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Expression of 9G4 idiotope on autoantibodies from patients with rheumatoid arthritis

TÂNIA DA CUNHA BRANCO DOS SANTOS

DISSERTAÇÃO

MESTRADO EM BIOLOGIA MOLECULAR E GENÉTICA

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Agradecimentos

A vida é feita de aprendizagens que se acumulam e moldam o que somos. Todos os caminhos que iniciamos estão sempre marcados, direta ou indiretamente, por pessoas que contribuíram para essa decisão, que nos apoiaram durante a trajetória e/ou estiveram presentes para partilhar o orgulho de se concretizar mais uma etapa na vida ou para nos apoiar quando a realidade se vai afastando do que sonhamos. Este trabalho que aqui apresento é um grande exemplo disto mesmo. Existem pessoas que, sem as quais, esta dissertação nunca teria sido possível e as minhas primeiras palavras têm de ser para elas.

Em primeiro lugar, quero agradecer à minha família que tanto amo! O meu pai, a minha mãe e a minha irmã foram sempre os primeiros a apoiar-me nesta aventura e nunca deixaram de mostrar o orgulho que sentiam no meu trabalho. Sem eles, nunca tinha chegado onde cheguei e não era o que sou! Sem o apoio deles, este mestrado não teria sido possível! Esta tese é mais deles que minha e, por todas as vezes que se sacrificaram por mim, quero dedicar-lhes esta dissertação!

Para além da minha família, existem pessoas cujo amor, carinho e amizade, foram essenciais para chegar até aqui e ser o que sou. Não é necessário que as coisas corram sempre como se planeia ou como se deseja, o que é mesmo importante é ter pessoas que acreditam em nós e lutam ao nosso lado. Não serão esquecidos e sempre terão um lugar especial na minha vida! A vocês dedico não só este trabalho mas todo o meu amor e amizade!

Um especial agradecimento é dedicado à equipa da University College London que me acolheu. Sem a Jo, sem os seus ensinamentos, sem a sua dedicação, sem o seu tempo para mim, sem as nossas discussões sobre teorias que poucos acreditam e sem toda a sua inteligência e mente aberta, não tinha aprendido tanto quanto aprendi seja a nível profissional como pessoal! Aprendi tanto com a Jo que lhe vou estar eternamente agradecida! Sem dúvida que é uma das pessoas que terei sempre como referência! À Maria, ao Venkat, à Elena e à Lina um grande obrigado por tudo o que fizeram por mim! Senti-me em casa ao vosso lado! O vosso apoio foi essencial e fez toda a diferença na aventura que foi este ERASMUS. Tenho-vos no coração!

Não posso deixar de agradecer à Rita, a grande “culpada” por esta oportunidade que única que tive na minha vida. Obrigada por tudo, Rita! Sempre estiveste presente quando precisei e esta dissertação não era o mesmo sem ti! À Professora Margarida Telhada, a primeira pessoa que me apresentou este projeto, deixo um enorme carinho, a Professora foi muito importante! Obrigado!

A toda a equipa da Unidade de Investigação em Reumatologia do Instituto de Medicina Molecular deixo o meu agradecimento por todo o apoio e ensinamentos.

Acknowledgements

Life is made of experiences which build up and shape what we are. Every path we begin is influenced, direct or indirectly, by people who contributed for that decision, who supported us during the trajectory and/or where present to share the pride of finishing one more step in life or to support us when reality is different from what we dreamed. This work here presented is an example of that. There are people, without whom, it wouldn't be possible. Thus, my first words go for them.

First of all, I want to thank my family who I love the most! My dad, my mom and my sister were always the first ones in supporting me in this adventure and never stop to show me how proud they were about my work. Without them, I would never reached what I got and I wouldn't be what I am! Without their support, this master wouldn't be possible! This thesis is more their than mine and, for all the times they sacrificed themselves for me, this dissertation is dedicated to them!

Besides my family, there are people whose love, caress and friendship, where essential to get here and be what I am. There is no need for things always go as we planned or desired, the most important is to have people who believe in us and fight by our side. You will never be forgotten and you will always have a special meaning in my life! To you, I not only dedicate this work but also all my love and friendship!

A special thanks is dedicated to the University College London team who welcomed me. Without Jo, her teachings, her dedication, without her time for me, without our discussions about theories few believe and without all her intelligence and open mind, I wouldn't have learned as much as I did as a person and as a professional. I have learned so much with Jo, I will be eternally grateful to her! She is one of the persons I will always have as reference in my life! To Maria, Venkat, Elena and Lina huge thanks for everything you did for me! I felt like home near you! Your support was essential and made all the difference in my ERASMUS adventure. I carry you all in my heart!

I also want to thanks to Rita, the main "guilty" for this unique opportunity in my life. Thanks for everything, Rita! You always were there when I needed and this dissertation wouldn't be the same without you! To Professor Margarida Telhada, the first person who introduced me this project, I leave a huge acknowledgement, the Professor was very important! Thank you!

To all the team from Rheumatology Investigation Unit from Molecular Medicine Institute I would like to thank you for all the support and teaching.

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Abbreviations

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AID	Activation-induced cytidine deaminase
APCs	Antigen presenting cells
BAFF-R	B-cell activating factor receptor
B-CLL	B cell chronic lymphocytic leukemia
BCR	B cell receptor
BM	Bone marrow
BSA	Bovine serum albumin
BT	Biological therapy
CA	Cold agglutinins
CDR	Complementary determining region
CR1	Complement receptor type 1
CR2	Complement receptor type 2
CRP	C-reactive protein
CSR	Class switch recombination
CTLA	Cytotoxic T-lymphocyte antigen
DAF	Decay accelerating factor
DMARDs	Disease-modifying anti-rheumatic drugs
ELISA	Enzyme-linked immunosorbent assay
ENRA	Early non-rheumatoid arthritis
ERA	Early rheumatoid arthritis
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FDC	Follicular dendritic cells
FR	Framework region
GC	Germinal centres
HLA	Human leukocyte antigen
H	Heavy
HC	Healthy controls
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
IL-6R	IL-6 receptor

L	Light
LT	Lymphotoxin
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MTX	Methotrexate
MZ	Marginal zone
NAL	*N*-acetyllactosamine
NET	Neutrophils extracellular traps
NSAIDs	Non-steroidal anti-inflammatory drugs
PAD	Peptidyl arginine deiminase
PBS	Phosphate buffered saline
PTPN22	Protein tyrosine Phosphatase non-Receptor type 22
***PTPN22* (1858T)**	Protein tyrosine phosphatase non-receptor type 22 gene variation C1858T
RA	Rheumatoid arthritis
RAG	Recombination activating gene
RBC	Red blood cells
RhF	Rheumatoid factor
RSS	Recombination signal sequences
RT	Room temperature
RTX	Rituximab
SD	Standard deviation
SE	Shared epitope
SF	Synovial fibroblasts
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
TCR	T cell receptor
TdT	Terminal deoxynucleotidyl transferase
TGF	Transforming growth factor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion molecule type 1
VERA	Very early rheumatoid arthritis
VENRA	Very early non-rheumatoid arthritis

Sumário

A artrite reumatoide é uma doença autoimune crónica, sistémica, que afeta principalmente as articulações e atinge cerca de 1% da população mundial. A artrite reumatoide é uma doença complexa, cujo desenvolvimento está associado a fatores genéticos, ambientais e/ou a eventos biológicos aleatórios, embora se desconheçam, exatamente, quais os mecanismos responsáveis pela sua origem e progressão. Esta doença autoimune, tem grandes implicações físicas para os doentes e económicas para a sociedade. Se a doença não for tratada, conduz a destruição das articulações, incapacidade funcional e redução da esperança média de vida.

A patofisiologia da artrite reumatoide está associada a interações complexas entre células do sistema imunitário inato e adaptativo. Em particular, as células B desempenham diversos papéis fundamentais para o desenvolvimento da doença. As células B podem funcionar como células apresentadoras de antigénios, são capazes de ativar as células T e são as responsáveis pela produção de autoanticorpos como o fator reumatoide e anticorpos anti-proteínas citrulinadas, que estão associados a um pior prognóstico e a uma maior gravidade da doença.

O fator reumatoide é um anticorpo que reconhece a porção Fc de imunoglobulinas da classe IgG. Nas pessoas saudáveis, estes anticorpos têm baixa afinidade, derivam de uma linha genética comum e pertencem ao isotipo IgM. Contudo, em doentes com artrite reumatoide, estes anticorpos podem pertencer a qualquer classe de imunoglobulinas, apresentam grande variabilidade genética e maturação de afinidade. As células B que produzam fator reumatoide são capazes de, através do seu recetor de superfície, capturar moléculas de IgG complexadas com qualquer antigénio, internalizar estes complexos, processá-los e apresentar os antigénios através do complexo de histocompatibilidade maior, recebendo ajuda das células T específicas para esses antigénios. Adicionalmente, os fatores reumatoides da classe IgG reconhecem outras moléculas de IgG e formam multímeros com capacidade de ativar o sistema complemento. Estes multímeros, quando reconhecidos pelo recetor de superfície da célula B produtora de fator reumatoide, fornecem os sinais de antigénio (molécula de IgG) e de complemento (sinal recebido através do recetor CD21 presente em todas as células B maduras) necessários para manter estas células ativadas. Outra característica destes anticorpos importante para a patogénese da artrite reumatoide é a capacidade de formarem dímeros de IgG-IgG, que não ativam o sistema de complemento, mas que têm dimensões físicas que lhes permite passar através do endotélio até ao espaço extravascular, onde ativam os macrófagos teciduais através dos seus recetores FcγRIIIa (CD16a). Quando os macrófagos são ativados, produzem fator de necrose tumoral e interleucinas 1 e 6, aos quais os fibroblastos sinoviais e células T são

altamente responsivos, o que estimula e favorece o processo inflamatório que pode levar à destruição da articulação.

Os anticorpos anti-proteínas citrulinadas estão, praticamente, ausentes nos indivíduos saudáveis. No entanto, em doentes com artrite reumatoide, estão amplamente associados a uma maior gravidade da doença. Saliente-se que, nos doentes com artrite indiferenciada, a presença destes anticorpos é um fator preditivo de evolução para o diagnóstico de artrite reumatoide.

Tal como no caso do fator reumatoide, nos doentes com artrite reumatoide, os anticorpos anti-proteínas citrulinadas pertencem a todas as classes de imunoglobulinas. De notar que estes anticorpos podem ser detetados em circulação vários anos antes do aparecimento dos primeiros sintomas clínicos da artrite reumatoide e, independentemente dos seus níveis em circulação, novas células B produtoras de anticorpos anti-proteínas citrulinadas estão constantemente a ser recrutadas da medula óssea. Contudo, o mecanismo responsável pela seleção destas células na medula óssea e pela sua sobrevivência na periferia é, ainda, desconhecido.

Os primeiros tratamentos administrados aos doentes com artrite reumatoide foram drogas anti-inflamatórias não esteroides sendo, posteriormente, substituídas por drogas anti-reumáticas modificadoras da doença como, por exemplo, o metotrexato, que são eficazes no controlo da atividade e progressão da doença. Todavia, a introdução das terapias biológicas como, por exemplo, drogas anti-fator de necrose tumoral, possibilitou atingir uma melhoria considerável na inibição da progressão da doença. Mais recentemente, têm sido adotadas terapias baseadas na depleção de células B, que demonstraram uma elevada eficácia no controlo dos níveis de autoanticorpos em circulação nos doentes com artrite reumatoide.

Os anticorpos produzidos pelas células B são codificados por um conjunto de genes constitutivos agrupados em famílias de diferentes complexidades. O gene VH4-34, em particular, tem sido associado a diversas doenças autoimunes devido à existência de diferentes autoanticorpos que expressam um idiotopo específico comum codificado por este mesmo gene, presentes em diversas doenças autoimunes. O anticorpo monoclonal 9G4, desenvolvido em rato, deteta todos os anticorpos derivados do gene VH4-34, assim como as células B cujo recetor de superfície é, também, derivado do gene VH4-34. Alguns exemplos de anticorpos que expressam o idiotopo 9G4 são, por exemplo, anticorpos contra o determinante antigénico *N*-acetil-lactosamina dos antigénios I/i dos glóbulos vermelhos, anticorpos anti-DNA, anti-lipido A e anti-cardiolipina.

As células B produtoras de anticorpos derivados do gene VH4-34 (células B 9G4+) existem naturalmente em pequenas quantidades em circulação nos indivíduos saudáveis, são autoreactivas, mas são excluídas das reações dos centros germinais. Contudo, em

doenças autoimunes como o lúpus eritematoso sistémico e a síndrome de Sjögren's, estas células escapam aos mecanismos de controlo do sistema imunitário, participam nas reações dos centros germinais, proliferam e, inclusive, iniciam processos de maturação de afinidade dos anticorpos fora das reações dos centros germinais, contribuindo significativamente para a patogénese das doenças.

Deste modo, o objetivo do presente trabalho foi determinar se os autoanticorpos associados a artrite reumatoide - o fator reumatoide e os anticorpos anti-proteínas citrulinadas - são derivados do gene VH4-34 e se expressam o idiotopo 9G4. Para tal, foram estudados doentes diagnosticados com artrite reumatoide em diversas fases da doença: doentes com artrite reumatoide inicial com menos de 6 semanas de duração de doença, doentes com artrite reumatoide inicial com menos de 1 ano de duração de doença e doentes crónicos com artrite reumatoide estabelecida com vários anos de duração da doença. Amostras de soro de todos os doentes incluídos no estudo foram processadas por *enzyme-linked immunosorbent assay* e comparadas com amostras de controlos saudáveis e outros doentes diagnosticados com outro tipo de artrites diferentes de artrite reumatoide. Adicionalmente, os níveis séricos e a classe destes anticorpos (IgM, IgG e IgA) foram, também, estudados para acompanhar a evolução da resposta dos anticorpos nestes doentes.

Neste trabalho, foram encontrados, pela primeira vez, fatores reumatoides e anticorpos anti-proteinas citrulinadas derivados do gene VH4-34 em doentes com AR desde as primeiras semanas de manifestação clínica da doença. Nos doentes estudados, a positividade para o fator reumatoide e/ou anticorpos anti-proteínas citrulinadas mostrou-se associada à presença de anticorpos derivados do gene VH4-34 numa relação dose-dependente. As combinações de anticorpos anti-proteínas citrulinadas da classe IgG com fator reumatoide e com anticorpos derivados do gene VH4-34 foram as mais prevalentes, detetadas em doentes com artrite reumatoide na fase inicial da doença. Com a progressão da doença, nos doentes com artrite reumatoide estabelecida foram detetados anticorpos de todas as classes e com níveis mais elevados na circulação, principalmente a classe IgM de anticorpos anti-proteínas citrulinadas. Além disso, é importante salientar que, nos doentes com artrite reumatoide estabelecida, o tratamento não teve qualquer influência sobre a produção de autoanticorpos.

Em conjunto, estes resultados sugerem um potencial papel na patogénese da artrite reumatoide para os anticorpos derivados do gene VH4-34 desde as primeiras semanas de evolução da doença contudo, estudos futuros são necessários para compreender qual a sua relevância.

Palavras-chave: Artrite Reumatoide, Células B, Autoanticorpos, VH4-34, idiotopo 9G4

Abstract

Rheumatoid arthritis (RA) is a chronic, systemic autoimmune disease that mainly targets the joints and affects approximately 1% of the world population. RA is a complex disease, whose development is associated with genetic, environmental and stochastic factors although the exact trigger mechanisms remain to be elucidated.

B cells play critical roles in RA physiopathology, namely through the production of autoantibodies such as rheumatoid factor (RhF) and anti-citrullinated protein antibodies (ACPA), which are directly related with disease severity.

B cells that use the germ line gene VH4-34 produce natural autoreactive antibodies. The rat monoclonal antibody 9G4 recognizes VH4-34 gene-encoded antibodies and the B cells that express these antibodies as surface receptor. VH4-34 gene-encoded antibodies have been found in some autoimmune diseases and are commonly linked with disease severity.

The main goal of the present work was to evaluate whether the 9G4 idiotope could be found on autoantibodies commonly associated with RA (RhF and ACPA). For that, patients diagnosed with RA were studied in different disease stages: very early RA patients (with less than 6 weeks of disease duration), early RA (with less than 1 year of disease duration) and chronic established RA patients (with several years of disease duration). Serum samples from all patients were processed by enzyme-linked immunosorbent assay and compared to healthy controls and other arthritis patients (non-RA).

It was found that RhF and ACPA autoantibodies express the 9G4 idiotope and these VH4-34 gene-derived antibodies could be detected in circulation since the first weeks of RA onset. Furthermore, it was observed that positivity for RhF and ACPA was dependent on the presence of VH4-34 gene-derived antibodies in a dose-dependent association.

Overall, these findings might suggest a possible role for VH4-34 gene-derived antibodies in RA triggering, but further studies need to be developed.

Key-words: Rheumatoid Arthritis, B cells, Autoantibodies, VH4-34, 9G4 idiotope

Chapter I – Rheumatoid Arthritis

1.1 – Introduction to the disease

Rheumatoid arthritis (RA) is a chronic inflammatory progressive autoimmune disease with articular and systemic features. RA inflammatory processes lead to synovial inflammation, pannus formation and joint loss of function but it may also lead to cardiovascular, pulmonary, psychological and other health disorders[1,2,3].

RA affects 0.5-1% of the population worldwide in a female/male ratio of 2.5/1, suggesting an association with specific hormonal components[3,4]. Patients with RA experience diminished quality of life and increased mortality compared with the general population[5]. The most common onset is between the ages of 30 and 50 years, but autoantibody production can be detected many years before the first clinical manifestation[3,6]. The autoantibodies mostly associated with RA disease are rheumatoid factor (RhF) and anti-citrullinated protein antibody (ACPA) but 20% of RA patients are seronegative for both antibodies[7,8,9,10,11].

Although disease etiology is unknown, there are strong environmental and genetic factors implicated in RA development and progression. It is believed that these factors together with stochastic events act in concert to cause the production of pathogenic autoantibodies[12]. The genetic contribution for RA is said to be around 50% and environmental and stochastic factors contribute for the remaining 50%[13,14].

1.2 – Risk factors and etiology

Importance of the genetic background

The idea that genetics could be implicated as a risk factor in RA pathology emerged from population studies that showed an increase in disease frequency in first-degree relatives of RA patients, especially among the ones with autoantibody production[15]. Since then, a wide range of studies found evidence of more than one gene possibly associated with RA susceptibility with different degrees of risk[16,17].

The strongest association between RA susceptibility and genetics has been assigned to the major histocompatibility complex (MHC) II – human leukocyte antigen (HLA) II – more specifically to its *DRB1* locus since it was found that an increased frequency of HLA class II type Dw4 was present among RA patients compared with normal individuals[18,19].

The *DRB1* locus encodes the classic DR specificities (DR1-14) and from these, DR4 (**04*) and in a less extent DR1 (**01*) are most strongly associated with RA[8,20]. DR4 possession is linked with an earlier disease onset and with higher mortality[21,22]. A conserved motif was found in the different HLA-DRB loci associated with RA, comprising the residues

67-74 at *DRB1* α helix wall which is glutamine[Q]-lysine[K]-arginine[R]-alanine[A]-alanine[A] or QRRAA and was named shared epitope (SE) by Gregersen *et al*[20,23]. Following this discovery, a strong association of RA susceptibility with SE was found which proved to be more significant than with the class II molecule as a whole. Furthermore, SE has been described as a risk factor more strongly associated with ACPA development than with RA itself, not only influencing their level in sera but also their fine specificity[24,25,26].

Protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) gene variation C1858T - *PTPN22* (1858T) is the second gene also associated with RA risk, although to a lesser extent[23]. The *PTPN22* gene encodes the cytoplasmic protein tyrosine phosphatase N22, which is a signal transduction inhibitor for the T cell receptor (TCR). Its variant has a potentiated catalytic activity inhibiting even more strongly the T cell activation upon TCR stimulation[27,28]. This gene is also expressed on B cells thus when its variant is present it interferes with their function in the same way as with T cells[29]. Possibly, these B and T cells are less responsive to death induced-activation (Fas/Fas-Ligand) promoting their mutual survival. In addition, *PTPN22* (1858T) as well as HLA-SE has been more closely associated with ACPA production than with RA itself and a combination of these two alleles seems to potentiate this effect suggesting a gene-gene interaction[30]. Other genes that have been associated with RA susceptibility even with a modest risk are the transcription factors *TRAF1-C5*, *STAT4* and *OLIG3*[31,32,33].

Environmental contribution for the disease

It is well described that RA, similarly to other autoimmune diseases, is more frequent in women than men. This raised questions about the contribution of the hormonal environment and its relevance for RA development specially when considering RA remission or relapse following hormonal changes induced by pregnancy, birth, breast feeding and/or oral contraceptives use[34,35,36,37]. Nevertheless, the pathways involved are not completely clear.

Smoking is another well-established environmental factor associated with RA development and severity. In fact, the risk of developing RA increases with dose and duration of smoking and is strongly associated with SE alleles, disease severity and autoantibody production[30,38]. Klareskog *et al* [39] proposed a model where smoking triggers mechanisms, which in persons with susceptibility to autoimmunity would be enough to promote the production of autoantibodies especially with ACPA specificity, and, consequently, form immune complexes and start an immune response leading to onset of RA.

In addition, infectious agents (e.g., Epstein–Barr virus, cytomegalovirus, Proteus species and *Escherichia coli*) and their products (e.g., heat-shock proteins (HSP)) have long

been linked with RA, and, although unifying mechanisms remain elusive, some form of molecular mimicry has been postulated (Appendix 1)[40,41,42].

Stochastic events as rheumatoid arthritis triggers

These factors are mostly related to inherent biologic processes. As pointed out by Pulendran *et al* [43] and further developed by Edwards and Cambridge[44,45], all antibodies are produced by chance, thus, autoantibodies may arise by random mutation during immune responses to any antigen. Consequently, the autoantibody produced may recognize and react with a self-antigen but production will not continue and the parent B cell will not survive and/or proliferate as specific T cell help will not be provided due to its self-reactive nature[44,45]. For that, the B cell needs an antigen binding site interacting with a self-antigen in such a way that triggers those processes or have other mechanisms to escape and avoid apoptosis[44,45]. This concept will be further developed on Chapter 2.1.

1.3 – Classification and diagnosis

From Garrod's initial definition of RA as a disease in 1859[46], classification criteria have been developed according to the evolution in the understanding about RA pathogenesis. Diagnostic criteria are extremely important for both epidemiological and clinical trials in order to standardise selection and clinical responses in patients[47]. The 1987 American College of Rheumatology (ACR) criteria and, more recently, its 2010 ACR revision in collaboration with the European League Against Rheumatism (EULAR) were created for this purpose[48,49]. For a patient to be included in the RA patients group, at least four of the features described on the criteria (Appendix 2) have to be fulfilled.

In the daily clinical practice, these criteria are limited since for the clinician it is more important to achieve a good differential diagnosis in order to exclude other diseases with symptoms in common with RA and to, therefore, treat the patient appropriately early in the disease. A number of studies show that a proper and efficient treatment during the beginning of RA improves considerably the outcome of the patient[50,51]. However, there is no single test to RA final diagnosis but a group of assessments which have to be taken in account. Firstly, objective evidence of joint pain, swelling and functional impairment, as well as mechanical joint problems and evidence of radiographic damage as well as evidence of symptoms of active disease should be obtained[50,52,53]. Additionally, it is suggested that baseline observations should also include a complete blood cell count with differential, RhF and ACPA determination as well as erythrocyte sedimentation rate (ESR) and/or C-reactive protein (CRP) and renal and hepatic function testes to guide treatment[50,53].

Chapter 2 – Clinical features of rheumatoid arthritis

2.1 – Pathophysiology

In 1998 Edwards and Cambridge proposed a model, which explained how RA could be triggered[45]. B cells with a class-switched (IgG) BCR recognising the Fc region of IgG molecules (RhF) may deceive the immune system, avoid apoptosis and trigger autoimmunity. The surface receptor from a B cell with the ability to produce RhF will pick up complexed IgG molecules attached to a variety of different antigens[54]. The B cell digests the antigen, presents it through its HLA class II and gets help from many different T cell which recognises a variety of peptide antigens captured in the form of immune complexes by the IgG-RhF B cell[54].

In normal individuals RhF is naturally present, belongs to IgM class, has low affinity, and tend to come from a common germ line[55,56,57]. On the other hand, RhF from RA patients belongs to any immunoglobulin (Ig) class, is derived from a wide range of Ig germ line genes and shows affinity maturation[44,55,57,58]. IgG RhF producing B cells, may become self-perpetuating because the IgG produced will bind to other IgG molecules[7,59], form complement-fixing multimers[7,60] and provide the antigen (IgG molecule) and complement signals (through the complement binding receptor CD21 on all mature B cells) to keep these B cells activated and avoid apoptosis[7,44,45,59]. Of note, IgM RhF producing B cells are not self-perpetuating but are also stimulated by IgG RhF complexes[44,45]. Nevertheless, these complexes may have other roles in addition to RhF B cells activation and self-perpetuation. Kunkel and colleagues[61] showed that RA patients had IgG RhF in circulation associated in oligomers but also, and predominantly, in dimers. Dimers of antibodies are small enough to escape clearance by red cell complement receptor type 1 (CR1) and to pass through endothelium and reach extravascular space[45,62,63]. Once there, these dimers will be able to activate tissue macrophages by the Ig Fc receptor FcyRIIIa (CD16a)[64,65], which is known to be expressed in high-levels only on macrophages from the synovial intima (Appendix 3) and other organs affected in RA as lungs and heart pericardial lining[66,67,68]. FcyRIIIa expression by macrophages is induced by transforming growth factor (TGF)-β which, in turn, is produced by many cell types in response to mechanical stress being in accordance with the sites and severity of inflammation in RA[69,70].

Around 70% of RA patients are also characterized by the presence of ACPA that recognize epitopes (e.g., vimentin and fibrinogen proteins) in which arginine is converted into citrulline by peptidyl arginine deiminase (PAD) during post-translational modifications[71,72,73]. Citrullination is a physiological process that occurs during keratinisation of epithelial cells, inflammation and apoptosis[73,74]. Although ACPA are almost absent from healthy individuals in RA patients these autoantibodies are associated with disease severity and are also a

predictive factor for RA development in patients with undifferentiated arthritis or arthralgia[6,75]. Indeed, ACPA are very specific for RA and highly cross-reactive recognizing a diverse set of citrullinated peptides with different specificities and usage of all antibody isotypes[47,76]. Moreover, ACPA can be detected not only when RA is established but also several years before disease onset[6].

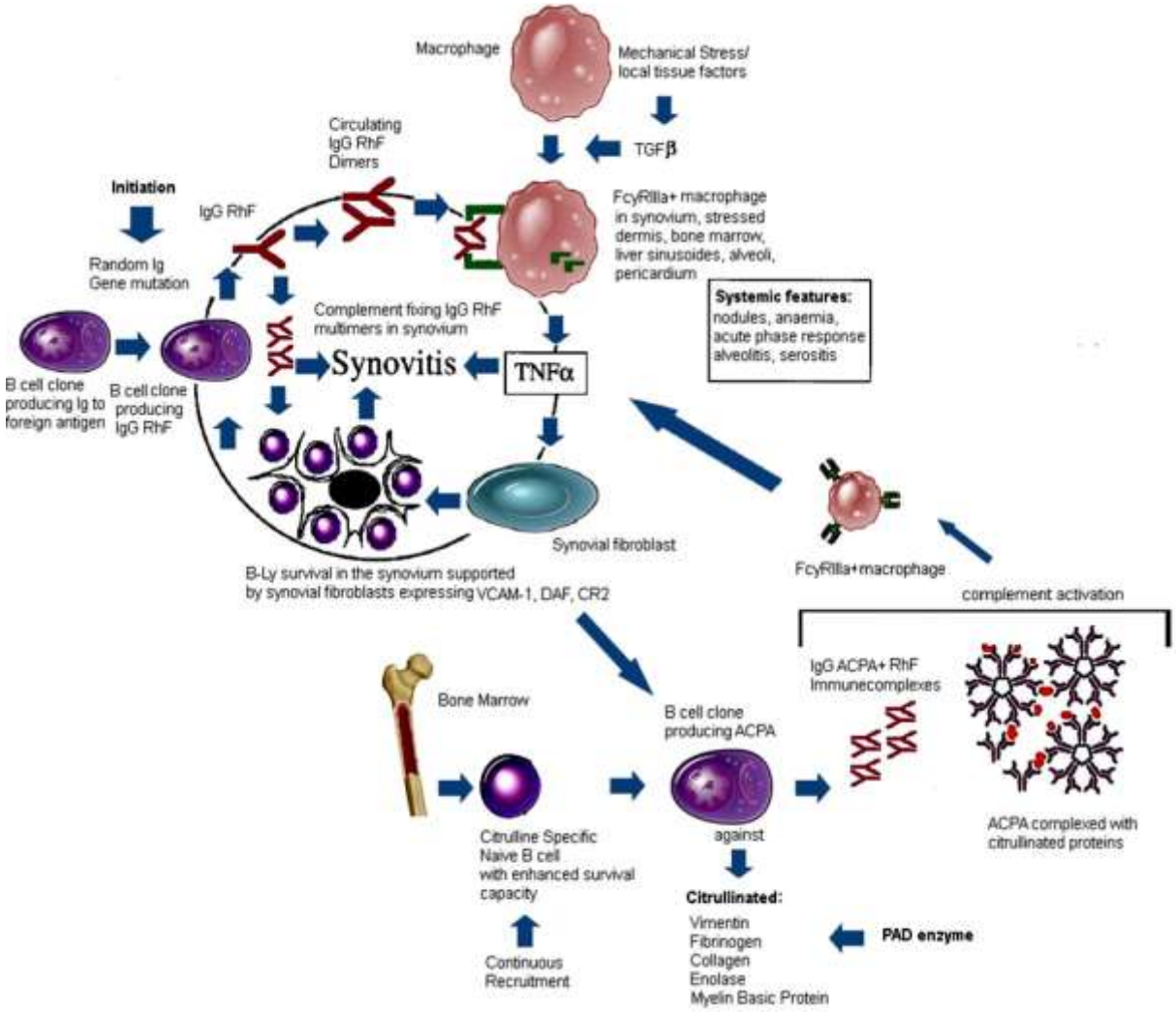


Figure 1 - B cell contribution for rheumatoid arthritis pathophysiology (Adapted from reference 45)

Generally, antibodies belonging to IgM isotype have a short half-life and are mainly produced by new B cells recruited from the naïve repertoire. Thus, the constant presence of IgM ACPA before RA onset and after disease establishment on RA patients seropositive for ACPA and the fact that IgM ACPA levels correlate with IgG ACPA ones, suggest a continuous renewal of auto-reactive IgM B cells developing into plasmablasts and/or undergoing class switch recombination (CSR)[6,77,78]. ACPA response is atypical and new cells are continuously recruited from bone marrow (BM) regardless the ACPA antibodies titres already present on sera and tissues[77,78,79]. Immune complexes formed by IgG ACPA and RhF, and by autoantibodies complexed with citrullinated proteins can trigger and

perpetuate the immune response through Fc receptor binding and complement system activation (Figure 1). The presence of ACPA before joint symptoms as well as disturbances of central B cell tolerance to citrullinated residues in both immature and mature B cells repertoire suggest the occurrence of mechanisms underlying the origin of B cells specific for citrullinated residues in RA patients most likely at sites distant to the joints.

Once activated, macrophages produce large amounts of tumor necrosis factor (TNF), Interleukin (IL)-1 and small amounts of IL-6[80]. Synovial fibroblasts (SF) are the most responsive to TNF and in its presence they will express vascular cell adhesion molecule type 1 (VCAM-1), decay accelerating factor (DAF) and complement receptor type 2 (CR2) which are molecules involved in B cell survival and differentiation that may facilitate the local accumulation of ectopic lymphoid tissue and plasma cells[67,81,82,83]. SF are also activated by IL-1 and secret large amounts of IL-6 and matrix degrading enzymes, which are principal contributors to synovial inflammation and joint destruction[81,84,85]. IL-6 also promotes inflammation through the expansion and activation of T cells, recruitment and differentiation of B cells, and induction of acute-phase reactants by hepatocytes[84,85]. The histological changes of acute inflammation within the synovial tissue include increased vascularity, endothelial swelling of vessels with some polymorphonuclear exudation, oedema and fibrin deposition on the synovial lining[86].

Accumulation of cells within the synovial intima mainly due to an increased influx of macrophages from the BM is also present as well as an increased cellularity at the subintima by a marked infiltration by plasma cells, dendritic cells, T cells, mast cells and neutrophils, which becomes progressively greater with continuing inflammatory activity[1,87,88]. As inflammation progresses, a cartilage thinning and invasion by a layer of vascular soft tissue known as “pannus” may also occur[84]. This stage is mediated, in part, by cytokines and matrix metalloproteinases (MMPs) that are released in addition to chondrocyte mediated destruction and failure of repair mechanisms[3,12]. Bone destruction may also occur and is mediated through enhanced action of osteoclasts[47,80]. Furthermore, in a minority of cases, a tendency for follicular aggregations of lymphocytes is also present with a consequent development of lymphoid follicles in the joint, which will potentiate and perpetuate the local inflammation through the secretion of autoantibodies and other inflammation-associated molecules[89,90]. In addition, nodules and vascular complications such as atherosclerosis may also be present in a small percentage of RA patients[45].

For years, T cells were considered to be the most important trigger of inflammation in RA due to their predominant infiltration observed in the synovium, to the association of RA with certain MHC class II loci and to their biological importance in activating macrophages[1,8,87,91]. Nevertheless, the T cell infiltrates are found in all chronically inflamed tissues, are absent from other tissues affected by RA, there is a lack of evidence supporting

the existence of a specific self-antigen recognized by T cells, and B cell depletion therapy used in RA patients was proved very efficient[87,92,93,94]. Thus, T cells have more recently been thought of as being important for RA pathogenesis, but not being specific for the development of the disease[9,92,95,96].

Overall, the main prognostic factors that are strongly linked with RA severity and considered key features underlying its development are the presence of ACPA and RhF, possession of HLA-SE associated alleles, early development of joint erosions, increasing number of affected joints and presence of extra-articular features, older age at onset and high CRP values[97,98].

2.2 – Drugs used in rheumatoid arthritis treatment

During the first half of the 20th century, symptoms of RA were managed with non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids[99]. These are efficient in relieving symptoms but cannot stop disease progression. Disease-modifying anti-rheumatic drugs (DMARDs) such as gold salts, sulphasalazine and methotrexate (MTX) were then proved as efficient in managing disease activity and started being used widely in RA patients[99,100]. With this new approach, joint damage was able to be retarded in these patients but not prevented and a loss of benefit with time was observed[99]. Cytotoxic chemotherapy as cyclophosphamide in combination with glucocorticoids was then used to potentiate the beneficial effect. However, the number of metabolic and immunosuppressive side effects resulted seriously and challenged the treatment guidelines[99,100]. By the 1990s, with evidence of MTX efficacy, safety and tolerability when given to patients with RA, it became the drug of choice to treat RA patients and DMARDs were prescribed earlier in disease[99]. Although, sustained MTX treatment was required to maintain clinical improvement, some patients still had persistent disease activity despite the treatment and often presented side effects such as gastrointestinal and pulmonary toxicity, headaches, fatigue and less commonly bone marrow suppression. MTX is also teratogenic[99,100].

An enormous advance in the treatment of RA came with the development of biological therapy (BT). The evolution of understanding about the key molecular mediators of RA disease alongside the monoclonal antibody production revolution created a large option of potential treatments. The earliest agents were TNF antagonists, which resulted in dramatic improvement in symptoms and inhibition of disease progression[99,100]. TNF antagonists include monoclonal antibodies targeting TNF (infliximab, adalimumab, certolizumab and golimumab), and a TNF-receptor Fc-fusion protein that binds TNF and lymphotoxin (LT) family members (etanercept)[12,99,100]. Although monotherapy with these drugs is effective, studies showed a consistent superior efficacy when used in combination with MTX[99].

Other BT have subsequently been approved for RA treatment including IL-6 receptor (IL-6R) antagonists, cytotoxic T-lymphocyte antigen (CTLA)-4 based drugs and B cell depletion therapy. The drug Tocilizumab (recombinant humanized antihuman IL-6 receptor monoclonal antibody of the IgG1 subclass) is the only available IL-6 antagonist and binds to both membrane-bound and soluble IL-6 receptors, preventing their activation by IL-6[99,100]. CTLA-4 is a molecule that binds with a high affinity to the CD80/86 ligand on antigen-presenting cells (macrophages, dendritic cells and B cells) and Abatacept (Ig fused to the extracellular domain of CTLA-4) is used to block the interaction between the antigen-presenting cells' CD80/86 ligand and the CD28 ligand from the T cell, which is necessary for T cell activation. The result is a decrease in T cell proliferation and reduction of the cytokines TNF, IL-1, and IL-6 having also implications for B cell activation[99,100]. B cell depletion protocols are currently based on Rituximab (RTX) (chimaeric monoclonal IgG1 anti-CD20 antibody). CD20 is a B cell surface Ig, which is expressed from the pre-B cell stage until differentiation into plasma cell[99,100].

Chapter 3 – B cells

The pathogenic role of B cells on RA triggering and maintenance is now well recognized and further understanding may elucidate about ways to prevent RA onset and to achieve clinical remission once disease is established.

3.1 – B cell development

During foetal development in humans, B cells are first generated in the liver and later in the BM[101]. After birth, BM is the only organ responsible for B-lymphopoiesis. In the BM, stem cells give rise to the earliest distinctive B-lineage cell, the B cell progenitor (pro-B cell), which then differentiates into B cell precursor (pre-B cell)[102,103]. This last is a large proliferating cell (pre-BI cell), which will become a small post-mitotic cell (pre-BII cell). This stage of B cell development culminates in the production of immature B cells with only IgM on its surface and these cells will pass through the induction of central tolerance to antigens present in the BM.[102,103] If not deleted or successfully achieving light-chain revision, the cell will acquire IgD on its surface and leave the BM as a naïve B cell. All B cell differentiation stages in the BM do not require antigen exposure to survive and, therefore, correspond to the antigen-independent phase of B cell development[101,102].

After leaving the BM, the naïve B cells circulate in the blood and lymphatic systems and are carried to secondary lymphoid organs (lymph nodes, spleen and Peyer's Patches) where, after encountering an antigen, will become large B-blasts mostly differentiating into short-living IgM producing plasma cells but a minority will differentiate to form germinal

centres (GC)[9,102]. Of note, the majority of the new B cells leaving the BM will die within few days presumably due to lack of appropriate survival signals (signals delivered through the B cell receptor (BCR) and B-cell activating factor receptor (BAFF-R) to naïve B cells). Once a B cell is activated by its specific antigen, a GC will form around it and interaction occurs with T cells able to deliver specific T-cell help[102,104]. GC B cell activation is dependent on the presence of follicular dendritic cells (FDC), which have a fundamental role in promoting B-cell recruitment, selection, proliferation and differentiation by trapping immune complexes to engage B cells that were trafficking through lymphoid follicles[102,104,105]. GC are specialized sites for memory B cell generation (although some memory B cells can be generated outside GCs), plasma cell differentiation and where affinity maturation of serum antibodies takes place[102]. Some memory B cells, particularly those formed in the spleen marginal zone, can re-circulate through the peripheral blood and secondary lymphoid tissues, in the same way as naïve B cells until encountering the same antigen which drove its origin in the first place and then participate in a secondary immune response[106]. Many, however, populate the mantle zones of secondary lymphoid organs. The plasma cells generated in the GC then go to reside mainly in the BM or in the lamina propria of the gut or other epithelial surfaces. Since B cell activation and differentiation in the periphery require antigen exposure, this stage comprises the antigen-dependent phase of B cell development[9,102].

3.2 – Generation of antibody diversity

Antibodies, or Ig, are the antigen-binding proteins present on the B cell membrane and secreted in soluble form by plasma cells[9]. Antibody diversity is achieved by V(D)J recombination (V-variable, D-diversity and J-joining), somatic hypermutation (SHM) and CSR[9,102]. The V(D)J recombination is an ordered site-specific DNA rearrangement process that occurs in developing lymphocytes in the BM , in which V(D)J gene segments are randomly combined at recombination signal sequences (RSS)[9,102]. RSS are recognized by an endonuclease coded by recombination activating gene (*RAG*)*-1* and *RAG-2*, responsible for DNA double-stranded breaks following the 12-23 bp rule[9,102]. The diversity generated by this process is further increased by terminal deoxynucleotidyl transferase (TdT) that adds nucleotides to gene segments junctions[9,102]. SHM is a mechanism that occurs within GC in secondary lymphoid organs and involves the introduction of point mutations, mainly nucleotide substitutions, as well as occasional deletions and duplications at a very high rate into the DNA of heavy and light chain variable region genes, at CDR[102]. This may alter the specificity of the encoded antibodies and increase antibody affinity – affinity maturation. CSR is an isotype switching deletional DNA recombination process that occurs in mature B cells and consists of replacing an expressed heavy-chain constant-region gene, usually IgM, with another one of a different biological function – IgG, IgA or IgE[102]. During this process, only

the effector functions of the antibody are changed. SHM and CSR are mediated by activation-induced cytidine deaminase (AID) that promotes the recombination events[107,108].

3.3 – Genes, immunoglobulins and autoimmunity

Heavy (H) and Light (L) chain from an Ig are assembled during B cell development by rearrangement of discontinuous germ line genes segments deriving from V(D)J recombination. A biased usage of some VH genes namely VH4-34 has been described in human autoimmunity[109]. The VH4-34 gene codes an idiotope (alanine[A]-valine[V]-tyrosine[Y]) within the framework region (FR)-1 and a tryptophan[W] at residue 7, which is close to the junction with the constant region, and is recognized by the rat monoclonal antibody 9G4 (Figure 2 and Appendix 4)[110,111,112,113]. Thus, all B cells expressing VH4-34 gene-derived Ig are called 9G4 cells (9G4+ B cells). These cells comprise 4-10% of the B cell naïve repertoire on normal individuals, but serum VH4-34 gene-derived Igs are rarely found[114,115,116].

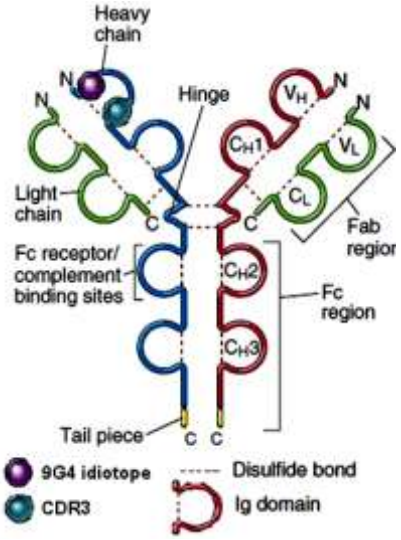


Figure 2 - Structure of a VH4-34 gene-derived antibody (Adapted from reference 129)

Nevertheless, the level of VH4-34 gene-derived Igs can increase transiently in response to certain bacterial and viral infections[117,118]. VH4-34 codes Igs that are inherently auto-reactive regardless of complementary determining region (CDR)-3 specificity and they may be advantageous in clearing effete or apoptotic cells or even tumor cells but also create a risk for the development of autoimmunity in susceptible individuals[119,120,121]. In fact, the germ line VH4-34 was found strongly associated with IgM cold agglutinins (CA) production as well as with anti-DNA antibodies in systemic lupus erythematosus (SLE) patients, and anti-myeloperoxidase antibodies in patients with vasculitis when in the presence of appropriate antigen binding specificity within the CDR3[122,123,124,125]. In normal individuals, 9G4 cells are located in the marginal zone (MZ) of spleen and mantle zone and cannot participate in a GC reaction[119,126]. Consequently, CSR is almost absent and VH4-34 gene-derived Igs belong to IgM isotype. On the other hand, 9G4 cells from SLE and human immunodeficiency virus (HIV) are not excluded efficiently from GC reactions[126,127,128]. Thus, VH4-34 class-switched antibodies with affinity maturation are found on these patients, which are strongly associated with disease evolution and severity[124]. These features suggest that further studies on 9G4 cells and VH4-34 gene-derived Igs might be necessary to clarify the underlying mechanisms responsible for autoimmune diseases.

Chapter 4 – Aims of the study

The main goal of the present work was to evaluate whether autoantibodies commonly associated with RA (RhF and ACPA) are VH4-34 gene-derived and which is their contribution to the disease. The rat monoclonal antibody 9G4 was used to study the RhF and ACPA derivation from VH4-34 in RA patients serum with few weeks of disease in comparison to those with many years. The levels and class of RhF and ACPA antibodies were also studied in order to track the evolution of the antibody response in these patients. Serum samples from healthy controls (HC) and from patients with non-RA polyathritis were also collected for comparison.

Chapter 5 – Patients and Methods

5.1 – Patients

The patients included in this study were untreated very early polyathritis patients (with less than 6 weeks of disease duration), untreated early polyathritis patients (with less than one year of disease duration) and established RA patients treated with MTX, TNF antagonists (etarnecept, adalimumab, infliximab and certolizumab), anti-IL6R (tocilizumab), CTLA-4 based drug (abatacept). All patients were attending the Rheumatology Department in the Hospital de Santa Maria and Hospital da Luz (Lisbon, Portugal), University College London Hospitals (London, United Kingdom) and Hospital Gregorio Maranon (Madrid, Spain). All patients with established disease from all centers had received a variety of treatments due to resistant disease. The hospitals ethics committees approved the study and all patients signed an informed consent prior to any protocol-specific procedure.

5.2 – Serum autoantibodies: experimental design and measurement

In order to measure the levels of autoantibodies in sera enzyme-linked immunosorbent assay (ELISA) tests were used. All the sequential samples from each patient were run simultaneously. The autoantibodies measured in this study included ACPA and RhF belonging to IgM, IgG and IgA isotypes. Binding of the rat monoclonal antibody 9G4 to ACPA and RhF autoantibodies was also determined by ELISA.

1) IgG, IgM and IgA ACPA: Sera diluted at 1/200 in RD6Q diluent (R&D Systems; Minneapolis, USA) were incubated for 1 hour in 96-well plates coated with CCP2 peptides (Axis Shield Diagnostics; Scotland, UK). After incubation with serum samples, plates were washed and anti-human horseradish peroxidase (HRP)-conjugated antibodies (listed on Table 1) were added, followed by a 40 minutes incubation period and washing. All conjugates (The Binding Site Group; Birmingham, UK) were diluted in RD6Q diluent. All incubations were at room temperature (RT) on a shaking platform and all washing steps

were performed three times using an automated plate washing machine that removes unbound antibodies/reagents by aspiration and washing with dulbecco's phosphate buffered saline (PBS) 10x with 0,05% of Tween-20 (PBS/Tween; Sigma-Aldrich; Missouri, USA).

Table 1 - Polyclonal antibodies for ACPA measurement

ELISA	Conjugate	Dilution
IgG ACPA	sheep anti-Human IgG-HRP	1/12800
IgM ACPA	sheep anti-Human IgM-HRP	1/3200
IgA ACPA	sheep anti-Human IgA-HRP	1/6400

The development of the colour reaction was performed using the peroxidase substrate tetramethylbenzidine (TMB) (Sigma-Aldrich; Missouri, USA) diluted at 1/4 in Citrate Phosphate Buffer (pH 5.0). This substrate develops a blue reaction product in a reaction catalised by peroxidase. The reaction was stopped with 2% sulfuric acid (H_2SO_4) originating a yellow end product which is stable for absorbance reading at 450 nm within 1 hour. The cut-off values for IgM and IgA ACPA were determined by calculating the mean±3 standard deviation (SD) from normal control sera. For IgG ACPA measurement, calibrators from a standard kit (Axis Shield Diagnostics; Scotland, UK) were used and the respective cut-off was 5 U/mL. For IgM and IgA ACPA, cut-off was determined from HC and all values were expressed as arbitrary units per mil calculated by linear regression curve created with values from an empty well and a high positive known serum. Results were considered as positive when exceeding the cut-off value.

2) 9G4 expression on ACPA: To determine the expression of 9G4 idiotope on serum autoantibodies recognizing citrullinated peptides, sera were diluted at 1/50 in RD6Q diluent and after incubation for 1 hour at RT in 96-well plates coated with CCP2 peptides, the rat IgG monoclonal 9G4 antibody (IGM; Palo Alto, USA), diluted at 1µg/mL in RD6Q diluent, was added. Following 1 hour incubation at RT, a sheep polyclonal IgG anti-rat conjugated with HRP (Amersham; Buckinghamshire, UK) was added and plates were incubated for another hour. The next step was reaction development, whose procedure as well as cut-off determination were the same as previously described above.

3) IgG ACPA – Pepsin digestion of sera: In order to exclude the possibility of interference from IgM RhF (often present in RA patient sera in high levels) on IgG ACPA ELISA assay, the measurement of IgG ACPA levels was also performed on sera digested with pepsin. For this, an adaptation of the commercial kit EL-RF/3 (TheraTest Labs; Chicago, USA) was used. The pepsin destroys IgM autoantibodies as well as a large proportion of IgA by digesting their Fc regions. IgG antibodies will also have its Fc portion digested but the end product (IgG $(Fab')_2$ fragments) keep their ability to react with the antigen-coated wells. Serum samples were digested following the manufacturer's instructions and were used for

IgG ACPA quantification by ELISA as previously described. The kit EL-RF/3 provided the conjugated antibody (rabbit anti-human IgG $(Fab')_2$ antibody conjugated with HRP) that was used in the assay according to manufacturer's instructions and no cut-off value was determined. Also, to determine the efficacy of digestion, samples were run in duplicate (one well to measure IgG ACPA and the other to measure IgM ACPA) in order to confirm the IgM ACPA destruction. This digestion technique with pepsin not only destroyed the Fc region of IgM antibodies, but also probably destroyed binding specificities and dissociated immune complexes, since the final product after neutralization of digestion had a high salt concentration.

4) IgM and IgA RhF: For IgM and IgA RhF autoantibody measurement, 96-well ELISA clear plates (R&D Systems; Minneapois, USA) were half-coated overnight at RT with anti-rabbit IgG (1µg/mL) (Sigma Aldrich; Missouri, USA) diluted in bicarbonate buffer (0.06 M, pH 9.6). The other half of the plates was only coated with bicarbonate buffer (0.06 M, pH 9.6) for background (unspecific binding) determination. After coating, the plates were blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich; Missouri, USA) in PBS/Tween and incubated for 1 hour at RT on a shaking platform. Sera diluted at 1/200 in RD6Q diluent were added in duplicate (one to coated and one to uncoated side of the plate) and incubated for 1 hour at RT on a shaking platform. After incubation, anti-Human IgM-HRP or anti-Human IgA-HRP conjugates were added for IgM RhF and IgA RhF determination, respectively (see Table 1 above). The reaction development and cut-off determination procedures were the same as for ACPA quantification.

5) IgG RhF – Pepsin digestion of sera: Similarly to IgG ACPA measurement, to avoid interference from IgM RhF on IgG RhF assay, an adaptation from the commercial kit EL-RF/3 (TheraTest Labs; Chicago, USA) was also used. Sera samples were digested with pepsin following manufacturer's instructions and were processed as for RhF ELISA as previously described (see 4). The conjugated antibody that was used in IgG RhF ELISA assay (rabbit anti-human IgG $(Fab')_2$ antibody conjugated with HRP) was provided in the commercial kit. Pepsin digestion efficiency was determined using the same procedure as described for IgG ACPA ELISA with pepsin digested sera samples. The reaction development and the cut-off determination procedures were the same as for IgM and IgA RhF measurement.

6) 9G4 expression on RhF: To determine the expression of 9G4 idiotope on serum autoantibodies recognizing the Fc region from an IgG antibody (RhF), antigen coated plates as used for RhF detection were utilized. Serum samples were diluted at 1/50 RD6Q diluent and after incubation the rat IgG monoclonal 9G4 antibody, diluted at 1µg/mL in RD6Q diluents was added and plates were incubated for 1 hour. After a washing step, a goat polyclonal anti-rat antibody conjugated with biotin (Abcam; Cambridge, UK) was added for 1

hour at RT, followed by incubation for 1 hour with streptavidin conjugated with HRP (R&D Systems; Minneapolis, USA). The reaction development and the cut-off determination procedures were the same as for IgM and IgA RhF measurement.

5.3 – Statistical analysis

Statistical differences were determined with nonparametric Mann–Whitney and Wilcoxon tests using GraphPad Prism (GraphPad, San Diego, California, USA). Differences were considered statistically significant for $p<0,05$.

Chapter 6 – Results

6.1 – Patients clinical information

Patients with a history of polyarthritis of less than 6 weeks of disease duration (n=40) were followed-up for a minimum of 3 months. Fourteen out of 40 patients fulfilled the 2010 ACR/EULAR criteria for RA[49] and were classified as very early RA (VERA) patients. The remaining early polyarthritis patients (n=26), who did not evolve into RA, were classified as very early non-RA (VENRA). VENRA patients evolved to: spondyloarthritis (n=6), SLE (n=5), polymyalgia rheumatica (n=4), microcrystalline arthritis (n=2), Sjögren syndrome (n=1), diffuse connective tissue disease (n=1), viral polyarthritis (n=1), arthritis associated with malignancy (n=1) and 5 patients entered spontaneously into remission before 3 months of follow-up, remaining without a specific diagnosis and were thus classified as presenting a self-limited form of polyarthritis. Additionally, untreated polyarthritis patients, who had less than one year of disease duration, but more than 6 weeks of disease duration (n=33), were also studied. After a minimum follow-up of 3 months, 28 patients fulfilled the 2010 ACR/EULAR criteria for RA[49] and were classified as early RA (ERA). The remaining 5 untreated early polyarthritis patients who did not evolve into RA were diagnosed with a self-limited form of arthritis and classified as early non-RA (ENRA). Serum samples from 21 HC were also collected for comparison. Clinical details of patients with untreated polyarthritis together with HC are shown in Table 2.

A total of 35 patients with chronic active RA (established RA) were also included in this study, organized into two groups according to their disease duration: patients up to 17 years (n=19) and patients up to 40 years, but more than 17 years (n=16). In addition, serial studies were also conducted in 7 patients from the VERA group who started treatment with MTX as well as in cohorts of patients with established active RA and under treatment with TNF antagonists (etarnecept, adalimumab, infliximab and certolizumab) (n=13), with CTLA-4 based drug abatacept (n=5) and also in patients treated with anti IL-6R tocilizumab (n=13).

Table 2 - Clinical details of early polyarthritis patients and healthy controls

	HC	VERA	VENRA	ERA	ENRA
	(n=21)	(n=14)	(n=26)	(n=28)	(n=5)
Disease duration (months)	NA	<6 weeks	<6 weeks	7.1±3.6	5.4±3.6
Age (years)	48±11*	52±19*	47±18*	47±13*	51±12*
Sex (% female)	67	93	73	82	80
Swollen joints	NA	13±11*	7±9*	8±7*	2±3*
Tender joints	NA	13±9*	9±8*	7±6*	3±2*
ESR (mm/1st hour)	NA	44.9±28.4*	44.6±35.6*	40.0±36.2*	15.2±11.9*
DAS28	NA	6.1±1.7*	4.9±1.9*	5.1±1.4*	3.8±1.3*

* mean±3SD from group data; NA=Not Applicable

6.2 – Autoantibodies expressing the 9G4 idiotope are secreted since the first weeks of rheumatoid arthritis

Sera from patients and HC were collected and processed in order to determine ACPA and RhF autoantibody levels from IgM, IgG and IgA isotypes, as well as the 9G4 antibody binding to these autoantibodies, using ELISA technique. The percentage of patients and HC positive for each autoantibody was assessed and results are shown on Table 3. ENRA patients and HC were seronegative for these autoantibodies. VENRA patients were seropositive for IgM ACPA (n=4, 15%), 9G4 ACPA (n=4, 15%) and IgM RhF (n=2, 8%) but the autoantibody levels were near the cut-off limit (Figure 3). In VERA and ERA patients, IgG ACPA, 9G4 ACPA and IgM RhF were found to be the most prevalent antibodies, being present on the sera of almost half of the patients studied. Nevertheless, ERA patients showed an increased frequency of IgM ACPA, IgA ACPA and 9G4 RhF levels when compared to VERA [n =13 (46%), n=11 (39%) and n=13 (46%) positive patients respectively on ERA group in comparison with n=3 (21%), n=3 (21%) and n=1 (7%) positive patients on VERA group, respectively]. Figure 3 shows a more detailed assessment of the ACPA and RhF autoantibody contribution to these early polyarthritis patients studied. In established RA patients, IgG ACPA, IgM ACPA, 9G4 ACPA and IgM RhF antibodies were present on the sera of the majority of the patients studied, as indicated in Table 3. Of note, the percentage of seropositive patients for 9G4 ACPA and 9G4 RhF antibodies increased with RA progression, since it was higher in both groups of established RA patients (<17 years and < 40 years of disease duration) in comparison with VERA and ERA patients.

For a further understanding about RA autoantibodies the percentage of patients containing up to three combinations of ACPA and/or RhF autoantibodies are shown on Tables 4 and 5. These tables show that the longer the disease duration the higher the

percentage of seropositive patients. The double combinations of 9G4 ACPA with IgG ACPA as well as 9G4 ACPA with IgM RhF, and also IgG ACPA with IgM RhF were the most common associations detected in the VERA group [n=6 (43%), n=6 (43%) and n=6 (43%) respectively]. These 3 combinations remained the most prevalent in established RA patients with up to 17 years of disease duration [n=16 (89%), n=16 (89%) and n=18 (95%) respectively]. The double combination of 9G4 ACPA with IgM ACPA was present in 100% (n=16) of patients with up to 40 years of disease duration, and all the other combinations were present in a higher frequency when compared to the other groups of RA patients.

Table 3 - Percentage of seropositive patients for ACPA, RhF and antibodies expressing the 9G4 idiotope

	HC	**VERA**	**VENRA**	**ERA**	**ENRA**	**Established Patients**	**Established Patients**
	NA	<6 weeks	<6 weeks	<1 year	<1 year	<17 years	<40 years
	(n=21)	**(n=14)**	**(n=26)**	**(n=28)**	**(n=5)**	**(n=19)**	**(n=16)**
IgG ACPA	0	43	0	50	0	100	94
IgM ACPA	0	21	15	46	0	63	100
IgA ACPA	0	21	0	39	0	47	81
9G4 ACPA	0	50	15	46	0	90	100
IgG RhF	0	0	NA	11	NA	5	38
IgM RhF	0	57	8	50	0	95	94
IgA RhF	0	9	0	19	0	26	63
9G4 RhF	0	7	0	46	0	53	88

NA=Not Applicable

Table 4 - Percentage of patients seropositive for double combinations of ACPA, RhF and/or antibodies expressing the 9G4 idiotope

	VERA	ERA	Established Patients	Established Patients
	<6 weeks	<1 year	<17 years	<40 years
	(n=14)	**(n=28)**	**(n=19)**	**(n=16)**
9G4 ACPA/IgG ACPA	43	43	89	94
9G4 ACPA/IgM ACPA	21	39	63	100
9G4 ACPA/IgA ACPA	21	39	47	81
9G4 ACPA/IgG RhF	0	7	5	38
9G4 ACPA/IgM RhF	43	36	89	94
9G4 ACPA/IgA RhF	9	11	21	63
9G4 ACPA/9G4 RhF	7	36	53	88
9G4 RhF/IgG RhF	0	7	5	31
9G4 RhF/IgM RhF	7	39	53	81
9G4 RhF/IgA RhF	0	18	21	56
9G4 RhF/IgG ACPA	7	36	53	81
9G4 RhF/IgM ACPA	7	29	37	88
9G4 RhF/IgA ACPA	7	29	32	69
IgG ACPA/IgM ACPA	21	39	63	94
IgG ACPA/IgA ACPA	21	39	47	81
IgG ACPA/IgG RhF	0	7	5	38
IgG ACPA/IgM RhF	43	39	95	88
IgG ACPA/IgA RhF	0	7	5	56
IgM ACPA/IgA ACPA	21	36	47	81
IgM ACPA/IgG RhF	0	7	0	38
IgM ACPA/IgM RhF	21	32	63	94
IgM ACPA/IgA RhF	0	7	11	63
IgA ACPA/IgG RhF	0	7	0	31
IgA ACPA/IgM RhF	21	29	47	75
IgA ACPA/IgA RhF	9	7	26	56
IgG RhF/IgM RhF	0	7	5	38
IgG RhF/IgA RhF	0	7	5	31
IgM RhF/IgA RhF	9	18	26	56

NA=Not Applicable

Table 5 - Percentage of patients seropositive for triple combinations of antibodies

	VERA	ERA	Established Patients	Established Patients
	<6 weeks	<1 year	<17 years	<40 years
	(n=14)	**(n=28)**	**(n=19)**	**(n=16)**
IgG/IgM/IgA ACPA	21	36	35	81
IgG/IgM/IgA RhF	0	7	4	31

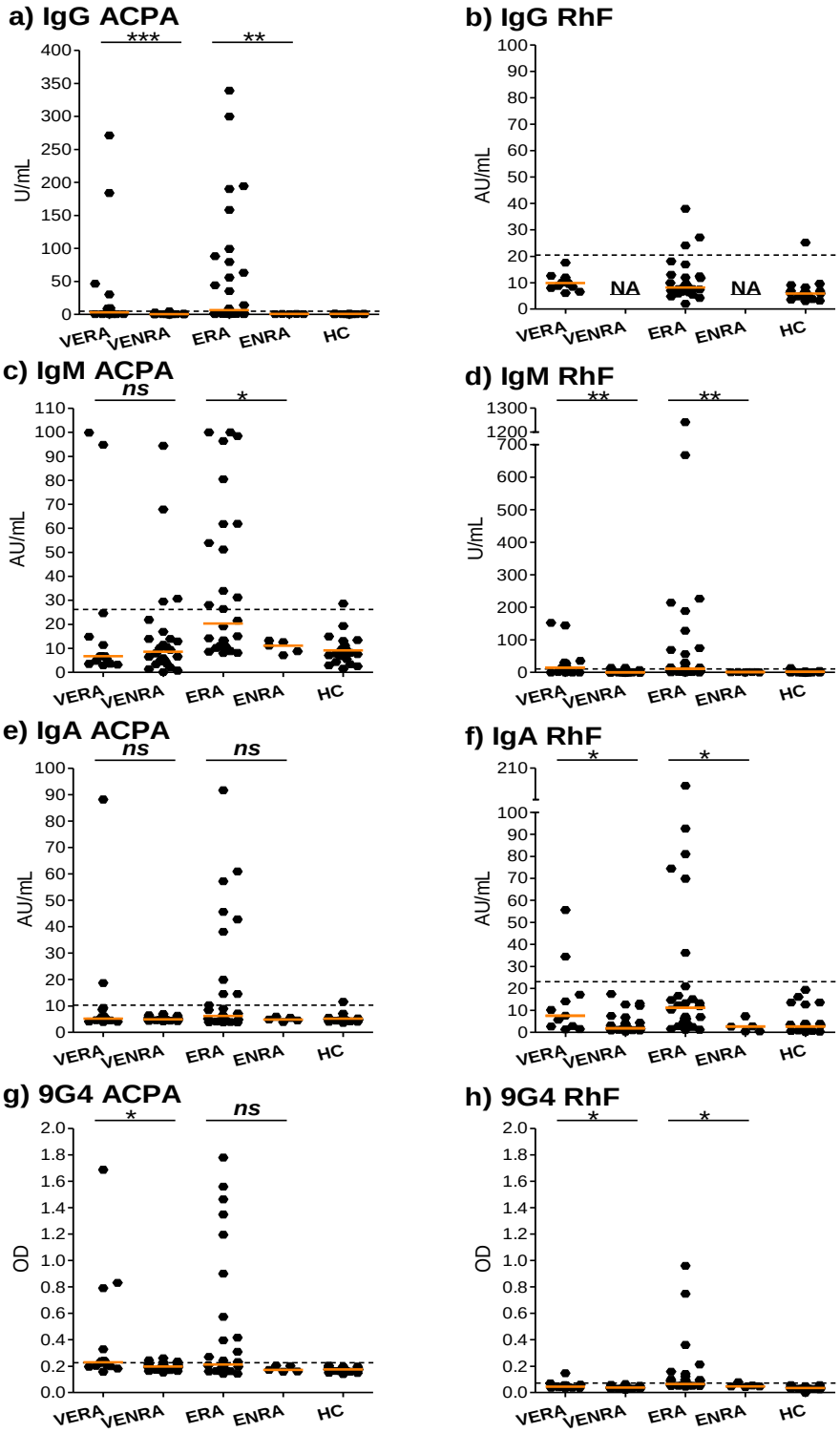


Figure 3 (a-h) – ACPA and RhF levels on early polyarthritis patients. Serum samples were processed by ELISA for **a)** IgG ACPA; **b)** IgG RhF; **c)** IgM ACPA; **d)** IgM RhF; **e**) IgA ACPA; **f)** IgA RhF; **g)** 9G4 ACPA and **h)** 9G4 RhF autoantibodies' quantification. U/mL=Units per mil; AU/mL=Arbitrary Units per mil; OD=Optical Density; NA=Not Applicable. Cut-off values are represented as a dot-styled line and median values as an orange filled line. *** = *p≤0,001*; ** = *p≤ 0,01*; * = *p≤0,05*; *ns*=not significant. Differences were considered statistically significant for *p≤0,05* using Mann-Whitney test.

6.3 – Assessment of IgM RhF interference on IgG ACPA Levels

In order to verify if the IgG ACPA levels obtained were being affected by IgM RhF interference (due to IgM RhF ability to bind to the Fc region from IgG antibodies), an ELISA was performed with serum samples digested with pepsin. This enzyme degrades IgM and destroys immune complexes. Therefore, ERA patients' serum was studied and both IgM ACPA and IgG ACPA levels before and after digestion with pepsin were determined. The resultant values are shown on Figure 4. After digestion, IgM ACPA levels decreased around 75% being significantly different from IgM ACPA levels before digestion ($p \leq 0,001$) and no significant differences were found in IgG ACPA levels before and after serum digestion with pepsin ($p > 0,05$).

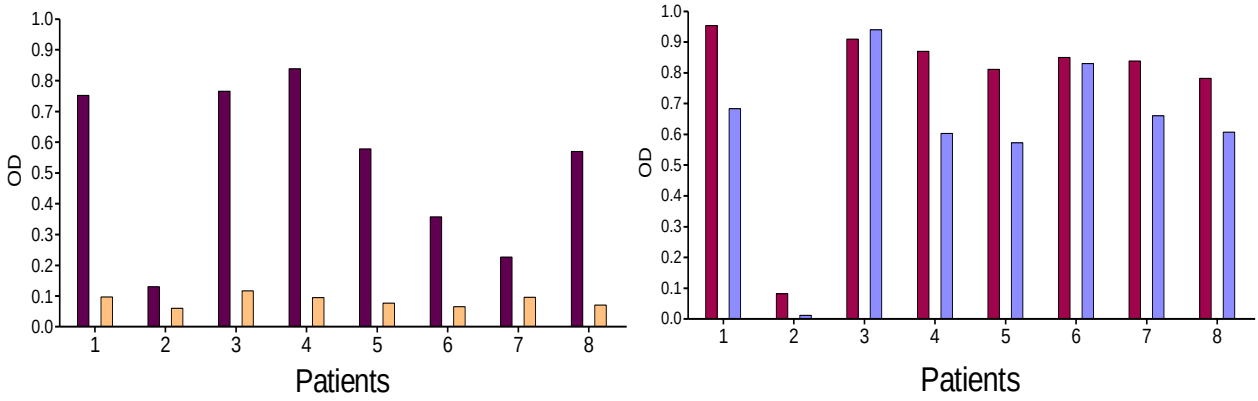


Figure 4 - Pepsin-digested sera IgM and IgG ACPA levels. Labels: IgM ACPA levels before serum digestion with pepsin; IgM ACPA levels after serum digestion with pepsin; IgG ACPA levels before serum digestion with pepsin; IgG ACPA levels after serum digestion with pepsin. OD=Optical Density.

6.4 – Autoantibody production analysis in individual patients

In the present work, the RA immune response has been evaluated by studying independent groups of patients, defined according to disease duration (Table 3-5 and Figure 3). The results from each patient from VERA, ERA, up to 17 years of disease duration and up to 40 years of disease duration groups were analyzed to determine whether there was any correlation between different autoantibody specificities in individual patients. Figures 5-8 show the autoantibody distribution of each patient from all groups analyzed. The patients were sorted according to their IgG ACPA levels (from the highest to the lowest). These results show a spread on isotype specificity with the increase in disease duration. It was observed a predominance of IgG ACPA, IgM ACPA, 9G4 ACPA and IgM RhF antibodies in all stages of RA, except in VERA patients. Of note, seropositivity for ACPA and RhF autoantibodies from IgM, IgG and IgA isotypes was only found in patients positive for 9G4

ACPA and/or 9G4 RhF, being this association dose-dependent irrespective of disease duration. In Figures 5-8 it is possible to see that the higher the levels measured for 9G4 antibody binding, the higher the levels of IgG, IgM and IgA ACPA and RhF detected. Furthermore, it was observed that 9G4 ACPA positive patients were more prevalent and globally had higher levels of this autoantibody than 9G4 RhF positive patients in all studied groups. The same result was found in IgG, IgM and IgA ACPA levels when compared to IgG, IgM and IgA RhF antibodies levels.

6.5 – Autoantibody levels in established rheumatoid arthritis patients

A characterization of the immune response at later stages of the disease (established RA patients) was also performed (Figure 9). All the RA patients studied were sorted in three main groups: “Up to 1 Year” – VERA and ERA patients, “Up to 17 Years” – Established RA patients with up to 17 years of disease duration and “Up to 40 Years” – Established RA patients with up to 40 years of disease duration. IgG RhF and IgA RhF were the only autoantibodies whose prevalence was not significantly different over time ($p>0,05$) and the opposite was found on all the remaining autoantibodies. It was observed that patients with longer disease duration (“Up to 17 Years” and “Up to 40 Years”) had higher autoantibody levels when compared with the early ones (“Up to 1 Year”) and the differences observed were significantly stronger when comparing the early RA patients (“Up to 1 year”) with the patients “Up to 40 Years” ($p\leq0,001$). Of note, IgG ACPA gave the most consistent increase over time. Furthermore, Figure 9 also shows that the group of patients “Up to 17 years” had significantly higher levels of 9G4 ACPA antibodies when compared to the early RA group (“Up to 1 year”) but no significant differences were found on 9G4 RhF levels between the same groups of patients.

6.6 – Effect of treatment on autoantibody production

Since autoantibodies play an important role in RA pathogenesis, the effect of treatment on autoantibody production was also analyzed. For that, serial studies of sera were conducted in cohorts of patients treated either with MTX, TNF receptor antagonists, TNF antagonists, CTLA-4 based drug or IL6-R antagonist. The mean time between the sample collection before and after treatment was 7.9 months, 4 months, 3.5 months, 3.8 months and 3.6 months, respectively. Figure 10 shows the results obtained. IgM RhF from patients treated with TNF receptor antagonists and 9G4 RhF from patients treated with IL-6R antagonist were the only autoantibodies significantly different after treatment ($p = 0,0117$ and $p = 0,0215$, respectively). Overall, the treatments analyzed had no statistically significant effect on ACPA, RhF or antibodies expressing the 9G4 idiotope.

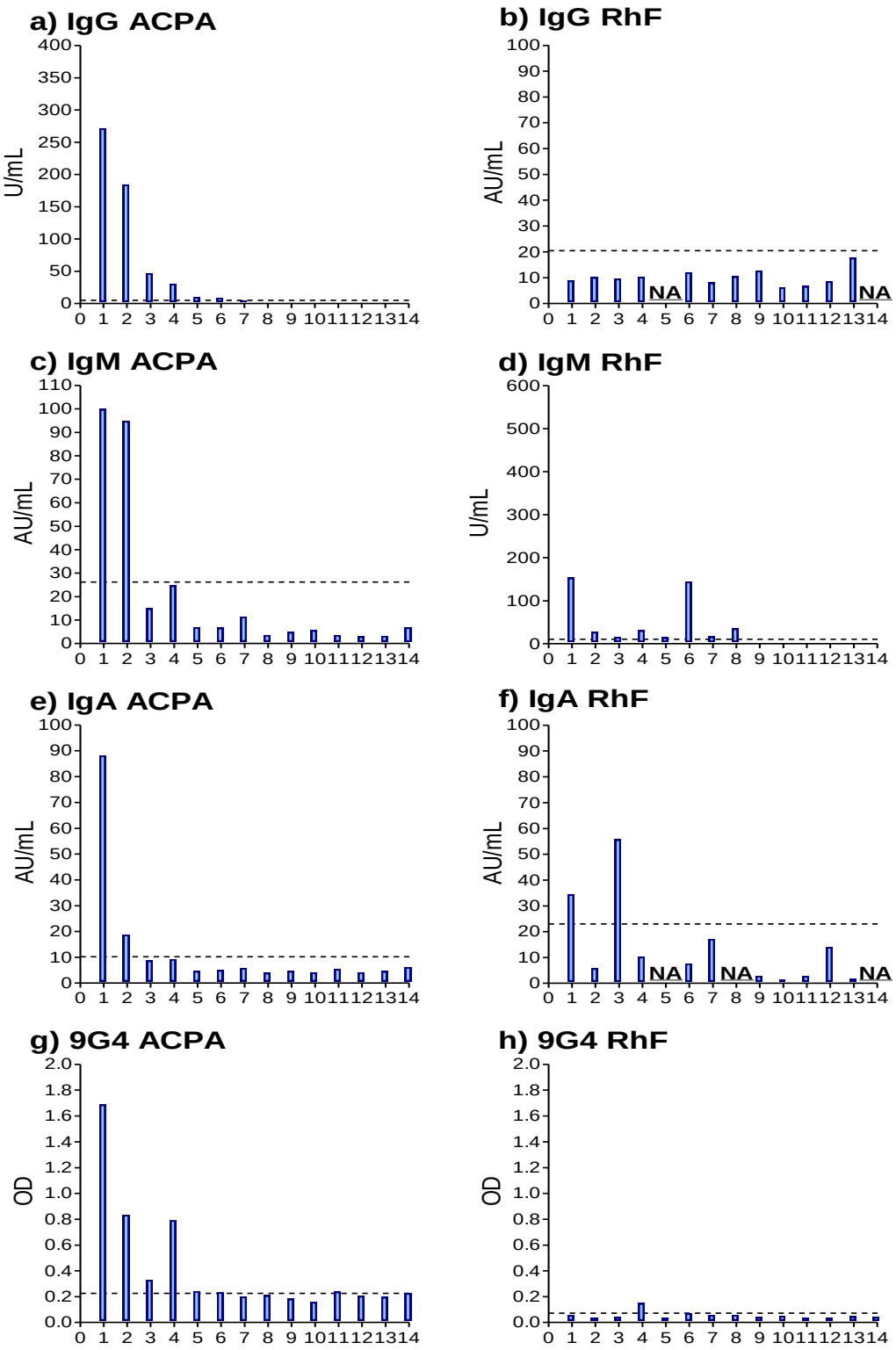


Figure 5 (a-h) – Autoantibody production in patients with less than 6 weeks of disease duration. Serum samples from 14 VERA patients were processed by ELISA for **a)** IgG ACPA; **b)** IgG RhF; **c)** IgM ACPA; **d)** IgM RhF; **e)** IgA ACPA; **f)** IgA RhF; **g)** 9G4 ACPA and **h)** 9G4 RhF autoantibodies' quantification. U/mL=Units per millilitre; AU/mL=Arbitrary Units per millilitre; OD=Optical Density; NA=Not Applicable. Cut-off values are represented as a dot-styled line.

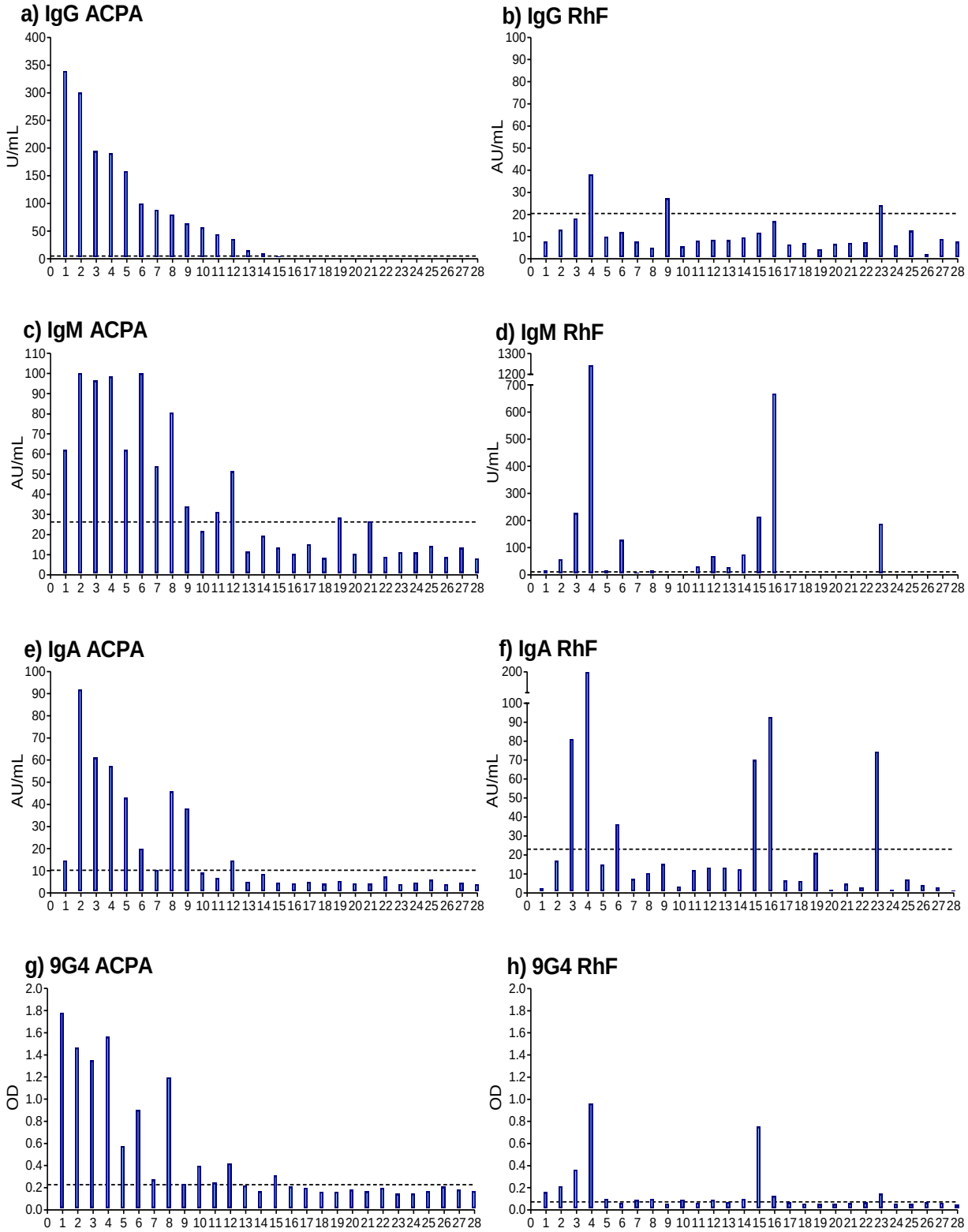


Figure 6 (a-h) – Autoantibody production in patients with less than 1 year of disease duration. Serum samples from 28 ERA patients were processed by ELISA for **a)** IgG ACPA; **b)** IgG RhF; **c)** IgM ACPA; **d)** IgM RhF; **e)** IgA ACPA; **f)** IgA RhF; **g)** 9G4 ACPA and **h)** 9G4 RhF autoantibodies' quantification. U/mL=Units per millilitre; AU/mL=Arbitrary Units per millilitre; OD=Optical Density. Cut-off values are represented as a dot-styled line.

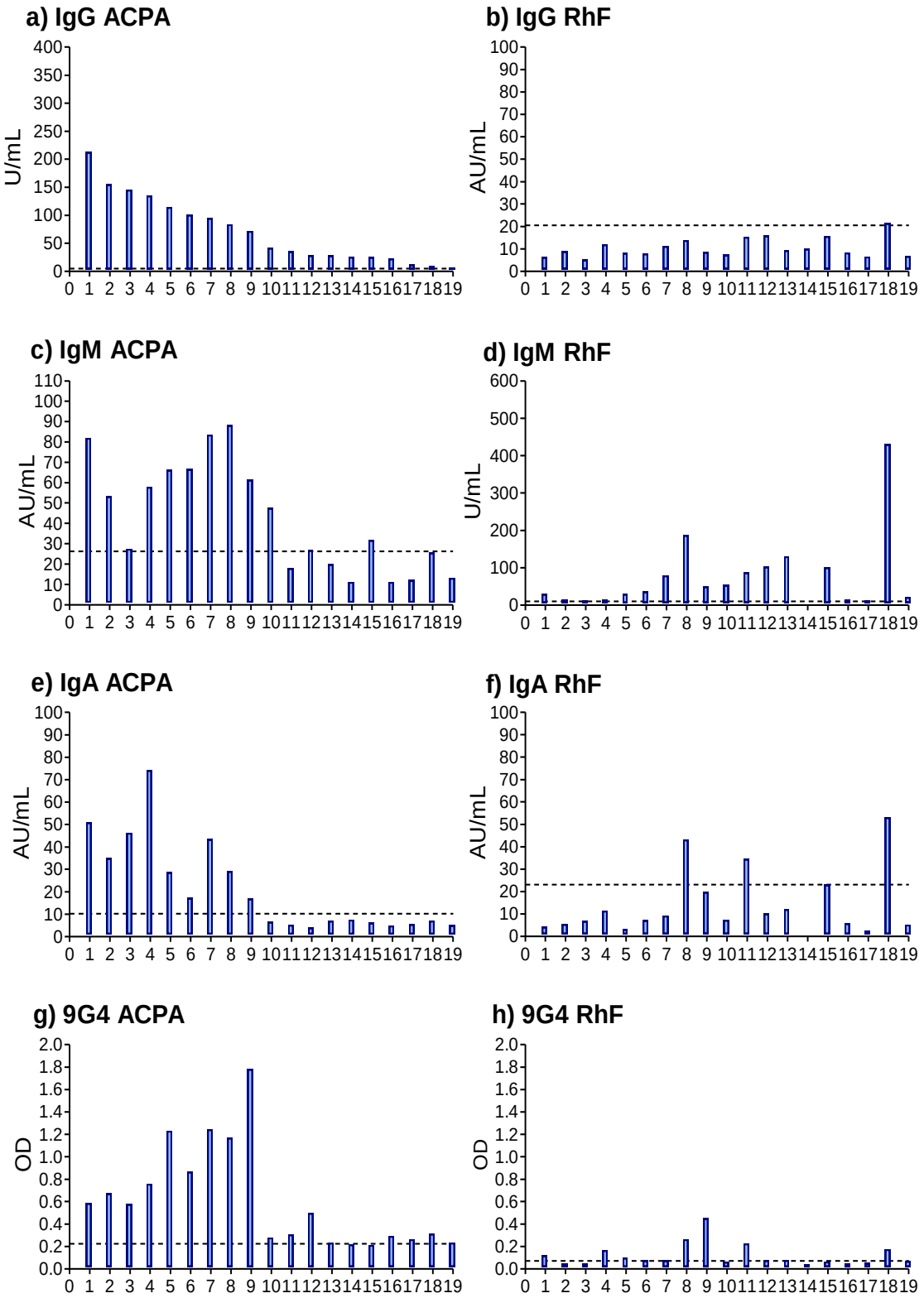


Figure 7 (a-h) – Autoantibody production in patients with up to 17 years of disease duration. Serum samples from 19 established RA patients were processed by ELISA for **a)** IgG ACPA; **b)** IgG RhF; **c)** IgM ACPA; **d)** IgM RhF; **e)** IgA ACPA; **f)** IgA RhF; **g)** 9G4 ACPA and **h)** 9G4 RhF autoantibodies' quantification. U/mL=Units per millilitre; AU/mL=Arbitrary Units per millilitre; OD=Optical Density. Cut-off values are represented as a dot-styled line.

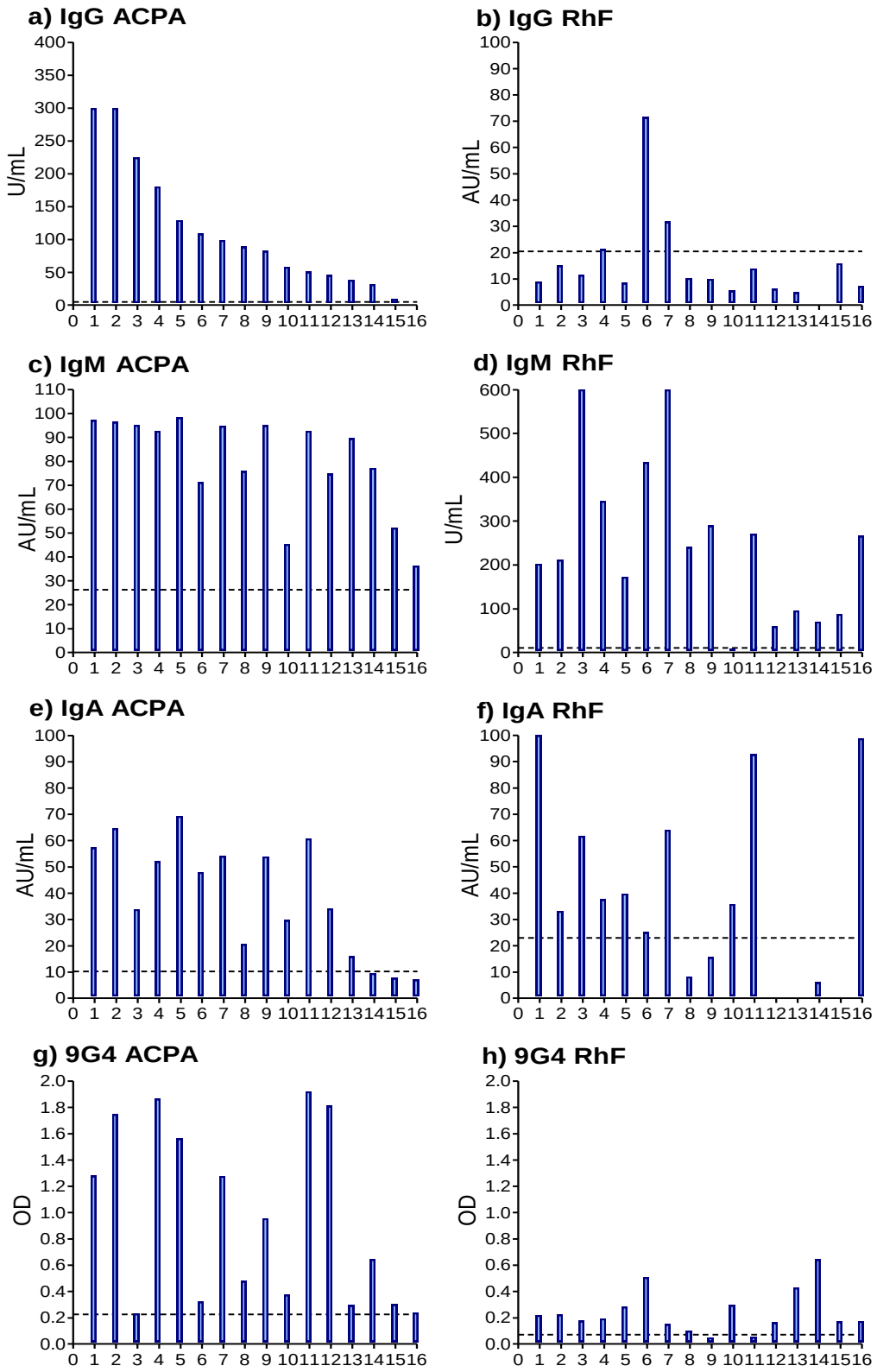


Figure 8 (a-h) – Autoantibody production in patients with up to 40 years of disease duration. Serum samples from 16 established RA patients were processed by ELISA for **a)** IgG ACPA; **b)** IgG RhF; **c)** IgM ACPA; **d)** IgM RhF; **e)** IgA ACPA; **f)** IgA RhF; **g)** 9G4 ACPA and **h)** 9G4 RhF autoantibodies' quantification. U/mL=Units per millilitre; AU/mL=Arbitrary Units per millilitre; OD=Optical Density. Cut-off values are represented as a dot-styled line.

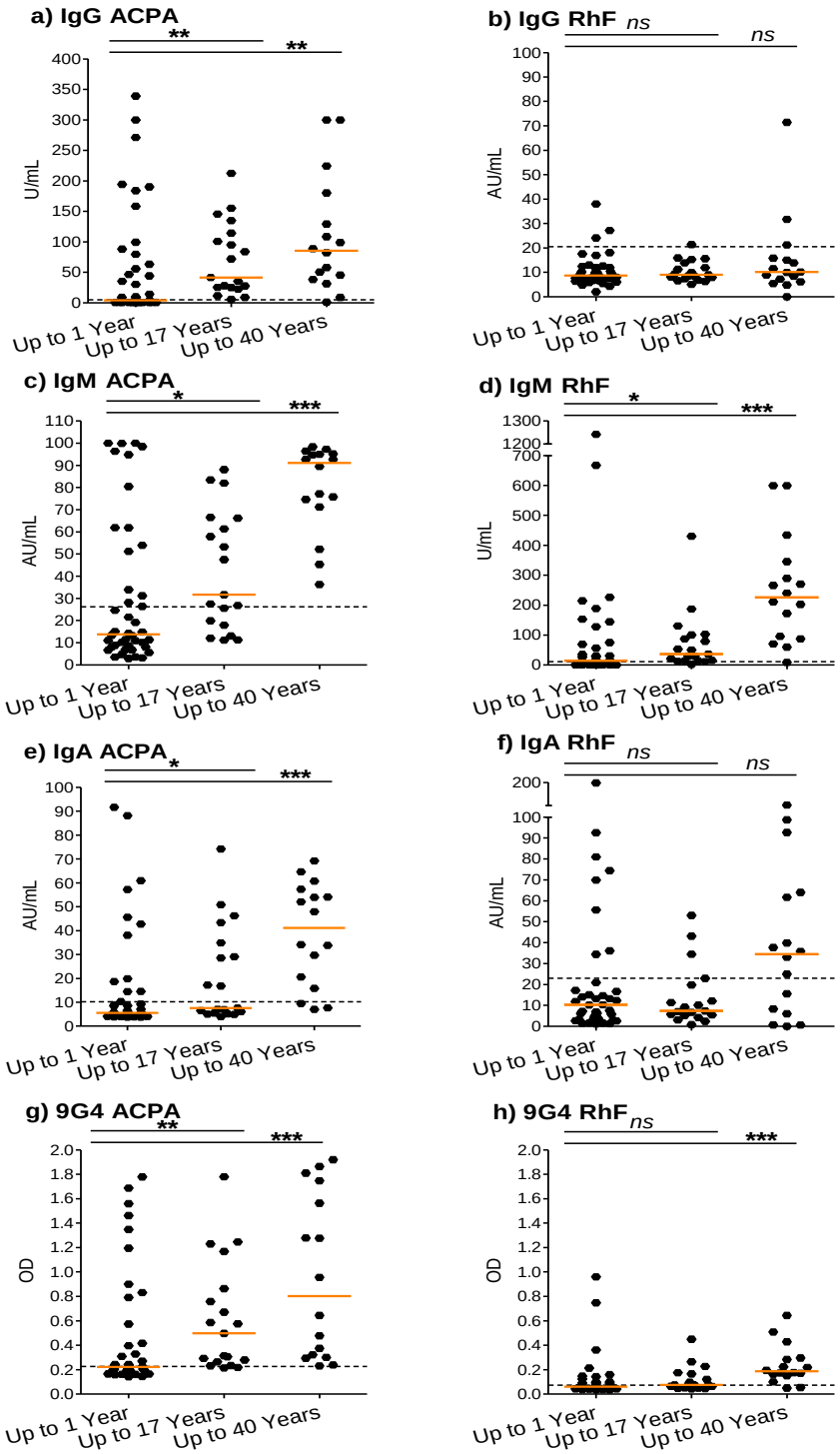
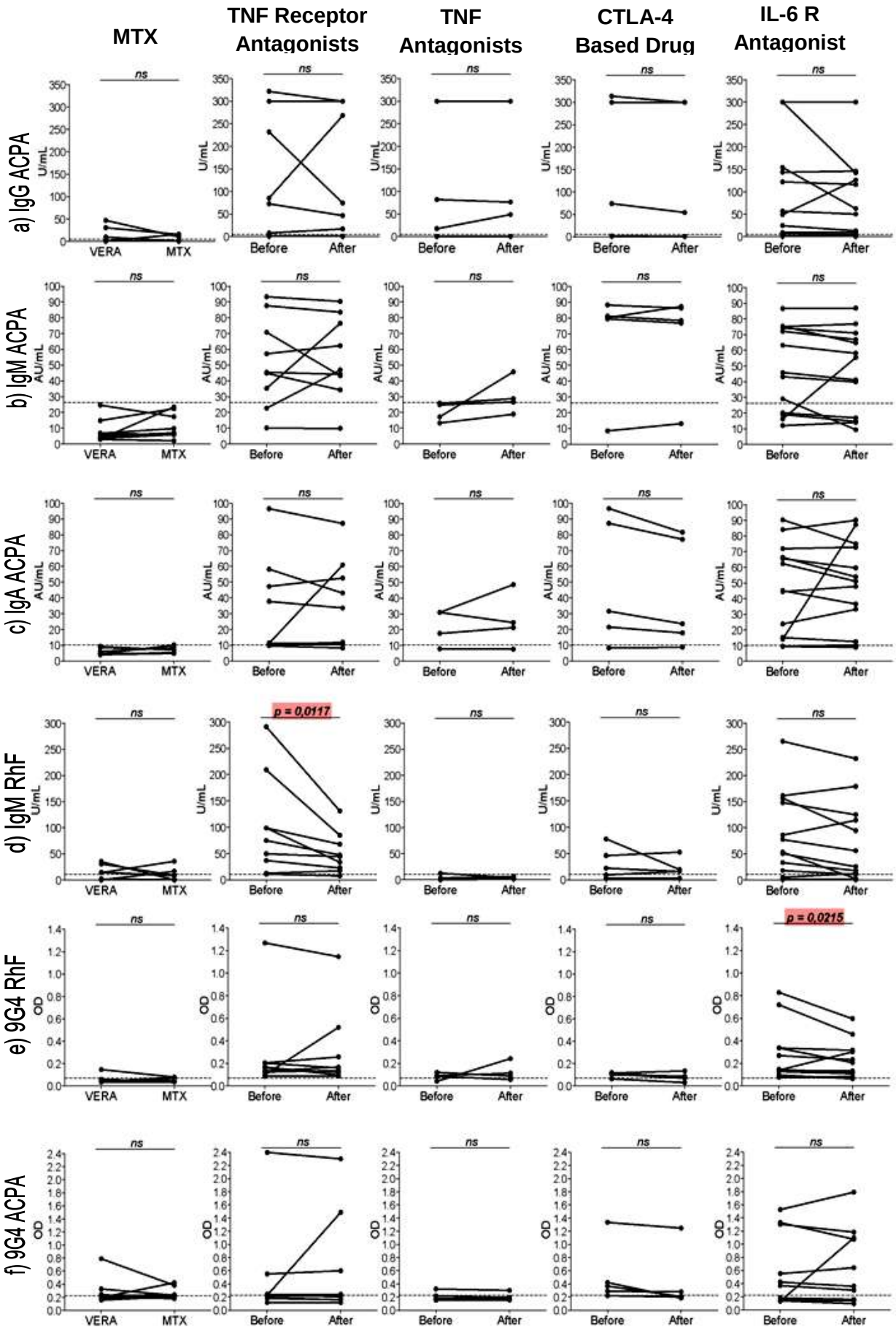


Figure 9 (a-h) – Autoantibody production according to disease duration. Serum samples from early ("Up to 1 year") and established ("Up to 17 years" and "Up to 40 years") RA patients were processed by ELISA for **a)** IgG ACPA; **b)** IgG RhF; **c)** IgM ACPA; **d)** IgM RhF; **e)** IgA ACPA; **f)** IgA RhF; **g)** 9G4 ACPA and **h)** 9G4 RhF autoantibodies' quantification. U/mL=Units per millilitre; AU/mL=Arbitrary Units per millilitre; OD=Optical Density. Cut-off values are represented as a dot-styled line and median values as an orange filled line. ****p≤0,001*; ***p≤ 0,01*; **p≤0,05*; *ns*=not significant. Differences were considered statistically significant for *p≤0,05* using Mann-Whitney test.


MTX
TNF Receptor Antagonists
TNF Antagonists
CTLA-4 Based Drug
IL-6 R Antagonist
a) IgG ACPA
b) IgM ACPA
c) IgA ACPA
d) IgM RhF
e) 9G4 RhF
f) 9G4 ACPA
U/mL
AU/mL
OD
ns
p = 0,0117
p = 0,0215
VERA
MTX
Before
After

← **Figure 10 - Effect of treatment on autoantibody production**. Serum samples were processed by ELISA for **a)** IgG ACPA; **b)** IgM ACPA; **c)** IgA ACPA; **d)** IgM RhF; **e)** 9G4 RhF and **f)** 9G4 ACPA autoantibodies' quantification. U/mL=Units per millilitre; AU/mL=Arbitrary Units per millilitre; OD=Optical Density. Cut-off values are represented as a dot-styled line. *ns*=not significant. Differences were considered statistically significant for $p \leq 0,05$ using Wilcoxon test.

Chapter 7 – Discussion

B cells are important in the immune response against pathogens through their role as antigen presenting cells (APCs), antibody production and cytokine release[101]. In autoimmune disorders, however, immune responses are erroneously directed towards self antigens[30,39]. The effectiveness of B cell depletion therapy with RTX in achieving remission in patients with RA, SLE and other autoimmune diseases characterized by autoantibody production, together with the lack of conclusive evidence supporting the existence of a specific self-antigen recognized by T cells supported the idea that B cells play an important role in RA development[92,93,130].

There are many reviews of the potential role of RhF and ACPA in RA pathogenesis but how the cells which produce these autoantibodies are selected and expanded remain to be elucidated. Recent studies developed at University College London demonstrated that auto-reactive B cells using the VH4-34 germ-line gene produce antibodies associated with RA diagnosis, which was considered as evidence of some connexion between RA and VH4-34 derived B cells[131]. The VH germline repertoire consists of 123 (44 functional) VH segments subdivided into seven VH families (VH1 to VH7)[132]. The usage of VH genes would be expected to correlate with their genomic complexity however, there is a biased use of VH4 genes[119,133]. The VH4 family has about 10 functional genes, which have been found over-represented in B cell chronic lymphocytic leukemia (B-CLL), they code almost all antibodies directed against the *N*-acetyllactosamine (NAL) antigenic determinant of the I/i antigens of red blood cells (RBC) (CA) and other polyreactive antibodies such as anti-DNA, anti-Lipid A and anti-cardiolipin[119,122,123,124,134]. The antibodies derived from VH4-34 gene are inherently auto-reactive without requiring somatic hypermutation and independently of the associated light chain[119,132]. VH4-34 gene presents a high degree of conservation among individuals and its expression under certain pathological conditions as well as its cytotoxicity to human B cells suggest that VH4-34 derived antibodies may play a role in immune regulation and/or in host defence against certain pathogens[116,117,118,119]. B cells producing VH4-34 derived antibodies are negatively selected in the peripheral blood from healthy individuals (through anergy and sequestration into MZ), are mostly naïve, scarcely represented in the GC and memory B cell compartments and excluded from plasma cell

compartment. This distribution is not observed among B cells using other VH genes[116,119]. However, in SLE and Sjögren's syndrome patients, VH4-34 derived B cells are deficiently excluded from GC reactions and around 50% of all SLE patients have IgG VH4-34 derived autoantibodies which are related with SLE severity, kidney and central nervous system involvement[123,124,135]. IgG VH4-34 derived autoantibodies and VH4-34 memory B cells are also increased in HIV patients and are consistent with the impaired B cell tolerance in HIV patients[127]. Furthermore, class-switched auto-reactive VH4-34 antibodies were found to be produced without a GC reaction and some auto-reactive B cells may pass through the pre-GC checkpoints and initiate a GC reaction[128,136]. Moreover, anergic VH4-34 B cells were found to overcome anergy during infections with i.e. *Epstein-Barr* Virus[137].

Thus, in this work, the rat monoclonal antibody 9G4 was used to evaluate the RhF and ACPA derivation from the VH4-34 gene in order to study their contribution to RA onset and progression.

In the cohort of RA patients studied, positivity for ACPA and RhF was associated with the presence of 9G4 ACPA and 9G4 RhF antibodies in circulation, respectively. This association was 9G4 dose-dependent regardless disease duration. This suggests that both ACPA and RhF producing B cells have been recruited and expanded in the periphery possibly through 9G4+ B cells help. In addition, positivity for ACPA was more prevalently found and globally with higher levels than positivity for RhF autoantibody, suggesting independent mechanisms between their respective B cells regarding recruitment and survival of these cells. ACPA and RhF were additionally found specific for RA patients. Although some VENRA patients were positive for 9G4 and IgM ACPA and IgM RhF, the percentage of these patients was not significant and these autoantibodies are known to be present in some infections as "cleaning mechanisms" and some autoimmune disorders as consequence of disease pathogenesis. The diagnosis of these patients was self-limited arthritis, psoriatic arthritis, reactive arthritis, early polyarthritis or SLE which may explain the subtle positivity for those autoantibodies.

IgG and 9G4 ACPA and IgM RhF were the autoantibodies mostly found on all VERA and ERA seropositive patients and the associations 9G4 ACPA/IgG ACPA, 9G4 ACPA/IgM RhF and IgG ACPA/IgM RhF were also the most common detected in these patients. Established RA patients ("Up to 17 years" and "Up to 40 years"), had generally higher levels of autoantibodies in circulation when compared to VERA and ERA patients, which suggests a continuous stimulation of survival mechanisms of both ACPA and RhF producing B cell clones. The majority of the patients included in the present work was seropositive for at least one autoantibody. In fact, no seronegative patients were found in the established RA patients' group with longer disease duration. Nevertheless, almost half of the VERA and ERA patients were seronegative. Seronegative RA patients present the same symptoms as

seropositive RA patients although with a less severe progression of the disease, possibly, due to slight differences in disease pathogenesis. However, some seronegative patients at the time of diagnosis may convert into seropositive with the progression of the disease.

The drugs for RA treatment studied seemed unable to interfere with the levels of autoantibodies. A single plasma cell can produce up to 10000 antibody molecules per second. Thus, if the treatment used cannot interfere with the naïve B cells in the periphery, or with the mature B cells from both periphery and tissues, and their maturation into plasma cells is not stopped, the autoantibody levels will not change and the disease will keep progressing. Nevertheless, the use of B cell depletion therapy with RTX has proved to be very efficient in RA, affecting autoantibody production, and is currently used in many autoimmune disorders characterized by autoantibody production[93,94].

Overall, these findings can lead to a hypothesis about how autoreactive B cells are selected. Citrullinated products derived from some infecting virus, cellular apoptosis, products from neutrophils extracellular traps (NET) and inflammation are recognized by autoantibodies derived from the germ line gene VH4-34 in a natural way[3,74,145,146] but, in the presence of a permissive biological background, can start recognizing self-proteins in a pathological way[119]. Furthermore, the epitope NAL recognized by CA is also expressed by CD45 in naïve B cells and apoptotic cells[116,138,139,141]. CD45 is a protein tyrosine phosphatase that functions to regulate Src kinases for T and B cells receptor signal transduction[140,141]. Its activity is dependent on a balance between monomeric and dimeric forms since CD45 is negatively regulated by dimerization[142,143] but VH4-34 anti-CD45 antibodies could interfere with the CD45 dimerization process[141]. Co-stimulation through the antigen receptor and CD45 is essential for B cell activation and proliferation. Thus, a naïve B cell with autoreactive potential could experience a diminished threshold of activation in the periphery by CD45 signaling mediated through VH4-34-derived autoantibodies and become IgG producing plasma cell[144]. Further work is needed to understand why ACPA producing B cells are selected in RA patients over B cells specific for other self-antigens and if this B cell selection mediated by VH4-34 derived antibodies could happen in other autoimmune diseases.

In summary, it was demonstrated, for the first time, the existence of autoantibodies commonly found in RA diagnosis (ACPA and RhF) expressing the 9G4 idiotope in RA patients' circulation since the first weeks of RA onset with serum levels increasin with disease progression. These observations might suggest that 9G4+ B cells have a relevant role since early RA onset, but future studies need to be developed.

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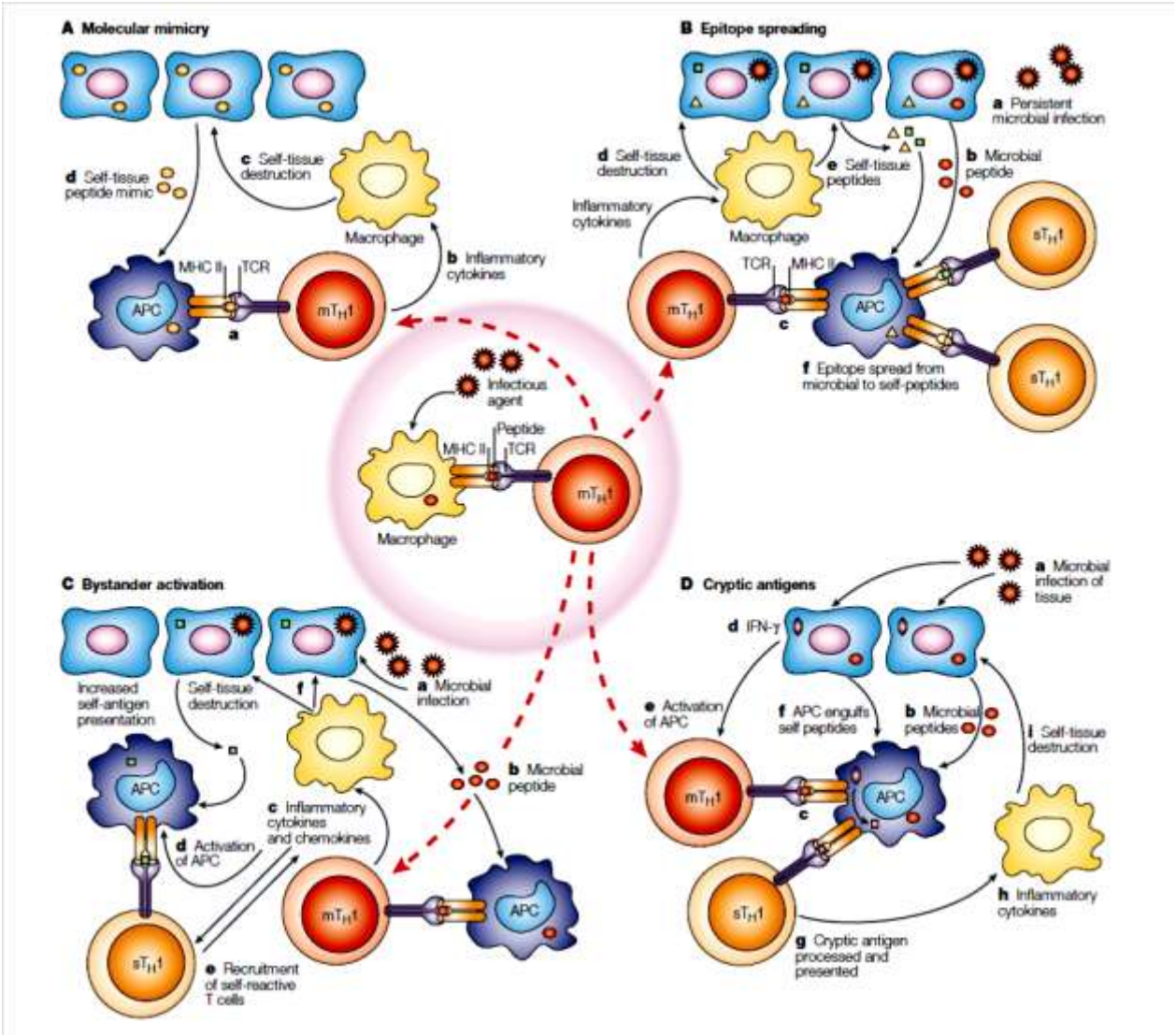
Appendix 1

Mechanisms of autoimmunity induction by infection[147]



After a microbial infection, activated microbe-specific TH1 (mTH1) cells migrate to the infected organ. **A** | Molecular mimicry describes the activation of crossreactive TH1 cells that recognize both the microbial epitope (mTH1) and the self epitope (sTH1) (**a**). Activation of the crossreactive T cells results in the release of cytokines and chemokines (**b**) that recruit and activate monocytes and macrophages, which mediate self-tissue damage (**c**). The subsequent release of self-tissue antigens and their uptake by APCs perpetuates the autoimmune disease (**d**). **B** | Epitope spreading involves a persistent microbial infection (**a**) that causes the activation of microorganism-specific TH1 cells (**b**,**c**), which mediate self-tissue damage (**d**). This results in the release of self peptides (**e**), which are engulfed by APCs and presented to self-reactive TH1 cells (**f**). Continual damage and release of self peptides results in the spread of the self-reactive immune response to multiple self-epitopes

(**f**). **C** | Bystander activation is the nonspecific activation of self-reactive TH1 cells. Activation of microorganism-specific TH1 cells (**a**,**b**) leads to inflammation (**c**,**d**) and results in the increased infiltration of T cells at the site of infection and the activation of self-reactive TH1 cells by TCR-dependent and -independent mechanisms (**e**) Self-reactive T cells activated in this manner mediate self-tissue damage and perpetuate the autoimmune response (**f**). **D** | Cryptic antigen model describing the initiation of autoimmunity by differential processing of self peptides. Following microbial infection (**a**) IFN-γ is secreted by both activated microbe-specific TH1 cells (**b**,**c**) and microbe-infected tissue cells (**d**). This activates APCs (**e**) and can lead to APC engulfing self-antigens (**f**). Cytokine activation of APCs can induce increased protease production and different processing of captured self-antigens, resulting in presentation of cryptic epitopes. The presentation of these cryptic epitopes can activate self-reactive TH1 cells (**g**), leading to self-tissue destruction (**h**,**i**). APC, antigen-presenting cell; MHC II, major histocompatibility complex class II; TCR, T-cell receptor.

Appendix 2

2010 ACR/EULAR classification criteria for rheumatoid arthritis[49]

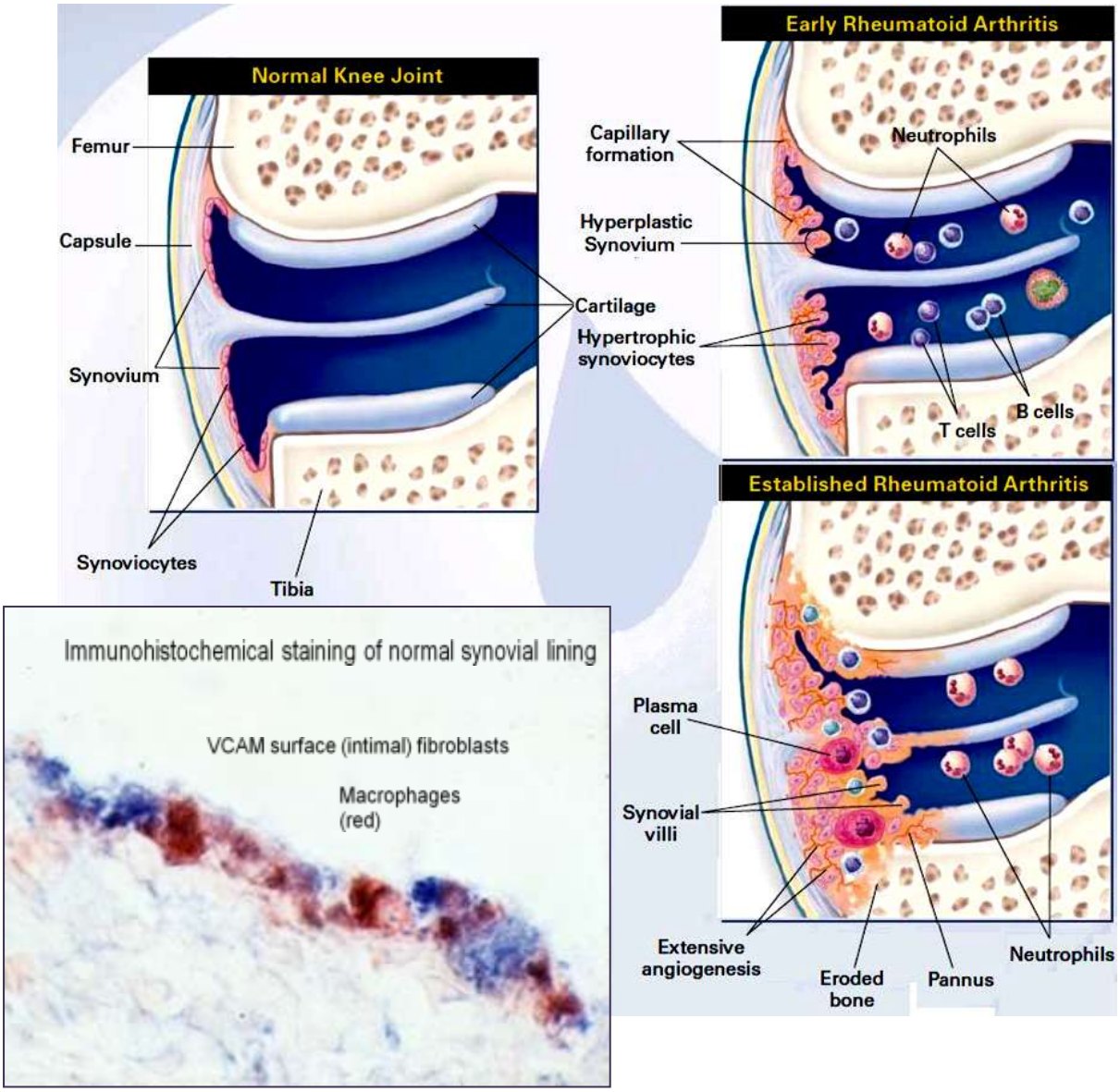
	Score
Target population (Who should be tested?): Patients who	
1) have at least 1 joint with definite clinical synovitis (swelling)*	
2) with the synovitis not better explained by another disease†	
Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of ≥6/10 is needed for classification of a patient as having definite RA)‡	
A. Joint involvement§	
1 large joint ¶	0
2 – 10 large joints	1
1 – 3 small joints (with or without involvement of large joints) #	2
4 – 10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint) **	5
B. Serology (at least 1 test result is needed for classification)††	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)‡‡	
Normal CRP *and* normal ESR	0
Abnormal CRP *or* abnormal ESR	1
D. Duration of symptoms§§	
<6 weeks	0
≥6 weeks	1

***** The criteria are aimed at classification of newly presenting patients. In addition, patients with erosive disease typical of rheumatoid arthritis (RA) with a history compatible with prior fulfillment of the 2010 criteria should be classified as having RA. Patients with longstanding disease, including those whose disease is inactive (with or without treatment) who, based on retrospectively available data, have previously fulfilled the 2010 criteria should be classified as having RA. **†** Differential diagnoses vary among patients with different presentations, but may include conditions such as systemic lupus erythematosus, psoriatic arthritis, and gout. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted. **‡** Although patients with a score of <6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time. **§** Joint involvement refers to any *swollen* or *tender* joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are *excluded from assessment*. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based on the pattern of joint involvement. ¶ "Large joints" refers to shoulders, elbows, hips, knees, and ankles. # "Small joints" refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists. ** In this category, at least 1 of the involved joints must be a small joint; the other joints can include

any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (e.g., temporomandibular, acromioclavicular, sternoclavicular, etc.). **††** Negative refers to IU values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF. **‡‡** Normal/abnormal is determined by local laboratory standards. **§§** Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status.

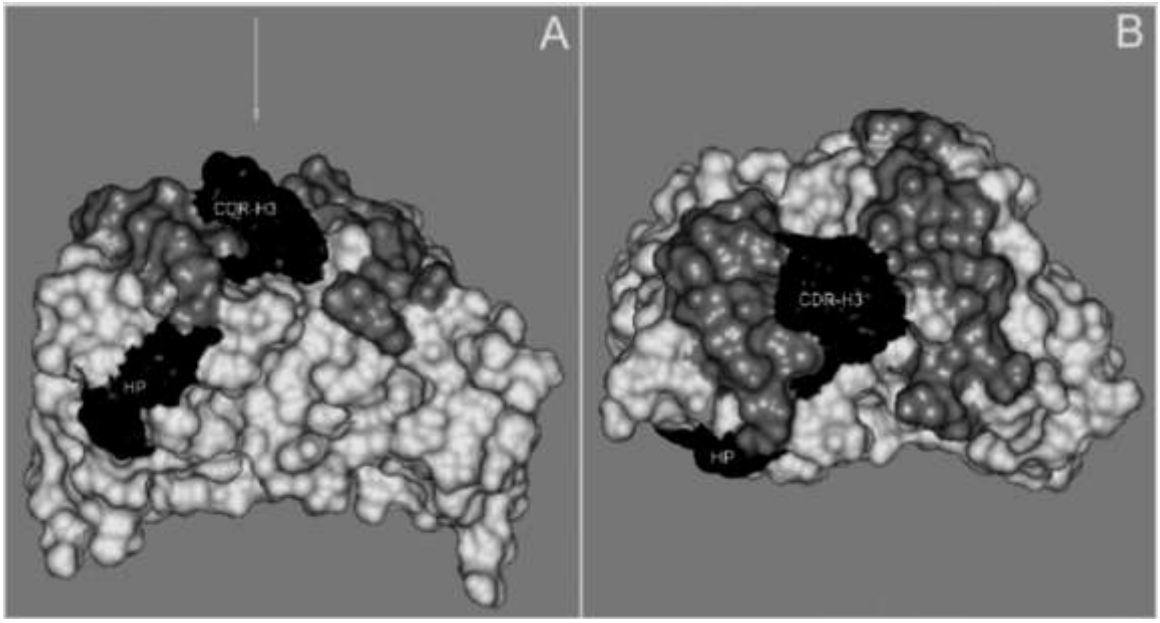
Appendix 3

Synovial structure and evolution through rheumatoid arthritis pathogenesis[82,148,149]



Appendix 4

Structure of the 9G4 idiotope from a VH4-34 germ-line gene derived antibody[112]



Crystal structure of CA showing the hydrophobic patch in FR1 in relation to the conventional antibody combining site. **A)** Space-filling representation of the surface of the VH and VL domains of CA (Brookhaven Protein Data Bank code 1DN0). The hydrophobic patch (HP) and the CDRH3 are depicted in black, the main body of the antibody is white, and the rest of the antigen binding site is gray. The arrow indicates the VH:VL axis and the location of the conventional antigen binding site. **B)** View almost perpendicular to A, looking down the axis of the VH:VL domain pair onto the conventional antigen binding site. Color code is as in A. Figures were produced using INSIGHT II.

At the FR1 in the CA anti-I crystal structure, W7 and AVY lie immediately adjacent to each other and form an extensive hydrophobic patch (A). The nature of this hydrophobic patch, with aromatic ring structures lying flat along the surface of the domain, and flanked by hydrophilic residues capable of hydrogen bonding, is similar to that of the oligosaccharide-binding hydrophobic patch on the Cγ2 domains of IgG Fc. In this structure, phenylalanine rings lie flat along the surface and the hexose residues of the oligosaccharide chains stack on top of them, while surrounding residues provide hydrogen bonding to the hydroxyl groups of the sugar rings. The limited database of crystal structures of antibodies complexed with carbohydrate antigens also reveals hydrophobic stacking interactions and hydrogen bonding. Further examination of the CA crystal structure reveals a number of surface-exposed residues of CDRH3 that point away from the conventional Ag binding site and lie in close proximity to surface residues of FR1. In particular, the exposed carboxyl-terminal residues of CDRH3 lie adjacent to the hydrophobic patch determinants W7 and AVY (A), and it is precisely these C-terminal residues that have been shown to affect I/i binding.