectable ADAb. These proportions might be underestimated, since many patients with obvious non-responses, potentially ADAb-positives, had already switched from initial therapy. Interestingly, in some cases, we were still able to detect ADAb against the previous biologic, several years after drug discontinuation. We did not find anti-etanercept antibodies,

which is in agreement with previous studies that used more specific methods to detect ADAb [478, 496, 539].

Patients with detectable ADAb had higher mean CRP values, reflecting the poor control of inflammation. Previous studies have revealed higher baseline CRP values in ADAb-positive patients, but its association with the development of ADAb is not clear [153]. Concomitant immunosuppressive therapies, particularly methotrexate, have been associated with decreased ADAb frequencies [496, 555]. In our study, almost all patients were receiving concomitant methotrexate and low dose corticosteroids, which limited us to confirm this.

Our study has important limitations, despite reflecting real-world evidence. This is a small-scale study, conducted at one single center, and not powered to assess all the branches described in our algorithm, as this was not our main purpose. We did not categorize detectable serum drug trough levels from low to high, since no robust studies have clearly defined those cut-offs. ADAb are unlikely to be detected in the presence of circulating drug by the most common assays, namely Bridging ELISA. We assessed immunogenicity of three TNFi approved for RA treatment. Extrapolations to other agents in the same class, such as golimumab and certolizumab should be done cautiously. We treated non-TNFi agents as a homogeneous group and did not evaluate the potential differences among them, due to limited data. Etanercept and abatacept did not reveal clinically significant immunogenicity and were both considered “less immunogenic”. Further studies, using the same methodology, should be conducted to better compare the immunogenic profile of biopharmaceuticals.

Many questions regarding immunogenicity remain to be elucidated. We are proposing strategies that are already approved for RA patients. This algorithm represents a preliminary tool to aid

decision-making among clinicians and how immunogenicity assessment can be integrated in the care for these patients, leading to personalized and more cost-effective strategies to RA treatment.

Table 4 – Patient's Baseline Characteristics

Characteristics	Total Patient Population (N=105)	Group A (concordant) (N= 54)	Group B (discordant) (N=51)	P value
Age, mean (SD), y	54(14)	53(13)	56(14)	0.006
Female, No. (%)	91(87)	47(87)	44(86)	ns
Dis. duration, median (IQR), y	10.1(4.6-12.6)	7.6(2.5-12.4)	8.7(2.6-13.6)	ns
Initial DAS28, mean (SD)	5.6(1.2)	5.5(1.2)	5.7(1.1)	ns
Biol. duration, median (IQR), y	2.6(0.6-5.3)	2.9(0.5-4.7)	1.9(0.6-6)	ns
Switchers, No. (%)	47(45)	31(57)	16(31)	0.01
Biologic at baseline, No. (%):				
Infliximab	24 (22.9)	8 (14.8)	15 (29.4)	
Etanercept	48 (45.7)	31 (57.4)	18 (35.3)	
Adalimumab	33 (31.4)	15 (27.7)	18 (35.3)	
Methotrexate use, No.(%)	102(97)	52(96)	50(98)	ns
Corticosteroids use, No.(%)	103(98)	53(98)	50(98)	ns
DAS28 study baseline, mean (SD)	4.1(1.3)	3.9(1.6)	4.2(1.1)	0.003
RF positive, No (%)	72(70)	34(63)	38(79)	ns
ACPA positive, No (%)	75(73)	38(70)	37(75)	ns
Erosive disease, No. (%)	56(62)	29(62)	27(63)	ns
ESR, median (IQR), mm/h	29(16-52)	29(19-41)	33(21-50)	ns
CRP, median (IQR), mg/L	2.9(1.2-4.5)	3.9(2-8)	3.6(2-7)	ns

Legend: Values shown are n (%), means (standard deviation [SD]) or median (interquartile range [IQR]).

Differences between patients' baseline characteristics were tested by Mann-Whitney U or chi-square tests; ns = non-singificant

Switchers = patients who have been previously exposed to biologic therapy and switch to a new agent

ACPA – Anti-Citrullinated Peptides Antibodies; Biol. – Biologic; CRP – C-Reactive Protein; DAS – Disease Activity Score; Dis. – Disease; ESR – Erytrocyte Sedimentation Rate; Initial DAS28 – DAS before biologic therapy; RF – Rheumatoid Factor

Table 5 – Response and Low Disease Activity in patients from Group A and Group B, over one year after therapeutic decision

	Group A (N=54)	Group B (N=51)	P value
Response	88%	49%	p<0.001
	OR= 7.91 (3.27-19.3)		
Low Disease Activity	69%	19%	p<0.001
	OR=9.77 (4.69-20.37)		

Legend: Values shown are n (%), odds ratio (OR) and 95% confidence intervals (95% CI). P-values were obtained from generalized estimation equation (GEE) with binary response.

Group A represents patients that followed the proposed algorithm (any branch) and Group B represents patients that followed different strategies.

Therapeutic response was assessed every 3 months over one year after therapeutic decision. Therapeutic response was defined as decreased DAS28>1.2 and DAS28≤3.2, improvement DAS28>1.2 and DAS28>3.2 or improvement 0.6>DAS28≤1.2 and DAS28≤5.1 and Low disease activity as DAS28≤3.2.

Table 6 – Therapeutic Response and Low Disease Activity among ADAb-negative and ADAb-positive patients, at study beginning

	Patients ADAb + (N=18)	Patients ADAb – (N=87)	P value
Biologic at baseline, No. (%):			
Infliximab	9 (37.5)	14 (58.3)	
Etanercept	0 (0)	48 (100)	
Adalimumab	9 (27.3)	24 (72.7)	
Therapeutic Response, No.(%):	4 (22.2)	54 (62.1)	0.003
Low Disease Activity, No. (%):	1 (5.6)	30 (34.5)	0.02

Legend: Values shown are n (%). Therapeutic response was assessed every 3 months and defined as an improvement DAS28>1.2 and DAS28≤3.2, improvement DAS28>1.2 and DAS28>3.2 or improvement 0.6>DAS28≤1.2 and DAS28≤5.1 (EULAR good and moderate response). Low disease activity was defined as a present DAS28≤3.2.

P-values were obtained from chi-square tests.

ADAb + - anti-drug antibodies positive; ADAb- - anti-drug antibodies negative

Table 7 – Patient's Baseline Characteristics among ADAb+ e ADAb-

Characteristics	Total Patient Population (N=105)	ADAb + (N= 18)	ADAb - (N=87)	P value
Age, mean (SD), y	54 (14)	57 (10)	54 (14)	0.02
Female, No. (%)	91 (87)	16 (89)	75 (86)	ns
Dis. duration, median (IQR), y	10.1(4.6-12.6)	9.6(6.6-14.6)	7.6(4.6-12.6)	ns
Initial DAS28, mean (SD)	5.6 (1.2)	5.4 (0.9)	5.6 (1.2)	ns
Biol. duration, median (IQR), y	2.6 (0.6-5.3)	1.1 (0.4-4.4)	2.9 (0.6-5.5)	ns
Switchers, No. (%)	47 (45)	3 (17)	44 (50)	0.008
Biologic at baseline, No. (%):				
Infliximab	24 (23)	9 (50)	14 (16)	
Etanercept	48 (46)	0 (0)	48 (55)	
Adalimumab	33 (31)	9 (50)	24 (28)	
Methotrexate use, No.(%)	102 (97)	17 (94)	85 (98)	ns
Corticosteroids use, No.(%)	103(98)	17(94)	86(99)	ns
DAS28 study baseline, mean (SD)	4.1 (1.3)	4.4 (0.9)	4.0 (1.3)	ns
RF positive, No (%)	72 (70)	13 (77)	59 (70)	ns
ACPA positive, No (%)	75 (73)	13 (72)	62 (73)	ns
Erosive disease, No. (%)	56 (62)	8 (44)	48 (65)	ns
ESR, median (IQR), mm/h	29 (16-52)	30 (19-62)	29 (16-49)	ns
CRP, median (IQR), mg/L	2.9 (1.2-4.5)	5.0 (3-10)	2.15 (1-4)	0.001

Legend: Values shown are n (%), means (standard deviation [SD]) or median (interquartile range [IQR]).

Differences between patients' baseline characteristics were tested by Mann-Whitney U or chi-square tests; ns = non-significant

ACPA – Anti-Citrullinated Peptides Antibodies; ADAb – anti-drug antibodies; ADAb- - Anti-drug antibodies negative; ADAb+ - Anti-drug antibodies positive; Biol. – Biologic; CRP – C-Reactive Protein; DAS – Disease Activity Score; Dis. – Disease; ESR – Erytrocyte Sedimentation Rate; Initial DAS28 – DAS before biologic therapy; RF – Rheumatoid Factor

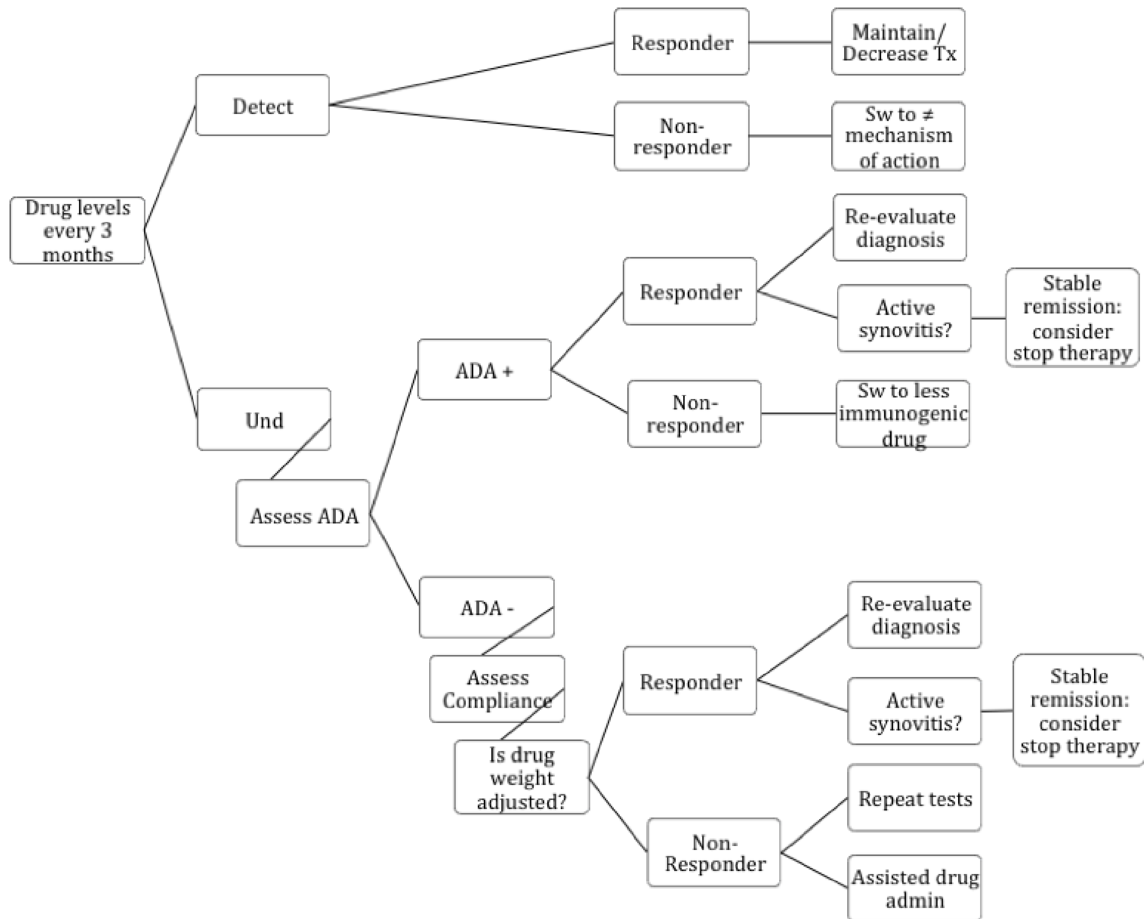

Figure 32 – Preliminary Algorithm to Approach RA Patients receiving TNF Inhibitor Therapies, introducing immunogenicity assessment

Legend: Serum drug trough levels and therapeutic response (EULAR criteria) assessed every 3 months

1) Serum drug trough levels detectable (Detect):
    a. EULAR responder - maintain therapy, eventually reducing dosage or increasing the interval between drug administrations
    b. EULAR non-response - switch (sw) to a drug with different mechanism of action
2) Serum drug trough levels undetectable (Und) – assess ADAb:
    a. ADAb positive:
        i. EULAR response:
            1. Re-evaluate patient (concurrent therapies; alternative diagnosis)
            2. Assess active synovitis – if remission, eventually consider withdrawal therapy
        ii. EULAR non-response - switch to a less immunogenic drug

b. ADAb negative:

   i. Assess patient's compliance

   ii. Verify if drug is adjusted to the patient's weight

   iii. EULAR response:

      1. Re-evaluate patient (concurrent therapies; alternative diagnosis)

      2. Assess active synovitis – if remission, eventually consider withdrawal therapy

   iv. EULAR non-response - switch to a less immunogenic drug

      1. Repeat tests

      2. Assisted drug administration

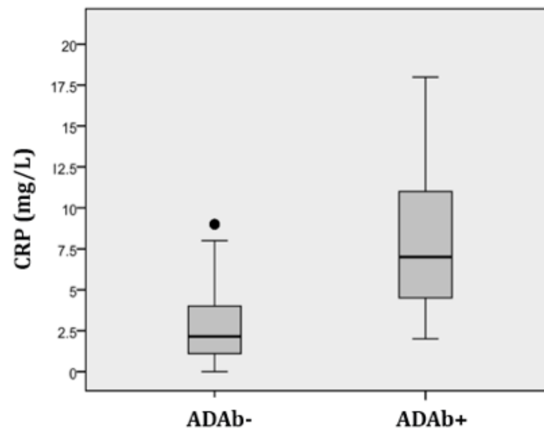

Figure 33 – C-Reactive Protein (CRP) concentration among ADAb-negative and ADAb-positive patients, at study beginning

Legend: Values shown are median (interquartile range [IQR]) of CRP: 5 (3-10) mg/L in ADAb+ patients and 2.15 (1-4) mg/L in ADAb- patients.

Differences between CRP median values from two groups were tested by Mann-Whitney U test, p=0.001.

ADAb – anti-drug antibodies; ADAb+ - anti-drug antibodies positive; ADAb- - anti-drug antibodies negative

4 GENERAL DISCUSSION

The introduction of biological therapies into clinical practice has greatly improved the treatment of chronic inflammatory diseases. The efficacy, effectiveness and safety of biological therapies have been extensively demonstrated in clinical trials and observational studies. There is a net positive balance where health care is concerned. However, this new class of drugs has also imposed several challenges for clinicians, biologists and economists. Those challenges are inherent to the very nature of these agents: large proteins (often antibodies), costly to produce and hard to reproduce under generic forms. Moreover, biologicals are replicates of natural compounds synthesized by the organism, administrated at doses far above physiological concentrations of their natural equivalents. Hence, their usage interfere with fundamental principle of life, that of physiological homeostasis. For over a century, immunologists have attempted to decipher the rules of immune tolerance, self / non-self discrimination (or assertion) and have proposed that variations from steady state (homeostatic conditions) alert the immune system and provoke adaptive immune responses. These fundamental notions are quite relevant to the challenge imposed by the use of biological therapies.

Over the last years increasing literature have revealed the ADAb as one of the main reasons behind therapeutic failure, also having impact on drug safety profile. Even though, the concept of drug immunogenicity has not reached medical community and clinical decision-makers. This overall neglect may owe to conflicting results from RCTs, which has higher impact near medical community than observational studies. However, immunogenicity induced by the long term of biologics in clinical practice has been poorly addressed by large RCTs, and never as a primary objective. Moreover, detection of ADAb is technically challenging and optimized assays were

only recently available. Therefore, we reavaluated all the available evidence, pooling all studies togheter in a meta-analysis, to formally established the clinical impact of ADAb. Posteriorly, we defined a convenient assay to monitor immunogenicity in clinical practice and we designed an algorithm introducing immunogenicity information into the current clinical management of patients receiving biologic therapies. We demonstrated the possibility of a personalized and scientifically-based approach to patients receiving biologic therapies, providing more safer and cost-effective strategies with obvious advantages for the patients and society.

4.1 THE IMPACT OF ADAB ON THERAPEUTIC RESPONSES

To formally document the impact of ADAb on therapeutic responses we performed a systematic review (2,082 studies) with a meta-analysis (17 included studies) of the literature. We assessed the effect of anti-drug antibodies on therapeutic response to infliximab, adalimumab and etanercept in patients with Rheumatoid Arthritis, Spondyloarthritis, Psoriasis and Inflammatory Bowel Diseases. We also scored the effect of concomitant immunosuppression on the detection of ADAb.

Meta-analysis is a statistical technique for combining data from independent studies, having higher statistical power to detect an effect than individual studies. Meta-analysis is also less influenced by local biases and it represents today the top of the evidence for clinical decision-making.

This approach revealed that i) anti-drug-antibodies significantly reduce drug effectiveness; ii) this effect can be attenuated by concomitant immunosuppression, which iii) reduces ADAb. Strikingly, detectable ADAb decreased drug response by as much as 80%. This result contrasted

with reports from most RCTs, which considered immunogenicity as a minor problem. Our analysis support the notion that lack of sensitivity and specificity in most ADAb assays has been the major misleading factor in these randomized clinical trials [326, 340, 485, 556]. Moreover, the appropriate time for sample collection was also not always respected. It is known today that in the majority of patients ADAb become detectable after 12-24 weeks of therapy, when ADAb titres exceed the circulating drug's concentration [153, 210, 213]. Clinical trials of short-term duration might underestimate the frequency of ADAb. Additionally, residual circulating drug significantly interferes with the detection of ADAb in the most conventional assays (ELISAs or RIAs) due to immune complex formation that prevents the binding or the capture of ADAb in those assays [209]. Therefore, to avoid drug interference, samples must be collected immediately before the next drug administration (trough), where it is expected the lowest drug concentration. Going back to those RCTs we may verify that many of these aspects were not always taking into account.

4.2 The Influence of Concomitant Immunosuppression and Other Possible Modulator Factors on ADAb Detection

We demonstrated that concomitant immunosuppression, mainly with MTX, attenuates the impact of ADAb on therapeutic response. This explains, in part, why combined therapy with biologic plus MTX is more effective than biologics in monotherapy. A sub-group analysis confirmed that in studies where higher proportion of patients was receiving concomitant MTX, the impact of ADAb on therapeutic response rates was attenuated to 68%. This result also suggests that in some cases clinical response is maintained by MTX and not the biologic anymore, when high ADAb titers and undetectable drug (biologic) levels are seen. We could not analyze the modifier effect induced by AZA or 6-MCP due to lack of data. However, our

results suggest that MTX may have higher modulating effect of drug immunogenicity than AZA or 6-MCP, which need to be confirmed in further large trials. This is of high clinical relevance for IBD patients, where AZA represents the main immunosuppressor. Similarly, in diseases where MTX is not approved, such as SpA or Ps, MTX although not having apparently significant role in disease pathogenesis, might be important to increase drug survival and efficacy. We verified that concomitant immunosuppression reduced detectable ADAb by 64%.

The exact mechanism by which MTX reduces ADAb detection and why that reduction is more evident in some patients, remain unknown. At lower concentrations, such those commonly used in RA (7.5-20mg/weekly), MTX has been shown to induce apoptosis of activated but not resting T-cells, thereby reducing cytokine production and inflammation [557-559]. As such, we might expect that MTX will suppress biologic-specific T cells when they became fully activated, i.e. shortly after biologic administration. Fully advantage of MTX efficacy may occur at this time point. Moreover, it is known that plasma bioactive MTX half-life is relatively short and some hours after MTX intake, which may also vary between individuals, its inactive metabolite rise in plasma [559-562]. In RA, MTX is given weekly and its intake is not usually coordinated with biologic administration. Differences in MTX metabolism and in the interval between its intake and biologic administration may explain, at least in part, inter-individual differences in the modulating effect of MTX on ADAb production.

We also assessed the influence of other factors that may modulate the clinical impact of drug immunogenicity, such as initial higher doses or scheduled regimens. Limited data prevent us to drawn robust conclusions. However, previous studies have demonstrated that initial higher doses lead to small numbers of memory T cells and less efficient immune responses upon re-

challenge with the same antigen, when compared with initial lower-doses [169, 170]. Small-scale studies have suggesting that higher initial infliximab doses (10mg/Kg compared with 1mg/Kg or 3mg/Kg) or higher initial adalimumab doses (160/80mg compared with 80/40mg at week 0 and 2, respectively, followed by 40mg eow) are associated with sustained better clinical outcomes, higher circulating drug levels and lower ADAb detection [159, 494]. However, induction doses are preconized only for some biologics and just for some diseases. This topic has not been specifically addressed, and although this approach may increase the initial therapy costs, if it lowers immunogenicity increasing drug survival, it might represent a cost-effective strategy over time. Scheduled administrations have also been associated with lower incidence of ADAb than episodic or on-demand regimens [160, 167, 168]. All these aspects have important clinical implications as they may reduce immunogenicity below its clinical significance. Therefore, further studies specifically addressing these topics are warranted.

4.3 Etanercept (fusion protein): Biologic Free of Significant Immunogenicity

Despite all the advances in biologics manufacturing, immunogenicity still represents one of the major drawbacks in the use of these therapies. However, important differences regarding immunogenicity exist among biologics. In our previous studies, no anti-etanercept antibodies were detected by using the most recent and optimized assays to detect ADAb. Very few studies in the Literature report the presence of anti-etanercept antibodies [331, 333, 487, 488]. In all of those studies, the detected antibodies were of low titer, transient, non-neutralizing and with no clinical impact. Because ELISAs of lower specificity were used in those studies to detect ADAb, false-positive results cannot be excluded. Consistent with this low immunogenic profile is the

fact that etanercept has consistently exhibited higher drug survival than infliximab or adalimumab in clinical studies [422, 432].

The reason why etanercept is of low/absent immunogenicity remains largely unknown. Some factors can, however, be hypothesized. Etanercept is a dimeric fusion protein comprised of two extracellular portions of p75-TNFR (75-kDa TNF receptors) linked to the Fc portion of a human IgG1. Only the junction between these two domains, the hinge region, is composed by few non-human a.a. residues. Etanercept does not contain the highly variable CDR regions present in Fab arms of mAbs, where resides the higly immunogenic epitopes [60]. On the other hand, etanercept contains a human Fcγ portion, where it is though to reside tolerogenic epitopes (Tregitopes) [102]. Possibly, the balance between lower Teffector and higher Tregitopes content may contribute to the low immunogenicity of etanercept. Consistently, abatacept, the CTLA4-Fcγ construct, has also been associated with low immunogenicity. However, lenercept, a fusion protein similar to etanercept, did not receive approval for clinical use due to high immunogenicity revealed during its development. In contrast to etanercept (p75-TNFR-Fcγ), lenercept consists in the extracellular part of p55-TNFR fused with Fcγ portion. Lenercept (i.v.) was also tested in RA, and anti-lenercept antibodies, IgM and IgG, were detected in the serum of 60% of RA patients [563, 564]. Subcutaneous formulations were subsequently developed but, still, anti-lenercept antibodies were associated with a faster drug clearance [565]. Differences in the structure and function of the two TNFR may account for differences in the immunogenicity between lenercept (p55-TNFR) and etanercept (p75-TNFR). While p55-TNFR becomes primarily internalized after ligation, p75-TNFR is rapidly shed [566-569]. The higher internalization of p55-TNFR may increase the possibility of its processing and presentation, which may constitute a pressure force for the selection and maintenance of specific T and B cell

clones and antibodies to p55-TNFR and not to p75-TNFR. Qualitative or quantitative differences in the thymic expression between p55- and p75-TNFR could be also hypothesized, although no suggestive evidence of that has been reported in the Literature.

Some studies have revealed that etanercept has lower affinity to transmembrane TNFa and induces significantly less antibody-dependent cell-mediated cytotoxicity (ADCC) than infliximab or adalimumab [329, 570]. Overall speaking, drugs that have membrane targets tend to be more immunogenic. By converting cell-binding antibodies into monomeric non-cell-binding mutants, tolerance can be induced to the therapeutic cell-binding forms, as demonstrated with the anti-CD52 CAMPATH [58]. Moreover, the complex etanercept-soluble TNFa cannot interact with transmembrane TNFR, at least with p75-TNFR, as both etanercept and transmembrane p75-TNFR is suppose to bind to the same TNF epitope. However, it is likely that infliximab and adalimumab recognize epitopes in the TNF molecule other than those recognized by the TNFR, not preventing the binding between the complex infliximab- or adalimumab-TNFa with transmembrane TNFR and its consequent internalization and processing. This aspect may also contribute for the lower immunogenicity of etanercept by comparison with infliximab and adalimumab. Finally, in addition to TNFa blockage, etanercept also blocks lymphotoxin-alfa (LTa), which among other functions is also important for germinal centre formation [489]. The role of LTa in drug immunogenicity is not known.

4.4 The Impact of ADAb on Drug's Safety Profile

Beyond the impact of ADAb on therapeutic effectiveness, drug immunogenicity may also interfere with drug safety profile, increasing the risk of adverse events [160, 210, 211, 213, 458, 495, 497-501].

The most common adverse events that have been associated with ADAb are the ”anaphylactoid-like” acute reactions after intravenous infusions of biologics such as infliximab. ADAb may also mediate subacute symptoms such as arthralgia, myalgia or thromboembolic phenomena [495]. However, in this case the temporal relationship is not so obvious between the symptoms and the drug’s administration, which may have prevented a more clear association.

Although IgE-ADAb have been reported, the great bulk of ADAb are of IgG isotype. This suggests an alternative pathway (IgE-independent) mediated by IgG, FcγRIII, macrophages, basophils, platelet activating factor (PAF) and complement activation with the consequent production of anaphylatoxins. Both IgE and non-IgE mediated acute IrAE might be clinically indistinguishable and tend to occur only from the second biologic’s infusion. Adverse reactions immediately during the first exposure to the drug have also been described, however they represent a minority. These reactions occurring at the first infusion are not related with ADAb, although the precise mechanism is not fully understood. A pre-existing sensitization towards some additives or cytokine release during cell apoptosis induced by the cross-linking of therapeutic monoclonal antibodies bound to target cells, may underlie these immediate reactions. These cytokine-release syndromes can be managed by short-term cessation of biologic’s infusion, restarting the infusion at a slower rate, or by the administration of histamine blockers and corticosteroids [534-536]. However, this approach has been employed indistinctly to all types of IrAE, irrespectively of the mechanism behind. This explains why in the majority of the cases pre-medication or slower infusion-rates do not prevent the recurrence of IrAE, as in the vast majority of the cases they are mediated by ADAb. Moreover, the persistence on the same therapy in the presence of ADAb may boost the immune response to biologic in some patients, which may increase the severity of adverse reactions without further clinical benefit.

The majority of the patients with ADAb-related IrAE end up loosing therapeutic response rapidly. Acute IrAE tend to be associated with high ADAb titres, which tend to increase with treatment continuation. ADAb can be detected at lower levels before the clinically overt IrAE, highlighting the predictive value of ADAb for such adverse events. Thus, immunogenicity monitoring may help us to better understand the mechanism underlying the adverse reactions, which have serious implications in their management. This type of approach can help us to prevent serious adverse events, namely through a timely therapy withdrawal.

4.5 Suitable Assays to Monitor Immunogenicity in Routine Clinical Practice

The detection of ADAb could be technically demanding and the quality of the assay may significantly affect the results. Newly developed assays have emerged to assess ADAb with better sensitivity and specificity. There are several optimized methods to detect ADAb. The most commonly used have been RIA-based methods. However, RIA requires high doses of radioactivity and special technical conditions, which prevent its use in the routine practice. In close collaboration with Sanquin Research Institute, Amsterdam, we developed a modified ELISA, known as Bridging ELISA, to specifically assess ADAb. Microtiter plates are incubated with the drug. After the incubation of the serum samples in the plates, the biotinylated drug are added and revealed through poly-HRP. This double recognition of ADAb leads to an ELISA of higher specificity.

We tested the performance of this Bridging ELISA in our cohort of patients by comparison with RIA-ABT, which have been considered by many as the *"gold-standard"* to assess ADAb. The same positive samples were detected by both assays. Bridging ELISA offers the advantage of

being a simple and cheap method, easy to automate and to implement in the routine practice. Some limitations also exist, namely drug interference and inability to detect ADAb of IgG4, which may represent a significant proportion of ADAb. Drug interference is one of the major drawbacks of both ELISA and RIA [209]. By using these methods, ADAb can only be detected when their concentration exceeds the concentration of the drug. In the presence of the drug, ADAb might be hidden due to immune complex formation. New methods are now available, which by promoting an acidic IC dissociation, allow the assessment of very low ADAb titres [462]. However, the clinical significance of such low titres remains unknown, as free drug remains available and no significant impact in therapeutic response has been seen. Both RIA and Bridging ELISA are, thus, able to detect clinically significant immunogenicity. The majority of ADAb are of IgG1 and IgG4 isotypes. Human IgG4 is able to exchange half of the Ig molecule with another IgG4 of different specificity, losing their bivalency and the ability to bridge. In these cases Bridging ELISA is not able to detect ADAb. However, we have not seen ADAb exclusively of IgG4 isotype. Strangely, some reports have shown patients IgG4-RIA-ABT positives, who tested negative in total IgG-RIA-ABT [209]. However, this might be owed to different drug interference between these two assays. Moreover, IgG4 represent a minority of total amount of IgGs and when a great proportion of ADAb is of IgG4 isotype is likely that they exchange half of the molecule with IgG4 of the same specificity, maintaining the bivalency. Therefore, the inability of detecting IgG4 by Bridging ELISA is a more theoretical than practical issue.

We concluded that drug levels monitoring through an ELISA format and the detection of ADAb through Bridging ELISA, in case of undetectable serum drug trough levels, represent a suitable strategy to monitor immunogenicity in routine clinical practice. This strategy will provide very

important information, namely: a) the reason for therapeutic failure (mechanism of drug action *versus* drug immunogenicity); b) the identification of non-adherent patients and c) possibly patients that are overtreated, in which dose drug reduction programs might be implemented.

4.6 INTEGRATING IMMUNOGENICITY INFORMATION IN THE MANAGEMENT OF PATIENTS RECEIVING BIOLOGIC THERAPIES

RA has been seen as a paradigmatic example of a chronic disabling disease, where cytokine manipulation through biologic therapies has revolutionized the prognosis of the disease. Given the successful results of biologics, an increased number of patients are receiving these therapies, which represents an enormous burden to our societies. Moreover, therapeutic failures to these drugs are frequent and therapeutic decisions in these cases, such as whether to increase the dose or to switch to another biological of similar or different target, are more an empirical rather than a scientific-based decision.

Until recently only TNFi were approved as first-line therapy in *naïve* RA-patients. In the 2013 updated EULAR recommendations, tocilizumab and abatacept was also included in the approved first-line agents for RA treatment. However, TNFi still represent the most commonly used mechanism of action. In case of therapeutic failure to the initial agent, a switch to any of the approved biologics is recommended, irrespectively of the reason behind the failure. Given the lack of direct comparative RCT, all biologics have been considered equally effective and safe. However, according to our work and an increasing body of evidence, non-responders definitively do not represent a homogeneous group, and according to the reason of failure patients might benefit switching to specific agents. Similarly, in responding patients, the same lack of objective guidance prevents an educated and desirable dose reduction program, as there

are recent reasons to believe that over-treatment is common in patients receiving biological therapies [571-575].

Through the routine immunogenicity monitoring of our patient's cohort, together with an exhaustive analysis of the literature, we designed an algorithm that integrates immunogenicity data in the current approach of patients receiving biologics - Figure 32.

This algorithm is a binary decision maker, where the first node is defined by the serum drug trough levels (at every three months) and, the second, by the detection of ADAb (in case of undetectable drug levels). The three months interval represents the usual and recommended schedule for clinical visits and therapeutic decisions in clinical practice.

We propose start divinding the patients according to detectable or undetectable serum drug levels, assessed by ELISA using a setup as described before. We did not categorize positive levels in an arbitrary low, normal or high level, as a paucity of studies exists to rigorously define the therapeutic range for each biologic and a high inter-individual variability has been reported. We purposefully choose this rough classification, as there is, today, no rigorous argument to define those cut-off values. Further analyses are needed to better discriminate those ranges and it is expected that routine assessment of serum drug levels will constructively contribute to this aim.

In the presence of no clinical response, despite TNF neutralization, we might assume that the drug mechanism of action might not be the most appropriate for those patients and a switch to a drug with different mechanism of action is advisable. Some patients are never able to achieve therapeutic response to TNFi and are, thus, designated as primary non-reponders or as patients

having a "TNF-independent" RA. The identification of these patients has not only practical interest, as it is also of high scientific relevance. A better characterization of these "TNF-independent" RA patients would be certainly important to better understand pathophysiologic mechanisms underlying RA and patient's heterogeneity. Cytokines are redundant and pleiotropic. The predominance of a given cytokine may change over time during the disease course, and also may be affected by the presence or absence of other cytokines. This may in part explain why some RA patients do not achieve clinical improvement with TNFi. Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine pattern, which is not found in patients with established disease [576]. The effect of some cytokines are often linked to the presence and effects of other cytokines. For example, TGFβ together with IL6 promotes the differentiation into Th17 cells, but in the relative absence of IL6 it can favours the induction of T regulatory cells [577]. It might be possible that in the near future different cytokines profiles will be identified in different patients and at different stages of the disease, where different therapies will have different efficacy.

Primary non-responders are currently defined as patients who cannot achieve DAS28 response as early as three months of therapy, in contrast to secondary non-responders, who loose initial DAS28 response at month six or later. This means that currently the distinction between these two groups of patients may rely only in DAS28 assessment at one single time point (3 months after the beginning of therapy). Considering that DAS28 is based on some subjective parameters and that therapeutic responses at month three may be affected by several bias, such as the placebo effect, it is conceivable to consider this classification of low reliability. However, some authors have proposed algorithms for therapeutic decision merely based on this classification, arguing that primary non-responses are associated with refractoriness to the drug's mechanism

of action, while secondary non-responses are likely mediated by ADAb development [549]. Not only this clinical classification is extremely fragile, as explained before, as non-responses at month three does not mean necessarily refractoriness to the drug's mechanism of action because they can have already developed significant immunogenicity by this time. Therefore, we propose to assess drug levels (and ADAb in case of undetectable drug trough levels) for a more reliable and objective distinction between primary and secondary non-responders. Primary non-responders will be those who, despite detectable and adequate drug levels, cannot achieve therapeutic response. We have then a possibility to easily and more rapidly discriminate the patients who benefit switching to different mechanism of action from those who will benefit from less immunogenic therapies.

We propose to assess ADAb only in case of undetectable serum drug trough levels. Due to drug interference, the most commonly used assays cannot detect ADAb in the presence of circulating drug. By forming immune complexes with the drug, ADAb also promote the drug's fast clearance from the circulation. Hence, detection of ADAb is predictably associated with very low/undetectable drug levels. When ADAb are produced at low titers, reduction in drug levels is not significant and virtually all ADAb remain in complex with the drug, preventing their detection [462]. Assays such as PIA-RIA-ABT, which promote the dissociation of immune complexes, allow the detection of such low levels of ADAb, otherwise hidden by high drug concentrations. However, in those cases the exceeding drug concentration remains free to neutralize TNF, as demonstrated in bioassays, and no significant clinical impact has been observed [193, 462]. Therefore, the clinical impact of ADAb, beyond their association with adverse events, occurs when ADAb concentration is enough to fully neutralize the drug. High sensitive assays are important to monitor ADAb responses and better understand the dynamic

of immune responses to biologics, but its added value in clinical practice remains to be established. Hence, we found Bridging ELISA a convenient method to monitor ADAb in clinical practice, in case of undetectable drug levels. Some authors have argued that ADAb is of little relevance, as their most important consequence is mediated through their impact on drug levels. However, non-adherence to the therapy may also lead to undetectable drug levels, which should not be interpreted as a consequence of drug immunogenicity. Reports of 50% non-adherence to chronic therapies have been revealed [540]. Treatment interruptions may have deleterious consequences. Poor therapeutic responses have been described after temporary therapeutic withdrawal, being more difficult to rescue response as good as previously. Episodic treatment regimens have been associated with higher immunogenicity as well. Hence, the assessment of ADAb in case of undetectable drug levels may provide added value in the identification of these patients, to whom an educational reinforcement regarding their disease and treatment might be advisable.

Other factors may possibly interfere with drug pharmacokinetics/pharmacodynamics, such as weight or fat distribution. However, none of those seem to be able to mediate a so huge and fast clearance of the drug from circulation, particularly after injecting so high amount of therapeutic protein, as it is normally injected during these treatments.

In case of clinical non-response and ADAb positivity, a switch to a less immunogenic drug, irrespectively of mechanism of action, is recommended. There is no cross-reactivity of ADAb with different biologics, as they are highly specific antibodies. However, patients who produce ADAb to one biologic have 2-fold higher probability to produce ADAb against a new biologic, when compared to biologic-*naïve* patients [455, 578].

There is a paucity of comparative studies evaluating immunogenicity of different biologics by using the same type of assays. The lack of standardization and huge heterogeneity among assays prevent direct comparisons between different studies. However, important information already exists regarding this topic. By using the same type of assays, it has been verified that: infliximab is able to induce more ADAb than adalimumab and, etanercept is free of significant immunogenicity. This is highly consistent with drug survival rates observed in clinical practice, which are higher for etanercept followed by adalimumab and lastly by infliximab [422].

These differences in immunogenicity among different biologics, may also explain conflicting literature evaluating the clinical outcomes after switching to drugs with the same versus different mechanism of action. In a swiss cohort study involving 116 RA patients, evolution of the DAS28 was more favorable in the group that received rituximab compared with the group that received an alternative TNFi. At 6 months, the mean decrease in the DAS28 was -1.61 (95% CI = -1.97, -1.25) among patients receiving rituximab and -0.98 (95% CI = -1.33, -0.62) among those receiving subsequent TNFi therapy [451]. Different results were obtained from a British cohort study involving 1328 RA patients, where no significant changes in DAS28 score or in the remission rate were verified among patients who switched to rituximab when compared to an alternative TNFi [372]. Any of these studies had taken into consideration which was/were the previous TNFi (mAb versus fusion protein), the reason of failure (presence versus absence of adequate drug levels) and which was the alternative TNFi (mAb versus fusion protein). By scrutinizing patient’s baseline characteristics in the previous studies, we verify that in contrast to the first one, in the later the majority of the included patients had failed one mAb (infliximab or adalimumab) and switched to etanercept (fusion protein). Thus, it is likely that in the second study the majority (although possibly not all) of patients had failed the previous TNFi due to

immunogenicity and, thus, a switch to a less immunogenic drug, TNFi or not, represent an effective strategy. In agreement to this, is a more recent study published by Gomez-Reino et al, evaluating the EULAR responses in 1124 RA switcher patients at 6 and 12 months after switching to an alternative TNFi (TNFi-group) versus rituximab (RTX group) [579]. By analysing these two groups, a significant higher proportion of EULAR good responses were verified in the RTX group. However, by subdividing the TNFi-group in 1) those who had previously failed a mAb (infliximab or adalimumab) and switched to etanercept and, 2) those who had previously failed etanercept and switched to mAb (infliximab or adalimumab), no significant differences in clinical outcomes were verified between RTX-group and those who switched to etanercept. In contrast, significant better outcomes were verified in the RTX-group when compared to those who had previously failed etanercept. As etanercept is free of significant immunogenicity, resistance to the mechanism of action is likely the cause of etanercept failures. In this case, the switch to a different mechanism of action represents a more effective strategy.

Overall speaking, fusion proteins are less immunogenic than mAb, in part explained by the absence of the highly immunogenic CDR regions, though many other aspects may be implicated, as previously discussed. No neutralizing antibodies have been also detected against abatacept (CTLA4-Fcγ construct), which lead us to define etanercept and abatacept as less immunogenic drugs. Overall speaking, as less as foreign sequences are present in a mAb, the lower is its immunogenicity, though this might be not entirely true. Immunogenicity does not simply rely on amino acid sequence and other factors contribute to drug immunogenicity. As stressed before, further studies comparing the immunogenicity of different biologics by using the same type assays are very important to better evaluate the immunogenicity of each biologic

induced by its long-term in clinical practice. In the absence of such information we treated all the other biologics as a homogeneous group but possibly they may have important differences regarding immunogenicity that need to be further explored. Consistent with this has been the lower secondary failures and higher remission rates reported under tocilizumab therapy, an humanized mAb against the alpha-chain of the IL6-receptor [379, 381, 580]. Moreover, in contrast to TNFi, the combination therapy of tocilizumab plus methotrexate has not demonstrated better clinical outcomes than tocilizumab monotherapy [581]. All together, this evidence suggests a possible lower immunogenic profile of tocilizumab. This lead us to hypothesize that drug mechanism of action may also influence drug immunogenicity and it may well be that TNF blockade influences differently ADAb production than IL6 inhibition.

4.7 Drug Mechanism of Action and ADAb Production

TNFa is known to have also immune-regulatory effects, which are believed to be mediated through p75-TNFR. In contrast to p55-TNFR, which have been associated with induction and maintenance of chronic inflammation and antibody responses to T cell-dependent antigens [309, 582-589], p75-TNFR signalling offers protective role in several disorders, including autoimmune diseases, heart diseases, and demyelinating/neurodegenerative conditions [590-596]. Consistently, several defects at the p75-TNFR signalling have been reported in both human and mouse models of autoimmune disorders, including Crohn's disease, Systemic Lupus Erythematous (SLE), Multiple Sclerosis (MS), Ankylosing Spondylitis (AS) and Type 1 Diabetes [597-601]. Interestingly, there is a predominant expression of p75-TNFR on human and mice CD4+FoxP3+ T cells. Approximately 70% of human CD4+FoxP3+ cells express p75-TNFR, compared with approximately 20% of CD4+FoxP3- cells [602-604]. P75-TNFR is constitutively expressed on human thymic T regulatory cells, but not on thymic T effector cells

[605]. Tregs expressing P75-TNFR generally exhibit a memory/effector phenotype and express higher levels of cytotoxic T-lymphocyte Antigen-4 (CTLA4), exhibiting a more potent immunosuppressive activity [603, 606, 607]. Even CD4+CD25- cells that express P75-TNFR have demonstrated considerable immunosuppressive activity [606]. It has been proposed that TNFa produced by activated T effector cells during inflammation activates Tregs through p75-TNFR [608-610]. Consistently, Treg cells deficient in p75-TNFR have shown lower immunosuppressive capacity and poor control of inflammatory responses *in vivo* [602]. Taken together, this evidence highlight the regulatory properties of TNF, known as a potent pro-inflammatory cytokine. It might be possible that TNFa assumes different properties (pro-inflammatory versus regulatory) according to its concentration and cytokine milieu. The impact of TNF blockade in immune-regulatory effects mediated by TNFa is more evident in chronic phases of the disease than during acute inflammatory phases. TNFi have shown higher anti-inflammatory efficacy and better clinical outcomes when administered at early acute inflammatory phases of RA. Similarly, TNF blockade in mice models of antigen-induced experimental autoimmune encephalomyelitis (EAE), which mimic acute inflammatory phases of Multiple Sclerosis (MS), was associated with significant disease improvement [309-313]. However, when administered to chronic MS patients, it was associated with deleterious effects [314, 315].

Prolonged treatment of RA or IBD patients with TNFi has been associated with autoimmune phenomena, such as high antinuclear antibodies (ANAs) titres, double-strand (ds)-antiDNA antibodies, lupus-like syndrome or neuroinflammatory conditions. TNFi in established SLE was also associated with increased titres of lupus-associated antibodies (ANAs, ds-aDNA) and severe nephritis, which may suggest a participation of TNFa in the control of those

autoantibodies. So far, no association have been found between ADAb and autoantibodies production (ANAS or ds-antiDNA antibodies) [611]. However, if we hypothesise that excessive TNFa neutralization will lead to increase titres of autoantibodies, we will then expect an inverse correlation between autoantibodies and ADAb, as the later prevents TNF neutralization. Further longitudional studies assessing both ADAb titres, ANAs and ds-DNA and ideally serum TNFa concentration are warranted to better understand a possible correlation between these biomarkers.

In contrast to TNFi, the use of tocilizumab in murine models of lupus and SLE patients has been associated with significant decrease of anti-dsDNA and disease activity [612-614]. Prolonged treatment with tocilizumab has not been associated with increased titres of ANAs, ds-DNA or lupus-like syndromes [615]. IL6 has been shown to play a pivotal role in driving the differentiation of naive T cells into pro-inflammatory T cell populations, such as TH17 cells, inhibiting the generation of T regulatory cells [616, 617]. IL6 has also been shown ability to inhibit the suppressive function of Tregs [618, 619]. The blockade of IL6 signaling has been associated with expansion of Tregs, in mice models and in humans [620-623]. It is possible that IL6 blockade will potentiate immune-regulatory mechanisms with impact on antibody responses and thus, on drug immunogenicity. Alternatively, we may also hypothesize that tocilizumab has higher and/or faster efficacy, inducing remission more rapidly. Once in remission, the development of immunogenicity would not have significant clinical impact. Further studies are warranted to better evaluate the immunogenicity of tocilizumab with its long-term use in clinical practice.

4.8 BIOLOGIC DOSE REDUCTION PROGRAMS BASED ON SERUM DRUG LEVELS

The EULAR recommendations consider the possibility of dose reduction in patients with stable remission [364]. In the last update of EULAR recommendations are not completely clear how long should remission be maintained before start the biologic dose reduction, but in the previous recommendations one year was the advised period [404]. However, such reduction has been proposed on an empirical basis, i.e. simply considering DAS28 remission and not taking into account circulating drug levels. Clinical remission may occur in the presence of low serum drug trough levels (low or high) or in the absence of detectable circulating drug levels. While in the former we can always imagine that remission is maintained by the treatment, in the later case it is conceivable to postulate that is not. Hence, we propose that biologic withdrawal might be considered earlier among these later subgroup of patients, saving costs and possible adverse events. Increasingly evidence about discontinuation and dose titration of biologics in RA patients has emerged over the last years. The results have shown that dose titration or even discontinuation of TNFi is feasible and leads to overall dose reduction without compromising the clinical outcomes, although no circulating drug levels have been taken into account [571-575]. In our cohort we verified the existence of patients with DAS28<2.6 with undetectable drug trough levels and detectable ADAb. Similarly, in another RA cohort, very low or undetectable drug levels were found in 33% of remission patients [624]. Those patients represent a subgroup of patients in which drug reduction can be proposed safely without waiting too long. Nevertheless, we recommend the use of ultrasonography or magnetic resonance imaging (MRI) before therapeutic withdrall, to exclude subclinical synovitis. Those imaging methods have revealed higher sensitivity and reproducibility than clinical evaluation and it is well known that

subclinical synovitis might lead to bone damage in patients who clinically seem to have controlled RA [552-554].

However, we have also found patients in remission or low disease activity in the presence of very high drug trough levels. Going back to previous studies we can verify that above 1ug/mL of serum infliximab levels, no significant differences in clinical outcomes (ACR responses) occur between those who had infliximab levels between 1-10ug/mL and those who had >10ug/mL [625]. Similarly, it was recently published that above 5ug/mL of serum adalimumab, the correlation between DAS28 and adalimumab levels is no longer present [626]. Further larger longitudinal studies are warranted to validate those cut-offs, but it is already possible to comfortably reduce biologic doses to values close to the previously mentioned, as often much higher drug concentrations are found among patients [153, 195, 519].

One of the main concerns of this strategy has been the possibility of flares and its management. However, considerable flare rates have been reported even despite standard dose regimens of biologic therapies [627, 628]. Those flares tend to be managed through the optimization of non-biologic DMARD therapies, chemical synovectomies or even biologic dose intensification [575, 629, 630]. Studies evaluating tapering strategies, even not considering circulating drug levels, have shown no significant differences in the number or severity of flares, when compared to standard treatment regimens [571-575]. The integration of serum drug levels assessment will offer even more safety, as it will prevent possible over-tapering. The dose reduction will be proposed comfortably just up to certain limit, below which some precaution is advised.

In conclusion, our algorithm, by having a first branching according to drug level that offers the possibility to readily identify responder patients who are overtreated or uselessly treated. The

assessment of drug levels in first place also prevents unnecessary ADAb assessments, as in presence of optimal drug levels, ADAb are unlikely to be revealed by the most common assays, such as Bridging ELISA or RIA [195, 209, 210, 484]. Progressive drug reductions or even therapy withdrawal can be done safer and more cost-effectively if based on serum drug levels. Further studies are now warranted to better discriminate the minimum effective drug level for each patient/subgroup of patients.

5 CONCLUSIONS

5.1 RELEVANCE (AND PERSPECTIVE) OF THIS WORK FOR CLINICAL PRACTICE

The clinical heterogeneity among patients is to be considered. Drug immunogenicity is one of the main reasons behind therapeutic failure, also increasing the risk of adverse events. Monitoring drug levels and anti-drug antibodies represent a very promising tool for an optimized and personalized usage of biological therapies.

The algorithm we proposed and tested operates along a qualitative classification of patients according to ADAb and drug level detection. It is expected that extension of this work to quantitative large-scale longitudinal studies, will provide the material for its improvement, notably for optimization of the effective drug level for each patient.

Our analysis of 105 patients, each followed for duration of 12 months after therapeutic decision, reveals that those who coincidently followed strategies concordant with our algorithm had about 10-fold higher probability to achieve low disease activity, when compared to those who followed different therapeutic strategies. Moreover, had our algorithm been fully applied, about 8 months would have been gained to achieve those beneficial outcomes.

Our proposal represents a personalized and scientific-based approach to patients receiving biologic therapies, providing more safe and cost-effective strategies.

5.2 RELEVANCE FOR ECONOMIC AND SOCIETAL VALUES

Biological therapies have revolutionized the prognosis of many chronic inflammatory diseases, which tend to affect young people at productive age. Despite the overall benefit of these novel therapies, some patients never achieve therapeutic response or, more often, do not sustain a good response to continuous therapy. The clinical identification of these subgroups is affected by several biases, which prevent fast and assertive therapeutic decisions. Moreover, accumulating evidence has now suggested that a considerable number of responder patients are indeed over-treated.

By using reliable bio-markers, such as levels of drug and of ADAb, these subgroups would be easily and rapidly identified. Fast and assertive therapeutic decisions could then be implemented, leading to a better control of disease activity and possibly clinical remission, which is today the main therapeutic goal, as defined by the European League Against Rheumatism (EULAR).

Our scientific-based strategy for clinical decisions would improve significantly the cost-effectiveness of biological therapies, a concern of modern societies. Comparing and improving immunogenicity tests, we demonstrated that monitoring immunogenicity and drug level can easily be implemented in routine clinical practice, for an estimate annual cost of 400€ per patient. Considering the modest cost of such a monitoring together with i) the very high cost of biological therapies (approximately 12 000€ per patient per year), ii) our evaluation that several months of inefficient therapies could be prevented by monitoring immunogenicity and iii) the amount of administrated drug could be reduced in a subgroup of patients, there is no doubts that implementing this in routine practice would be cost-effective. In addition to direct and

immediate costs reductions, this strategy would also provide indirect gains on a longer scale, by reducing disability and promoting patients' functionality and productivity.

In conclusion, our findings could guide decisions at a National regulatory level as it allows revision of the costs engaged in biological therapies while keeping as a priority the welfare of patients.

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APPENDIX