B-cell depletion in Rheumatoid Arthritis:

clinical, biochemical and synovial predictors

of response

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Declaration

I, Arti Mahto, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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Abstract

Despite a growing armamentarium of therapies available to treat rheumatoid arthritis (RA), predicting response to therapy remains a clinical challenge. Witnessed variability in treatment outcomes translates to a large unmet clinical need; with significant risk of disease progression, exposure to side effects and health economic burden. Clinical heterogeneity in RA is reflected at the tissue level as diseased synovium displays variation in both degree and organisation of immune cell infiltration. Transcriptomic analysis suggests that synovial cellular heterogeneity is mirrored by a diversification of molecular profiles which may affect response to therapy. B-cells play a critical role in the pathogenesis and propagation of RA. Treatment response to the B-cell depleting agent rituximab in RA is varied and clear predictors of response are yet to be elucidated.

The first aim of this work was to develop a pathological scoring system for the rheumatoid synovium to determine 'B-cell rich' and 'B-cell poor' pathotypes and investigate whether these subgroups can predict clinical response. I establish that despite the degree of reduction in synovial B-cells following rituximab therapy, clinical response remains indeterminate.

The second aim was to investigate whether a molecular signature associated with response to rituximab therapy. Critically, I demonstrate that an upregulation of genes associated with ectopic lymphoid structures (ELS) and those which orchestrate Th17/interleukin 17 (IL-17) pathways are associated with diminished response to rituximab. Furthermore, these genes can be utilised to create a model which has high sensitivity to predict response to rituximab.

Finally, I investigate whether changes in synovial cellular infiltration and gene expression are associated with changes in ultrasound (US) synovial thickening (ST) and power doppler (PD) signal, demonstrating a key role of fibroblast and IL-17 associated genes. Synovium remains the site of insult in RA; this work enhances the notion that synovial biopsies may be pivotal stratification tools of the future.

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List of Abbreviations

ACPA	anti-citrullinated protein antibodies
ACR	American college of Rheumatology
ADAMTS	A-disintegrin and metalloproteinase with thrombospondin-1-like domains
AID	activation-induced Cytidine Deaminase
APC	antigen presenting cell
APRIL	proliferation-inducing ligand
ARA	American Rheumatology Association
BAFF	B-cell activating factor
BAFF-R	B-cell activating factor receptor
BCMA	B-cell maturation antigen
BCR	B-cell receptor
Bip	Binding immunoglobulin protein
BLyS	B lymphocyte stimulator
CC	CC-chemokine
CCL -	CC chemokine ligand -
CCR	CC chemokine receptor
CD21L	Long isoform of CD21
CDAI	clinical disease activity index
CDR	complementarity determining regions
CRP	c-reactive protein
csDMARD	conventional synthetic disease modifying antirheumatic drug
CSR	class switch recombination
CTLA4	cytotoxic T-lymphocyte associated antigen 4

CXC	CXC-chemokine
CXCL	CXC chemokine ligand
DAMPs	danger associated molecular patterns
DAS-28	28 joint count disease activity score
DIA	digital image analysis
DMARD	disease modifying antirheumatic drug
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ELN	ectopic lymphoid neogenesis
ELS	ectopic lymphoid structures
ENA-78	epithelial-neutrophil activating protein 78
ESR	erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
Fab	fragment antigen binding
Fc	crystallised fragment
FCYR	Fc gamma receptor
FDC	follicular dendritic cells
FGF	fibroblast growth factor
FLS	fibroblast like synoviocytes
micro-rna	micro-ribonucleic acid
Foxp3	forkhead box p3
G-CSF	granulocyte-colony stimulating factor
GATA	erythroid transcription factor
GC	germinal centres

GITR	glucocorticoid induced tumour necrosis factor receptor family-related protein
Gp	glycoprotein
GWAS	genome wide association study
H&E	haematoxylin and eosin
HAQ	health assessment questionnaire
HEV	high endothelial venules
HLA	human leucocyte antigen
HSP	heat shock protein
ICAM	intracellular adhesion molecule
ICC	intra-class correlation coefficients
ICOS	inducible co-stimulator
IFN γ	interferon gamma
IFNα	interferon alpha
IFNβ	interferon beta
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IHC	immunohistochemistry
IL	interleukin
JAK	janus kinase
JSN	joint space narrowing
kDA	kilodalton
LTα	lymphotoxin alpha
LTβ	lymphotoxin beta

MC	manual count
MCP	metacarpophalangeal joint
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTP	metatarsophalangeal
Mtss	modified total sharp score
MTX	methotrexate
NETs	neutrophil extracellular traps
NFKB	nuclear factor kB
NICE	National Institute of Clinical Excellence
NK	natural killer
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OMERACT	Outcome measures in rheumatology
PADI	Peptidyl arginine deiminase
PAMPs	pathogen associated molecular patterms
PB	plasmablasts
PBMC	peripheral blood monocytes
PC	plasma cells
PCR	polymerase change reaction
PD	power doppler
PDGF	platelet derived growth factor

PIP	proximal interphalangeal
PRGA	physician reported global activity
PtGA	patient assessment of global activity
PTPN22	protein tyrosine phosphatase N22
QMUL	Queen Mary University of London
STAT	signal transducer and activator of transcription
R4RA	Randomised, open labelled study in anti-TNFα inadequate responders to investigate mechanisms of response-resistance to Rituximab vs Tocilizumab in RA
RA	rheumatoid arthritis
RAG	recombinase activating genes
RAMRIS	rheumatoid arthritis magnetic resonance imaging score
RANK	receptor activator of nuclear factor k-B
RANKL	receptor activator of nuclear factor k-B ligand
RCTs	randomised controlled trials
RF	rheumatoid factor
RNA	ribonucleic acid
RNP	ribonucleoproteins
SCID	severe combined immunodeficiency
SD	standard deviation
SDF-1	stromal cell derived factor 1
SE	shared epitope
SHM	somatic hypermutation
SJ	swollen joint

SJC	swollen joint count
SL	sublining
SLE	systemic lupus erythematosis
SNP	single nucleotide polymorphism
SOP	standard operating procedure
SQ	semi-quantitative
SSB	single stranded break
ST	synovial thickening
STRAP	'Stratification of biologic therapies for Rheumatoid arthritis by pathobiology'
TACI	transmembrane activator and CAML interactor
TB	tuberculosis
Tbet	T cell transcription factor
Tbx21	T-box transcription factor
TGFβ	transforming growth factor beta
Th17	Th17 cells
Th2	Th2 cells
TJC	tender joint count
TLR	toll like receptors
TNFAIP3	tumour necrosis factor alpha-induced protein 3
TRAF1	TNF receptor-associated factor 1
Tregs	regulatory T-cells
tsDMARD	targeted synthetic Disease-modifying antirheumatic drugs
UIP	usual interstitial pneumonitis
NSIP	non-specific interstitial pneumonitis

US	Ultrasound
UK	United Kingdom
VAS	visual analogue scores
VCAM	vascular adhesion molecule
VEGF	vascular endothelial growth factor

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Chapter 1: Introduction

1.0 Rheumatoid Arthritis: an overview

Rheumatoid arthritis (RA) is an autoimmune, progressive, multisystem inflammatory disease that primarily affects the synovial lining of diarthrodial joints. It is associated with significant and progressive morbidity, increased mortality and high socioeconomic costs [1]. Despite recent advances in the understanding of disease pathogenesis, early diagnostic accuracy, changes in classification, improved clinical assessment scores and the continued development of novel therapeutics, the true aetiology of the disease has not yet been fully elucidated. The complex interplay between genetics, the environment and immune system lead to a diversity of clinical phenotypes, which, despite a growing wealth of new therapies, continue to provide treatment challenges for those who suffer from the disease and for the physicians of today.

1.1 Epidemiology of RA

RA affects approximately 0.5-1% of the world's population, with an estimated annual incidence of 3 per 10000 adults [2]. The disease has a prevalence of 0.02-0.05%, with the peak incidence in the fifth to sixth decades of life [3, 4]. Geographic variations have been reported, however, with a prevalence of 0.02-0.07% in Southern European countries [5-7] and lower rates in developing countries [8] [9, 10]. Interestingly, some particular ethnic subgroups show higher rates of the disease, such as Pima Indians and other native American Indian populations [11, 12]. Other studies have suggested that such observations may, in part be attributable to methodological differences in survey methods and reflect changes between classification criteria between 1958, 1987 and 2010 [4, 10, 13, 14]. Even accounting for this, there appears to be a distinguishable difference between population groups worldwide.

In the United Kingdom, the prevalence of RA is estimated at 1.16% in women and 0.44% in men [15] using the 1987 American College of Rheumatology (ACR) classification criteria. The incidence of the disease has been reviewed more recently using the more recent 2010 ACR/European League of Action against Rheumatism (EULAR) criteria using data from the Norfolk Arthritis Register, with rates of 54/100,000 for women and 25/100,000 for men [13]. This was higher than if using the 1987 criteria; however, similar rates were achieved if individuals were followed up to five years suggesting the new criteria has increased sensitivity for early disease detection [13].

Some studies have shown a decrease in the incidence of RA over time, particularly in females [16-20]. There are several plausible explanations for this observation. Firstly, there may be a true decline in the incidence of the disease. This has been attributed to changes in environmental factors. Secondly, as discussed by both Silman [10] and Ulhig and Kvien [21], methodological variations in data collection may affect results, as repeated surveys differ from prospective notifications, with varying levels of reporting or classification bias. The findings from these studies and perceived general decline may indicate that a shift in environment or infectious agents may play an important role in the pathogenesis of the disease.

1.2 RA: Clinical Features, Disease Activity and Treatment Modalities

1.2.1 Articular signs and symptoms

RA is the most common form of inflammatory joint disease. It is characterised by articular stiffness, pain and swelling which is usually symmetrical. Clinical onset is usually insidious and gradual but although less frequent, some individuals can present with a more acute onset with systemic symptoms over days [22, 23]. Other forms of onset include predominant tendon and bursa involvement and palindromic RA, where there is spontaneous remission of symptoms after a few hours or days of pain and swelling. A polymyalgic onset of RA has also been described more commonly in the elderly, where systemic symptoms predominate and are associated with proximal myalgia [24, 25].

Joints typically involved at the onset of disease are the metacarpophalangeal (MCP) or proximal interphalangeal joints (PIP), wrists or metatarsophalangeal joints (MTP) which are affected in a polyarticular manner, although occasionally the disease starts as an oligo- or pauciarticular disease and develops over time. The temporomandibular joint and cervical spine may also be involved, with relative sparing of the lumbosacral joints [22, 26, 27]. Tendon sheath and bursal inflammation can cause distension or rupture of structures and entrapment neuropathies. Ongoing inflammation within the synovium can result in a loss of joint space, degradation of cartilage and bone erosions. Left untreated, this leads to a reduction in the range of movement, subluxation and deformity of the joints which results in physical disability.

1.2.2 Extra-articular manifestations

RA is a multisystem disorder with varying levels of systemic involvement. Extra-articular manifestations are thought to associate with the severity of the disease and excess mortality [28, 29]. Systemic findings are summarized in table 1.1, with some more details of specific organs provided below.

Previously, rheumatoid nodulosis occurred in up to 25% of patients with RA who had detectable autoantibodies were thought to be pathognomonic of disease [27]. More recent data suggests the figure is somewhat lower, with 7% presenting with nodules at the time of diagnosis [30]. They occur typically over areas of pressure such as the Achilles tendon and olecranon bursa, however have been described in several other areas including the larynx, lungs, eyes and heart. The size of the nodules may increase or wane over time through the course of the disease, sometimes paradoxically worsening with treatment.

Lung involvement is another common finding in RA and can include airway, parenchymal, pleural or vascular disease. Airway involvement manifests in the form of bronchiectasis [31] or bronchiolitis. Arthritis of the cricoaytenoid joints can result in hoarsenes or stridor. Bronchiectasis or interstitial lung disease are usually associated with a gradual onset of dyspnea and cough and detected on high resolution computed topography scanning. Patterns in RA include usual interstitial pneumonia (UIP), non-specific interstitial pneumonia (NSIP) and organising pneumonia, with some other rare forms described in the literature [32]. Rheumatoid nodules may also be present within the lung and also may change in size over time. Inflammation of medium sized blood vessels can result in a pulmonary vasculitis, although this is a rare finding. Pleuritis and pleural effusions are also frequently encountered,

characterised by exudative fluid with multinucleated giant cells and elongated or 'tadpole' macrophages [33].

Cardiovascular risk in RA has been studied in detail in the last decade. Studies show that patients with RA have an increased mortality of up to 50% when compared with the general population [34-38]. The increased risk has been shown to be directly linked to high levels of circulating cytokines and subsequent inflammation, promoting endothelial dysfunction, vessel abnormalities, a shift in lipid levels and oxidative stress [39-41]. In light of these findings, specific recommendations have been made for tight disease control in order to reduce the burden of cardiovascular disease. The European League of Action against Rheumatism (EULAR) guidelines recommend an annual cardiovascular risk assessment in all patients with rheumatoid arthritis and to apply a multiplier to certain subsets of patients when using traditional risk scores such as the Framingham or Systemic Coronary Risk Evaluation model [42].

Table 1.1 Extra-articular and drug associated manifestations of RA

Organ Involvement	Features
Cardiovascular	Increased Cardiovascular risk, pericarditis, pericardial effusion, myocarditis
Constitutional Symptoms	Weight loss, fever, asthenia
Dermatological	Nodules, leucocytoclastic vasculitis, ulceration
Gastroenterological	Gastroduodenal ulceration, usually drug related
Haematological	Anaemia, thrombocytocytosis, thrombocytopenias, other blood dyscrasias, Felty's syndrome, hyperviscosity, cryglobulinaemia
Malignancy and Infection	Increased risk of malignancy and infection
Muscle, bursae and bones	Muscle weakness and atrophy, Osteoporosis
Neurological	Entrapment neuropathy, mononeuritis multiplex, cervical myelopathy
Ocular	Keratoconjunctivitis sicca, scleritis, episcleritis, corneal perforation
Pulmonary	Pleuritis, interstitial pneumonitis, nodular lung disease
Renal	Glomerulonephritis, Tubular interstitial nephritis (usually drug related), secondary amyloidosis
Vasculitis	Skin, lung, renal, peripheral neuropathy

1.2.3 Classification criteria

To date, there have been three clearly defined criteria for RA. The first were adopted by the American Rheumatism Association (ARA) in 1958 and divided patients into possible, probable and definite RA [43]. These guidelines were revised in 1987 in an attempt to streamline the diagnosis and build a platform for evidence based medicine within the field and are presented in Table 1.2 [44].

Table 1.2 1987 ARA Classification Criteria for RA [44]

Morning Stiffness*
Arthritis of 3 or more areas*
Arthritis affecting hand joints*
Symmetrical Arthritis*
Rheumatoid Nodules
Positive Rheumatoid Factor (RF)
Radiographic changes

At least 4 out of 7 criteria must be satisfied for RA, * must be present for at least 6 weeks

Although these criteria performed better than their 1958 predecessors, a shift towards early treatment of the disease indicated that the updated criteria were limited in both sensitivity and specificity, especially in early disease without radiographic changes [18, 45, 46]. A large meta-analysis of 7438 patients by Banal et al showed a sensitivity and specificity of 77% in early RA vs 79% and 90% in established RA, which was deemed to be significant [46]. Furthermore, the discovery of anti-citrullinated peptide antibodies (ACPA), which are a prognostic marker for more erosive disease were not included in the 1987 criteria. The

criteria were therefore reviewed and revised by the American College of Rheumatology (ACR) / European League of Action against Rheumatism (EULAR) taskforce in 2010 and are summarized in Table 1.3 [47]. Several studies have since been performed to validate the sensitivity and specificity of the new criteria, summarized by Radner et al [48] which gave a pooled sensitivity of 0.81 in early disease.

Table 1.3 2010 ACR / EULAR Classification Criteria for RA[47]

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 small joints	5
Serology	
Negative RF and ACPA	0
Low positive RF or low positive ACPA	1
High positive RF or high positive ACPA	2
Acute phase reactants	
Normal ESR or CRP	0
Abnormal ESR or CRP	1
Symptom Duration	
< 6 weeks	0
≥ 6 weeks	1

A score of ≥6 fulfils the criteria for definite RA

Abbreviations: RF = rheumatoid factor, ACPA = anti-citrullinated protein antibodies, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein

1.2.4 Assessment of disease activity

Alongside early recognition and prompt treatment of RA, there has been a move towards a "treat to target" approach [49]. In order to define a clear 'target' however, well defined models of disease activity are required, which incorporate both clinical findings and overall patient well-being and that can be used as a measure in clinical trials and routine practice. To this end, a multitude of disease assessment criteria have been developed and validated, those used commonly in clinical trials are briefly described below. More extensive information is provided in a comprehensive review by Anderson et al [50].

Disease Activity Score: 28 joints (DAS-28 Score)

The DAS-28 score is the most frequently used disease activity index in routine clinical practice. It was developed by EULAR and is endorsed for disease activity measures in clinical trials [51]. The original DAS score incorporated 44 joints, however this was reduced to 28 to facilitate feasibility and ease of use within the clinical setting following studies that the two scores were comparable[52, 53]. It consists of four different domains: a tender joint count (TJ) , swollen joint count (SJ), patient assessment of global activity (PtGA) by the means of a visual analogue score (VAS) and a biological marker of inflammation through the ESR or CRP. The formula is calculated as follows: $0.56 \times V(28TJC) + 0.28 \times V(28SJC) + 0.70 \times \ln(ESR) + 0.014 \times PtGA$ and modified according to which acute phase reactant is used [54]. A high score reflects high disease activity. Pre-defined values give an indication of remission, moderate or high disease activity and changes after treatment can be utilised to select 'responders' and 'non-responders', summarised in Table 1.4[55].

Table 1.4 The EULAR response criteria using the DAS-28 score [55]

	Improvement in DAS-28 from Baseline →		
DAS-28 at Endpoint ↓	**>1.2**	**<0.6 and ≤1.2**	**≤ 5.1**
≤ 3.2	**Good**	**Moderate**	**None**
>3.2 and ≤5.1			
>5.1			

Abbreviations: DAS-28 = Disease Activity Score using 28 joints

Simplified Disease Activity Index (SDAI)

The simplified disease activity index is similar to the DAS-28 score, varying in that it includes a provider assessment of global activity (PrGA) and utilises the CRP value. It correlates well with the DAS-28 score and the score is the sum of the included domains 28SJC + 28TJC + PrGA + PtGA + CRP[56].

The clinical disease activity index differs from the SDAI in that the laboratory values are not included: 28SJC + 28TJC + PrGA + PtGA. It is useful due to its simplicity and in trials involving biologic agents where acute phase reactants are suppressed due to the pharmaceutical agent and not necessarily a reduction in disease activity[57].

ACR Core Set measures

The ACR core dataset was first proposed in the 1993 in order to facilitate standard measurements across clinical trials. It consists of seven domains: tender joint count, swollen joint count, patients' assessment of pain, PtGA, PrGA, patient's assessment of physical function and evaluation of one acute phase reactant. Measurements are described as percentage improvements, with ACR 20, 50 and 70 responses equating to 20%, 50% and 70% improvement in the core set data, respectively [58]. Although this measure is commonly used in the reporting of clinical trials, it is often difficult to incorporate into routine clinical practice.

Boolean Remission

Despite varying indices for low disease activity, it was felt that several of the disease measures did not encompass the true definition of remission in RA. For this reason, Felson and colleagues attempted to redefine this in the context of clinical trials [59]. The Boolean based remission criteria therefore were defined as follows: TJC ≤1, SJC ≤1, patient global assessment ≤ 1 and CRP ≤1.

The role of imaging in RA treatment and prognosis

Structural damage can occur early in RA. Imaging modalities include plain radiographs, high sensitivity doppler ultrasound (US) and magnetic resonance imaging (MRI). Recent recommendations by the EULAR task force consisting of rheumatologists, radiologists, methodologists and rheumatology practitioners recommended the use of MRI or US in order to confirm disease in case of clinical uncertainty, and to aid the diagnosis of early RA from undifferentiated arthritis [60]. Imaging is better at detecting residual synovitis than

examination alone, figures of 2.21-fold and 2.18-fold differences in synovitis detection have been reported for MRI and US, respectively [60].

US is more readily available, quicker and easier to perform than MRI. Several studies have shown that US is more sensitive at detecting subclinical synovitis than clinical examination alone [61-64] which formulates an argument to include imaging in disease activity scores and indeed to define remission. Recently, however, two large trials have concluded that targeted US remission is not associated with significant improvements in clinical or patient reported functional outcomes[65, 66]. A further randomised controlled study (Targeted Ultrasound in Rheumatoid Arthritis) which is expected to complete this year aims to address whether clinical outcomes will vary based upon treatment guided by clinical or US remission (clinical trials identifier: NCT02056184).

1.2.5 Treatment Strategies in RA

The "window of opportunity" or the need for early treatment has been well recognised for several decades [47]. Untreated, RA results in both functional and economical disability. Early control of inflammation is crucial to try and prevent clinical and radiographic progression. A 'treat-to-target' or 'tight control' strategy has been recommended by EULAR [67], defined as clinical remission or low disease activity. A recent systematic literature review incorporating trials where a treat-to-target approach was used, demonstrated that all trials brought more patients into remission or low disease activity quickly, with improvements in pain scores and functional abilities [68]. An important message here is that the methodologies employed focus on the intensity of treatment without specifically commenting on any particular disease modifying anti-rheumatic drug (DMARD), with the

exception of methotrexate. I have outlined the treatment modalities below and will go on to describe B-cell depletion therapy in more detail.

Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids

NSAIDS inhibitors have been ubiquitously used for several decades. Despite ameliorating symptoms, they do not affect disease progression and are often limited by their side effects, particularly the risk of gastrointestinal bleeding and cardiovascular disease [69].

Corticosteroids are still a cornerstone of treatment in RA, particularly during 'flares' of disease. Current EULAR recommendations consider low dose glucocorticoids as initial treatment in combination with conventional synthetic DMARDs (csDMARD) [70] but with a view to taper them as quickly as possible. This is largely supported by the COBRA trial, which demonstrated sustained radiographic progression when steroids were incorporated into a combination regimen with csDMARDs [71] and the observation that low dose steroids may decrease radiographic progression when given in early RA [72].

Conventional synthetic disease modifying antirheumatic drugs (csDMARDs)

Current EULAR recommendations suggest that patients should initially be commenced on conventional synthetic disease-modifying anti-rheumatic drugs, which include methotrexate, sulfasalazine, leflunomide or hydroxychloroquine. Methotrexate (MTX) should be preferentially used, except in the advent of intolerance or reaction [70]. Utilisation of combination therapy in early disease remains under debate.

Biologic DMARDs

Failure to achieve a low disease activity or remission should prompt treatment with a biologic DMARD (bDMARD) or targeted synthetic DMARD (tsDMARD), as non-response to 2 or more cDMARDs is likely to result in disease progression [73]. If possible, methotrexate should be continued, as greater efficacy is achieved in combination [74]. bDMARDs include the tumour necrosis factor (TNF) inhibitors (infliximab, etanercept, adalimumab, certolizumab and golimumab), anti-CD20 monoclonal antibody (rituximab), inhibitors of interleukin 6 (IL-6) (tocilizumab and sarilumab) and CD80/CD86 blockade through abatacept. Since the advent of biosimilars, further nomenclature changes have been made, with the terms bio-originator and biosimilar to distinguish between the parent drug and those which have the same protein structure and biologic functions.

Targeted synthetic DMARDs

TsDMARDs modulate specific targets within the immune cascade. Currently, inhibitors of janus kinase (JAK), tofacitinib and baracitinib are licensed for the treatment of RA [74]. Their placement in the treatment algorithm is alongside bDMARDs. Baricitinib has been shown to be superior to adalimumab[75] in combination with methotrexate, although this was not demonstrated with tofacitinib [76].

They key to optimal treatment lies in early recognition of disease and flares. The number and choice of drugs available to rheumatologists continues to grow – yet efficacy rates between drugs remain similar [74], once again highlighting the need for biomarkers or predictors of response.

1.3 The aetiology of RA: genetic and environmental factors

RA is a complex autoimmune condition that appears to rely on the interplay on both endogenous and exogenous factors, outlined in figure 1.1. The presence of circulating autoantibodies years before any clinical manifestations and not always associated with subsequent disease development suggests that environment factors play a critical role in RA pathogenesis. This includes exposure to pathogens, lifestyle factors and in the case of females, differences in levels of circulating hormones. Factors influencing this transition from being 'at risk' to disease manifestation are yet to be fully understood and may in the future lead to a shift in focus from disease treatment to prevention.

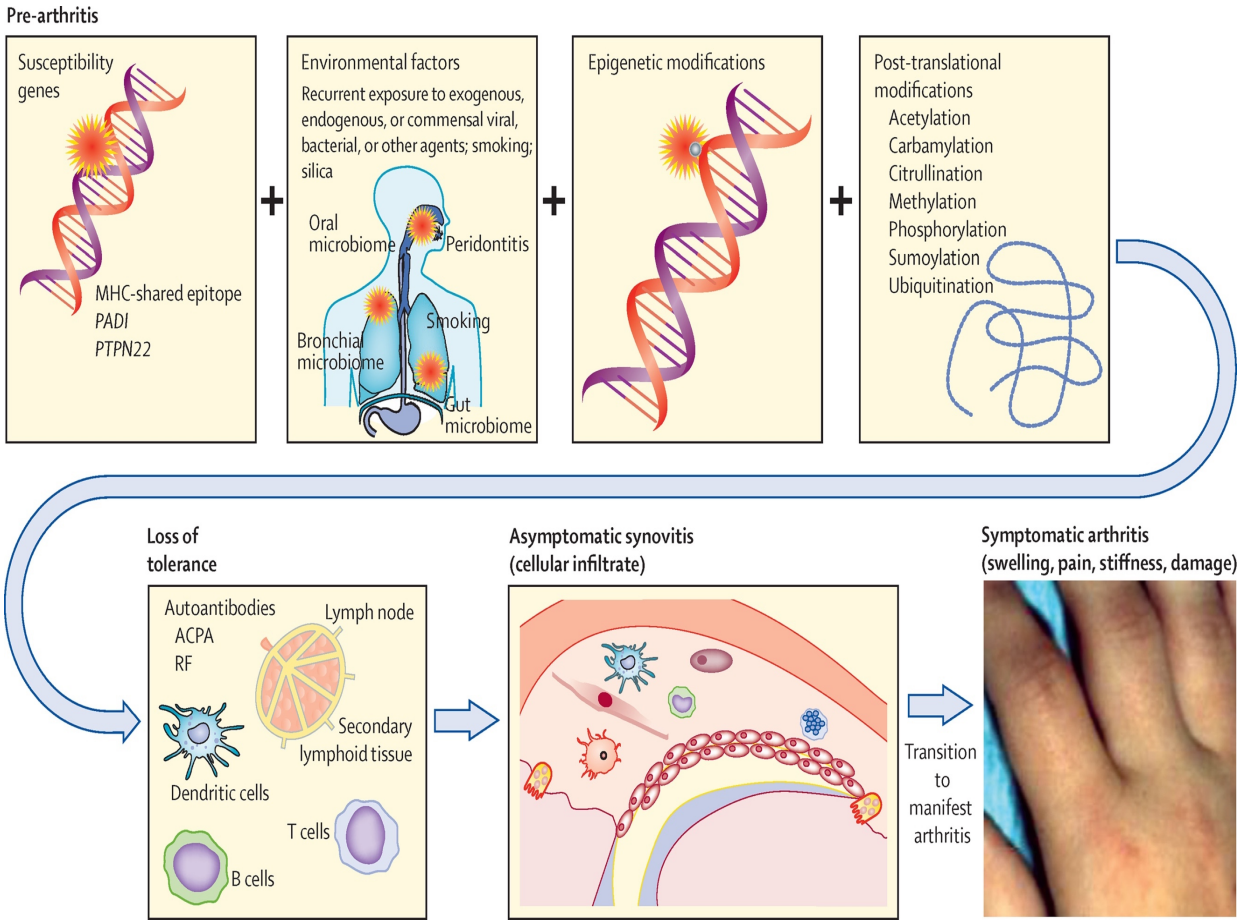


Figure 1.1 Pathways to RA

In a genetically predisposed host with susceptibility genes, environmental insults, epigenetic modifications, and post-translational modifications can lead to loss of tolerance with subsequent asymptomatic synovitis, ultimately leading to clinically overt arthritis. ACPA = autoantibodies against citrullinated peptides. RF=rheumatoid factor.

From [77], with permission from Elsevier.

1.3.1 Genetic factors

The notion of genetic susceptibility in RA is well established. This was first noticed through familial clustering and increased concordance between monozygotic twins [78]. Overall predisposition to RA through genetic factors is thought to range from 20-65% with a discernible variation between ACPA positive and negative disease [79-81]. These can largely

be grouped into genes which directly involve the major histocompatibility complex (MHC) and those outside this region. In recent times, genome-wide association studies (GWAS) have identified >100 loci which may be associated with RA susceptibility [82]. Furthermore, several susceptibility genes have been also been associated with severity of disease [77].

Human Leucocyte Antigen (HLA) DRB-1 locus and the shared epitope hypothesis

An association between MHC genes and RA has been known for approximately 40 years [83]. Human leucocyte antigens (HLA) are a group of proteins expressed on cell surfaces which allow the immune system to differentiate between 'self' and external antigens. Six genes, including HLA-DRB1, encode the MHC class II genes, which influence interactions with proteins found on certain cells of the immune system. Studies identified multiple RA risk alleles found within the same HLA-DRB1 region on chromosome 6(6p21.3) displaying common, conserved amino acid sequences. These sequences are found at positions 70-74 in the third hypervariable region of the HLA-DRβ1 chain and associate with the HLA DRB1*01 and *04 alleles [84, 85]. This led to the development of the 'shared epitope hypothesis which confers that these polymorphisms, which occur at the antigen binding site of the MHC molecule, are directly involved in T-cell activation and the initiation of the immune response [84]. Different combinations of alleles confer varying susceptibility to disease across populations and may also result in higher disease severity [85-87]. Du Montcel and colleagues proposed a further classification for HLA-DRB alleles, by which the amino acid at positions 70 or 71 can further subdivide the shared epitope alleles into S1, S2 S3P and S3D subsets [88]. The presence of these alleles confers, to varying degrees, susceptibility to the development of ACPA and therefore may contribute directly to disease pathogenesis [89].

The contribution of non-HLA genes to RA

The contribution of the HLA complex to the disease is approximately 37% [84, 90]. This leaves approximately two thirds of the genetic component of RA to be due to non-HLA genes. The last two decades have seen unprecedented advances in whole genome screening and subsequent single nucleotide polymorphism (SNP) analysis. Recent GWAS studies have confirmed multiple loci conferring susceptibility to disease in individuals with European ancestry; common ones are discussed herein [91].

Protein tyrosine phosphatase non receptor type 22 (*PTPN 22*)

PTPN22 resides on chromosome 1. (1p13.2) and confers the second strongest risk association with RA [92]. Several studies and metanalysis have shown a positive association between polymorphisms and RA [93-95]. The gene encodes for a tyrosine phosphatase LYP that binds to c-terminal src tyrosine kinase (CSK) and acts as a negative regulator of T-cell activation. The 1858 C/T polymorphism has been widely observed, changing an arginine residue to a tryptophan and subsequently disrupting interactions between LYP and CSK, leading to an increased T cell response [92]. Furthermore, variant *PTPN22* expression can further promote autoimmunity through alterations in B-cell tolerance and receptor signalling. Data from both murine models and human studies suggest that risk variants can result in an increased number and positive selection of transitional B-cells, with a subsequent increased proportion and range of autoreactive B-cells found at critical checkpoints[96-98]. Furthermore, Menard and colleagues demonstrate an upregulation of genes *CD40*, *TRAF1* and *IR5*, involved in B-cell receptor (BCR) signalling [96].

Cytotoxic T lymphocyte associate antigen 4 (*CTLA4*)

Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) is a member of the immunoglobulin superfamily and found at location 2q33.2. It is homologous to CD28, which binds to CD80 on antigen presenting cells and maintains the T-cell response [93]. The interaction of CTLA-4 and CD80 inhibits T-cell activation. Associations have been observed with polymorphisms, particularly 49 A/G and CT 60 G/A, which decreased the inhibitory function of CTLA-4 and therefore may have a role in the reduction of peripheral tolerance [93]. The putative role of this gene is also further defined by good therapeutic response to Abatacept therapy, a CTLA4/ Ig fusion protein licensed for the treatment of RA [29].

Pepitdylarginine deaminase (*PAD*)

The discovery of ACPA in RA suggested involvement of a family of citrullinating enzymes known as peptidylarginine deaminases (PAD). The PAD4 enzyme is responsible for the post translational conversion of arginine to citrulline, which can result in the production of pathogenic autoantibodies. The gene encoding this enzyme was found by linkage disequilibrium methods to reside at 1p36.13.[99] and later confirmed by GWAS [100]. Some meta-analyses suggest that pathogenic polymorphisms may be more common in the Asian population than those of European descent [101, 102].

Signal Transducer and Activator of Transcription 4 (*STAT4*)

Inflammation in RA is driven by immune cell interactions and the production of systemic cytokines. The *STAT4* gene encodes a transcription factor that mediates intracellular interactions by type 1 interferons and several cytokines, namely interleukin (IL)-12, IL-23 and

IL-27. Of these, STAT 4 plays a crucial role in IL-12 dependent Th1 helper cell differentiation and interferon dependent natural killer (NK) cell proliferation [93]. STAT4 also plays a role in systemic lupus erythematosus (SLE) and has been shown to confer increased risk in RA [103].

Tumour necrosis factor (TNF) receptor associated factor 1 (TRAF1)

TRAF 1 is an adapter protein that links the TNF to downstream signalling networks, inducing cell survival[92]. Polymorphisms have been linked to alteration in toll-like receptor (TLR) signalling, changes in messenger RNA (mRNA) levels and an overall increased risk of RA [104-106].

Tumour necrosis factor alpha-inducible protein 3 (*TNFAIP3*)

Tumour necrosis factor alpha-inducible protein 3 is a gene responsible for the production of a ubiquitin editing protein A20. This protein regulates and inhibits nuclear factor kappa B (NFκB). Polymorphisms in this gene have been associated with RA in studies and subsequent meta-analyses [107, 108].

1.3.2 Epigenetics in RA

The discordance rates for monozygotic twins in RA suggest environmental differences may play a role in development of the disease. Epigenetics relates to changes outside encoded genes which directly affect gene expression, such as deoxyribonucleic acid (DNA) methylation and post translational histone modifications [78]. Such changes may be related to environmental or metabolic factors. In unrelated individuals, nine differential clusters of methylation in the HLA region have been identified [109]. Hypomethylation of both T-cells and fibroblast like synoviocytes (FLS) has been observed in individuals with RA when

compared with controls [110] [111]. Genome wide evaluation of FLS revealed nearly 2000 differentially methylated loci [112] between individuals with RA and osteoarthritis (OA), many relating to cell trafficking and adhesion. Whitaker and colleagues furthered these findings by investigating the stability of DNA methylation, concluding that persistent alterations within the genome could lead to diversity of FLS phenotypes that contribute to disease pathogenesis [112]. Effective targeting of epigenetic modifications in disease remains challenging due to wide variations at the individual level.

1.3.3 The role of micro-RNAs

Micro-ribonucleic acids (miRNAs) are short single stranded non-coding RNA's that have been implicated in both autoimmunity and cancer. These post transcriptional regulators pair with the 3'end of a target mRNA resulting in degradation or inhibition of translation and have roles in both physiological and pathological processes [78]. In RA, some examples include the upregulation of miRNA-115 and miRNA 203 in FLS, which lead to increased production of matrix metalloproteinase-1 (MMP1) and IL-6 [113]. miRNA-14a has also been found to be higher in peripheral blood monocytes in patients with RA than OA and may play an important role in IL-17 expression [114]. A summary of other miRNA's identified in RA is presented in an extensive review by Miao et al [115].

Despite extensive genome-wide studies attributing causality to specific findings remains difficult due to the number of patients required to adequately power studies. Nevertheless, miRNAs remain a potential target for therapy in an era where genomic platforms and computational analysis continue to expand.

1.3.4 Environmental factors in RA

The role of smoking

The link between smoking and RA was first described by Vessey and colleagues approximately 30 years ago [116]. There is a strong association between cigarette smoking and RA, although this finding is more established in seropositive disease and in males. A meta-analysis of 16 studies showed that the odds ratio for developing RA in males who had ever smoked was 1.89 and 1.27 for females. This ratio increased to 3.2 in males who were positive for rheumatoid factor [117]. A further meta-analysis suggested that this risk may be dose dependent up to 20 pack years [118].

The combination of certain shared epitopes and smoking confers a higher risk for developing RA with both increased production and heterogeneity of ACPA proteins, particularly citrullinated-α enolase 1 and anti-vimentin antibodies[119]. The presence of ACPA is highly predictive of disease, with PADI enzymes required for the post-translational change from arginine to citrulline. Higher levels of PAD2 enzymes and subsequently citrullinated proteins have been found in bronchoalveolar lavage fluid from smokers, indicating that this may be an important explanation for the higher incidence of RA [120]. This is also the case for lung tissue, where investigators found higher levels of citrullinated proteins than in other tissues [121], although in this study levels were similar in smokers and non-smokers leading the authors to propose a “two-hit hypothesis”, where molecular mimicry is followed closely by antibody production and epitope spreading.

Cigarette smoke also contains a multitude of toxic substances, many of which have the ability to modulate the immune system. There is data to suggest that smoking results in a shift from

Th1 to Th2 type 2 immune responses, blunts immune reaction to foreign antigens and results in the formation of mutated tumorigenic cells [118]. Additionally, macrophage responses in the alveoli are also diminished, demonstrated through both animal models and in cultured human cells in vitro [122, 123]. Tobacco found in cigarettes has also been implicated in dysregulation of apoptosis and sequestration of intracellular antigens, which may also induce a break in tolerance. Direct release of free radicals increases rates of DNA mutagenesis, which again may lower the threshold for developing autoimmune disease.

The role of cigarette smoking is both complex and multifactorial with links to infection, inflammation and DNA methylation and mutagenesis. Direct and indirect modulation of the immune system through smoke and chemical exposure modulates the host response to microbes, local inflammation and the citrullination of proteins which when combined are likely to cumulatively increase the risk of developing RA.

The role of microbes

The possibility of a link between bacteria and the development of RA has been discussed for many years, with a particular focus in recent times on periodontal disease and the gut microbiome. Bacteria thought to be involved include escheria coli, proteus mirabilis, mycoplasma, porphyromonas gingivalis (*p.gingivalis*); important viruses include the human immunodeficiency virus, Epstein barr virus, parvovirus B19, hepatitis B and C and chikungunya [124, 125]. The 'molecular mimicry' hypothesis arises from the notion that bacteria have similar epitopes to the host with a resultant loss in tolerance as self-antigens are no longer recognised. This theory, however has never been completely proven in RA, and previous

authors have postulated that a “double hit” or prior susceptibility is still necessary for development of the disease [125, 126].

The role of periodontal disease

The postulation that oral sepsis plays a role in the pathogenesis of several different diseases, including RA, dates back several centuries. Periodontitis is a condition that is caused by bacteria which progressively destruct structures surrounding the tooth. The key pathogen in this process is porphyromonas gingivalis (p.ginigivalis); others thought to play an important role are trepnoma denticola and tanerella forsythia which together, are also known as the red complex [127] .

Recent epidemiological studies have shown that despite accounting for common risk factors such as smoking, HLA status and other environmental factors, individuals with periodontitis are at an increased risk of developing RA [128-131]. By the same vein, periodontal disease is more common within the RA population. There are several lines of evidence to suggest a pathogenic role for periodontal disease in RA; for example levels of antibodies to p. ginigivalis correlate with ACPA and RF in humans [132] and in animal models, infection with the organism results in increased severity of collagen induced arthritis [133]. The question arises as to the mechanisms by which the presence of bacteria can result in a break of immune tolerance. Infection by the organisms described above results in gingival inflammation and the recruitment of neutrophils, activation of the complement cascade and cell death, promoting the release of human PADI and citrullination of local proteins within the gum. P gingivalis is unique in that also expresses PAD. Release of the bacterial enzyme may result in further changes in protein conformation and the formation of a neo-epitope [129]. This

hypothesis is supported by findings that bacterial DNA is present in the sera and synovium of patients with RA [134] and that human PAD, PPAD and citrullinated proteins are also found in the oral mucosa [135].

The role of the gut microbiome

Intestinal flora may also play a role in the development of RA. Murine models of arthritis can be improved or exacerbated by alterations in the levels of gut bacteria [136, 137]. In humans, dysbiosis of gut bacteria has been implicated in several diseases and are being increasingly investigated following the advent of sequencing initiative such as the National Institutes of Health human microbiome project [138]. Analysis of the faecal microbiota of patients with newly diagnosed RA showed lower diversity and overall counts than patients with fibromyalgia. [139] A higher prevalence of prevotella capri and lower numbers of bacteroides species within the gut has also been described in patients with early RA [140]. The link was further investigated in mice that exhibited a more severe phenotype when exposed to prevotella. Association is further supported by the clinical response to pharmaceuticals directly modulating microbial action such as sulfasalazine and tetracyclines [141]. The mechanisms by which gut flora may induce arthritis are likely to involve dysregulation of TH17 and T cell balance [136, 142]. Toll like receptors (TLR) are also likely to be implicated in shaping the T-cell repertoire in the intestine, possibly resulting in self-reactive cells. Migration of these cells to the joint may promote inflammation and destruction, resulting in the overall clinical phenotype of RA [141]. Further work is, however, needed to establish a definitive pathogenic link and dissociate causality from chance co-existence.

1.3.5 Sex hormones and gender

It is well recognised that there is female preponderance in autoimmune conditions and RA is no exception. Observation that the disease improves in pregnancy also suggests a role for hormonal factors. Sex hormones are involved in the immune response and there are several putative mechanisms by which they may influence the course of disease.

Androgen and oestrogen receptors are found on synovial macrophages and monocytes [143, 144] indicating that these cells are sensitive to circulating sex hormones. Oestrogen stimulates the Th2/humoral response and increases levels of interferon gamma (IFN-γ) and interleukin (IL) -10, which are found in high levels in the sera of patients with RA [145]. Progesterone also results in a shift from Th1 to Th2 responses which may decrease levels of circulating Th17 cells, which may account for the improvements in the prepartum period [146]. Oestrogens can also modulate B-cell function. Oestradiol is involved in antigen presentation and results in an increase in the survival of autoreactive B cell and release of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, leading to a shift towards autoimmunity [147, 148].

Androgens may play an anti-inflammatory role in RA. Serum testosterone levels are inversely correlated with disease activity [149]. Their potential protective effect is further showcased by the observation that administration of anti-TNF agents downregulate the conversion of androgens to oestrogens [150]. Lower levels of testosterone have been found in the joints of patients with RA, leading to studies investigating the role of androgen replacement therapy in males with RA, although this avenue was not followed commercially [151].

Demarcating the influence of individual roles of genetic, environmental and endogenous hormonal factors in the pathogenesis of RA is an arguably difficult, perhaps even impossible, task. The current model of RA suggests that in a genetically susceptible individual, environmental or bacterial triggers can cause alteration of self-proteins that lead to autoantibody formation, dysregulation of the immune system and the activation of the inflammatory cascade resulting in the clinical phenotype that is RA.

1.4 The Aetiology of RA: the role of the immune system

1.4.1 The role of the innate immune system in RA

The role of Toll like receptors in RA

The innate immune system provides the first line of defence against pathogens. Following exposure to an antigen, the immune response is mediated by TLRs which activate other innate immune cells, including macrophages, monocytes and dendritic cells. TLR activation results in the release of pro-inflammatory cytokines that act to recruit and retain macrophages and monocytes which take up the pathogen and travel in circulation until components of the adaptive immune system are met. The pathogen is then cleared, with a resolution of inflammation. In RA this process is defective, resulting in high levels of ongoing inflammatory responses which lead to joint swelling and destruction.

TLR's are a family of receptors found in a number of cells that recognise pathogen associated molecular patterns (PAMPs) found on microbes or danger associated molecular patterns (DAMPs) which are expressed when cells are under stress. TLR 1, -2, -4, -5 and-6 are expressed extracellularly, whereas -3,-7,-8 and -9 are intracellular and require ligand binding

and endocytosis in order to activate downstream cells. The adaptor-ligand complex results in the activation of two pathways[152]. The first, the MyD88 dependent pathway activates NFκB and results in cytokine expression and mitogen activated protein kinases whereas the second, which is MyD88 independent, results in interferon release [152]. Additionally, binding of endogenous ligands such as heat shock proteins (HSP), fibronectin, fibrinogen and endoplasmic reticulum stress protein (gp96) may also result in TLR activation.

Patients with RA show an increased expression of TLR 2 and TLR 4 in both peripheral blood and synovial macrophages, as well as being present on synovial fibroblasts [152-154]. Within the synovium, there is expression of TLR 3, 7, 8 and 9 which have been found to be elevated when compare with patients who have OA – stimulation can result in the production of cytokines and matrix metalloproteinases (MMPs) [155] and furthermore result in osteoclastogenesis. There are numerous other reports of expressed TLR and examples of putative pathogenic roles in RA reviewed in [152].

Neutrophils: the role of NETosis Recently it has emerged that polymorphonuclear cells have the propensity to generate neutrophil extracellular traps (NETs), a process in which autoantigens and immune modulators are externalized. These have been recognised in several autoimmune diseases and are thought to play an important role in the citrullination of protein. Citrullination of histones is an important step in NETosis and these are displayed externally on the NETs. Levels of NETosis have been found to correlate with ACPA and inflammatory markers, and are induced potently by anti-citrullinated vimentin antibodies. [156, 157] It is therefore suggested that presence of these traps has the propensity to result in a cycle of chronic citrullinated autoantibody formation and subsequent inflammation,

potentially making NETosis a target in pre-clinical disease or to break the cycle of ACPA production in early RA.

Monocytes and Macrophages

Macrophages are key orchestrators of the immune system. They play a key role in RA, evidenced by the success of conventional therapies which act to decrease levels of cytokines produced by this subset of cells, such as TNFα blockade. Additionally, it has been shown by several groups that a reduction of the number of residing macrophages in the synovial sublining layer correlates with treatment response [158-160]. They also, through the production of cytokines, play a chemotactic role for other cells of the immune system. Targeting macrophages specifically in RA is difficult as they form one of the first and most important lines of host defence; therapy would need to preferentially target activated macrophages.

Dendritic Cells

Dendritic cells play an important role in the rheumatoid synovium, particularly in the formation of ectopic lymphoid structures (ELS). Following an antigenic stimulus, they migrate and act as mature antigen presenting cells in lymphoid organs. In RA they have been shown to be resident in the perivascular infiltrate [161]. They have also demonstrated to produce cytokines including IL-23, IL-12, BAFF and IL-18 [162]. Dendritic cells are therefore able to activate naïve T-cells to recognise self-epitopes and differentiate into activated T-cells, thus initiating the immune cascade[163].

Fibroblast like synoviocytes (FLS)

Inflamed hyperplastic synovium contains a high number of activated FLS which normally reside in and are crucial to the normal architecture of the joint[164]. FLS respond to a pro-inflammatory environment through the production of cytokines, proteases and alterations in metabolic pathways through cellular crosstalk, leading to progressive joint destruction[165]. Activated FLS are also thought to display an inflammation independent pattern of destruction, demonstrated through ongoing cartilage degradation when RA fibroblasts were implanted in mice homozygous for the severe combined immune deficiency spontaneous mutation (SCID)[166, 167]. Targeting FLS in conjunction with alterations to circulating immune cells may therefore be an option in a subset of patients with RA.

Mast cells

The role of mast cells in RA has been debated, with potentially pro and anti-inflammatory functions[168]. Recent work from our laboratory suggests that high numbers of mast cells were associated with disease activity and local inflammation, particularly in patients with lymphoid aggregates. Furthermore, they were able to demonstrate that mast cells promote the activation and differentiation of B-cells in vitro and in vivo through an antigen induced mouse model of arthritis [169]. This is in line with previous studies that have demonstrated expansion of mast cells in early disease[170, 171]. Mast cells may exert their effects by a number of different mechanisms through the production of IL-10, a pleitropic cytokine with both anti and pro-inflammatory effects, and IL-33 and as regulators of cellular recruitment and tissue permeability [172]. Whether their increased presence in RA is causal or effectual

remains to be clearly established and therefore we remain some way from these cells becoming potential disease modifying targets.

1.4.2 The role of the Adaptive Immune System in RA

The adaptive immune response consists of cellular and humoral responses by T and B lymphocytes. Since the focus of this work is on B-cell pathways, I will describe the role of T cells and key cytokines first, with further dedicated emphasis on B-cells and subsequent B-cell depletion therapy in the next section.

1.4.2.1 The role of autoantibodies in disease pathogenesis

Autoantibodies in RA can be identified years before the disease manifests. Studies have suggested that this time is usually 3 to 5 years, although up to 15 years prior to onset has been observed. [173] To date, a number of autoantibodies have been described, the most important and common of which are RF and ACPA, and which are included in the new ACR classification criteria.

Rheumatoid Factor

RF was first described by Waaler in 1940 [174]. These are a family of antibodies directed again the crystallised fragment (Fc) portion of immunoglobulin (Ig) subclass G, detected in 60-80% of patients with RA. They can also be found in disease-free individuals, ranging from 4% in Caucasians to higher levels of 30% in certain ethnic groups such as Pima Indians [175]. They also co-exist in other diseases such as SLE , Sjogren's syndrome and tuberculosis and therefore are sensitive, but lack specificity [176]. They exist in different isoforms, IgE, IgM, IgG and IgA, but IgM is the isoform routinely detected in clinical practice. A difference has been reported

between RF's from RA patients and from healthy individuals, as the former bind with a much higher affinity, having undergone T-cell driven affinity maturation with more sense mutations. The latter bind to antigens weakly and tend to be polyreactive [177].

Circulating antibodies can be pathogenic by their ability to form immune complexes and activate the complement cascade, enhancing joint destruction. A recent study has also shown that IgM-RF increases cytokine production by macrophages, dependent on the number of engaged FcγR [178]. Furthermore, RF is a prognostic marker in RA, as patients with higher circulating levels are more prone to develop erosions and extraarticular manifestations of disease [176, 179].

Anti-Citrullinated Protein Antibodies (ACPA)

In 1964, Nienhuis and Mandema identified a new class of autoantibodies in the sera of patients with RA [180] which reacted to anti-perinuclear granules in the buccal mucosa. Several years later it was reported that these antibodies co-localised with filaggrin [181]. Serre and colleagues noted that filaggrin in buccal mucosa and fully differentiated epithelial was different to profillagrin found in cultured cells. The main modification of the protein was found to be the presence of citrulline. This was subsequently shown to be essential for antigenicity of these antibodies in RA [182] and led to the identification of cyclic citrullinated antibodies (CCP), which showed better specificity than the original linear enzyme linked absorbent assays (ELISAs). This was further refined in 2002 with the advent of the CCP2 assay which was derived from screening approximately 12 million synthetic peptide libraries with sera of patients with RA [183, 184].

In normal physiology, citrullination occurs in the presence of high calcium and usually accompanies cell death. Apoptotic cells are then usually cleared by the innate immune system [184]. If these are ineffectively cleared, active PAD enzymes and citrullinated proteins are exposed to the immune system, with the ability to citrullinate more local proteins within joints, such as collagen or fibrillin. As discussed earlier, the presence of the HLA DRB1 epitope greatly increases the risk of developing RA when the immune system is exposed to citrullinated proteins. This "spreading" of the ACPA pool is a step recognised prior to the onset of disease [185]. Like RF, the presence of circulating ACPA can result in immune complex formation and the release of pro-inflammatory cytokines to stimulate the inflammatory cascade and result in joint damage.

ACPA positivity is associated with more erosive disease and presence of circulating antibodies is a strong predictor of erosions, shown by a recent meta-analysis of 25 studies [186]. Positivity is also associated with worse clinical phenotypes and cardiovascular disease[184, 187] indicating a direct pathogenic role in the disease. How this is achieved is yet to be fully elucidated, but animal models of the disease show that transfer of synthetic antibodies enhances collagen induced arthritis in mice and citrullinated collagen or fibrinogen can induce joint symptoms in the animals. [187-190] In vitro, similar to changes induced by RF, macrophages produce more cytokines, particularly TNF-α, propagating a pro-inflammatory environment[191]. Additionally, activation of the complement cascade is responsible for recruitment and retention of inflammatory cells to the synovium.

Anti carbamylated antibodies (Anti-CarP antibodies)

Akin to citrullination, carbamylation is another post-translational modification process that results in the generation of a different amino acid, in this case, homocitrulline, which differs from citrulline in the length of its side chain, resulting in a conformational change [192]. The physiological role of carbamylation has not yet been fully ascertained, but it has been described in kidney disease, uraemia, atherosclerosis and inflammation [193]. These antibodies have recently been recognised as a separate entity in RA, although approximately 20% do overlap with ACPA positive patients [192].

Protein 14-3-3

14-3-3 are a seven-isoform family of proteins that act as chaperons and have the capacity to interact with over 200 intracellular proteins, thus regulating several processes including cellular signalling, protein trafficking and cytoskeletal transport[194, 195]. 14-3-3η has been shown to be present in higher levels in the sera of patients with RA, and up to five times normal levels in synovial fluid. Levels correlate strongly with MMP-1 and MMP-3, suggesting that they may play a role in joint damage. [196] A study of 619 patients with RA showed that identification of the protein was associated with a diagnosis of RA with an associated sensitivity and specificity of 77% and 92.6% respectively [197, 198]; adding this test to ACPA antibodies alone increased disease identification rates to 72% compared with 59%, and addition to both RF and ACPA increased the detection rate from 72% to 78%. There are currently studies underway to enhance the knowledge of this protein and its potential as a disease biomarker.

Nuclear ribonucleprotein complex Anti A2/ anti-RA 33 antibodies are directed to the heterogenous ribonuclearprotein A2 (hnRNP-A2), a protein found in nuclei that is involved in mRNA splicing and transport[199]. These occur in approximately 33% of patients with RA but can also be found in connective tissue diseases such as lupus and mixed connective tissue disease. If these diseases, however are ruled out, clinically and through the exclusion of other lupus associated antibodies, specificity for RA may rise to 96%[200].

Anti-Bip antibodies

Anti-stress protein immunoglobulin heavy-chain binding protein (Bip) antibodies have been demonstrated to be present in approximately two-thirds of RA patients with a specificity of 96%. They have been reported to be overexpressed in the synovium and may form a target for autoreactive T-cells [201].

Anti-SA antibodies Anti-SA antibodies are directed against a protein of unknown function and are found more frequently in patients with established disease. A study investigating antibodies in early arthritis found that these were associated with more destructive disease in males, indicating that they may be of prognostic benefit [202].

1.4.2.2 The role of T-cells in RA

T-cells comprise of two major subsets: CD4+ T-helper cells and CD8+ cytotoxic T- cells. CD4+ T-cells are activated by engaging with a complex of (possibly arithritogenic) antigen fragments presented by professional antigen presenting cells (APC's) and MHC class II molecules. Additionally, a co-stimulatory molecule of CD80/CD86 expressed on the APC is required to interact with CD28 found on T cells for activation to take place [203]. Once activated, T cell

clones proliferate to become effector T cells and secrete cytokines that initiate the inflammatory cascade.

CD4+ T-helper (Th) cells were traditionally divided into Th1 and Th2 subtypes, however in recent years evidence has emerged that Th17 cells also play a critical role in RA. Th1 cells, associated with the transcription factor STAT1, STAT4, Tbet (TBX1) and IL-12, secrete interferon-γ (IFN-γ) and tend to protect against intracellular bacteria; Th2 cells appear to combat extracellular organisms such as parasites through the production of Il-4, -5 and -13 and are associated with the expression of transcription factor GATA 3, and STAT 6 [204]. There is a further functional difference related to various target antigens, Th1 cells link to cellular immunity, largely provided by CD8+ cells, whereas the latter lead to activation of B-cells and humoral immunity[205]. Initial ideas regarding the role of T-cells hypothesized that a shift towards a preponderance of Th1 cells resulted in an abnormal release of associated cytokines resulting in disease; however this was challenged by a paucity of IFNγ and IL-2 in the synovium, resulting in a shift in focus yo the potential role of TH17 cells and CD4+CD25+ regulatory T-cells (Tregs) [203].

Th17 cells

In the last decade, a new lineage of T effector cells has been described, known as Th17 cells based upon their ability to produce IL-17. The importance of this cytokine and subsequently subset of cells was first described in models of autoimmune encephalitis and in experimental animal models. CD4+ effector T cells were shown to be pathogenic in a collagen-induced arthritis model, as IL-23 (required for production of IL-17) deficient mice did not develop

disease, whereas the same was not observed in mice deficient in IL-12, which is required for Th1 IFNγ producing cells, who developed arthritis[206, 207].

Th17 cells require specific cytokines for proliferation, including IL-1, IL-6 and IL-23 and transforming growth factor (TGF)-β [208]. In normal physiology, several naïve Th cells dwell at mucocutaneous barriers such as the skin, gut and respiratory tract, differentiating to TH17 after encountering bacterial and fungal infections [209]. The differentiated cells express IL-17A and IL-17F, IL-21, IL-22, IFNγ and granulocyte colony stimulating factor (G-CSF) [209]. IL-17 binds to its receptor to trigger an intracellular signal that leads to the production of IL-6, CXC chemokines, resulting in synovial inflammation, cartilage damage and bone resorption [210].

The role of T regulatory cells (Tregs)

Regulatory T-cells, akin to their name, dampen and regulate inflammatory responses towards self-antigens through cell to cell interactions and cytokine production. Tregs express the regulatory transcription factor Foxp3, CD25, CTLA 3 and glucocorticoid induced tumour necrosis factor receptor family-related protein (GITR). Although CD4+ an CD8+ are the prime targets of Tregs, they can also have suppressive effects on B-cells, natural killer cells, dendritic cells and other APCs [211]. They are also responsible for the production of IL-10 and TGFβ [212] and have been found in both the peripheral blood and synovium.

There is currently debate over the numbers of T-cells found in RA sera compared with healthy controls, as studies have shown conflicting results of increased, decreased and normal circulating Tregs [213-217]. Less conflicting, however, is the notion that Tregs are dysregulated in the presence of abnormal levels of cytokines, as in the rheumatoid joint.

TNFα and 1L-17 diminish the suppressive effects of Tregs [217], which can be reversed to normal levels by anti-TNF therapy [215]. It has also been suggested that Tregs are less able to regulate CD4+ effector cells within the synovium which appear to be more resistant to suppressor effects, particularly TH17 cells. These cells are in balance with Treg cells and depending on cellular signals can be derived from the same precursor[217, 218]. A change in this delicate ratio can therefore lead to inflammation and has been implicated in the development of certain tumours and in acute lung injuries [219, 220].

1.4.3 Mechanisms of inflammation: the roles of cytokines and chemokines

TNF-α superfamily

TNF-α plays a critical role in inflammation and a pivotal role in RA. It is a multifunctional cytokine with downstream pro-inflammatory effects on several cells of the immune system, including macrophages, synoviocytes, chondrocytes and osteoclasts, promoting synovial immune cell proliferation and tissue damage. It is produced by activated macropahges and T-cells, and exerts function after binding to p55 (TNF-R1) or p75 (TNFRII.) Both receptors have an intracellular portion for signal transduction; cleavage of the extracellular domain results in soluble TNF receptors which are capable of sequestering anti-TNF [221]. High levels of TNF have been found within the rheumatoid joint. It is a potent inducer of several other inflammatory cytokines, including IL-1, IL-6, IL-8 and GM-CSF. Additionally, within the joint it stimulates fibroblasts to produce cell adhesion molecules, thus regulating critical pathways in immune cell recruitment and pannus formation. It also regulates the receptor activator of

nuclear factor kappa-B ligand (RANKL) and affects the RANKL / osteoprotegerin balance, favouring osteoclast formation, leading to the increased presence of erosions.

Interleukin -1 (IL-1)

Another important interleukin that forms part of the inflammatory milieu is IL-1. IL-1 is a 17 kd protein produced by monocytes and macrophages, but also endothelial cells, B cells and activated T-cells [222]. It promotes leucocyte activation, endothelial cells, osteoclasts and chondrocytes and release of circulating MMPs, resulting in joint damage. IL-1 is bound by two types of cell surface receptors, of which only type 1 are capable of intracellular signalling. Type 2 receptors bind IL-1 in the circulation but do not have any downstream intracellular affects. Like TNF, levels of IL-1 are high in the synovial fluid of patients [223]. Despite its potent pro-inflammatory actions, IL-1 blockade through Anakinra has only proved to be of modest value in RA [224].

Interleukin-6 (IL-6)

IL-6 is a key mediator of inflammation in RA, with both local and systemic effects. IL-6 signals via a protein complex that has a membrane bound receptor unit and two signal transducing gp130 subunits. IL-6 trans signalling utilises a soluble receptor which binds to membrane bound gp130 subunits. Gp130 is found on a wide range of cells, such as endothelial cells and synoviocytes, broadening the repertoire of cells that will respond to IL-6 [225]. Local effects of IL-6 include neutrophil recruitment, Vascular endothelial growth factor (VEGF) production, osteoclast activation, B-cell proliferation and antibody production. It also plays a role in T – cell proliferation and differentiation. In addition, systemic effects include the production of acute phase proteins, acceleration of cardiovascular disease through alterations in oxidative

stress and lipid dysregulation, anaemia and changes in the hypothalamic pituitary axis resulting in fatigue and depression, commonly found in patients with RA [225]. Clinically, inhibition of the IL6 pathway with biologic therapies such as Tocilizumab and Sarilumab, which competitively bind to the IL6 receptor, is an effective therapeutic approach for RA patients [226, 227].

Interleukin-17 (IL-17)

IL-17 is produced by Th17 cells and has, in recent years been shown to have a key role in inflammation. IL-17A is similar to IL-17F, and these two isoforms appear to be predominant in human peripheral blood monocytes (PBMCs) [228]. They bind to a receptor complex composed of at least two subunits, IL-17RA and IL-17RC. IL-17 cytokines activate pathways linked to the innate immune system, hence acting as a potential link between the primary host defenses and the adaptive immune system. IL-17 stimulates the expression of multiple genes involved in inflammation, including IL-6, G-CSF, IL-32 and chemokines CXCL1, CXCL2, CXCL5 and CCL20. IL-17 also has the ability to upregulate receptors for TNF in synoviocytes and therefore in the presence of TNF is a potent pro-inflammatory mediator[229]. This synergy is also displayed with IL-1 and IFN-γ via a positive feedback mechanism which perpetuates inflammation. Trials utilising secukinumab and ustekinumab, which block IL-17 and the IL-12/IL-23 pathways respectively, have so far not conferred clinically significant benefits[230-232].

Several other cytokines are known to play a role in RA including IL-15, IL-32, IL-33, GM-CSF, IL-12, macrophage inhibitory factors. Many of these are being currently characterised and investigated for the utility of potential treatment targets.

Chemokines

Chemokines are low molecular weight proteins that are required for chemotaxis and recruitment of immune cells such as leucocytes and angiogenesis. They are expressed both constitutively and can be upregulated during inflammation or stress. Chemokines have been divided into four supergene families: CXC, CC, C and CX3C with L denoting the ligand of each specific chemokine [233].

IL-8/CXCL8, epithelial-neutrophil activating protein 78 (ENA-78)/CXCL5 and gro-alpha/CXCL1 are well characterised in RA and are found in high levels in the sera and synovium of patients with RA [234-236]. The main producers of these chemokines are synovial macrophages; however, they may also be found in fibroblasts and resident synovial cells. A role in disease pathogenesis has been demonstrated through induction of synovitis following an intraarticular injection to rabbit knees [237].

Potentially some of the most important roles of chemokines in the immunopathogenesis of RA are witnessed as they orchestrate production of ELS within the synovium. Stromal cell derived factor 1 (SDF-1)/CXCL12 is a ligand specific for CXCR4. SDF-1/CXCL12 is involved in organization of the lymphoid compartment, it has been found to be expressed by synovial endothelial cells and therefore implicated in lymphoid neogenesis. It affects T-cell migration and adhesion; in SCID mice transplanted with human synovial tissue, monocytes were preferentially recruited to transplanted human synovium injected with CXCL12, indicating a chemotaxis following exposure to the ligand complex [238]. Adhesion of T-cells to fibroblasts upregulates production, resulting in leucocyte migration, osteoclastogenesis and erosions [154, 234, 239].

Formation of ELN within the joint also relies on the expression of CXCL13 and CCL21. Manzo and colleagues provided direct evidence that CXCL13 and CCL21 were produced in the joint and this was associated with the formation of lymphoid aggregates within the synovium, mirroring the homeostatic structural and compartmental changes in secondary lymphoid organs [240]. A role for ELN in the pathogenesis of disease and resistance to treatment will be discussed further in this work.

Over 50 chemokines have been described to date, many of which have homeostatic roles which change in the presence of inflammation or injury. Only a few have been mentioned here. Several chemokines are known to be upregulated in RA, the dynamics and subsequent cell movement models, however are limited to animal or cultured cells. Ubiquitous expression of chemokines and their role in homeostasis also makes it difficult to target them as potential therapeutic agents, although efforts have been made to modulate the CCR2 receptor with little clinical benefit [241].

1.5 The role of B-cells in RA

1.5.1 B-cell development and function

Following the discovery of RF by Waaler, B-cells were initially postulated to be key players in the pathogenesis of RA. The lack of RF specificity shifted attention towards alternate cells of the immune system, with increased focus on the dysregulation of T-cells and macrophages [242]. The clinical response seen to the anti-CD20 antibody, Rituximab reignited interest in B-cells and their critical role in the development and propagation of the disease process.

B-cells are part of the adaptive immune system. They are produced continuously throughout life, arising first from the foetal liver and later from hematopoietic stem cells in the bone marrow. Transgression from an immature B-cell to a fully functioning mature B-cell requires several tightly regulated checkpoints and transcription factors as well as factors contained in the stromal environment.

Figure 1.2 details the stages of B-cell development and is described herein. B-cell development is initiated when lymphoid progenitor cells receive the appropriate signal from bone marrow stromal cells, under the influence of Terminal deoxinucelotidyl transferase and recombinase activating genes (RAG-1 and RAG-2) [243]. Additionally, the presence of stem cell factor, kit and IL-7 are required for transition signalling.

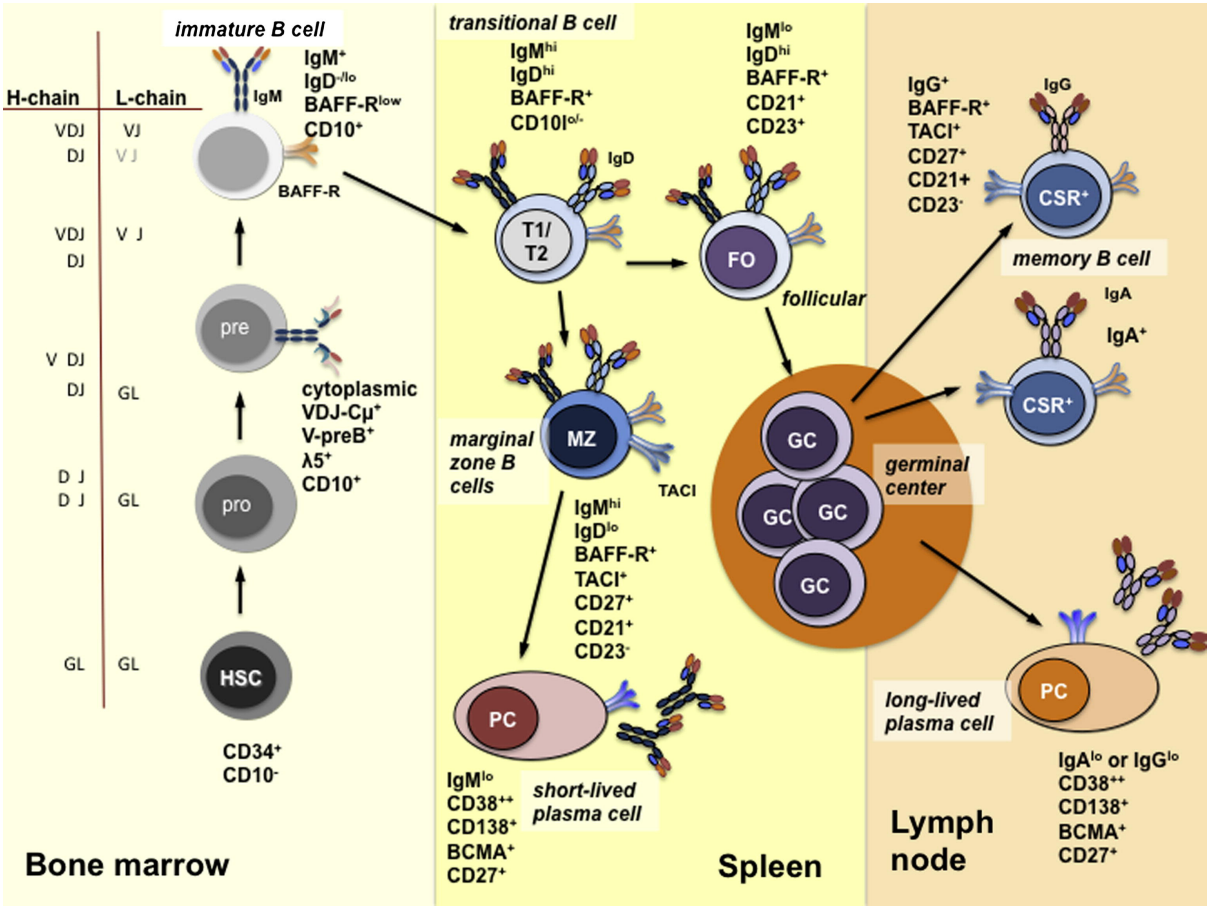


***Figure 1.2 Stages of B-cell development**; used with permission [244] from the Journal of Allergy and Clinical Immunology*

Abbreviations: HSC haematopoetic precursor cells, GL germline, BAFF-R B cell activating factor receptor,
GC germinal centre, PC plasma cells, CSR class switch recombination

Each change in the stage of development is dependent of the rearrangement of immunoglobulin gene segments that result in antibody formation, with variable recombination of the variable (V), diversity (D) and joining (J) regions. Early pro-B cells are formed following a D-J recombination on the immunoglobulin heavy chain gene (IgH) and are characterised by the expression of CD45 and MHC class II molecules. Late pro-B cells form following addition of an upstream V segment [244]. VDJ rearrangement and expression of a membrane bound surrogate light chain in the B-cell receptor defines the pre B-cell

population; early and late stages are differentiated by size (early pre-B cells are dividing and larger) and expression of the membrane chain in the cytoplasm and on the surface. The cells now express CD45, CD19 and CD40 [245].

Immature B-cells are formed following V-J recombination of light chain genes, a process which exhibits allelic exclusion. If a functional light chain is produced and paired with a heavy μ chain this is expressed on the cell surface as IgM. Alternative mRNA splicing will result in the formation of IgD (IgD+/ IgM+) and membrane bound IgG which completes the transition to a naïve mature B-cell. At this stage it is ready to leave for the periphery. VDJ recombination which occurs prior to antigen exposure is the first step towards developing an almost infinite repertoire of diverse antibodies [246].

1.5.2 B-cell maturation and the germinal centre reaction

Naïve mature B-lymphocytes migrate continually through the bloodstream and secondary lymphoid organs in search of potential antigens. They circulate through primary lymphoid follicles and following antigen encounter undergo further expansion to form secondary follicles which contain germinal centres (GCs) [247]. GCs are found in the follicular B-cell zones of lymphoid organs such as the spleen and lymph nodes. GCs have been subdivided into 'zones' based upon histological findings from tonsils and lymph nodes, named the dark and light zones which are surrounded by follicular mantle B-cells. The organization and migration of cells is dependent on an array of tightly regulated cytokines and chemokines. The formation of compartments relies on CXCL12, which is more abundant in the dark zone, and CXCL13 which is present in the light zones. These differences depend on the expression of

CXCR4 by centroblasts in dark zones and CXCR5, the ligand for CXCL13 in light zones [248, 249].

Dendritic cells present the antigen to Th cells followed by clonal expansion of these antigen specific cells. Following encounter of a mature B-cell with a specific antigen, upregulation of the chemokine receptor CCR7 and migration to its ligands CCL19 and CCL21, results in the congregation of B-cells at the border of B-cell and T-cell rich areas in order to further engage Th-cells [250, 251]. These encounters are responsible for GC reactions, particularly through binding of CD40 to its ligand, CD40L, expressed on Th2 cells [252]. Rapid clonal expansion of these B cells results in the formation of the secondary follicle. B-cells then undergo somatic hypermutation in order to increase affinity for the antigen. This process is aided by the presence of antigen as immune complexes held by FDC's. Cells with high affinity undergo positive selection and continue to proliferate to recirculate and become B memory cells; those with low affinity return for a further cycle of SHM or if after several rounds low affinity persists, cells undergo apoptosis and are taken up by tingible body macrophages.

1.5.3 Somatic hypermutation

B-cell Receptor (BCR) maturation and diversification is a [253] achieved through a process called somatic hypermutation (SHM). As cells proliferate within GC's they undergo high rates of mutations in the IgV genes, usually in the form of single nucleotide exchanges. The enzyme activation induced cytidine deaminase (AID) is vital for this process and is found in the dark zone of germinal centres[254, 255]. Its action is to deaminate cytidine to uracil. Random changes are introduced by excision of deoxycytidine bases by uracil DNA glycosylase and subsequent addition of bases by a DNA sequence error prone polymerase. Replacement

mutations are inserted in preference to silent changes. Most mutations occur at complementarity determining regions (CDRs) which will result in a conformational change at the binding site of the antigen. B-cell variants with high affinity and specificity are then selectively expanded through repeat cycles of GC selection. [249, 256, 257].

1.5.4 Class-switch recombination (CSR)

Antibodies are divided into five different classes determined by the heavy chain constant region, which determine their effector function. The process of CSR is an intra chromosomic deletional recombination event in switch (S) regions upstream of VH regions, resulting in a switch in immunoglobulin classes, defined as IgM, IgG, IgA , IgD and IgE isotypes. IgG is further subdivided in four subclasses: IgG1, IgG2, IgG3 and IgG4; similarly IgA is subdivided into IgA1 and IgA2 [258]. S regions are characterised by short, 20-80bp G-rich sequences which are unique to each isotype. CSR can occur anywhere within or near these regions [259].

There are several distinct steps in CSR. The first step required is CD40 ligation/ TLR stimulation which is required to initiate CSR and release cytokines and chemokines which will drive the process. Like SHM, AID is necessary for the steps that follow. Deamination of cytosine to uracil in the S and variable Ig regions is the first to occur, followed by the formation of single stranded breaks in DNA by uracil DNA glycosylase (UNG). The formation of double stranded breaks occurs by two mechanisms; the first occurs if another single stranded break (SSB) is sufficiently near, or if the SSB is more distal, then mismatch repair mechanism is employed [253]. The empty base sites are repaired by enzymes known as apurininc or apyrmidinic endoculeases [260]. Mismatch repair occurs if SSBs are not in close proximity to one another.

Any 5’ or 3’ overhangs are subsequently excised or completed using polymerases or endonucleases. Following this S regions are joined by non-homologous end joining.

1.5.5 Plasma Cell Development

Plasma cells from mature B-cells and are capable of producing mature, high affinity antibodies. B-cells which leave GCs acquire continue as memory B-cells or develop into short-lived plamsa cells. Plasma cells from mature B-cells and are capable of producing mature, high affinity antibodies. Plasma cells can develop from either outside the follicle or within GCs [261].

First, they can develop from marginal zone B-cells and follicular cells. These cells tend to be short lived and are required for the immediate immune response. Within a few hours of exposure to antigen, marginal zone cells move to the red pulp and bridging channels within the spleen and undergo proliferation, resulting in IgM secreting plasmablasts [262]. Secondly, circulating mature follicular cells can receive Th2 help after they have encountered an antigen and differentiate to plasmablasts and plasma cells. These have not, however, undergone the process of SHM and therefore tend to be short-lived, undergoing cell-death in situ after a few days [263]. Post GC plasma cells arise from B-cells who have undergone rounds of SHM and CSR are able to exit the germinal centre and produce antibodies. Memory B-cells display high affinity BCR on their surface and following re-encounter with an antigen have the ability to rapidly proliferate to form antibody producing plasma cells.

Long-lived plasma cells are formed in GCs and preferentially migrate to reside in the bone marrow and can produce ongoing antibodies for years, even in the absence on ongoing antigenic stimuli [264, 265]. They are also found in various organ-specific niches such as gut

mucosa and sites of ongoing inflammation [266, 267]. This is an important finding in autoimmune diseases, where autoantibodies are differentially reduced between patients and therapies, suggesting that there are niches of development and residence for these cells, including the bone marrow, lymphoid tissue and the synovium.

1.5.6 The roles of BAFF and APRIL

B-cell activation factor (BAFF) of the T-cell family (also called BLyS) and A-proliferation inducing ligand (APRIL) regulate lymphocyte activation and survival. BAFF has three receptors, the BAFF-Receptor (BAFF-R), Transmembrane activator and CAML interactor (TACI) and with lower affinity to BCMA (B-cell maturation antigen). APRIL also binds to the latter two receptors, but additionally interacts with proteoglycans. These ligands play several roles in the modulation of lymphoid cells, with functions differing between receptors. They play a role in the survival of peripheral B cell survival and in the induction of self-reactive B-cells [268].

BAFF is necessary for B-cell survival signals, however in the absence of B-cells, BAFF is continuously expressed by constitutive cells [269]. It is also a player in the expansion of autoreactive B-cells, as B–cells bound to self-antigens can escape cell death in the presence of high levels of circulating BAFF, which has been postulated as a mechanism for ongoing autoimmunity [270]. BAFF and APRIL also have varying roles in different B-population subsets, as responses in mice to a T-cell dependent antigen and a molecule blocking both TACI and BAFF was a reduction of circulating plasma cells but long term immunity appeared to remain intact, suggesting that these cytokines may have little role on memory cells [271]. BAFF is responsible for the cell surface markers CD21 and 23. BAFF and APRIL may also play

a role in antibody switching via T-cell independent mechanisms. Lastly, BAFF also had the ability to stimulate T-cells through IFN-γ and IL-2 production[268].

1.5.7 The pathogenic role of B-cells in the pathophysiology of RA

B-cell tolerance checkpoints

Autoreactive B-cells undergo negative selection at two distinct checkpoints. The first is centrally, within the bone marrow, where the majority of naïve auto reactive B-cells are eliminated. A further peripheral checkpoint in the spleen also counter selects against transitional B cells which display poly- or autoreactivity [246]. It has been suggested that these checkpoints are deficient in patients with RA, following a small study of six patients by Samuels and colleagues in 2005 [272]. The same group showed that these were not changed by administration of methotrexate, concluding that the break of tolerance occurs early in RA [273]. Peripheral checkpoints are also defective, and may be due to a combination of dysregulated Tregs and relative resistance to FAS-mediated apoptosis [274, 275].

Autoantibody production

The importance and formation of autoantibodies in RA has already been discussed. Antibodies act against foreign and self-antigens in several ways including the formation of immune complexes and complement activation, subsequent recruitment of immune effector cells and a direct tissue or cytotoxic effect through binding of the Fcγ receptors.

Antigen presentation

B-cells can act as antigen presenting cells to stimulate the CD4+ T-cell population. Takemura and colleagues showed that in SCID mice, B-cells were necessary for the development of T-cell responses after adoptive transfer of CD4 cells[276]. This finding is mirrored after the administration of Rituximab, a monoclonal antibody directed against CD20; treatment response was associated with a decrease in the number of activated CD4+ T-cells [277]. Another study showed that this was particularly relevant for the Th17 subtype and did not affect other CD4+ cell numbers [278]. These findings suggest a level of T-cell / B-cell immune crosstalk, indicating that B-cells are important mediators of the ongoing inflammatory process in roles other than just autoantibody production.

Cytokine production

B-cells secrete several cytokines. One of the most important is the production of IL-6 which has an effect on the regulatory roles of suppressive Tregs and the balance with proinflammatory Th17 cells [274]. There is also evidence to suggest that B-cells play an important role in forming specific subunits required for the activation of certain cytokines, and are a source of RANK-L in the synovium. More recently, the presence of RANK-L has also provided a putative mechanism by which B-cells play a direct role in bone destruction as patients who exhibit ACPA positivity have a higher erosive load, indicating a direct role in the disease process [279].

1.6 B-Cell depletion therapy

Several clinical trials have highlighted the efficacy of B-cell depletion therapy with Rituximab for patients with RA and are summarised in the next section. Despite these findings, however, only 40-60% of patients exhibit a meaningful response to therapy. Much work has been done to try and elucidate patterns of response to therapy and the factors that drive disease relapse, which will be further discussed in this section.

1.6.1 Rituximab mechanism of action

Rituximab is a chimeric mouse / human monoclonal antibody directed against B-cells expressing CD20. CD20 is a 33-37 kilodalton (kDA) protein that is found on the surface of most B-cells (except precursors and plasma cells). Binding of the drug causes internalization or shedding of CD20 at the membrane. Its mechanism of action is dependent on binding its Fc receptor which mediates antibody dependent cellular toxicity and complement mediated cytotoxicity. Rituximab also results in direct apoptosis of CD20 cells [280, 281]. It does not display an effect on stem cells, pro-B cells and mature plasma cells. It causes near complete depletion of circulating peripheral blood B cells, with variable depletion in the synovium, discussed later in this chapter.

1.6.2 Clinical Trials of Rituximab: an overview

Rituximab first came to light in RA after patients receiving the drug for haematological malignancy experienced alleviation in their joint symptoms [282]. Edwards and colleagues had also started using Rituximab in 1998 to treat patients with RA, resulting in the publication of the first open label trial [283]. Since then, efficacy has been proven through several

randomised controlled and observational studies. A summary of randomised controlled trials is provided in Table 1.4.

Edwards et al published initial Rituximab data in 2001 as a small, open label study of 5 patients showed a significant improvement in symptoms following a combination of cyclophosphamide, Rituximab and prednisolone[283]. This led to the development of the first randomised controlled trial where 161 patients with established RA who had failed methotrexate therapy were randomised to receive: Methotrexate monotherapy, Rituximab monotherapy, Rituximab and methotrexate or Rituximab and cyclophosphamide [284]. The dose of Rituximab was 1000mg administered intravenously 14 days apart. The results showed that at week 24, ACR 50 responses in the arms that contained Rituximab with or methotrexate or cyclophosphamide were higher than controls ($p = 0.005$). A higher number of patients exhibited ACR 20 responses at 24 weeks in all rituximab groups and the a statistically significant proportion in the rituximab and methotrexate group reached an ACR 70 response ($p=0.048$).

The DANCER trial was a phase IIB dosing study, where 465 DMARD refractory patients were randomised to receive placebo, or 500 mg or 1000 mg of Rituximab in combination with methotrexate. Both Rituximab arms showed better clinical response than placebo [285]. The MIRROR trial was another dose related study of 346 RA patients on stable doses of concomitant methotrexate who received varying dosing regimens of Rituximab at baseline and again at 24 weeks: 2 x doses of 500 mg and 2 x 500 mg, 2x 500 mg and 2 x 1000mg and finally 2 x of 1000 mg and 2 x 1000mg. There were no significant differences in ACR 20 response at week 48. The higher dose regimen however showed a significant improvement in EULAR response ($p=0.0495$). Numerically, patients receiving the higher dose regimens

maintained or improved response at week 24 [286]. The SERENE trial was a similar study, where 511 patients resistant to methotrexate were randomised to receive two 500 mg doses or two 1000 mg doses or placebo, in combination with methotrexate. Both doses showed superior efficacy to placebo but no significant differences were demonstrated between the two groups [287].

An early randomised controlled trial of Rituximab therapy exploring radiographic damage was the IMAGE trial of 748 patients. Patients were randomised to receive placebo, 2 doses of Rituximab at 500mg, 2 doses at 1000mg in combination with methotrexate. At one year both Rituximab arms showed better ACR50 responses when compared to methotrexate alone, but the higher dose group showed significant limitation of joint damage, which continued for further 12 months [288]. The RA-SCORE trial randomised RA patients who had failed anti-TNF therapy to placebo, 2x 500 mg and 2 x 1g Rituximab at days 0 and 14. It demonstrated a significant reduction in MRI erosions scores and cartilage loss in both rituximab groups compared with placebo[289].

Four trials have investigated Rituximab in patients with established RA who failed anti-TNF therapy. The REFLEX trial randomised patients, in combination with methotrexate to receive rituxumab, two doses of 1000mg or placebo and showed better responses in all ACR core set measurements at 24 weeks. Radiological outcomes were also better in the Rituximab group at 2 years, making it a pivotal phase III trial leading to drug approval [290, 291]. The other three trials were open label observational studies. 559 patients who had failed anti-TNF therapy received Rituximab two doses of 1000mg with methotrexate in the SUNRISE study, with randomisation to a second course for those who failed to achieve a DAS-28 response at week 24[292]. At week 48, those who had received Rituximab had better outcomes than

methotrexate alone. The MIRAR trial was a non-randomised study where 1124 patients were allocated to another anti-TNF therapy or Rituximab; clinical outcomes were better in those who switched therapy [293]. The RESET trial was an open label study where patients received Rituximab following failure to TNFi with an improvement in ACR responses at week 24 [294].

In terms of further observational studies, Assous et al[295] retrospectively assessed 50 patients who had failed or had a contraindication to TNF-alpha agents, demonstrating a EULAR good or moderate response of 82% following treatment with 1g of rituximab at days 0 and 15 in conjunction with methotrexate. A study where rituximab 375 mg was administered weekly for four weeks showed a clinical improvement in DAS-28 in 73% in 46 patients who were had failed DMARD and TNF-alpha therapy[296]. Keystone and colleagues demonstrated in an open-label extension study that further cycles of rituximab in 1039 patients resulted in sustained improvement in both ACR and EULAR outcomes with no added safety concerns[297]. The same group reported a reduction in progressive joint damage with rituximab treatment over five years[298].

Registry data from the CORRONA register showed in 296 patients who had failed anti-TNF therapy that administration of Rituximab resulted in favourable CDAI outcomes, but more so in patients who had only failed one-anti TNF therapy previously [299]. Additionally, a recent Cochrane meta-analysis of Rituximab therapy recommended that 2 doses of 1000mg in combination with methotrexate therapy was more efficacious than methotrexate alone in RA [300].

Authors	Study design	Arms	n	Length	Retreatment	Primary outcome
Cohen, 2006 (REFLEX)	Randomised, double-blind, placebo-controlled phase III	PBO+MTX RTX 2x1000mg +MTX	209 308	24 weeks	Y(rescue therapy)	ACR 20 response 51% vs 18% p<0.0001
Edwards, 2004	Randomised, double-blind controlled Phase IIb	PBO +MTX RTX 2x1000mg RTX 2x1000mg +CTX RTX 1000mg +MTX	40 40 41 40	48 weeks	Y	ACR 20 24 wk: 38% vs 65% (p=0.025), 76% (p=0.001) and 73% (p=0.003) respectively 48 wk: 20% vs 33% (ns) vs 49% (p=0.01) vs 65% (p<0.001), respectively
Emery, 2006 (DANCER)	Randomised, double-blind, double-dummy, placebo-co ntrolled, dose-ranging Phase II B trial	PBO+MTX RTX 1000mg+MTX RTX 500mg +MTX	149 192 124	24 weeks	N	ACR 20 28% vs 55% (p<0.001) vs 54% (p<0.001) respectively
Emery, 2010 (SERENE)	Randomised, placebo-controlled, double blind, parallel group	PBO+MTX RTX 1000mg +MTX RTX 500mg+MTX	172 170 167	48 weeks	Y	ACR 20 24 weeks: 22.3% vs 54.5% (p≤0.0001) vs 50.6% (p≤0.0001) 48 weeks: vs 55.7% and 57.6% (p≤0.0001)
Owczarczyk, 2008	Randomised, open-label trial	RTX 1000mg RTX 1000mg + MTX	20 20	24 weeks	N	DAS-28 DAS-28 4.54 vs 4.43 (ns)
Robert Roth, 2010 (MIRROR)	Randomised, double-blind phase III trial	RTX 2 x 500mg, 2x500 mg +MTX RTX 2 x 500 mg, 2x1000mg +MTX RTX 2 x 1000mg, 2x1000 mg +MTX	134 119 93	48 weeks	Y	ACR20 64% vs 64% vs 72% (ns)
Tak, 2010 (IMAGE)		PBO+MTX RTX 2x 500 mg + MTX RTX 2x1000 mg +MTX	249 249 250	104 weeks	Y	Change in mtss from baseline to week 52 Week 24: 0.70 vs 0.58 vs 0.33 (p<0.05) Sustained at week 52 0.38 vs 0.06 vs 0.03 (p<0.01)
Peterfry, 2014 (RA-SCORE)	Randomised, double-blind, placebo controlled	PBO + MTX RTX 2x500mg +MTX RTX 2x1000mg + MTX	63 62 60	24 weeks	Y	Change in RAMRIS score from baseline to week 24 0.47 vs 0.18 (p=0.003) vs 0.16 (p=0.001)

Table 1.5 Summary of controlled trials using rituximab with or without combination methotrexate
Abbreviations: PBO = placebo, MTX = methotrexate, RTX = rituximab, CTX = cyclophosphamide, mg milligrams, ACR = American college of rheumatology, mtss = Mean total sharp score, RAMRIS = RA MRI scoring system

1.6.3 Rituximab: dosing and retreatment considerations

A full understanding of mechanisms driving the amelioration of symptoms following B-cell depletion is yet to be fully elucidated. Rituximab may exert some effects by depletion of B-cells in peripheral blood and more variably in the synovium. The initial concept driving B-cell depletion as potential treatment in RA was presumed to be the elimination of autoreactive B-cells and subsequent pathogenic plasma cells. B-cell depletion appears to have wider effects, as clinical efficacy may perhaps also be achieved through a reduction in B-cell associated cytokines and a reduction in their function as antigen presenting cells [301].

In most cases, rituximab treatment results in a reduction in circulating B-cells [302], however most patients will go on to relapse, with complete remission observed in only a minority of patients. The time to relapse, however varies and can be classed as concordant, where the return of clinical symptoms correlates with B-cell return in the periphery, or discordant where relapse is delayed [303, 304]. Despite these observations, two key questions remain unanswered when considering rituximab treatment: 1) the optimal dose and 2) the point in time at which patients should be retreated.

Early trials demonstrated significant improvements in both the 2x 500 mg and the 2 x 1000 mg dosing regimens [285-287, 290]. A recent systematic review concluded that 2 x 500 mg doses of rituximab was non-inferior to 2 x 1000 mg, although there is a statistically significant difference between doses and radiographic outcomes, measured through the mean total sharp score [305]. Mariette and colleagues randomised patients who had received an improvement in disease activity six months after one cycle of standard dose rituximab to receive either 1x1000mg rituximab infusion or 2x1000mg [306]. They demonstrate non-

inferiority of the single-dose regimen with similar improvement in DAS-28 scores and time to retreatment. They noted higher but non-significant levels of circulating IgG in the group receiving the lower dose, although there was no difference in the rates of serious infections, a trend further confirmed by the aforementioned systematic review. A further report of seventy patients who had achieved a EULAR response following three cycles of a standard dose regimen found no differences in disease activity if the subsequent dose was decreased to 2 x 500 mg [307]. There has been a small open-label study of low dose 1 x 100mg [308] and three reported case studies [309-311] of 1x 50 mg and 2 x 100 mg which have resulted in B-cell depletion and disease amelioration. It should be noted, however, that all three case reports are from the same centre. To address this further there is currently a randomised, double-blind controlled trial underway to assess ultra-low dose, 1x 500 mg, 1 x 200mg compared to standard low dose 1x 1000 mg rituximab [312]. These studies suggest that in a subset of patients, low dose B-cell depletion may be enough to establish a clinical response. Based upon previous studies and the reduction in radiological damage, however, the current recommended dose remains 2 x 1000 mg at fixed intervals [313].

The second question that remains largely debated is the time of retreatment. Options include a fixed treatment regimen (eg every six months), treatment at disease relapse or individualised treatment regimens to prevent relapse. The mean time for retreatment in a prospective study was approximately 8.5 months [294], but for some individuals remission is achieved for longer [302], which suggests that perhaps retreatment intervals should be based on individual profiles in order to prevent full relapse of the disease. The risk of disease relapse and radiographic progression is offset by the increased theoretical risks of infection and low

levels of circulating immunoglobulins witnessed with repeat cycles[297, 304, 314]. A retrospective study of 177 individuals, in the absence of previous steroid or cyclophosphamide therapy, repeat cycles were not associated with an increased risk of hypogammaglobulinaemia [315] and infections were not necessarily linked levels of immunoglobulins. Serum immunoglobulin levels are therefore checked and monitored with each cycle and reviewed if levels are progressively lower, especially in high risk individuals [313].

B-cell reconstitution is characterised by newly generated immature B-cells and re-emergence of plasma cells. Naïve B-cells increase and tend to predominate the peripheral compartment with a delayed repopulation of CD27+ memory cells, which can remain at low numbers for a significant period of time [316]. A concomitant decrease in autoantibodies has also been observed following rituximab, indicating that these are likely to be produced by short-lived plasma cells which are interrupted by B-cell depletion[317]. The degree of efficacy of rituximab has been linked to the degree of depletion[318] and disease relapse is associated with the return of memory B-cells [319, 320] as determined by flow cytometry analysis – serial measurements however are not feasible in routine clinical practice.

1.6.4 Current markers of response / resistance to Rituximab

Response or resistance to rituximab therapy may be modulated by several individual and drug-related factors. Depletion depends on the total number of B-cells and perhaps the total dose of rituximab administered, which I have discussed above. The immune effector mechanisms of rituximab are linked directly to endocytosis and binding on the number and type of Fcγ receptors, which in turn can influence serum drug levels [321, 322]. Differences

in internalization between types of CD20 monoclonal antibodies have been described in vitro in cells from both SLE and RA patients [323]. Polymorphisms in the FcγR have been associated with a diminished response to rituximab [324] and in the development of side effects such as late-onset neutropenia[325]. The presence of certain complement regulatory proteins have been associated with faster B-cell repopulation[326] and may potentially vary depending on the type of monoclonal antibody used[327].

B-cell apoptosis induced by crosslinking of CD20 molecules with rituximab is dependent on intact lipid rafts within the cell membrane [328], which are disrupted by statin therapy. The link between response and statin use is yet to be clearly elucidated in RA – one small study suggested that statin use may result in diminished response[329], although others, included a pooled retrospective analysis have not demonstrated and differences in clinical response[330, 331].

Studies have shown that the presence of ACPA or RF increases the probability of achieving a favourable response to Rituximab therapy. Early data from 10 European registries which included 2019 patients [332] showed that patients who were RF, ACPA or double positive achieved better DAS-28 responses than those who did were seronegative. Further adjusted regression analysis confirmed that ACPA positivity and lower DAS-28 at baseline were significant predictors of response. Another Italian study in 110 patients showed that the number of prior anti-TNF agents, RF and ACPA positivity associated with an ACR 50 response by univariate analysis; further multivariate analysis confirmed that the number of TNF agents and RF positivity conferred a more positive response to therapy [333]. The SMART study, a Rituximab retreatment study involving 208 patients showed that high baseline ACPA

antibodies, RF, serum immunoglobulin levels, kappa and lambda light chains (all indicators of B-cell activity), were associated with a favourable response to therapy [334].

Further retrospective work on RA patients showed that high ACPA titres resulted in a good response to Rituximab therapy with a significant association with a decrease in DAS-28, EULAR good response criteria and a EULAR remission criteria [335]. A meta-analysis conducted by Isaacs et al, which included 2177 patients from four placebo-controlled phase II or III trials showed that seropositive patients respond more favourably than seronegative patients to rituximab[336], although titres did not affect the change in DAS. A meta-analysis which included 14 studies and 2103 patients treated with Rituximab showed more mixed results [337].

Several other studies have investigated other clinical parameters and response. Couderc et al explored retrospective registry data in 1709 patients and found that although men appeared to display higher remission rates than women gender played no role in EULAR response.[338] One retrospective study has explored whether body mass index can predict response to Rituximab therapy. Data analysed from 114 patients failed to show that BMI affected response to B-cell depleting therapy, contrary to results from anti-TNF data [339]. Other studies have looked at less commonly measured serological markers as predictors of response to Rituximab, including alternative autoantibodies and circulating protein levels. Lindenberg et al demonstrate that a decline in antibodies against mutated citrullinated vimentin was predictive for response to Rituximab in 50 patients [340].

High sensitivity flow analysis has shown that residual peripheral blood B cells and the return of memory B cells are associated with a poorer response and relapse [319, 320]. Early studies showed that B cell depletion was variable [319, 320] at six months, and the presence of B cells resulted in less favourable outcomes than in those in whom B cells were undetectable. RA patients have increased circulating of activated memory B cell populations prior to treatment, which seem to persist even after therapy in patients who fail to respond adequately[341]. Additionally, RA patients with a lower baseline percentage of circulating CD27+ memory cells [342] seem to have an enriched response to Rituximab therapy, as do those with a lower mRNA plasmablast signature[343]. This may be due, in part, to variable depletion in the synovium or lymph nodes.

As alluded to previously, B-cell depletion in different compartments is variable [321]. A question that remains to be answered is whether migration of B cells from peripheral blood to sites of inflammation affects clinical response to rituximab therapy and indeed whether this is measurable. Chemokines play a crucial role in the development and trafficking of B-cells, in particular CXCL12, CXCL13 and CCL19. Sellam and colleagues reported a subgroup of patients with a lower baseline level of memory B-cells and plasmablasts that showed a good clinical response to rituximab [334, 342]. The group went on to explore whether levels of circulating chemokines could be used as predictors of response to B-cell depletion therapy [344]. Patients with RA had significantly higher level of B-cell chemokines and that these were inversely correlated with CD27+IgD- switched memory B-cells. CXCL12 and CXCL13 levels were not associated with clinical response to Rituximab at baseline, whereas they observed an association between the log-transformed CCL19 level with a EULAR response. This study however did not include matched synovial tissue or fluid, leading the authors to speculate

that the higher levels of circulating chemokines in the peripheral blood lead to an increased proportion of activated B cells being recruited to the site of inflammation.

Lending support to this hypothesis is the presence of activated B-cells and ectopic lymphoid structures in the synovium. Mononuclear cell aggregates at the interface of the synovium and bone marrow have been found to contain mature memory-switched B-cells and numerous plasma cells [345]. Tertiary lymphoid organs, or ectopic lymphoid structures are also found at sites of inflammation in both infection and autoimmune diseases and have been well characterised in RA synovium. Previous ex vivo data has supported a role for B-cell aggregates in disease pathogenesis via functioning as survival niches for autoreactive B cells [346]. Subsequent in vivo data supported this concept demonstrating that response to rituximab is associated not simply with degree of synovial B cell infiltration but suggesting that modulation of functionality is important. Certainly synovial plasma cell depletion [347] and modulation of immunoglobulin synthesis [348] have been associated with clinical response whilst persistent circulating pre plasma cell precursors (presumably originating from solid tissue such as synovium) associated with treatment resistance [320, 349, 350]. Further analysis from the VC gene repertoire within the synovium also shows accumulate plasma cell clones suggesting that these are produced within locally generated niches [351].

1.6.5 Synovial depletion of B-cells

Several small, open label studies have been undertaken to ascertain the effects of Rituximab on the synovium. A study of 17 patients with refractory RA treated with Rituximab showed that at 4 weeks, there was significant depletion of synovial B-cells. 3 biopsies demonstrated no B-cells prior to treatment. 3 patients showed complete B-cell depletion, whereas this was

incomplete in 11. The early timepoint of the biopsy precluded any correlation within clinical response and specifically the relationship of B-cell presence to clinical response was not assessed. [352]

The same group extended this study to 24 patients (including those in the original study) [347], with biopsies taken at baseline, 4 weeks and 16 weeks post treatment. They reported that baseline B-cell levels did not correlate with clinical response, although there was an association noted with progressive plasma cell depletion at the 6-month primary endpoint. Additionally, there was a decrease in T-cell numbers and lymphocyte aggregates that did not correlate with clinical response.

In the ARISE study, 13 patients underwent biopsies at baseline and 8 weeks post treatment (without peri-infusional steroids). This group demonstrated a non-significant trend associated with B-cell depletion and clinical response and a significant reduction in immunoglobulin synthesis in clinical responders [348].

A further study from Leiden with 25 patients who underwent biopsies at baseline and 12 weeks post-treatment (with perinfusional corticosteroids) found no association with clinical response and the level of B-cell depletion (CD79a+ cells). This group followed this with a study of 20 patients with biopsies at baseline, 12 weeks and 21 weeks post treatment. They demonstrated a gene expression signature within the synovium that enriched for clinical response. Of particular relevance, poor responders showed increased expression of interferon alpha and remodelling genes. They also found that a low disease activity state correlated with low synovial levels of plasma cells [353, 354].

All of these studies demonstrate that treatment with Rituximab, at least in the early phase,

does result in depletion of B-cells in the joints of RA patients. To determine correlation with clinical response, however, proves more problematic. This can be attributed to the small numbers of patients included within these studies, the variations in biopsy sampling time-points and the differences in the use of peri-infusional medications which makes it difficult to extrapolate findings to the clinical setting. No studies have directly assessed the question of the number of baseline B-cells and overall clinical response. Finally, B-cell depletion within the synovium may not be the primary determinant of overall clinical response, although it may be associated with resistant disease or relapses within individual joints.

1.7 Synovial histology: a new biomarker of response?

1.7.1 Methods of synovial tissue acquisition

Recent advances in the acquisition of synovial tissue have enhanced our understanding of disease pathogenesis and have allowed the identification of biomarkers of prognosis and response[355, 356]. Arthroscopy has been utilised heavily in clinical trials and has to date been the gold standard for synovial tissue sampling [357] but is limited by the specialist training that is required, high cost and complications [358]. Arthroscopy is limited to large joints, which preclude its use in the earliest phases of disease where often small joints are more likely to be affected. Minimally invasive techniques include the blind needle biopsy, portal and forceps technique and US guided procedures, which are both cheap and require less technical expertise to perform. Furthermore, they increase access to both large and small joints[359]. Following the insertion of a guidewire under US guidance, the portal and forceps technique utilises a Hartmann's forceps for tissue extraction [360]. A minimally invasive technique using a 14- or 16-gauge QuickCore (Cook Medical) needle has been described as a

safe and tolerable procedure which results in sufficient tissue for both histopathological analysis and mRNA extraction [361, 362]. Of importance, the technique does not alter subsequent pain scores or power doppler scores [363] and can therefore be reliably used in serial biopsies to monitor disease progression before and after treatment. Figure illustrates the different methods of synovial tissue acquisition.

A recent multicentre study [364] has validated the use of US guided techniques using 159 procedures from five different centres. 25 arthroscopic, 35 US guided portal and forceps, 11 US needle biopsies and blind needle biopsies were performed on large joints; there were no significant differences in the amount of graded tissue samples or total gradeable tissue area between the different techniques, with the exception of the blind needle technique. 41 procedures were carried out on small joints (11 US guided portal and forceps, 20 US-guided needle biopsies and 10 blind needle biopsies). 34 sequential biopsies were also included for evaluation. Again, no significant differences were observed between the groups. This supports the notion that US guided synovial biopsies can be incorporated into clinical trials to provide reliable synovial tissue for analysis and crucially, for sequential biopsies following therapeutic intervention. The authors do note limitations, however, as only limited tissue is available for RNA extraction and therefore dependent on study design, arthroscopy may be preferential for several experiments which require tissue for molecular analysis.

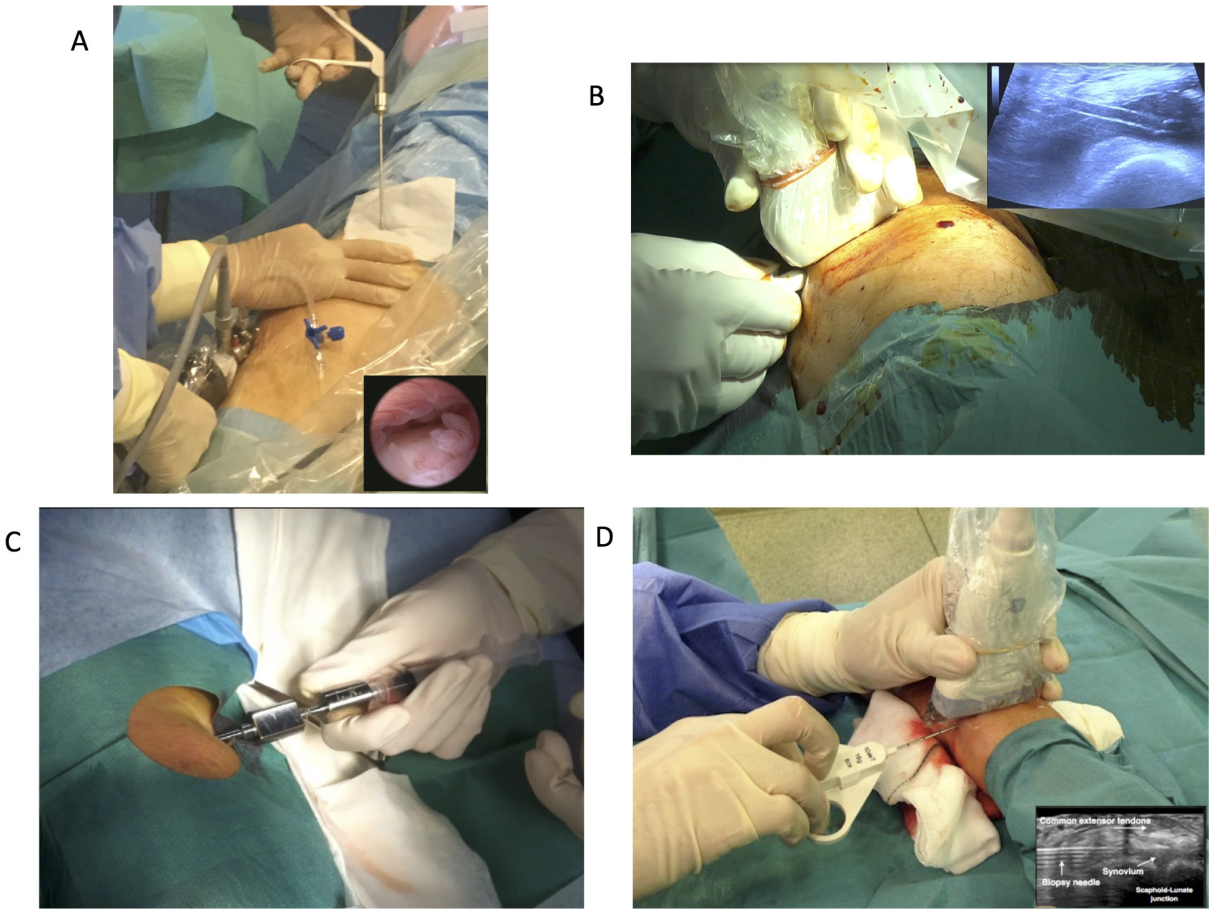


***Figure 1.3 Methods of synovial tissue acquisition**, from [364] with permission from John Wiley*

A Arthroscopic sampling of the knee joint, inset depicts direct vision of synovial tissue, B Ultrasound guided portal and forceps technique, inset depicts the needle in the suprapatellar pouch, C Blind needle biopsy of the knee joint D US-guided synovial biopsy of the wrist joint, inset depicts the needle within the joint space

1.7.2 The Normal Synovium

The synovium is the primary site of insult in RA. It is an ectoderm derived soft tissue lining which covers the surfaces of diarthrodial joints, tendons and bursae. It consists of an intima, characterised by the presence of macrophage-like and fibroblast-like synoviocytes and a subintimal layer composed of fibroblasts, collagen-containing extracellular matrix and a supply of nerve fibres, blood and lymphatic vessels. The presence of hyaluronic acid between intimal cells ensures fluidity of movement between tissues. [365] Normal synovium is only 1-

2 cells (20-40mm) thick and is associated with a relatively acellular subintimal layer with few resident immune cells and vascular structures. Thickness varies across different tissues, bursae and tendons, mirrored by varying amounts of synovial fluid.

Three different patterns of synovial tissue have been described: fibrous, areolar and adipose, based upon the content of the subintimal layer. [365] CD163 and CD68 macrophages, derived from circulating monocytes are found in the normal intima, expressing a FcIIIγ receptor. These cells are also known as type A synoviocytes. CD55 expressing fibroblasts, or type B synoviocytes, are also found in the intima and subintima, with interspersing dendritic cells that can act as antigen presenting cells. These cells are usually derived locally. The latter cell population also express adhesion molecules such as vascular cell adhesion molecular-1 (VCAM-1), intracellular adhesion molecules (ICAM)-1, CD44 and integrins. [365] Small numbers of CD3+ cells and occasional B lymphocytes may also be found within the joint [366]. The normal cytokine milieu in the joint favours suppression of inflammation and maintains joint homeostasis. Low levels of IL-1, IL-5 and TNFα and receptor activator of NFKB/rank ligand are detectable within the joint; however appear to be countered by higher levels of inhibitory antagonists and osteoprotegerin [365, 366]. The findings described are significantly altered in the presence of disease.

1.7.3 Changes in the diseased synovium

So far, I have discussed risk factors, the break of tolerance and the critical roles of antigen processing, T and B cell differentiation in the disease. The hallmark of RA, however, is synovial inflammation. Innate immune cells and those from the adaptive immune system infiltrate the joint to create a pro-inflammatory environment, propagated by cross talk between cells and the influx of cytokines, chemokines and newly differentiated immune cells. The result is hyperplasia of the synovium and conglomeration of immune and inflammatory cells, termed pannus formation. The synovial lining thickens following macrophage and synovial fibroblast proliferation; predominantly infiltrating Type A fibroblasts. Bone and cartilage destruction are mediated by the release of matrix metalloproteinases, serine proteases, aggrencases released by macrophages, further enhanced by osteoclast activation and subsequent bone and cartilage loss. The synovium becomes hyperplastic, resulting in both pain and swelling.

Influx of inflammatory cells is accompanied be neoangiogenesis within the sublining layer to allow transfer of oxygen and nutrients. Numerous growth factors, chemokines and cytokines regulate vessel formation. The presence of vascular growth endothelial factor (VGEF), platelet derived growth factor and hepatocyte growth factor has been demonstrated in synovial tissue and in fluid. This is countered by inhibitors of angiogenesis such as endostatin and thrombospondin 1 and 2, indicating that there may be dysregulated balance between neovascular promotion and angiostasis. [237, 367]

1.7.4 Patterns of synovial infiltration

Three distinct patterns of immune cell infiltrate have been described in the synovium. Of most critical relevance are highly organized tertiary lymphoid structure (30-40%), the second

pattern is a diffuse pattern of infiltrating cells and the third, most recent pattern that has been described is a pauci-immune synovitis [368, 369]. These patterns are not mutually exclusive and it has not yet been ascertained whether they are dynamic at different stages of the disease or distinct subtypes of RA.

What is of interest, however, is that these synovial pathotypes have been shown to segregate with specific transcriptomic signatures. Three recent patterns have been described: Lymphoid, myeloid and fibroblast associated patters as determined by specific gene sets and appear to correlate with histomorphological subtypes [356]. Gene expression tends to associate with specific patterns or families; for example, B-cell associated genes segregate predominantly with the lymphoid phenotype. Furthermore, there are some signals indicating that they may be predictive of response to treatment[356].

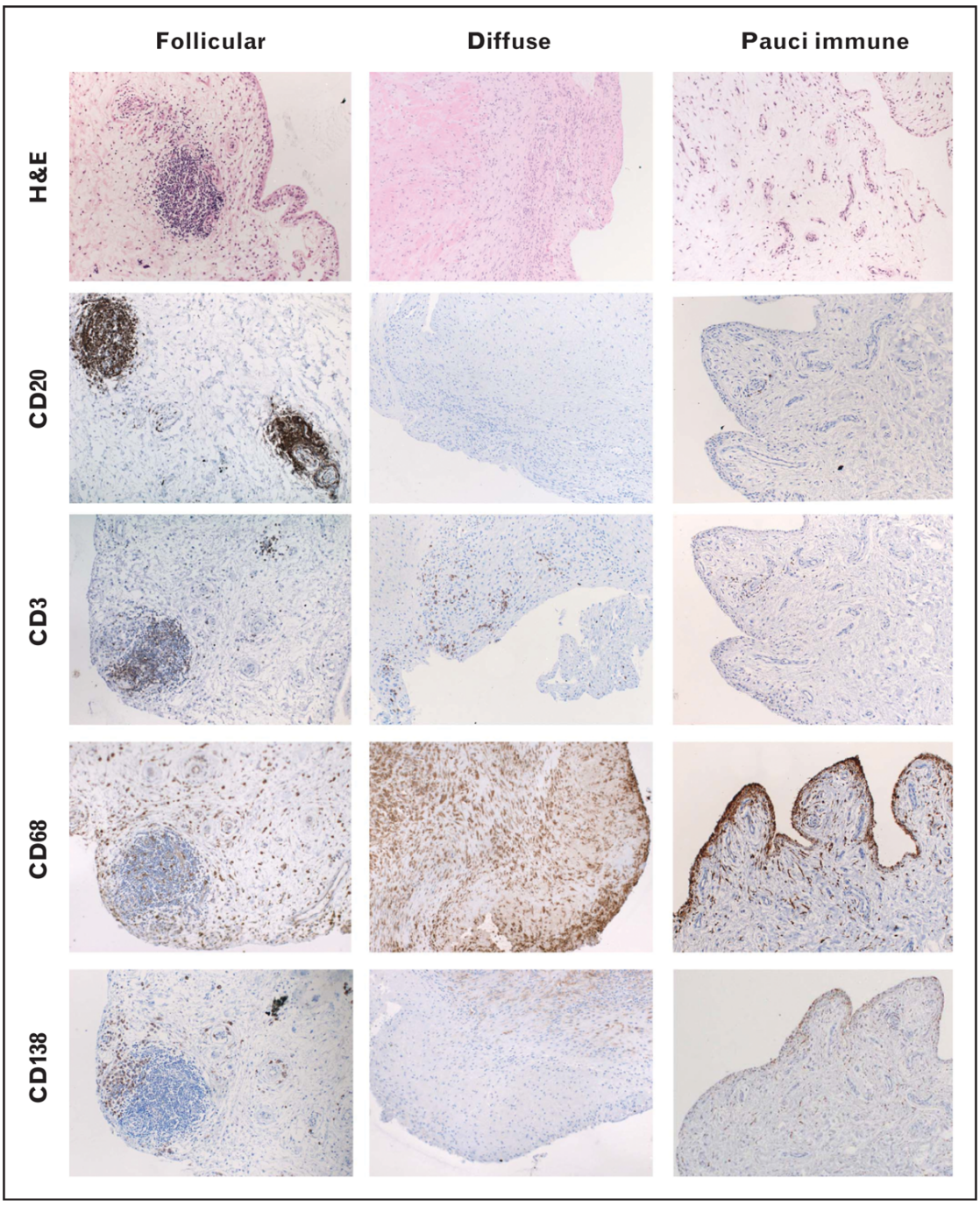


Figure 1.4 Synovial heterogeneity in RA

Examples of follicular, diffuse and pauci-immune synovitis. RA synovial tissues stained by haemotoxylin and eosin (H&E), and by immunohistochemistry H&E for B cells (CD20), T cells (CD3) macrophages (CD68 and plasma cells (CD138). From [369] with permission from Wiley materials.

1.7.5 Ectopic Lymphoid Structures (ELS): pathogenic players or bystanders?

The lymphoid pattern of synovitis is characterised by the presence of B and T cells which are found in up to 40% of individuals with RA. ELS form at sites of chronic inflammation and have been described in several diseases, including RA and Sjogrens syndrome in synovium and salivary glands, respectively [370]. Within the joint, they are characterised by aggregates of T and B cells, the presence of high endothelial venules and may or may not express CD21 follicular dendritic cells and the expression of AID, when they are coined tertiary lymphoid organs.

Within the synovium, B-cells have a crucial role in the regulation of lymphoid tissue architecture within lymphoid organs and at peripheral sites. Ectopic lymphoid structures (ELS) are present in approximately 5-25% of patients with RA within the synovium. Key cytokines and chemokines such as IL-7, IL-21, LTα, LTβ, TNFα, RANKL, CXCL13, CCL19 and CCL21 are known to be instrumental in their formation. It has been demonstrated that synovial ELS are functional, and support the self-sustaining differentiation/survival of B cells and plasmablasts/plasmacells (PB/PC) that continue to produce class switched IgG ACPA when ELS+ve synovial tissues are transplanted into Severe Combine Immunodeficent (SCID) mice, in the absence of new cells coming into the grafts [346]. It is therefore possible to postulate that one mechanism for resistance to Rituximab may be due to the "escaping" of auto-reactive B cell PB/PC clones surviving in "protected" niches within the joint.

The pathogenic role of ELS in disease has been debated. However, there are now several lines of argument that exist to suggest functionality for lymphoid structures within the synovium. It has been shown that ELS within the joint express AID and are capable of ongoing SHM and

CSR, suggesting that the synovium could form a niche where there is ongoing activation and differentiation of autoreactive B-cells [346]. Furthermore, circular transcripts resulting from CSR continued to be expressed when engrafted into SCID mice, indicating that ongoing antibody production is possible even in the absence of new immune stimuli [346]. This finding is also supported by the recognition that analysis of the V-gene repertoire displays expanded clonal subsets with highly mutated V regions, suggestive a local antibody mediated process.

1.7.6 The synovial biopsy as a potential biomarker

Whether distinct synovial pathotypes can predict outcome to treatments remains unknown. Interpretation of previous synovial biopsy-based studies is difficult, as studies have varied in methods of tissue acquisition and the timing of biopsies in relation to the disease process. Furthermore, there is no agreed method of grading lymphocytic infiltrates and infiltrating immune cells. Finally, there are few published gene expression studies specifically addressing modulation of both imaging and the synovium following therapy.

1.8 Summary and hypotheses

RA is a heterogenous disease, characterised by distinct synovial and molecular phenotypes. Despite an increase in imaging modalities, new technologies and a growing number of therapies, tailoring treatment at an individual level remains a desirable goal. Treatment is still based on 'trial and error', resulting in progressive damage until the right therapeutic target is met. Although a successful treatment for some, a sizeable proportion of patients fail to respond to Rituximab therapy.

The aim of this thesis is to examine the hypothesis that distinct histological and molecular pathoypes, i.e. B-cell or lymphoid rich within the synovium can segregate responders and non-responders following B-cell depletion.

The specific aims are to identify whether:

1. A specific B-cell rich synovial pathotype can predict response to Rituximab therapy and whether response is associated with changes in immune cell infiltration following treatment
2. The molecular signature of the synovium can predict response to Rituximab therapy and to explore the immunomodulatory effects of Rituximab on the synovium
3. Treatment with Rituximab correlates to changes in synovial thickening and power doppler graded using ultrasound and whether this associates with histological and molecular changes
4. The synovium can be used as a predictive tool to predict response to Rituximab therapy by combining clinical, transcriptomic and imaging data

Methods

Methods

General protocols are described within this chapter, more detailed descriptions will be provided in each experimental chapter.

2.1 Study Population

25 patients with active RA were enrolled to an open-label observational study which formed a pilot cohort for a **R**andomised, open labelled study in anti-TNFα inadequate responders to investigate the mechanisms for **R**esponse-Resistance to **R**ituximab versus Tocilizumab in **R**A (R4RA) trial which is led by Professor Pitzalis at Barts Health National Health Service Trust. One patient withdrew from the study. All patients had to be over 18 and meet the 1987 American College of Rheumatology criteria for RA. Patients were included if they were eligible to receive Rituximab therapy according to the National Institute of Clinical Excellence (NICE) Guidelines. All patients had failed traditional DMARD therapy. To be eligible for Rituximab therapy patients had to have active disease DAS-28$\geq$ 5.1, been deemed a non-responder or have a contraindication to anti-TNF therapy. Inclusion and exclusion criteria are fully described in table 2.1.

As this was an observational study, patients had no restrictions on the use of methotrexate or other DMARDs. Oral prednisolone was permitted at a dose of $\leq$10 mg per day provided this was stable for four weeks prior to any biopsy visits. All patients underwent an ultrasound guided synovial biopsy of a clinically inflamed joint at baseline. Optional repeat biopsies were performed at 16 weeks and at 52 weeks. Patients were given 1g of paracetamol, 10 mg of chlorphenamine intravenously and intravenous methylprednisolone 100mg as pre-medication. Rituximab 1000mg was administered at day 0 and day 15. The cycle was

repeated at physician's discretion at flare. The study received local ethical approval and all patients gave informed consent. (REC 10/H1109/23).

A flow-chart of patients that were available for each section of the analysis is described in figure 2.1.

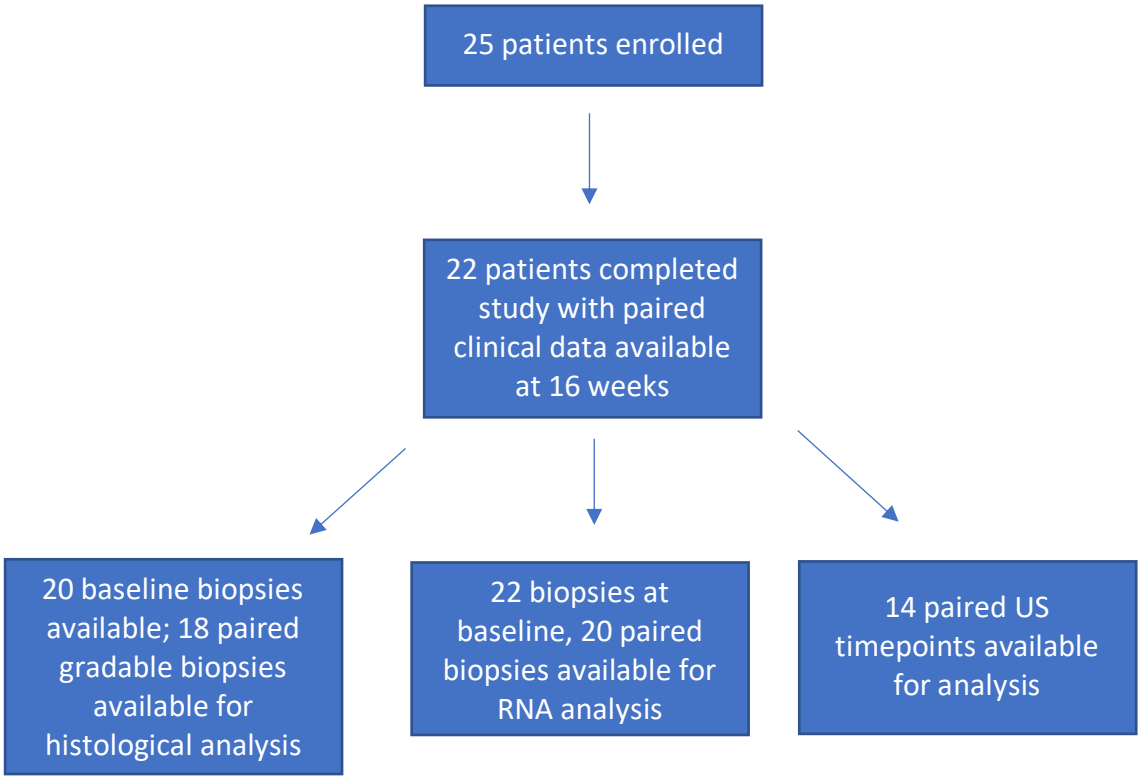


Figure 2.1 Flow diagram of patients enrolled in the study

25 patients were enrolled, 1 patient withdrew before the start of the study, 2 patients withdrew prior to the end of the study. Biopsies were deemed gradable if there was a presence of a clear lining layer in the tissue, 18 paired samples were available for comparison. 20 samples had sufficient RNA available for Fluidigm gene analysis. Only 14 patients had paired images available for analysis of ultrasound data, this was due to a change in ultrasound machines and storage during the study and due to patients who attended for the biopsy, but did not stay for US assessment.

Table 2.1 Inclusion and Exclusion criteria for the R4RA pilot study

Inclusion Criteria	Exclusion criteria
Patients with 1987 ACR classification for a diagnosis of RA	Women who are pregnant or breast-feeding
Patients who have failed or have a contra-indication to anti TNF therapy	History of current inflammatory joint disease or other autoimmune disease other than RA
Eligible to receive Rituximab according to NICE guidelines	Treatment with any investigational agent ≤ 4 weeks prior to baseline
Over 18 years of age	Intrarticular or parenteral corticosteroids ≤ 10mg prior to baseline
Capable of giving informed consent and comply with study visits and procedures	Active infection
	Septic arthritis within a native joint in the last 12 months
	Sepsis of a prosthetic joint within 12 months or indefinitely if joint in situ
	Known HIV of Hepatitis B/C infection
	Latent TB
	Malignancy (other than basal cell carcinoma in the last ten years)
	New York Heart Association grade 3 or 4
	Demyelinating disease
	Latex allergy or allergy to rituximab
	Presence of a transplanted organ
	Known recent substance abuse
	Poor tolerability of venepuncture of lack of adequate venous access for blood sampling
	Patients unable to tolerate synovial biopsy or on oral anticoagulants
	Patients recruited to other clinical trials
	Other severe acute or chronic medical or psychiatric condition which would impart excess risk (investigator's judgement)

Abbreviations: ACR = American College of Rheumatology, RA = rheumatoid arthritis, NICE = National Institute of Clinical Excellence

2.2 Clinical Assessments

Patients were seen at baseline and then followed-up monthly for twelve months. Clinical assessments included the number of tender joints, swollen joints, a patient visual analogue scale assessment, a patient's pain score and a physician's global activity of disease assessment score. Health assessment questionnaires (HAQ) were performed at baseline, 3,6,9 and 12 months. The primary end point of the study was improvement in DAS-28(ESR) scores at 16 weeks.

2.3 Laboratory assessments

At baseline, blood was sampled for erythrocyte sedimentation rate, c-reactive protein, Ig-M Rheumatoid Factor determined by nephelometry and ACPA determined by a second-generation enzyme-linked immunoassay. Immunoglobulin levels of IgA, IgM, and IgG and B and T- cell subsets were measured prior to Rituximab infusion. Full blood count, urea and electrolytes, liver function tests, ESR and CRP were performed monthly. Additionally, blood was sampled and stored for peripheral blood mononuclear cells isolation at weeks 0, 12, 16 and 54.

2.4 Radiographic assessments

Plain x-rays of hands and feet were performed at baseline to assess the presence or absence of erosions.

2.5 Ultrasound Assessments

At baseline and at 16 weeks standard longitudinal images of the 1st to 5th metacarpophalangeal joints and radial, midline and ulnar views of both wrists were acquired

by myself and two other investigators using a GE logic 9 ultrasound machine with a 2-dimensional M12L transducer (14MHz) at room temperature (20-22 degrees). Images were assessed by myself and a blinded assessor (Ilias Lazarou) on a 0-3 scale for synovial thickening and power Doppler activity in accordance with EULAR-OMERACT synovitis scores [371], with excellent interobserver correlation coefficients. Any differences were resolved by mutual agreement and by an independent assessor, Dr. Stephen Kelly. Minimal probe pressure was applied in order to maintain at least 1 mm gel between the probe and skin. A region of interest was selected using the power doppler colour box facility, with doppler settings adjusted to the lowest possible pulse repetition frequency. Detailed description of scoring is seen in Figure 2.2. Briefly, for each patient baseline and 16 week scores were calculated by a 0-3 scoring system to quantify synovial thickening (ST) and power doppler (PD) at the single joint level. Addition of the total MCP score and mean score at the wrist, giving a total of 12 joints with a minimum score of 0 and a maximum score of 36. To summarise, settings were as follows: Grey scale frequency 14 Mhz, Power doppler frequency 7.5 MHz, gain 41, PRF 1.4 kHz and wall filter 127 Hz. Images were stored in a DICOM format and transferred to ImagePro, an imaging processing program.

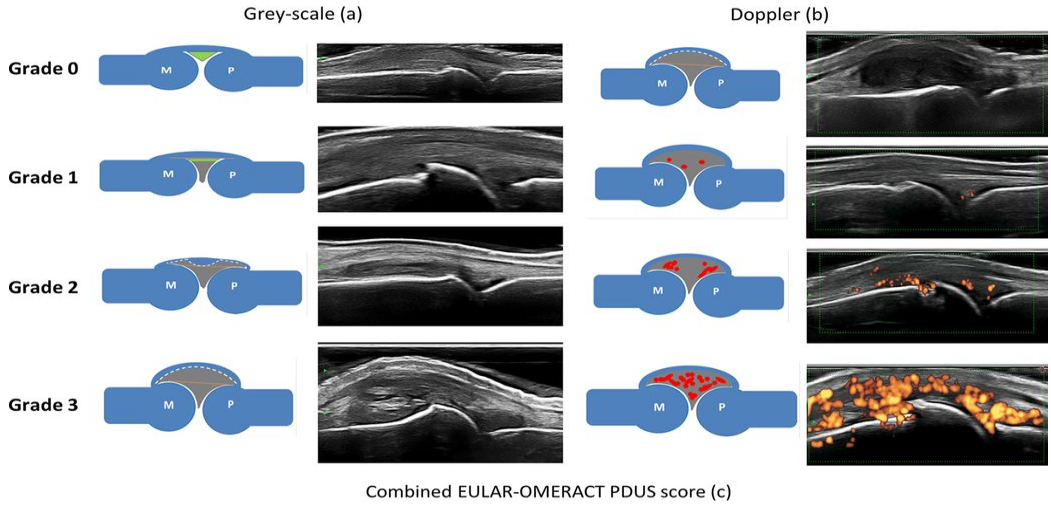


Figure 2.2 Semi-quantitative US scoring methods using the recommended EULAR-OMERACT scoring methods *from [371], with permission from BMJ publishing group*

(a) depicts scores of grey scale synovitis; G0 = no hypoechoic synovial thickening, G1 = minimal hypoechoic synovial thickening, G2 = Hypoechoic synovial thickening extending beyond the joint line but with upper surface convex or flat G3 = Hypoechoic synovial thickening extending beyond the joint line with upper surface convex, bulging over the periarticular bones

(b) depicts scores of PDUS PD 0 = no flow in the synovium, PDUS 1 = up to 3 single spots or 2 confluent spots, or 1 confluent and 2 single spots, PDUS 2 = Signals in less than 50% of the synovial area, PDUS 3 = signals in >50% of the synovial area

2.6 Ultrasound guided synovial biopsy

This procedure was performed as detailed [361] by trained operators: myself, Dr. Nora Ng, Dr. Frances Humby and Dr. Stephen Kelly. Briefly, using a GE Logic 9 ultrasound machine with 2-dimensional M12L transducer , 1-3 mls of of local anaesthetic are injected into the soft tissues until the joint capsule under direct ultrasound guidance. A further 2-5 ml of local anesthetic may be infiltrated into the joint, up to 15 mls may be used for large joints. A Quick-Core® Biopsy Needle (Cook medical, Limerick, Ireland) is then placed inside the joint under direct vision. A minimum of 6 biopsy samples were retrieved and fixed in 4% formaldehyde.

A further 6 samples were placed in RNA-Later (Ambion) RNA stabilisation solution for subsequent RNA extraction [361], stored at -80°Celsius until ready for use.

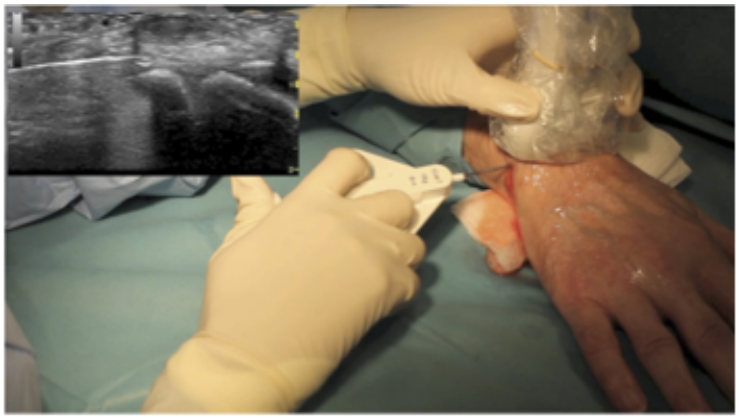


Figure 2.3 Ultrasound guided biopsy of the wrist joint

Inset shows the needle within the joint space above the scaphoid-lunate junction.

Picture courtesy of Dr. Stephen Kelly.

2.7 Sample fixation and staining

The following methods are currently utilised within the experimental medicine and rheumatology laboratory and have been previously optimized for similar work. They make up part of the standard operating procedures (SOP) within the laboratory. This part of the work was undertaken by Dr. Vidalba Rocher, Dr. Sudeh Riahi and Dr. Alessandra Nerviani.

Following synovial biopsy, fresh tissue was fixed in 4% formaldehyde and kept at room temperature for 24 hours. This tissue was then placed into embedding cassettes and the following protocol was used for processing: 70% ethanol (two changes, 1 hour each), 80% ethanol (two changes, 1 hour each),95% ethanol (two changes, 1 hour each), 100% ethanol (two changes, 1 hour each), Xylene, (three changes, 1 hour each). The tissue samples were then embedded in paraffin wax and left on a cold plate for approximately 20 minutes until

completely set. Once embedded, samples were stored at room temperature in boxes until use.

2.8 Sample cutting and sectioning

Tissue samples were placed on ice to cool for two hours before being cut to 3 μm thick sections on a Leica microtome (Knowlhill, UK). The sections were floated on a warm water bath at 40°C prior to being picked up on sequentially numbered Superfrost plus slides. Excess water was permitted to drain off before being placed on a hot plate at 60°C for thirty minutes. These are then removed and stored at room temperature.

Prior to any staining, tissue must be deparaffinised and rehydrated, allowing the tissue to become permeable again. Slides were first immersed in xylene (2 changes, 10 minutes each) followed by 100% ethanol (2 changes, 5 minutes each). They were then rinsed with distilled water and placed in Tris buffer saline until used.

2.9 Haemotoxylin and Eosin Staining

Haemotoxylin and eosing (H&E) staining allows both the differentiation and visualization of nuclei and the cytoplasm of cells under light microscopy. Haematoxylin stains chromatin found within the nuclei of cells a purple, whereas eosin stains cytoplasm and connective tissue pink. Following rehydration, slides were immersed in Mayers haematoxylin for 4 minutes, rinsed and placed in 1% alcohol for a few seconds to remove any excess haematoxylin. Slides were then washed with distilled water and placed in eosin for 5 minutes. This was followed by washing in tap water, dehydrated with absolute ethanol (2 changes, 3

minutes each), followed by xylene (2 changes, 3 minutes each). Any excess solvent was blotted. The slides were then fixed using DPX mounting medium. (Sigma, UK).

2.10 Immunohistochemistry

This technique relies on the binding of an unlabelled primary antibody to a target antigen in the tissue. A further labelled secondary antibody reacts with the primary antibody and allows localization of the antigen of interest. The secondary antibody can be 1) either directly linked to an enzyme or 2) conjugated to several biotinylated secondary antibodies that can recruit enzyme linked avidin complexes. The latter requires a chromogen which results in light emission on reacting with the enzyme, resulting in colour precipitation at the antibody antigen binding site. Sequential sections underwent immunohistochemical staining and semi-quantitative scoring (SQ) (0-4) for CD3+ T cells (F.7.328 Mouse, DAKO), CD68+ lining and sublining (sl) layer macrophages (KP1 mouse, DAKO), CD20 (L-26 mouse, DAKO) CD138+ plasma cells (M115 mouse, DAKO) and CD21+ follicular dendritic cells (1F8 mouse, DAKO) using our laboratory protocols.

2.11 Scoring of synovial tissue

Semi-quantitative (SQ) scores from 0-4 were performed using a standardised atlas by two observers (BH and VR) blinded to patient identification and any discrepancies in scores resolved by mutual agreement. I also scored each sample independently. H&E samples were first scored according to the presence and/or size of lymphocytic aggregates as described by Manzo and colleagues [240]. Grade 1 (G1) display a radial cell number between 2 and 5, grade 2 (G2) between 6 and 10 and grade 3 (G3) were defined as greater than 10 cells. Germinal centres were identified on IHC staining, characterised by the presence or absence of CD21+

FDC networks. Sections stained for immune cell markers then underwent SQ scoring (0-4) for CD3, CD20, CD68L, CD68 SL and CD138. A score of 0 represents minimal cellular expression and 4 high expression. The total synovitis score was determined by the method described by Krenn [372]. Briefly, three features – the thickening of the lining cell layer, cellular density and cellular infiltration of leucocytes were graded on a 0-3 scale, from absent to strong. Summation of the three scores then provides a synovitis score from 0-9.

Samples were further classified as 'lymphoid' based on large numbers of G2/G3 aggregates, 'myeloid' based on the absence of aggregates but the abundance of macrophages and 'pauci-immune' based upon the absence of aggregates and immune cell infiltrate.

Samples were also further stratified into 'B-cell poor' (CD20 0-1) or 'B-cell rich' (CD20 2-4) following a new algorithm which was upon previous scoring methods [373]; detailed description of this will follow in the next chapter.

2.12 RNA extraction

This was done in accordance with our laboratory SOP by Dr. Vidalba Rocher. Briefly, tissue samples were homogenized in TRIZOL reagent using a power homogenizer after shearing with a 26 guage needle. Samples were centrifuged according to instruction at 20,000 rpm for 10 minutes at 4°C. The supernatant contained the RNA. Chloroform was added and after shaking for 30 seconds, incubated on ice for 2 minutes and recentrifuged. The sample further separated into a phenol-chloroform phase, interphase and an aqueous RNA containing phase. This was removed and added to isopropyl alcohol, incubated, recentrifuged for 40 minutes at 4°C. RNA precipitation was followed by a 70% ethanol wash which was then removed and

the RNA pellet left to dry. This was then resuspended in Baxter water, quantified and stored at -80°C.

All RNA samples were quantified using spectrophotometric analysis performed by a Nanodrop-1000 v. 3.2.I Spectrophotometer (Thermo Scientific). RNA integrity was determined by electrophoresis on tissue samples using the Agilent 2100 Bioanalyzer. (Agilent Technologies, California, USA).

2.13 High throughput quantitative real-time polymerase chain reaction (PCR)

High-throughput quantitative real-time PCR for 190 genes, pre-selected according to known role in RA/B cell biology (Appendix 1), was performed using the Fluidigm© platform (Fluidigm Corporation, South San Francisco, California, USA) as per manufacturer's protocols, described further in Chapter 4. PCR was carried out on the samples by the MedImmune laboratory.

2.1 Statistical Analyses

Statistical analysis is further described in each chapter in detail. Ordinal variables are described as median (interquartile range), continuous variables are expressed as means ± standard deviation. Categorical variables are expressed as whole numbers and percentages.

Analysis of clinical and histological data was performed using the Wilcoxon signed rank test, Spearman rank correlation coefficient, Fishers' exact test and Mann-Whitney U-test as appropriate. Analysis of gene expression data was performed using R programming language and is described in more detail in chapter 5.

Unless stated, the cut-off for significance was $P<0.05$ for all tests.

Chapter 3: B-cell depletion: clinical, biochemical and synovial predictors of response

3.1 Introduction

Despite a growing number of therapies available to treat RA, predicting response to treatment remains a clinical challenge. Rituximab has been shown to be an effective and safe therapy for patients who have active RA and have not responded to or have contraindications to anti-TNF therapy, yet up to 40% of patients remain resistant to therapy [286]. Several studies have examined clinical predictors of response; however, data remains limited and clear clinical parameters at baseline, other than RF and ACPA positivity, are yet to be elucidated. A key question that arises, however, is whether the presence of B-cells in the synovium in the absence of organized structures is of pathogenic relevance to the underlying disease process, and what score of infiltration suggests a 'B-cell rich' pathotype.

As alluded to in the introductory chapter, B-cells play a critical role in the pathogenesis of RA through the production of autoantibodies, as antigen presenting cells and through the production of proinflammatory cytokines and chemokines which drive inflammation[242]. B-cell infiltration in the synovium is heterogenous and can range from a complete absence of cells to tightly arranged aggregates which are present in 40% of patients with RA[369, 374]. Furthermore, active germinal centres with CD21+ FDC networks can be seen in up to 25% of synovial biopsy samples [276]. ELS produce B-cell specific chemokines such as CXCL13, CXCL12 and CCL19 which are critical in promoting migration of B and T cells within the synovium and for continued immunological responses [240].

Despite observations that rituximab, an anti-CD20 monoclonal antibody effectively depletes CD20+ B-cells in the peripheral blood majority of patients, clinical outcomes remain variable. The key questions here are whether a 'B-cell rich pathoype' can predict response to rituximab

and whether response correlates with the number of pre-treatment B-cells and the presence of ectopic lymphoid structures or with levels of depletion following therapy. Studies exploring the role of B-cells in the synovium in response to therapy have thus far suggest that synovial depletion of B-cells is variable and may or may not correlate with clinical response.

An early synovial biopsy-based study of 17 patients demonstrated a significant change in B-cell depletion 4 weeks after the administration of rituximab. Complete depletion was seen only in 3/13 patients who had pre-treatment synovial B-cells but no definite associations with response could be drawn due to the early end-point of this study [352]. In a longer study with biopsies taken at 16 weeks the authors reported that baseline B-cell numbers did not correlate with response, although there was an association with a reduction of plasma cells [347]. Kavanaugh and colleagues demonstrated a trend in synovial B-cell depletion in 13 patients with biopsies undertaken at baseline and 8 weeks after treatment [348], however this was not replicated in a further study of 25 patients where there was no difference in responses in patients who had complete versus incomplete synovial CD20 B-cell depletion[353]. The latter group did note, however, that high pre and post treatment expression of CD79a B-cells predicted non-responsiveness to rituximab and that ACPA IgM levels correlated with CD79a/ CD20- B-cells in the synovium, leading to the speculation that incomplete depletion of B-cell subsets or local plasmablasts may produce in-situ autoantibody production with continued synovial inflammation [354].

The studies mentioned above are observational with limited sample sizes and varying timepoints, making interpretation and applicability to larger trials challenging. Given these conflicting results, there remains potential to use the synovium as an assessment or

prognostic tool to guide optimal treatment. In addition, the application of minimally invasive US-guided techniques to sample synovium [361] offer significant benefits in terms of tissue accessibility and thus minimise recruitment bias to clinical trials whilst maximising opportunities to serial sample synovial tissue following therapeutic intervention [375], as a larger number of joints can be sampled and does not depend on those undergoing surgery as in previous studies. Therefore, in order to address whether baseline synovial pathotype can predict response to rituximab therapy I evaluated clinical outcomes from a historical cohort of patients recruited for an observational non-blinded pilot study in which patients underwent an US guided synovial biopsy pre and post rituximab treatment.

Before using the synovial biopsy as a stratification tool, however, it was critical to formulate and validate different scoring methods within the synovium, which I will describe herein. Synovial biopsy specimens have been utilised to investigate the underlying disease process in inflammatory arthritides for approximately five decades. More recently, they have also been used in order to create molecular signatures of disease and assess response to both DMARD and biologic therapy. Several scoring systems have been described over this time period, developed in order to reliably compare both the degree of synovial organization and cellular infiltrates. To the best of my knowledge, no study to date has defined a 'B-cell rich' pathotype. Defining this was therefore formed the initial part of this work.

The earliest scoring systems correlated macroscopic changes on arthroscopy with histological change. Yates and Scott[376] provide the early descriptions of histopathological scoring on a 0-4 scale in accordance with descriptions of surface fibrin deposition, synovial cell hyperplasia, subsynovial mononuclear cell infiltration, perivascular cellular infiltration and

perivascular oedema. Additionally, they used three random high-power fields to grade the intensity of lymphocytic infiltration. They found a significant correlation between the total histological score and total visible synovitis, as well as a correlation between the number of active villi and lymphocytic infiltrates but do not comment upon inflammatory cells individually. The same six criteria were utilised to grade synovial biopsies on a 0-10 scale by Rooney[377], although the authors themselves comment that such a wide scale increases interobserver variability, making it difficult to score. The Rooney score was further tested in a prospective study, where it did not associate with joint progression in established disease[378].

A Japanese pathology group led by Koizumi and colleagues[379] used a 0-3 scale to grade RA synovium using eight different domains: 1) proliferation of synovial cells; divided into 2) degree, 3) palisading and 4)the presence of giant cells, 5) proliferation of synovial stroma, 6) presence of synovial granulation tissue, 7) synovial fibrin and 8) synovial haemosiderosis. This study, however used the scoring technique to confirm a histological diagnosis of RA rather than OA in synovial tissue samples, rather than to quantify histological severity of the disease. They group went on to find no correlation between clinical symptoms and the previous Rooney scoring system, although they did find an association with radiographic progression in a cross-sectional cohort [380]. The degree of variation and lack of agreement between the two scoring techniques proved problematic in terms of utility and application to large clinical trials.

Tak and colleagues describe a five-point 0-4 scale according to the cellular infiltrate (0 being no inflammatory cells and 4 being significant infiltration) which they proved to be both

reproducible and sensitive to change [381-383] for a number of different immunohistochemical markers. Furthermore, SQ scoring was comparative to quantified analysis undertaken through manual counting (MC), although may not be as sensitive to small changes over time [384]. It is widely accepted that MC is both sensitive and accurate, but is onerous and time-consuming when the analysis of several samples is required[385]. The advent of digital image analysis provided a further quantitative option for researchers. DIA allows standard image acquisition and can provide quantification of the areas stained as well as the intensity, although the process itself is also dependent on the programs and expertise available, uniform staining and tissue quality. Kraan and colleagues demonstrated that the three methods were comparable with strong correlations for T-cells, subintimal and intimal macrophages with no discernible differences between SQ, DIA and MC except for the time taken to score samples [386].

Normal synovium consists of an intimal lining layer 1-2 cells thick and a synovial sublining. It is thought to be relatively acellular with minimal infiltration of inflammatory cells, although variations have been described [365]. Studies focus mainly on the presence of adhesion molecules and perivascular lymphocytic infiltrates; little work has been done on the presence of B-cells in uninflamed synovium. Smith and colleagues [366] took normal synovium from 20 clinically and arthroscopically normal knees and scored the inflammatory infiltrate using a semi-quantitative 0-4 score and by digital image analysis. With specific regards to B-cell and plasma cell infiltrate, they found the mean SQ score of 0.1, with a range of 0-1. They did the same with cell count per mm2 using DIA which resulted in a score of 0.6, with a larger standard deviation of 1 and range of 0-4 cells per mm^2 taken from six high power fields. Another study of 12 patients with normal knees undergoing needle-biopsy did not reveal any

L26+ B-cells [387]. An older study did show the occasional IgM positive staining lymphocytes but does not quantify how many samples or the quantity of circulating B-cells [388]. Post mortem studies prior to this are not reliable, given the limitation of available staining-techniques.

Arguably the presence of ELS, characterized by B and T-cell organization, follicular dendritic cell networks and high endothelial venules equates with B-cell rich pathology. A scoring system based upon the number of radial cells from a central blood vessel as grade 1 (G1) aggregates displaying 2-5 radial cells, grade 2 (G2) between 6-10 cells or grade 3 (G3) with >10 cells has been previously described [346, 389] and the same definitions have been used for the purpose of this study. A key question that arises, however, is whether the presence of B-cells in the synovium in the absence of organized structures is of pathogenic relevance to the underlying disease process, and what score of infiltration suggests a 'B-cell rich' pathotype.

3.2 Hypothesis and Aims

The hypothesis of this chapter is that a baseline or change in synovial B-cell signature can predict response to Rituximab therapy. In order to do this, it is important to elucidate and validate the presence of absence of a 'B-cell rich' pathotype. I will then explore whether any clinical parameters and synovial pathotypes can be used to discriminate between responses to rituximab therapy in RA.

The specific objectives were to:

1. Ascertain whether B-cell SQ scoring was similar in this cohort to MC and DIA in order to validate scoring methods used for the remainder of this work
2. To agree a 'cut-off' value for a B-cell rich pathotype through review of synovial samples and further expert opinion
3. Determine any baseline clinical predictors of response to Rituximab therapy at 12 and 16 weeks
4. Describe the pathological characteristics of the synovium in an established RA cohort
5. Determine whether different synovial pathotypes in this cohort correlate with clinical phenotypes
6. Determine whether the presence or absence of B-cells in the synovium, and in particular germinal centres, correlates with clinical response to Rituximab therapy
7. Describe pathological changes in the synovium after Rituximab treatment and explore correlations with response to therapy

3.3 Materials and Methods

3.3.1 Study Population

25 patients all fulfilling the revised 1987 criteria for RA were enrolled to the R4RA pilot study between 2009-2014 at Barts Health NHS Trust as described in the methods chapter. One patient withdrew prior to baseline assessments, 2 patients withdrew prior to the study completing. 22 patients (18 females, 4 males) completed the study and had paired biopsies for analysis. Of these 18 paired biopsies were available for comparison after excluding those where a clear lining layer was not visible, therefore deemed ungradeable.

3.3.2 Patient Assessment

Clinical parameters including number of swollen and tender joints, DAS-28 scores and c-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were recorded at baseline and then monthly for a total of 52 weeks. A health activity questionnaire (HAQ) were undertaken at 0, 12, 24 and 52 weeks. Clinical response to rituximab were assessed using the EULAR response criteria at 12 and 16 weeks. Disease duration, previous number of DMARDs, prednisolone dosage and concomitant methotrexate dosage were all recorded at baseline.

3.3.3 Laboratory assessments

Levels of rheumatoid factor, ACPA, ESR, CRP, immunoglobulins and CD19 levels were recorded at baseline. ESR and CRP were recorded monthly with the remaining variables repeated at 16 weeks. Repeat CD19 levels were taken if a further cycle of Rituximab was planned.

3.3.4 US guided synovial biopsy

Standard US images of 5 MCPs and wrists were acquired immediately prior to synovial biopsy. Synovial thickness (ST) and power Doppler (PD) scores for each joint were assessed on a 0-3 scale by a blinded assessor. The maximum score of 12 joints was determined for ST and PD. Synovial samples were taken using ultrasound guided synovial biopsy from all patients with a clinically involved joint at baseline. The same joint was biopsied16 weeks using the technique described in the methods section. A minimum of six synovial samples were retrieved and immediately immersed in paraformaldehyde for subsequent paraffin embedding as described in the methods chapter.

3.3.5 Formulation of a B-cell Rich Score: comparison of imaging techniques

The first aim of this chapter was to create and validate a cut-off for 'B-cell rich vs B-cell poor samples. SQ, MC and DIA techniques were used to score 22 baseline samples.

Semi-quantitative analysis

SQ analysis was performed by an independent observer (VR) and myself. The expression of CD20+ cells was scored using a five-point scale as described by Tak and colleagues [381]. Minor differences were resolved by mutual agreement. A standardized scoring atlas for CD20 staining present within the Experimental medicine and rheumatology laboratory at Queen Mary University is demonstrated in figure 3.1.

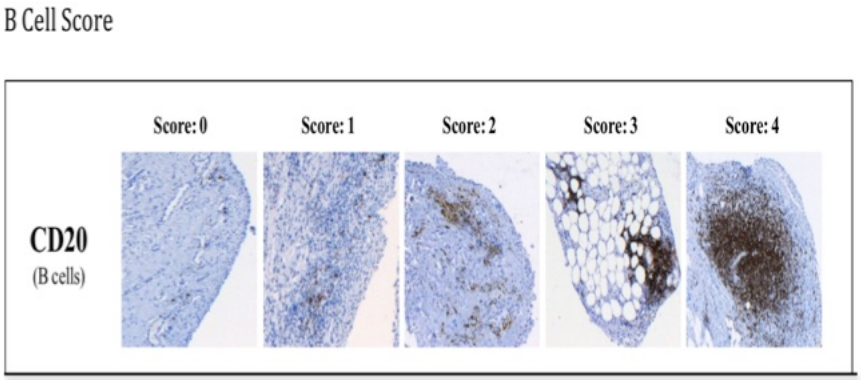


Figure 3.1 A semi-quantitative scoring atlas to grade CD20

Score 0 = no staining, Score 1 = minimal CD20 staining, Score 2 = confluent areas but no aggregates, Score 3 = moderate staining beginning to resemble aggregates, Score 4 = dense CD20 staining with the formation of aggregates.

Manual counting

MC was performed on all sections containing both synovial lining and sublining layers. Five high power fields at x20 magnification (Olympus light microscope) were selected as the most inflamed area of each specimen and the cells were counted by eye. The final cell number recorded was the most extreme for each individual block and specimen.

Digital Image Analysis

In order to determine the area stained by DIA, I processed the images obtained using an Olympus microscope and Cellsens dimension software. Briefly, an overview of each tissue section at x20 magnification was constructed using the stage navigator function. Five high power fields which contained both synovial lining and sublining layer were selected and a region of interest was drawn around the selected areas. Using the 'count and measure function' colour threshold technique I calculated the fraction of synovial tissue stained in relation to the total area of tissue selected. The final result was the mean score of the stained area. Ideally, the threshold should be the same across all samples and pre-determined to be

applicable to each sample. Due to variances in tissue quality and staining intensity, I was unable to define a minimal colour threshold that could be reliably calibrated and used on all samples and therefore this had to be reset for each individual sample.

Expert Opinion

Based upon this pilot work, our group launched the **R**andomised, open labelled study in anti-TNFα inadequate responders to investigate the mechanisms for **R**esponse-Resistance to **R**ituximab versus Tocilizumab in **R**A (R4RA) trial. As part of this work 22 samples from the new study and the samples from this study were also regraded and analysed by an independent consultant histopathologist (HR) and the cut off for B-cells were discussed. Interobserver correlations were calculated and a threshold for 'B-cell rich' or 'poor' specimens was discussed.

3.3.6 Statistical Analysis

Continuous variables are expressed as mean $\pm$ standard deviations (sd) and ordinal variables as median (interquartile range). Spearman's correlation coefficient was used to determine correlations between the three scoring methods. Comparison of pre-treatment and post-treatment clinical data and histological analysis was performed using the Wilcoxon signed rank test for paired data. For unpaired data, the Mann-Whitney U test was used to analyse for differences in median and rank. Associations were performed by deriving the Spearman rank correlation coefficient. Fishers' exact test of Chi-squared was used to test for associations between baseline synovial infiltrate scores and treatment EULAR treatment response.

All statistical analysis and graphs were derived using Prism (version 7, compatible for Macintosh systems). The cut-off value for significance was <0.05 for all tests.

3.4 Results

3.4.1 SQ scoring significantly correlates with MC and with DIA

Of the 22 samples analysed, 7 samples were identified as containing B-cells for manual counting. Of these, three had easily identifiable germinal centres, whilst the remainder had a diffuse infiltration of B-cells. SQ scoring and DIA were compared to MC which was considered the gold standard. Using manual counting as the gold standard there was a significant correlation between semiquantitative scoring and manual cell counts, r=0.95, p=0.01. A similar observation was made when using the DIA method, r=0.8, p=0.03.

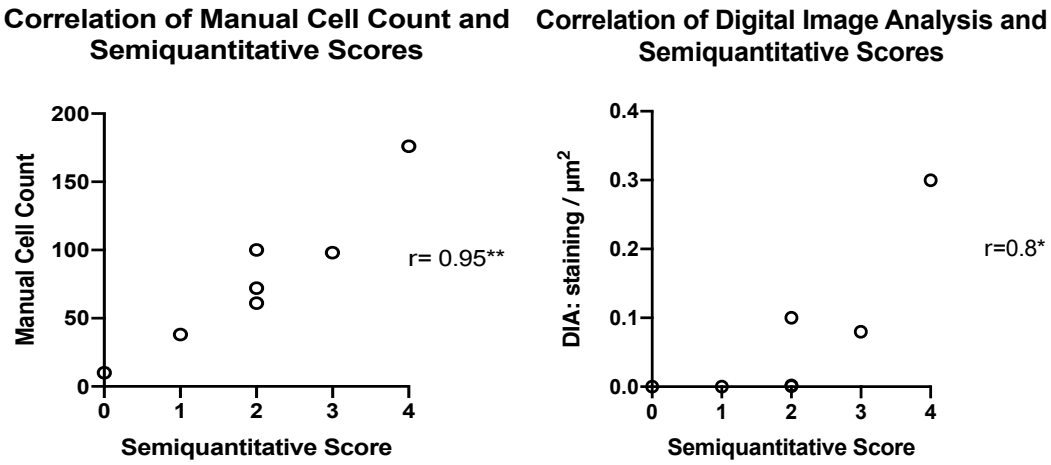


Figure 3.2 SQ scoring correlates significantly with both manual counts and digital image analysis

Spearman rank correlation plots of SQ scoring vs manual cell count (p=0.01) and DIA p=0.03, * represents p<0.05.

Abbreviations: DIA = digital image analysis

3.4.2 Manual cell count does not significantly correlate with DIA

In contrast to the SQ scoring method, I observed a trend towards correlation between the manual cell count and SQ scores (p=0.07) but this did not reach significance.

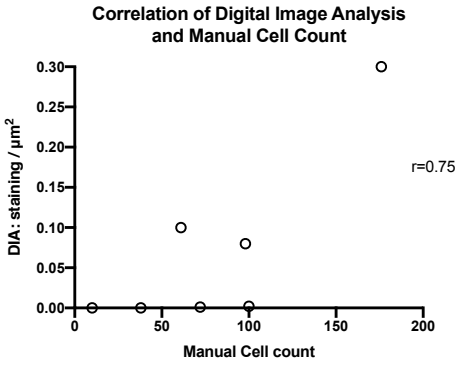


Figure 3.3 There is a trend towards correlation between DIA and MC

Spearman rank correlation plots of MC vs DIA, p = ns

Abbreviations: MC = manual count, DIA = digital image analysis

3.4.3 Expert opinion and grading in the NHS laboratory defined a B-cell rich pathotype as over 50 cells or the presence of aggregates

An independent expert consultant histopathologist (Dr Hasan Rizvi) also graded 23 samples as described above (these were a combination of the pilot data and some samples from the R4RA study), with a strong interobserver correlation coefficient (kappa value of 0.87) for CD20. Any differences were reviewed through mutual agreement. The presence of over 50 cells by manual counting, with or without the presence of aggregates, were deemed to be 'B-cell rich', as it was concluded that it was unusual to find B-cells in normal uninflamed synovium. This was applied retrospectively to all of the samples within the pilot cohort – all had a SQ score of 2.

3.4.4. A Semi-quantitative B-cell score of 2 correlates with the presence of plasma cells

I next utilised the CD138 marker to score the newly defined 'B-cell rich' group for plasma cells, summarised in table 3.1. Plasma cells were seen in biopsies from five patients, indicative of B-cell driven immune infiltration. Furthermore, the presence of plasma cells was exclusive to the B-cell rich cohort, leading to the formulation of a new scoring procedure, illustrated in figure 3.4.

	CD20 SQ score (0-4)	CD138 SQ score (0-4)
Sample 1	2	2
Sample 2	2	3
Sample 3	2	3
Sample 4	3	3
Sample 5	4	4

Table 3.1 Concordance of SQ scoring of CD20 and CD138 scores

Grading Analysis Requirements
Tissue must have a visible cell lining layer in order to be graded. The radial cell count is estimated by counting the number of cells from the centre of the aggregate not from the vessel as vessels are not always central to the aggregate. A vessel must be present either in or next to the lymphocytes (Perivascular). Mixed infiltrates have to be mainly lymphocytic and it must be noted if there is diffuse inflammatory infiltrate present in the tissue. **Grading Categories:** Uniflammed, Diffuse, Grade 1 (between 2-5 cell aggregate), Grade 2 (between 5-10 cells), Grade 3 (>10 cells), Ungradable (necrotic/adipose/muscle tissue)

H&E grading requirements

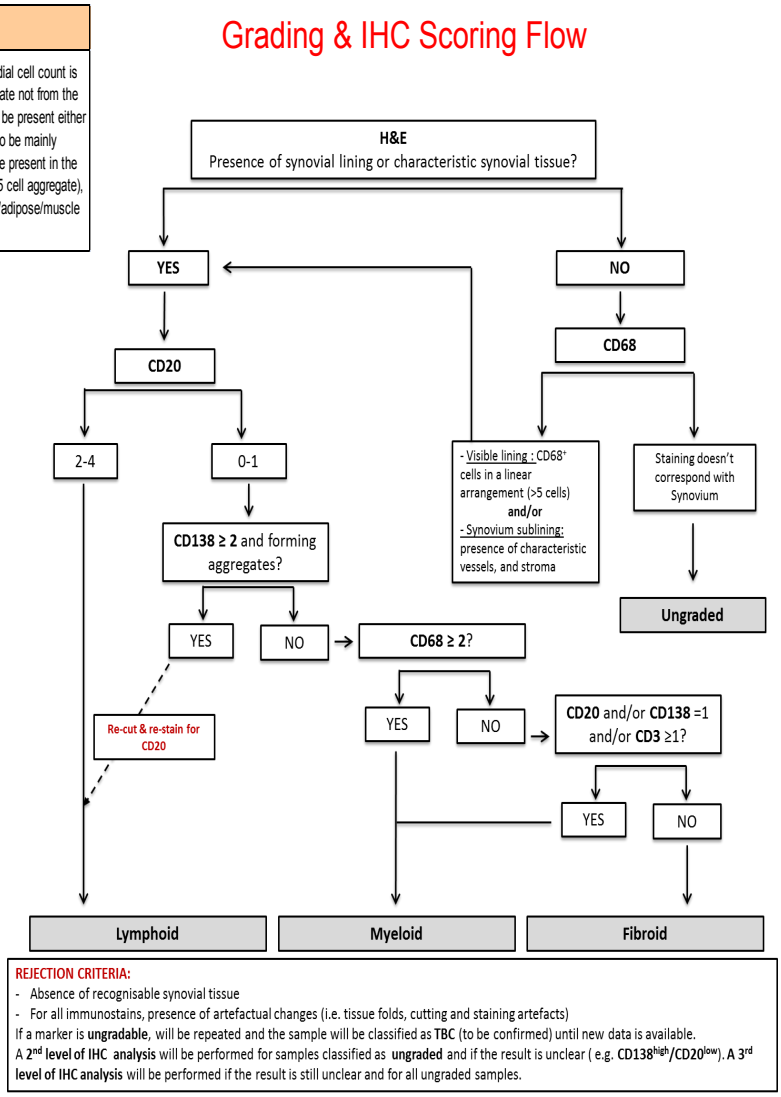


Figure 3.4 Formulation of a new Standard Operating Procedure (SOP) for grading Immunohistochemical samples

used courtesy of Dr. Rebecca Hands and the experimental medicine and rheumatology laboratory

All samples must have a visible synovial lining layer for further CD20 staining to be valid. If there is discordance with the presence of CD138 (plasma) cells then the tissue should be recut and re-stained for CD20. In the absence of aggregates, a CD68 score of ≥ 2 defines a myeloid pathotype. The absence of immune cells but the presence of a visible lining layer correlates with a fibroid pathotype.

3.4.5 Clinical data: Patient Demographics

The demographics and clinical characteristics of the 22 patients who completed the study and had paired synovial tissue available for analysis is shown in table 3.2. 82% (18/22) patients were female with a mean age of 52.3 years (SD 13.73). 77% (17/22) were positive for RF and 86% (19/22) were positive for ACPA. The median disease duration was 120 months, the median number of previous anti-TNF drugs was 1 (IQR 0-4). 32% (7/22) received rituximab as first line therapy following DMARDs due to contraindications, including prior malignancy or history of multiple sclerosis. 55% (12/22) were taking concomitant methotrexate, the median dose was 5 mg. 36% (8/22) were taking a single DMARD and 27% (6/22) were taking more than one DMARD at the time of enrolment. 36% (8/22) received Rituximab monotherapy. 45% of patients (10/22) were taking oral steroids, with a median dose of 0 mg (0-8.75).

Table 3.2 Demographics and clinical characteristics of cohort at baseline

Age (yrs) mean ± SD	52.4 ± 13.7
Female, n, (%)	18 (82)
Median disease duration months (IQR)	120
RF positive, n, (%)	17 (77)
ACPA positive, n, (%)	19 (86)
RF and ACPA negative, n, (%)	2 (9)
ESR mm/hr median, (IQR)	26 (9-58)
CRP mg/l median, (IQR)	9 (0-19)
DAS-28 mean ± sd	5.7±1.0
SJ median (IQR)	6 (3-9)
TJ median (IQR)	11 (7.7-22)
VAS mean ± sd	65.67± 23.98
HAQ mean ± sd	2.1 ± 0.72
Erosive≥1 joint n, (%)	18 (82)
ST median (IQR) (n=21)	26 (23-30.5)
PD median (IQR) (n=21)	3 (2-5.5)
Median number of previous anti-TNF drugs, IQR	1 (0-2)
No of patients receiving Rituximab as first line therapy, n (%)	7 (32)
No of patients on methotrexate n (%)	12 (55)
Median methotrexate dose, mg (IQR)	5 (0-17.5)
No of patients on prednisolone, n, (%)	9 (41)
Median prednisolone dose, mg (IQR)	0 (0-8.75)

Abbreviations
RF=rheumatoid factor, ACPA= anti-citrullinated protein antibody, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, IQR=interquartile range DAS-28= 28 joint count disease activity score, HAQ = Health assessment questionnaire, SJ = swollen joints, TJ = tender joints, VAS = visual analogue score, ST = synovial thickening, PD = power Doppler, EULAR = European union league of action against rheumatism, TNF = tumour necrosis factor

3.4.6 Treatment with Rituximab results in a significant reduction in DAS-28 scores at 12 and 16 weeks

Data was available for 22 patients at 16 weeks. 1 patient withdrew from the study prior to the biopsy and 1 patient was lost to follow-up. A significant decrease in mean DAS-28 scores was observed across the group at 12, 16, 24 and 52 weeks, shown in figure 3.5. The overall mean DAS-28 score at baseline was 5.73 (sd $\pm$ 1.55). There was a significant reduction in DAS-28 scores between baseline and 12 weeks (post-treatment mean DAS-28 4.93, mean Δ DAS-28 0.81, p=0.0082) and at 16 weeks post treatment (post treatment mean DAS-28 5.01, mean Δ DAS-28 0.072, p=0.027). At 12 weeks 6/22 (27%) showed a 'good' EULAR response, 5/22 (23%) showed a moderate response. At 16 weeks this was 2/22 (9%) and 9/22(41%), respectively. Treatment effects continued at week 24 and 52, although these figures are harder to interpret as rescue steroids and retreatment were permitted. 16/22 (73%) patients were retreated "at flare" during the 52-week follow up period and 14 (64%) patients remained on Rituximab therapy at 52 weeks.

Next, I looked at specific components of the DAS-28 scores which improved at each timepoint. The number of tender joints did not significantly change from baseline, (median 11, IQR 7.7-22) to 12 weeks, (median 9, IQR 5-20). Similarly, there was no significant change at 16 weeks (median 7.5, IQR 3-16.25). There was significant reduction in the number of swollen joints from week 0 (median 6, IQR 3-9) to week 12; (median 4, IQR 1.5-7), p=0.003 but this was not seen at week 16 (median 5.5, IQR 2-8). Rituximab treatment resulted in a significant reduction in pre-treatment CRP (median 9, IQR 0 -19.3) at week 12 (median 0, IQR 0-11.75, p=0.002) and week 16 (median 0, IQR 0-8, p=0.016). A significant reduction from pre-treatment ESR (median 25.5, IQR 9-58) was only seen at the 16 week timepoint (median 22, IQR 9-37),

p=0.03. The VAS also reduced in both groups from baseline (mean 65.7 ± 23.98) to 12 weeks (mean 42.85 ± 20.61, p= 0.004) and to 16 weeks (mean 44.86 ± 28.35, p 0.01). These values are summarised in Figures 3.5 and 3.6.

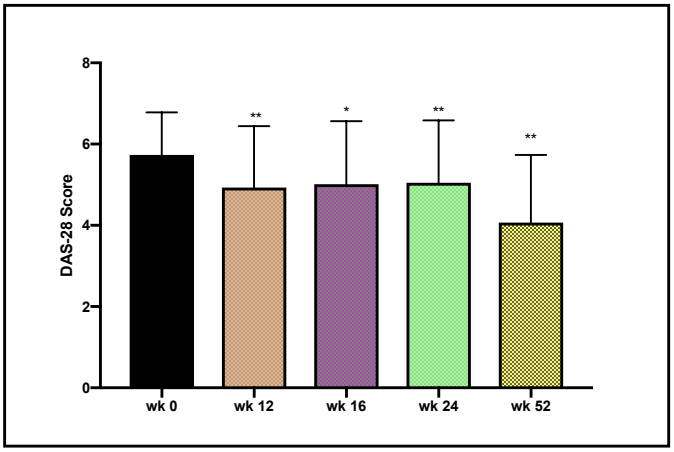


Figure 3.5 Change in DAS-28 score following treatment with rituximab

Bars represent mean score, error bars represent standard deviation. Wk corresponds to the number of weeks after first infusion of rituximab. *p<0.05, **p<0.01, Wilcoxon matched paired signed rank test

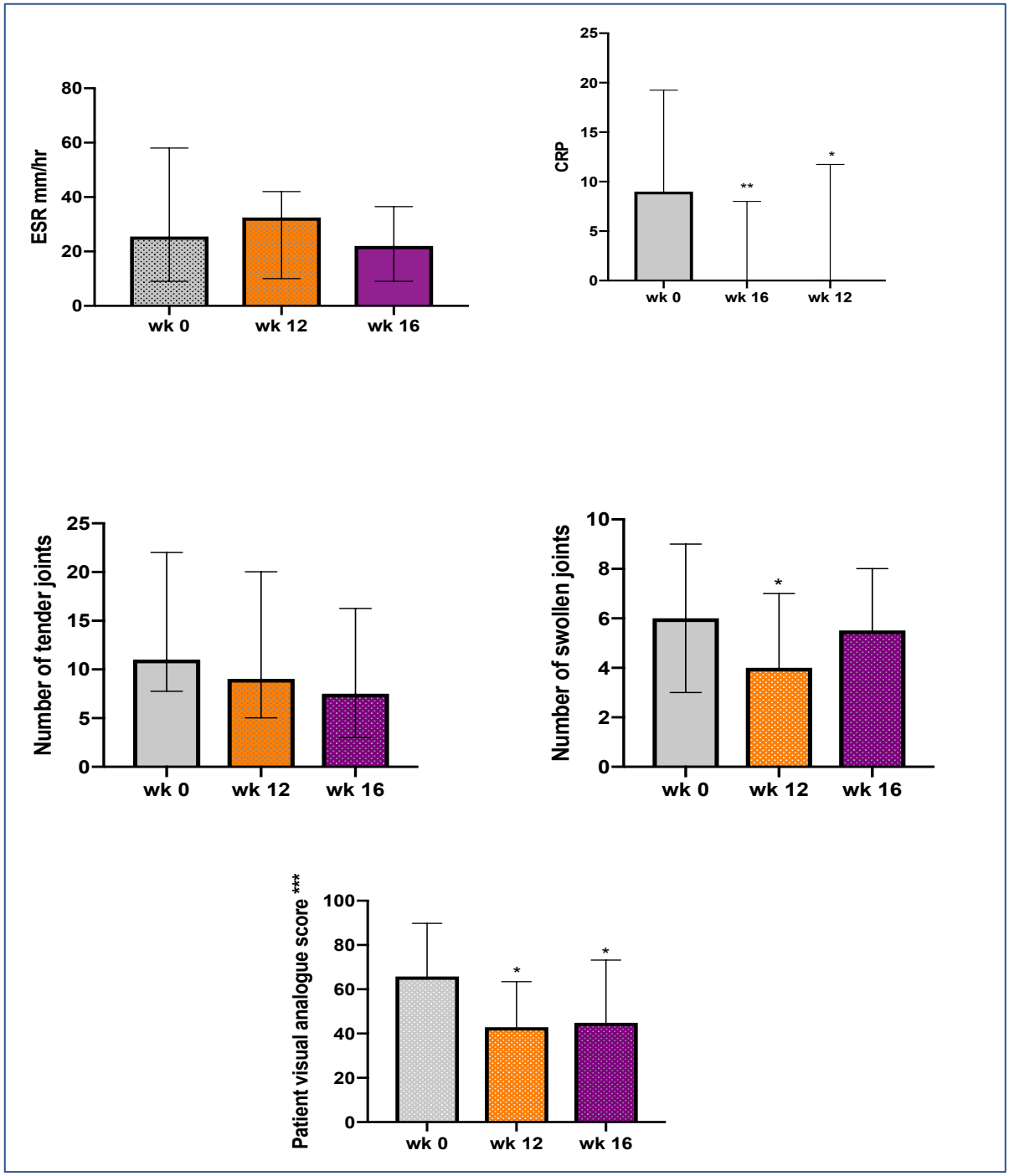


Figure 3.6 Individual components of the DAS-28 score following treatment with rituximab

Bars represent median, lines present interquartile range except *** where bar represents mean and standard deviation. Wk corresponds to the number of weeks after first infusion of rituximab. Abbreviations: *ESR* = erythrocyte sedimentation rate; *CRP* = c-reactive protein

*p<0.05, Wilcoxon matched paired signed rank test

3.4.7 Treatment with rituximab improves quality of life scores up to 52 weeks

In line with a reduction in DAS-28, a significant reduction in both mean HAQ (pre-treatment mean 2.03 $\pm$ 0.73, post treatment mean 1.55 $\pm$ 0.82) and VAS (pre-treatment mean 63.8 $\pm$ 24.3, post treatment mean 42.6 $\pm$ 20.61) were noted at 12 weeks. This trend continued for patients who persisted on rituximab therapy at 52 weeks (post treatment mean 1.66 $\pm$ 0.95, p=0.02), illustrated in figure 3.7.

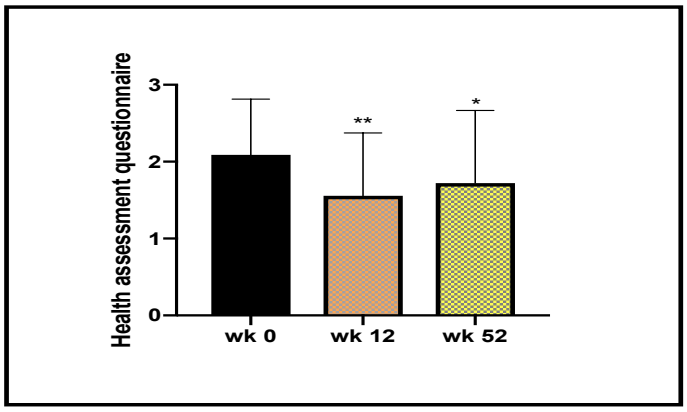


Figure 3.7 Change in health assessment questionnaire scores following treatment with rituximab

Bars represent mean score, error bars represent standard deviation. Wk corresponds to the number of weeks after the first infusion of rituximab.

*p<0.05, **p<0.01, Wilcoxon matched paired signed rank test

3.4.8 There are no differences in clinical parameters at baseline between responders and non-responders at 16 weeks

No clinical differences were found between responders and non-responders at baseline. Table 3.3 shows p-values comparing baseline variables between the two groups. Although responders appear to have a higher mean level of rheumatoid factor and CCP at baseline, this was not significant. Interestingly, 8/9 patients who responded to Rituximab had only had one anti-TNF prior to therapy, but there was also a high level of non-response in similar patients. No differences were observed in DAS-28 or HAQ at all timepoints between patients who received rituximab as monotherapy and those on concomitant methotrexate. Additionally, no significant correlations were noted between levels of baseline variables and Δ DAS-28 at 16 weeks.

	Responders (n=9)	Non-responders (n=13)	p-value
DAS-28, mean (sd)	5.82 (0.75)	5.67 (1.23)	0.832
RF, median (IQR)	215 (11.5-417)	66 (17-271)	0.459
ACPA, median (IQR)	354 (95-600)	66 (17-271)	0.240
ESR, mm/hr median (IQR)	16 (7.5-44)	38 (9-77)	0.194
CRP mg/l, median (IQR)	9 (0-17.5)	9 (0-45)	0.592
IgG g/l, median (IQR)	13 (9.3-14.3)	12.05 (9.73-14.9)	0.931
CD19, median (IQR)	211 (156-221)	205 (134-337)	0.669
VAS, mean, sd	70.33 (20.95)	62.6 (23.1)	0.544
Previous anti TNF (<1) n, (%)	8 (88)	9 (69)	0.606
Concomitant Methotrexate, n, (%)	5 (55)	11 (85)	0.607
Methotrexate dose, mg, median (IQR)	5 (0-13.75)	17.5 (0-18.75)	0.39
Concomitant prednisolone, n (%)	3 (33)	8 (62)	0.39
Prednsiolone dose, mg, median (IQR)	0 (0-8.75)	5 (0-10)	0.51

Table 3.3 There are no differences in baseline variables between responders and non-responders based on the EULAR response criteria

Unless stated, all values are expressed as the median (IQR) or mean (sd) of each variable. P-values calculate using Mann-Whitney test.

Abbreviations: DAS-28 = disease activity score, RF = rheumatoid factor, ACPA = anti-citrullinated protein antibodies, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, IgG = immunoglobulin G, CD19= peripheral blood B cell marker, VAS visual analogue score, TJ = tender joints, SJ = swollen joints

3.4.9 Synovial pathotype may be a predictor of response at 12 weeks, but this is dependent on the cutting level of tissue

Initial analysis of this data revealed 5 patients that were deemed to be B-cell rich. Analysis of these five patients resulted in a trend towards response in the B-cell rich group (p=0.09) at 12 weeks using Fisher's exact test. Tissue samples noted to have plasma cells but no B-cells were then recut at further levels, resulting in a regrading of three samples, now deemed to be B-cell rich, thus highlighting the importance of adequate tissue acquisition and cutting levels and the need for development of a clear scoring technique. It should be noted that not all the tissues were recut at this point, which would have added further validity to the study.

3.4.10 Synovial pathotype at baseline does not predict clinical phenotype or response to rituximab therapy at 16 weeks

At week 0, 20/22 patients had synovial tissue suitable for histological analysis. The sites of synovial biopsy are represented in figure 3.8. Patients were divided into 'B-cell rich' or 'B-cell poor' groups as described. Aggregates were scored from G1-G3 as described in the methods section and further scores as lymphoid, myeloid or pauci-immune, summarised table 3.4. 11/20 (55%) patients were classified as myeloid, 4 were lymphoid (18%) and 5 samples were classified as diffuse or 'pauci-immune'. There were no associations between synovial pathotype and response to rituximab therapy (p=0.46, Chi-squared). 8/20 (25%) of patients were classified as B-cell rich. Of the B-cell rich category 4/8 (50%) displayed features of G2/G3 aggregates but none stained positively for CD21. Out of these 4 patients, two patients did not have either RF antibodies or ACPA. No differences were found in clinical parameters between B-cell rich and B-cell poor groups at baseline.

Of patients categorised as B-cell rich, 3/8 (37.5%) and 5/12 (42%) B-cell poor achieved a EULAR moderate / good response at 16 weeks. (p>0.99) Further classification of the B-cell rich group into those with and without aggregates also failed to show an association with response to therapy (p=0.76). Baseline histological classifications therefore did not predict response to rituximab therapy (table 3.3).

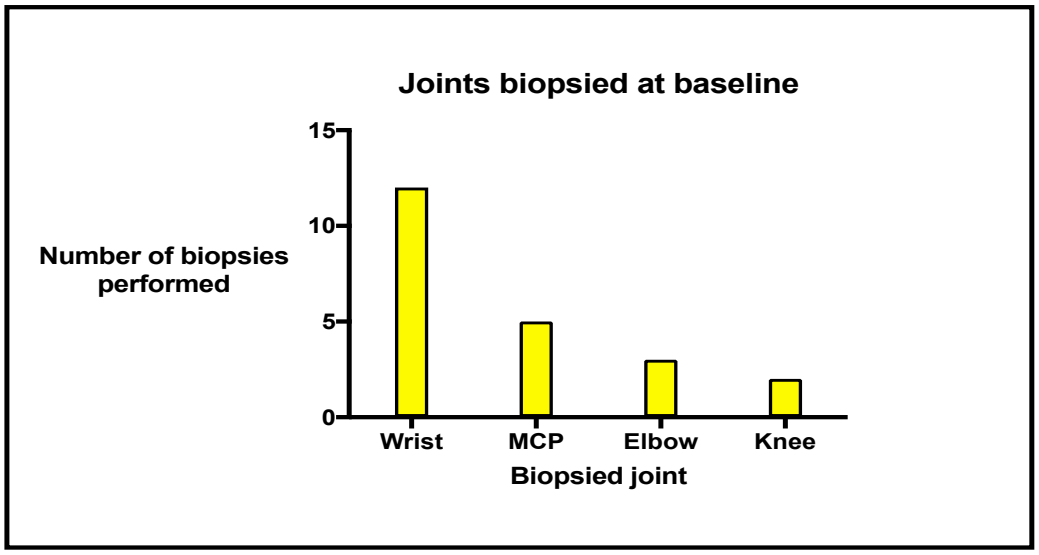


Figure 3.8 Distribution of joints that underwent US-guided synovial biopsy

Abbreviations: *MCP* = metacarpophalangeal joint

Synovial Pathotype	EULAR response following Rituximab	
	Responders, n, (% of subgroup)	Non-responders, n (% of subgroup)
Lymphoid	3 (60)	2 (40)
Myeloid	3 (27)	8 (73)
Pauci-immune	2 (40)	3 (60)

Table 3.4 Synovial pathotype does not predict response to Rituximab

p = 0.46, Chi-squared, 2 degrees of freedom.

Histological classification	EULAR response following Rituximab	
	Responders, n, (% of subgroup)	Non-responders, n (% of subgroup)
B-cell poor	5 (42)	7 (58)
B-cell rich	1 (25)	3 (75)
B-cell rich + aggregates	2 (50)	2 (50)

Table 3.5 Histological B-cell classification does not predict response to rituximab

B cell poor = semiquantitative score of 0-1; B-cell rich = semiquantitative score of 2-4, B-cell rich + aggregates = SQ score of 2-4 AND the presence of lymphoid follicles. p=0.76, Chi-squared, 2 degrees of freedom

3.4.11 B-cell rich patients have a significantly higher degree of synovial cellularity and are more likely to be on higher doses of methotrexate and prednisolone

Next, I went on to examine whether the degree of synovial inflammation differed between the B cell rich and poor groups, displayed in Figure 3.9. No differences were noted in median (IQR) semi-quantitative scores for CD68 lining layer macrophage number, B-cell rich 2.75 (1.25-3) vs B-cell poor 2 (1-3). There were significant differences in the median synovitis score ($p=0.013$) between the two groups with a higher synovitis score, median 5.5 (3.25-7.7) in the B cell rich patients compared with B-cell poor patients median 2.5 (2-3.75). In addition, there were significantly higher numbers of CD3 T-cells, 1.5 (1-2) vs 0.75 (0-1); CD68 SL macrophages, 2.75 (2-3) vs 1 (1-1.88) and most strikingly in CD138 plasma cells, 3 (2.25-3.75) vs 0 (0-1) in patients with a B-cell rich lymphoid pathotype, exhibited in figure 3.9. This did not, however, correlate to clinical phenotype as no significant differences were found between the two groups except for the prednisolone dose which was slightly higher in the B-cell rich group, $p=0.048$ and interestingly, median methotrexate dose, $p = 0.03$ was also higher in the B-cell rich group, shown in table 3.6.

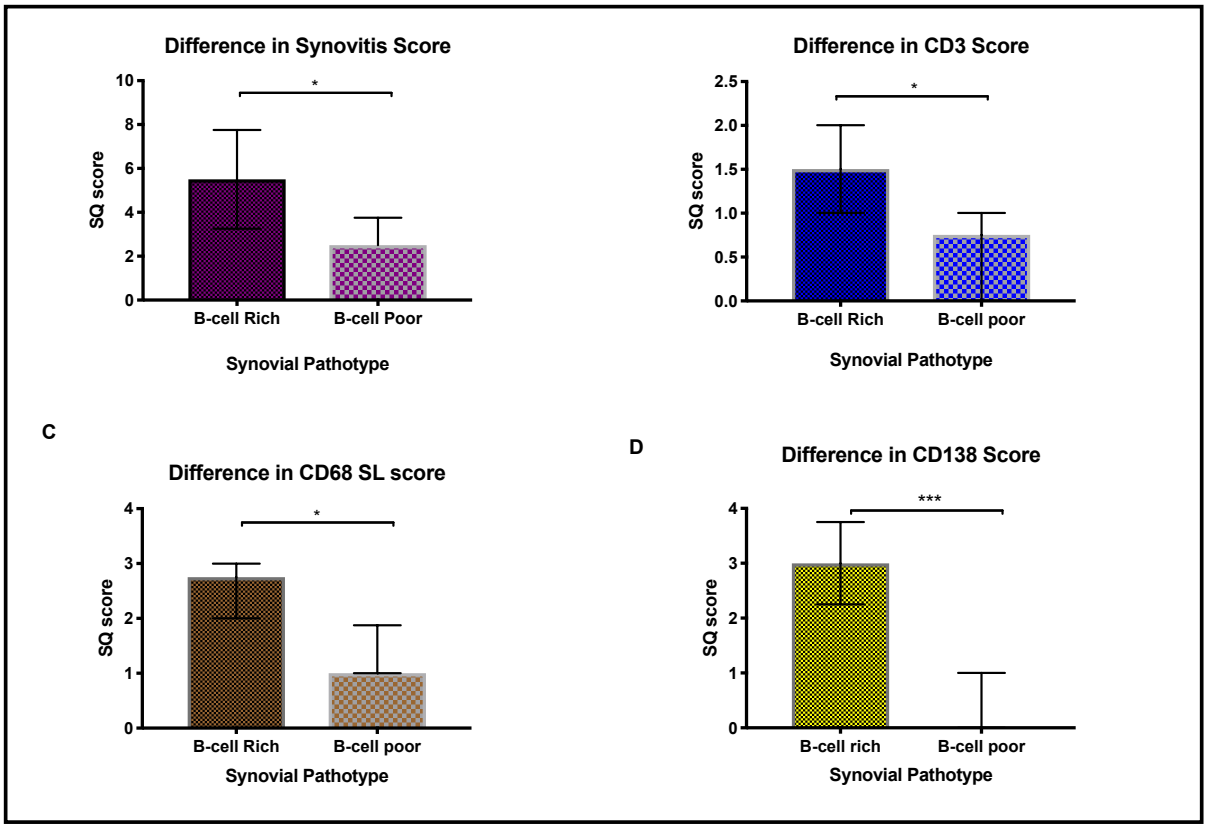


Figure 3.9 B-cell rich patients display a higher degree of cellularity in the synovium

Bars represent the median and lines represent the interquartile range

A Difference in synovitis score between B-cell rich and B-cell poor subgroups, *p<0.05

B Difference in CD3 T-cells SQ scores between B-cell rich and B-cell poor subgroups, *p<0.05

C Difference in CD68 sublining macrophages between B-cell rich and B-cell poor subgroups, *p<0.05

D Difference in CD138 plasma cells between B-cell rich and B-cell poor subgroups

***p<0.0001, Mann-Whitney test

	B-cell Rich	B-cell poor	p-values
DAS-28 mean, sd	5.66, 0.57	5.99, 1.15	
RhF, median, IQR	27.5 (3.75-294)	165 (20.5-514.8)	0.33
ACPA, median, IQR	315.5 (11-600)	116 (71-600)	0.98
IgG, g/l median, IQR	11.9 (8.6-11.9)	13 (9.87-14.4)	0.79
ESR mm/hr, median, IQR	31 (10.25-56.5)	30 (8.5-63.5)	0.97
CRP mg/l, median, IQR	4.5 (0-16.75)	13 (0-32.75)	0.62
TJ, median, IQR	12 (7.5-20.5)	12 (8.5-22.75)	0.72
SJ, median, IQR	7 (3.75-8.75)	6.5 (3.75-9)	0.96
VAS, mean, sd	66 (20.77)	68.35 (29.9)	0.74
Median disease duration	102 (39-120)	120 (27-120)	0.67
Number of anti-TNF, median, IQR	1 (0-1)	1 (0.25-2)	0.49
Median methotrexate dose, IQR	17.5 (8.13-19.38)	0 (0-6.88)	0.03*
Median prednisolone dose, IQR	7.5 (1.88-10.0)	0 (0-3.75)	0.049*

Table 3.6 There are no differences in clinical variables between B-cell rich and B-cell poor groups except for methotrexate and prednisolone dose

Unless stated, all values are expressed as the median (IQR) or mean (sd) of each variable. P-values calculate using Mann-Whitney U-test, *p<0.05

Abbreviations: DAS-28 = disease activity score, RF = rheumatoid factor, ACPA = anti-citrullinated protein antibodies, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, IgG = immunoglobulin G, CD19 = peripheral blood B cell marker, VAS visual analogue score, TJ = tender joints, SJ = swollen joints

3.4.12 The synovial cellular modulatory effects of Rituximab

In order to determine the effects of rituximab on synovial immune cell infiltration I analysed differences in the synovitis score and changes in immune cell infiltration at baseline and 16 weeks. Paired data was available for 18 patients. I observed a significant reduction (reported as median, IQR) in pre and post CD20 score (pre 0.5, 0-2.25, post 0, 0-125), p = 0.031 and a strong trend for reduction in CD138 plasma cells (pre 1, 0-3, post 0.75, 0-2), p= 0.088). There was also a significant reduction in sublining macrophages (pre, 1-3, post 1.25 (1-2), p= 0.039). There were non-significant reductions in the overall synovitis score, CD3 and CD68L macrophages. Findings are summarised in figure 3.10.

Next, patients were segregated into EULAR responders and non-responders in order to determine whether responders had differences in immune cell infiltration at baseline or a significant reduction in immune cell infiltration following treatment. No differences were observed between the two groups, shown in figure 3.11.

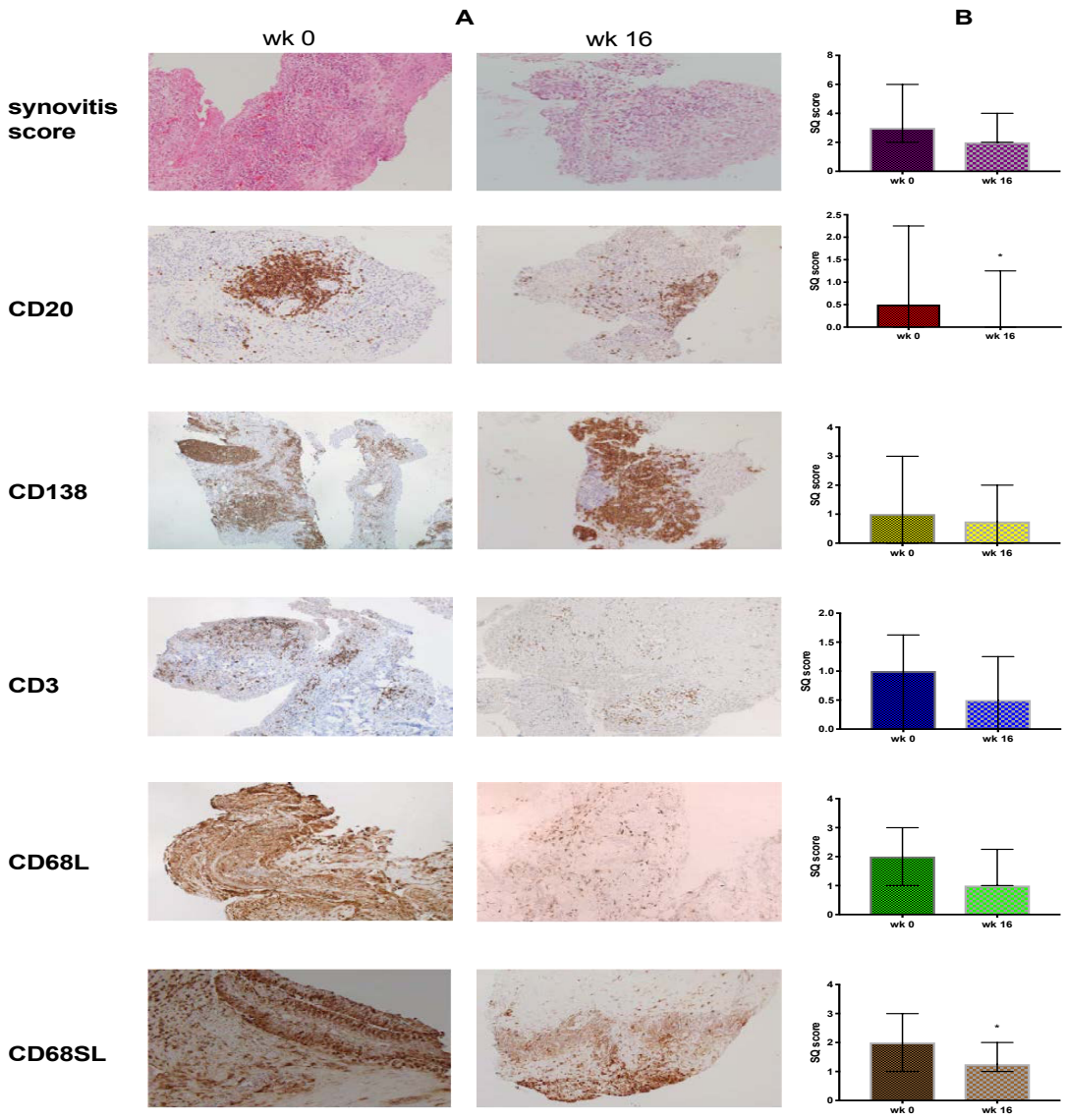


Figure 3.10 Effect of rituximab on the histological characteristics of synovial tissue and distribution of cell populations in synovial biopsy samples obtained pre and post therapy

A Immunohistological findings in representative synovial tissue samples obtained at week 0 and 16 weeks after rituximab therapy (original magnification x20)

B Results of semiquantitative evaluation obtained in 18 paired samples (obtained at week 0 and week 16). Each bar represents the median and lines denote the interquartile range.

*p<0.05 using the Wilcoxon matched paired signed rank test.

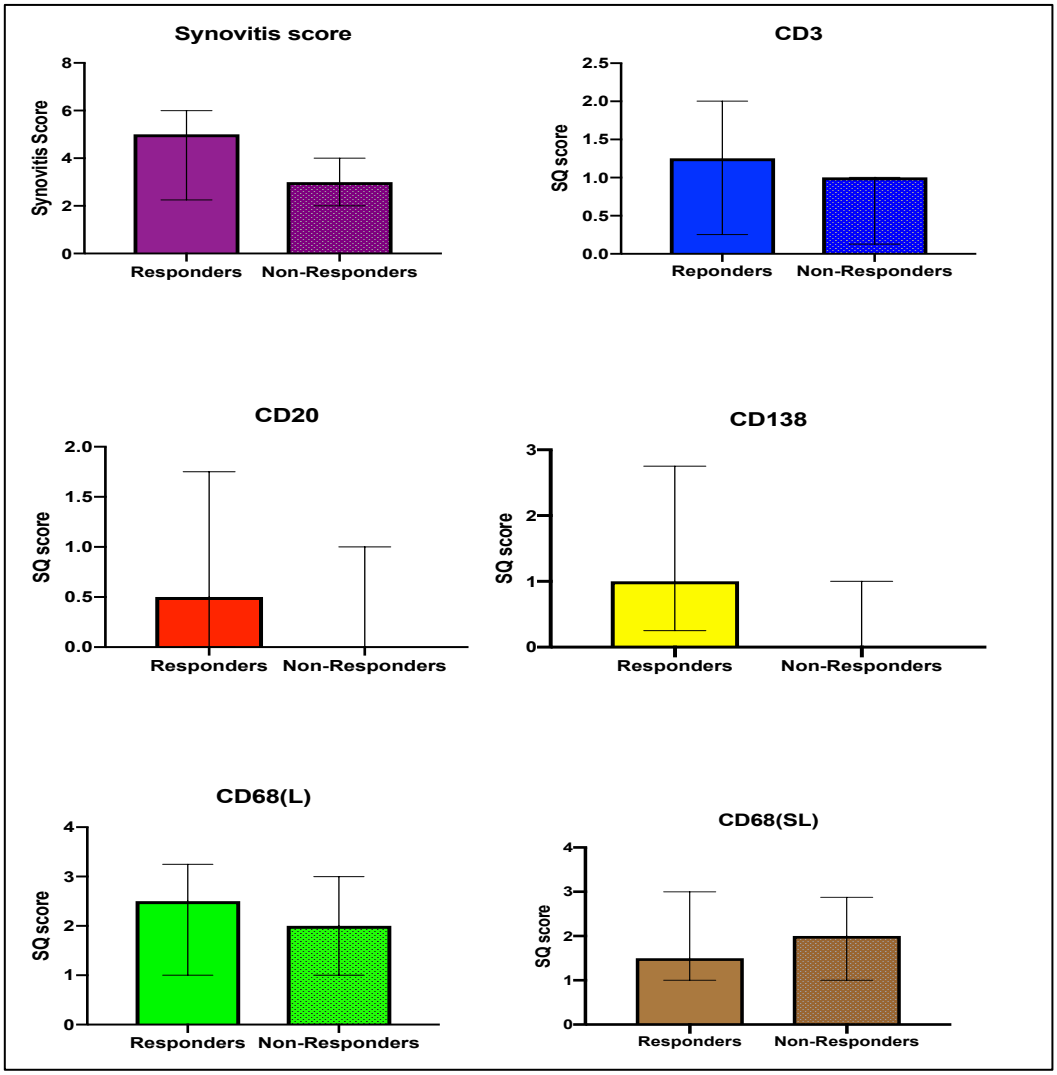


Figure 3.11 There are no differences in cellular infiltrates at baseline between responders and non-responders

Bars represent median and interquartile range. No differences were observed in synovitis score, p=0.34, CD3 score p=0.26, CD20 score p=0.61, CD 138 score p=0.18, CD 68(L) score p=0.71, CD68(SL) score p=0.86 between EULAR good/moderate responders and non-responders. Abbreviations SQ = semi-quantitative

3.4.13 A trend towards a reduction in plasma cells correlates with Δ DAS-28

In order to analyse whether a change in each specific synovial cellular marker correlated with a change in DAS-28 score I calculated Spearmans rank correlation co-efficients for each cell type and for the change in DAS-28 scores. In the B-cell rich group, there was a trend towards a decrease in plasma CD138 cells and a decrease in DAS-28 (r 0.65, p=0.09). When further analysed in only the moderate / good EULAR responder group no further associations were seen.

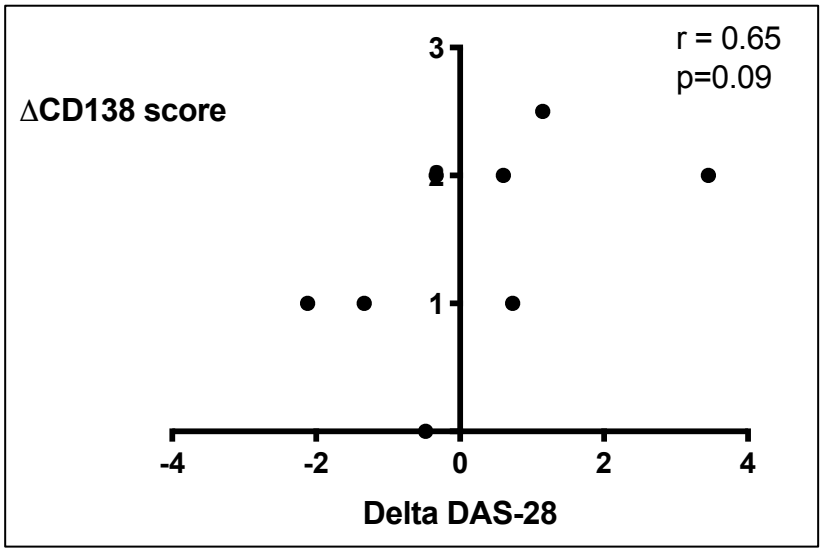


Figure 3.12 A trend towards reduction of plasma cells correlated with a reduction in DAS-28 scores

Spearman's rank correlation coefficient, p=ns

3.5 Discussion

Ascertaining clinical and synovial biomarkers of response to rituximab therapy is challenging. RA synovium is a heterogenous entity. Distinct histological subtypes (fibroid, myeloid and lymphoid) have been observed within the synovium but few studies have explored whether these can be used as a stratification tool to guide response to therapy. I have shown that at baseline, there were no clear clinical, laboratory or immunohistochemical parameters that associated with response to therapy in this cohort of patients. Treatment with rituximab does, however, lead to global changes in the cellular infiltrate although I did not show that this correlated with clinical outcomes. The data presented here therefore adds to existing observational studies, showing that there are changes in cellular infiltration after Rituximab administration and that the modulatory effects of B-cell depletion extend beyond peripheral blood B-cell depletion.

One of the main aims of this chapter was to validate the best method of scoring for the samples in this study in order to create a 'B-cell rich' or 'B-cell poor' classification. Firstly, I validated the use of DIA and MC and have shown that these methods correlate significantly with SQ scoring. It is however, important to consider significant limitations of these comparisons in synovial tissue analysis that would attract criticism.

Firstly, there were only seven samples in total which had a significant number of B-cells to allow comparison. The last 'B-cell rich' patient was not included as this sample was recut at the end of the study. More samples would be needed to make this applicable to large scale studies, although others have previously demonstrated correlations between scoring methods [386]. Both manual counting and DIA became significantly more difficult when

determining the number of nuclei in samples with dense aggregates, particularly if only adhering to one magnification. I faced difficulty in utilising 'pre-set' thresholds for DIA of synovial tissue samples due to the fact that compared with arthroscopic samples, the tissue obtained is less in both size and sometimes quality, and therefore staining was often not uniform. The maximal number of cells in all the sections visualised was taken as count number. This inevitably leads to the discussion of whether the maximum number of cells obtained from different biopsy specimens and cutting levels is reflective of the diseased tissue status in 1) the biopsied joint and 2) the disease process as a whole. This is further highlighted by the change in results when the tissue samples were recut. Initial analysis suggested that a B-cell rich pathotype could predict response to therapy. This highlights the significant limitation of this sample size and whether synovial tissue should be cut at all possible levels to gain an accurate reflection of tissue staining.

Previous studies have addressed the heterogeneity of immune cell marker expression in synovial biopsies. Scire and colleagues [390] demonstrated that a cumulative area of 2.5 mm^2 from at least three biopsy specimens allows an estimate of cell number within 10% mean for the overall tissue. In this study, a minimum of six (maximum of twelve) ultrasound-guided biopsy specimens were obtained and cut at different levels prior to staining for CD20. Rooney and colleagues also describe considerable immune cell homogeneity within one joint [377], as do Kirkham et al who also looked at variability in immune cell and cytokine expression within the knee joint[391]. The degree of T-cell infiltration within and between biopsy sites has also been explored, with the authors concluding that variation arises predominantly between different pieces of tissue and a reduction in variability could be attained by studying multiple slides[392]. Applying this method by calculating the mean number of cells per slide

to this work was challenging, as limited tissue meant some sections had little or no B-cells. This also proved to be testing when applied to the DIA method as often aggregate staining in a large tissue section resulted in a small area fraction as seen in Figure 3.3. It was here where expert opinion from a consultant histopathologist guided the decision to take the maximal count following the notion that uninflamed synovium does not display a significant B-cell infiltration. The positive correlations observed between SQ scoring which was based upon all tissue sections, MC and the DIA method as described lend support to this decision.

Kraan and colleagues demonstrated in nine patients that synovial biopsies taken from an inflamed knee joint have similar histopathological changes to an uninflamed small joint at the same timepoint, demonstrating similar numbers of immune cell markers in each biopsy[393]. Immune cell infiltration was also witnessed in tissues from clinically uninvolved joints [394] in patients with RA and in Rhesus monkeys induced with RA [395], suggestive that tissue from one joint is likely to be reflective of the disease process as a whole. Furthermore, several studies have reported associations between histological features of inflammation and clinical paramaters disease activity [373, 396-398]. These observations have been extrapolated to this cohort to establish a subgroup of histologically 'B-cell rich patients'.

Finally, in order to further validate a SQ score of 2 I examined whether this subset of patients also had co-existing plasma cells within the synovium. I found that all patients with a score of 2 had an equivalent or higher number of plasma cells present, indicating similar B-cell dependent processes within the joint lining, although it remains unclear if B-cell differentiation occurs *in situ* from autoreactive within the synovium[346, 399] or if similar trafficking processes leads to the co-existence of both cell lines.

In summary, I determined two subgroups within this cohort: 'B-cell rich', determined by a SQ ≥2 and a 'B-cell poor' pathotype determined by a SQ score of ≤1. I have shown that SQ scoring correlates with both MC as the gold standard and the with DIA analysis – but is time-effective, accurate and reproducible. Expert opinion from a specialized histopathologist adds value to this study in that it allows us to cross medical disciplines, reflecting that synovial scoring techniques can be used in 'real-world' NHS settings.

Next, I examined clinical responses to rituximab. In this cohort, rituximab was successful at ameliorating disease as evidenced by an improvement in DAS-28 scores pre and post treatment (p=0.027). Compared with previous trials that reported EULAR good / moderate response rates of 89% at week 48 (MIRROR)[286] and 65% at week 24 (REFLEX) only 41% of patients were deemed to be moderate / good EULAR responders in this cohort. The British Society of Rheumatology Biologics Registry report a 60% attrition rate in 1629 patients, of which 1371 had been exposed to a previous biologic DMARD, with RF positivity being associated with continued therapy [400]. In line with these findings, 16/22 patients (73%) of this cohort were retreated with a further cycle of Rituximab and 14/22 (63%) remained on Rituximab at 52 weeks. This highlights the difficulties in the universal utility of clinical trial outcome data, as in this case treatment was led by physician and patients' opinions of a 'partial response' and knowledge that repeat cycles of rituximab may improve clinical outcome. The differences observed in EULAR response could potentially be explained by the population being reflective of a cohort with long-standing disease duration and high numbers of DMARD and TNF failures. This finding also highlights the limitations of the DAS-28 itself, as high numbers of tender joints persisted in this cohort, which may in part be driven by an

upregulation in pain pathways and not ongoing inflammation itself. In support of this, a recent systematic review showed that baseline characteristics of RA patients in observational studies differed significantly from those enrolled in RCT's with regards to age, disease duration, DMARDs and DAS-28, leading to the conclusion that RCT data may not be reflective of efficacy in the 'real-world' setting[401].

Another reason for the discrepancy in observed response rates may be the limited use of methotrexate, which was only 58% in this cohort. 6/22 (27%) of patients received Rituximab as monotherapy. Early trials supported more favourable outcomes when Rituximab was given in conjunction with methotrexate, supported by a systematic Cochrane review[300]. Recently more observational data is emerging to suggest similar levels of efficacy in those not receiving concomitant methotrexate. In a six-year study of 2484 patients on Rituximab therapy, 22.7% received the drug as monotherapy and achieved similar response rates to those on combined therapy [402]. This is further supported by similar findings [403, 404] in early disease, although higher levels of ACR response were noted in combination therapy. This once again highlights that this cohort is more reflective of registry than clinical trial data, including patients intolerant of cDMARD therapy.

The improvement in DAS-28 was mirrored by an improvement in patient reported outcome measures. Amongst the multitude of patient reported outcomes available, the HAQ has strong predictors of economic outcomes including work disability, loss of income and finally as a predictor of mortality [405-407]. Estimates for the ability to detect change range between 0.02-0.87, although 0.22 is widely accepted figure for meaningful change. [407] There was a mean reduction in HAQ at 12 weeks which continued to 52 weeks in those patients who

stayed on Rituximab therapy. This is in keeping with published registry data [408, 409], although no differences were observed between patients receiving rituximab monotherapy and those taking concomitant methotrexate.

Despite the heterogeneity of synovial subtypes and associated molecular signatures, clinical phenotypes in RA remain similar. B-cell rich or 'lymphoid' predominant with ELS have been observed in 44-58% of patients with up to 25% displaying CD21 positive germinal centres[276, 410]. In this cohort 40% had a B-cell rich pathoype, with 20% showing evidence of aggregates, lower than previously reported, which may be due to the limited sample size. Another important consideration here are studies that report reversal of ELS after the administration of TNF therapy [411, 412]. Given that the patients in this cohort had received varying numbers of cDMARDs and anti-TNF agents prior to Rituximab therapy, it is possible to speculate that this may have resulted in lower numbers of aggregates. Finally, there has been debate over the relevance of ELS at different stages of RA. In early RA it has been postulated that they contribute to the initial break in tolerance, which can either resolve through clearance of the antigen over time or continue to perpetuate inflammation.

In a prospective study, Van de Sande and colleagues observed that no differences in cellular lymphoid infiltration between patients with undifferentiated arthritis, psoriatic arthritis and established RA could be demonstrated, concluding that the presence of ELS was of limited diagnostic value. Furthermore, they noted that the patterns of infiltration were dynamic over time [413]. The patients in this cohort had a long disease duration with universally high levels of disease activity at baseline yet considerable variation of baseline B-cell infiltration. There were no discernible differences in clinical parameters at baseline between the B-cell rich and

B-cell poor groups, supporting the hypothesis that ELS may not play a crucial role in driving systemic inflammation in established disease.

In keeping with previous observations [347, 411, 414] B-cell rich pathotypes were associated with a higher synovitis score and immune cell infiltrates of macrophages, T-cells and plasma cells. Studies have suggested that synovial lymphoid neogenesis has been associated with higher levels of CRP, ESR, TNF[411, 414] although this does not necessarily result in a more aggressive phenotype[414]. I did not demonstrate any significant association between the B-cell rich pathotype and autoantibody status, laboratory markers of inflammation or disease activity. One confounding factor for the lack of any observed associations is that the B-cell rich group were more likely to be on methotrexate and prednisolone, which may in itself be indicative of more aggressive disease. Of interest, 2/4 patients that exhibited lymphoid aggregates in the synovium were seronegative, in line with previous studies suggesting that aggregates do not correlate to circulating levels of antibodies[412] and are seen in seronegative inflammatory diseases such as psoriatic arthritis[413, 415]. None of the samples in this study stained positively for CD21 and therefore I was unable to provide evidence at the histological level that these aggregates displayed any features of active germinal centres.

In line with previous studies [347, 348, 353], I have shown that treatment with rituximab results in a significant decrease in B-cells in the synovium and a strong trend towards decrease in plasma cells but this does not always correlate with response. Interestingly, a decrease in plasma cells did show a trend for correlation with Δ DAS-29 ($p=0.09$). Sequencing studies have shown that clonally expanded B-cells / plasma cells reside within the RA synovium[416] and it is therefore possible that improvement in disease activity results from depletion of autoreactive B / plasma cells. I did not test specifically for correlations of changes in ACPA or

RF levels at 16 weeks, however previously a decrease in plasma cell numbers has been correlated with a reduction of ACPA levels, which may be again be due to the depletion of short-lived plasma cells, providing an alternative explanation for improvement [347]. Rituximab results in B-cell depletion through antibody-dependent cytotoxicity, complement dependent cell death and B-cell apoptosis [280, 319], yet its precise mechanism of action in ameliorating clinical disease remains incompletely understood. This work adds support to the observation that B-cell depletion therapy within the synovium is unlikely to be the most important mechanism in response and disproves the hypothesis that those with a lymphoid or 'B-cell rich' pathotype at baseline are more likely to respond to therapy.

A reduction in CD68SL macrophages has previously been reported to as a biomarker of clinical response in the synovium [158, 160]. This change has been observed in response to several cDMARDs and bDMARDS, including prednisolone, gold, methotrexate, leflunomide and infliximab[159, 417, 418]. Whilst I observed a reduction in CD68SL in the group as a whole following Rituximab therapy, this change was not significantly different between responders and non-responders. This may again be attributable to the small sample size and the limited number of responders at the 16-week timepoint. The data does, however support the notion that the synovium is sensitive to changes induced by therapy and that subintimal macrophages may play a role in a common pathway that leads to disease progression or amelioration.

This work is limited by the small sample size and numbers of paired synovial biopsies available. It is reflective of 'real-world' data as patients had long-standing disease and had failed a number of different therapies. The concomitant use of prednisolone may also have had an effect on the changes seen within the synovium. It would have been interesting to

collect more data on reduction in rheumatoid factor and ACPA. (Flow cytometry data has not been included in this work as it is forming part of a different thesis which is currently being undertaken by another student in our laboratory).

Taken together however, this data illustrates salient findings, many of which support the current literature. Despite marked B-cell depletion in the peripheral blood, synovial B-cells persist in most patients and indeed depletion is variable. This may be due to local chemokines which remain active in the synovial compartment, promoting the formation of germinal centres and subsequent "resistance" to therapy. Decrease in other cell types suggests that B-cells may be key players in mediating synovial inflammation and that somehow a reduction in circulating pathogenic B-cell results in a secondary reduction of macrophages and plasma cells. Additionally, the results presented here are in line with the other synovial- biopsy based studies, showing that minimally invasive ultrasound-guided synovial biopsies are able to reproduce comparable tissue and similar findings. In summary, these findings show that B-cell repletion is variable and does not correlate with clinical response. These findings suggest that immunohistochemistry alone is not sensitive enough to act as a baseline biomarker for response to B-cell depletion.

Chapter 4

Rituximab: Molecular effects on the synovium

4.1 Introduction

The use of genomic and metabolomic technologies are creating new opportunities to allow more targeted treatment in RA. The advent of high throughput technologies in transcriptomics, metabolomics and proteomics is both exciting and promising for both the understanding of RA pathogenesis and response to therapy. Currently, however, clear molecular markers of response have not been identified for utilisation in the clinical setting. Elucidating biomarkers of response to rituximab therapy remains a therapeutic challenge. Response to therapy is measured over an extended time period during which time there is continuation of disease activity and joint damage in those who fail to respond. Additionally, there is a risk of exposure to unwanted side effects such as anaphylactic reactions, blood dyscrasias and a reduction of circulating immunoglobulins. Rapid recognition of molecular subtypes, akin to certain malignancies, that may predict response to different therapies would transform the RA landscape. The first step towards this process relies on the identification of molecular markers, both in blood and synovium.

Several studies have focused on the identification of response gene signatures in peripheral blood. The first whole-genome microarray expression profiling study assessing B-cell depletion therapy was conducted in 2009 by Julia and colleagues [419] who took RNA from whole blood, B cells and CD4+ T-cells from 9 patients treated with Rituximab followed by validation with real-time PCR for identified genes. They identified seven genes in whole blood which differed between responders and non-responders according to the relative improvement DAS score. Whole blood overexpression of *ARG1*, an important modulator of immune response through the nitric oxide pathway and subsequent downstream production

of citrulline, was associated with non-response to Rituximab. Overexpression of carboxypeptidase D that cleaves arginine residues was also associated with non-response. The authors also demonstrate a high TRAF1:ARG1 ratio in whole blood and overexpression of TLR4 in CD4+ T-cells correlate with response.

To date, the type 1 interferon response signature is the most widely reported biomarker of response to Rituximab therapy in peripheral blood gene expression studies. The expression of 3 IFN response genes was associated with a poor clinical response in 20 patients[420] and further validated in a genome-wide microarray study in 14 patients where a low level of IFN-type 1 gene signatures associated with a good clinical response[421]. Furthermore, individual patients have differential regulation of type 1 interferon response following B-cell depletion therapy whereby an increased in IFN type 1 response gene confers better clinical outcomes[422]. This leads to two putative mechanisms of resistance to rituximab therapy; firstly that those with high interferon signatures have a disease regulated by pathways not controlled by B-cell activation and processes, or that high interferon activity associates with resistant pathogenic B-cell subsets that survive in niches in the bone marrow or synovium and continue to promote inflammation [421, 422]. Recently, a multi-parameter response model to Rituximab therapy has been described including eight interferon genes to form a baseline interferon score which has an AUC of 0.82, thus highlighting the potential for personalised medicine based upon gene expression data.[423]

Sellam and colleagues identified a molecular signature of 143 genes which correctly classified 89% of EULAR responders at 24 weeks. They again found that down-regulation of the interferon pathway associated with response. Upregulation of inflammatory genes including

IL33, STAT5A was noted in responders. B-cell regulatory and functional genes were downregulated in the whole cohort but with no significant difference in clinical outcomes, reflective of CD19 depletion in peripheral blood not correlating with response to therapy [424].

In another study, downregulation of non-tissue specific regulatory genes MTOR, ULK1, p21, caspase 3m TNFα and IL-1β we associated with an improvement in clinical and laboratory outcomes after one cycle of Rituximab therapy[425]. Treatment with Rituximab induced a significant decrease in MTOR and MMP-9 levels.

Studies assessing synovial gene and transcriptional profiles have also identified particular signatures or processes that associate with response. Kavanaugh and colleagues used pre-selected genes in 13 patients from the ARISE trial which demonstrated no change in synovial expression of TNFα, IL-6, or MMP1 but witnessed a change in IL-8 pre and post Rituximab treatment, although this change was noted more in non-responders.[348]

Analysis of the synovial transcriptional profile in 20 patients using a GeneChip microarray revealed 549 differentially expressed genes before and after B-cell depletion therapy[426]. Downregulated genes were enriched in immunoglobulin genes, immune responses and leucocyte activation, whereas genes which control developmental processes, wound healing and cell development were upregulated. Of interest, this pattern of upregulation is similar to those seen in response to TNF inhibition with adalimumab and iL-6 inhibition with tocilizumab [427, 428] indicating that perhaps response to therapy is linked to the balance of active inflammation versus tissue repair and restoration. The authors also found that a higher baseline synovial immunoglobulin gene profile and baseline IgG levels associated with

response to therapy, lending support to findings that patients with RF and ACPA positivity with B-cell driven immune responses have favourable outcomes.

Finally, a study of synovial gene expression using high throughput quantitative real-time PCR in 20 RA patients showed two distinct pre-treatment populations characterised by different expression of immune response genes and remodelling / interferon α genes which associated with response[429]. The authors of this study used 16 genes which correlated most strongly to the three month change in DAS-28 to construct a composite baseline gene score which correlated with both early and late response to treatment, ESR, pain score and the histological thickness of the synovium lining layer. Those who had higher baseline macrophage and T-cell genes were more likely to respond, whereas those who had genes associated with remodelling and type I IFN had poorer outcomes. Distinguishing a pre-treatment synovial gene profile in this case was able to discriminate between clinical outcomes based upon EULAR response criteria.

The observation that there appear to be at least two patterns of synovial tissue gene expression; broadly, high inflammatory and low inflammatory, correlates to different patterns of synovitis that have been previously been described[430]. The high inflammatory group may be defined by genes related to the myeloid or lymphoid inflammatory compartments whereas the low inflammatory may reflect the 'diffuse / pauci-immune' group[369, 431].

To date, there has been no study exploring specifically whether the presence of ELS or lymphoid associated genes affects outcomes in patients treated with B-cell depletion therapy.

Two studies have suggested that a synovial gene signature characterised by lymphoid inflammatory pathways did not associate with response to the anti-TNF agents infliximab or adalimumab [356, 428]. At the tissue level, however, there are conflicting reports on the presence of lymphoid gene signatures (including ELN) and response to therapy[411, 412].

The functionality of ELS in the synovium has been demonstrated by our group by a series of elegant experiments described by Humby at al[346]. Activation-induced cytidine deaminase (AID), encoded by the *AICDA* gene is required for ongoing somatic hypermutation and class-switch recombination (CSR) of immunoglobulin genes[432], as detailed in the introductory chapter. Humby and colleagues demonstrated that AID was only expressed in tissues with CD21 positive FDC networks and particularly in GC B-cells, confirmed by quantitative PCR. AID expression was also noted in the cytoplasm of large interfollicular B-cells. Furthermore, AID was associated with CXCL13 and LTβ expression and the presence of ACPA producing plasma cells around the periphery of GCs, suggesting a role for local cell differentiation within the synovium. Finally, continued AID expression, B cell proliferation and production of human ACPA when GC positive synovial tissue was transplanted into severe combined immunodeficiency (SCID) mice provided further evidence that GCs are capable of sustaining chronic inflammation in the absence of peripheral B-cells. Bugatti and colleagues, in conjunction with our laboratory, also demonstrate a correlation between CXCL13, LTβ and AID expression. They demonstrate an association between the presence of active follicles and abnormal bone remodelling, but did not find an association with clinical parameters[399]. Further evidence for functionality of ELS is evidenced by recombinant antibodies generated from B-cells within ACPA positive synovial ELS that can target citrullinated proteins and

recognise neutrophil extracellular traps from peripheral blood or synovial neutrophils, thus perpetuating the immune response within the synovium[433].

A study performed on peripheral blood showed significantly higher levels of *AICDA* expression in patients with RA with a positive correlation with serum RF, ACPA, IFNγ and IL-17[434]. The same group also demonstrated AID in the synovium of patients with GCs in RA, but not in the synovium of patients with osteoarthritis. The critical role of AID is further highlighted by the observation that overexpression in the BXD2 mouse model of arthritis resulted in increased germinal centre activity and the production of pathogenic autoantibodies, which could be blocked by inhibiting the CD28-CD86 interaction[435]. Inhibition of the functional activity of AID resulted in decreased GC formation and autoantibody production[436].

Conflicting with this data, Cantaert and colleagues report the presence of GCs occurring in similar frequencies in both seronegative and seropositive individuals and report similar ACPA levels in SF regardless of the inflammatory pathotype[437]. IgVh sequencing demonstrated multiple clonally related sequences, but of these only one sample was suggestive of antigen driven B-cell maturation and in situ somatic hypermutation, occurring independently of synovial lymphoneogenesis. This is supported by a previous study which demonstrated that local plasma cells were not clonally related to intrafollicular B-cells related sequences despite the identification of similarly expanded clones within the CD20+ B-cell population[438]. This could lead to the conclusion that despite B-cell clonal expansion within the synovium, only a few cells take part in the GC reaction. It is important to note, however that the latter study only used synovial tissue from one patient with RA.

Whether ELS in the synovium are related to the degree of inflammation or whether they act as fully functioning secondary lymphoid organs perpetuating the immune response remains unclear. I have demonstrated that in the present cohort, the histological presence of synovial B-cells or ELS did not discriminate between response and non-response to Rituximab therapy. This may be due to small sample size, variation in synovial sampling or that immunohistochemical analysis may not be a sensitive marker to change, which I have discussed in the previous chapter. In order to explore the molecular effects of Rituximab, and in particular the effects of ELS, the expression of AICDA and B-cell associated genes I examined whether differences in gene expression would provide a more sensitive marker of clinical outcome.

4.2 Hypothesis and aims

The hypothesis of this experimental chapter was that patients with a 'B-cell rich' molecular signature, characterised by the expression of ELS associated genes, would aid stratification of response to therapy.

The specific aims were to:

1. Assess the global molecular effects of rituximab on the synovium by identifying significant changes in gene expression pre and post therapy
2. Assess if there are any differences in gene expression in responders and non-responders
3. Create a 'B-cell rich' molecular signature based upon genes which are known to be involved in B-cell developmental processes and in the development of ELS
4. Ascertain whether the expression of *AICDA* affects response to B-cell depletion therapy
5. Utilise the findings to create a unique prediction model for response or non-response to therapy

4.3 Methods

4.3.1 Study Population

A subset of 20 patients from the original cohort described in Chapter 3 who had paired biopsies with >1ug of RNA available were chosen for this analysis.

4.3.2 RNA extraction

Synovial samples were homogenised in TRIzol (Invitrogen, Carlsbad, California USA) using a power homogenizer following shearing with a 26 gauge needle. Total RNA was isolated as described in the methods chapter and stored at -80°C. All RNA samples were quantified using a spectrophotometric analysis performed by a Nanodrop-1000 v.3.2.1 Spectrophotometer (Thermo Scientific). RNA integrity was determined by electrophoresis on tissue samples using the Agilent 2100 Bioanalyser. (Agilent Technologies, California, USA.)

4.3.3 High throughput quantitative real-time PCR

High throughput quantitative real-time PCR for 190 genes, pre-selected according to known roles in RA / B cell biology was performed using the Fluidigm© platform (Fluidigm Corporation, South San Francisco, California, USA) as per manufacturer's protocols. Per sample Ct values were converted to ΔCT values for genes using *GAPDH* and *UBC* as controls. To normalise across all samples, ΔCT values were converted to $\Delta\Delta$CT values by subtracting the mean ΔCT of each gene across all samples and then to a negative $\Delta\Delta$CT. Genes that fell under the lower 10% bracket of the variance range (cut-off $\delta 2$=4.007) were then removed to reveal a uniform dataset. In total, 69 genes were removed. A full list of genes is available in Appendix A.

4.3.4 Statistical analysis

B-cell rich and poor populations were identified by histopathology scoring or by splitting the *MS4A1* Fluidigm gene expression range in half, with the lower half being defined as $MS4A1^{lo}$ and $MS4A1^{hi}$ populations. Fishers' exact test was used to compare molecular and histological classifications. For identifying markers of response to rituximab in 16 week EULAR responders versus non-responders, we fitted a linear model to the expression values at baseline, firstly comparing B-cell rich and B-cell poor using both histological and molecular parameters and subsequently responders and non-responders by changes in gene expression at 16 weeks. Statistical values were then adjusted using the empirical Bayes approach. Genes which segregated with *MS4A1* were identified as B-cell signature genes. The baseline B-cell signature genes ($p<0.05$) were then used to conduct a regression and correlation analysis with 16 week ΔDAS-28 as the dependent / response variable. Spearman correlation coefficients and their associate p-values were used to determine B-cell signature genes that significantly correlated with response. The signature was then extended to create a further model based upon the expression of *AICDA*, with samples segregated into tertiles as follows: $MS4A1^{lo}$, $MS4A1^{hi}AICDA^{lo}$, and $MS4A1^{hi}AICDA^{hi}$ according to levels of mean *MS4A1* and *AICDA* expression across the whole cohort.

All clustering and heatmap generation was performed using the heatmap.2 (gplots) function in R Programming Language (R), with either Euclidean or correlation distance and Ward's linkage. To define B-cell signatures at baseline, linear models were used using limma and ebayes. A likelihood ratio test was conducted to develop a signature at baseline for the three groupings based on the levels of expression of *AICDA* and *MS4A1*. Correlation analysis was conducted using the cor.test function in R. Also in R, linear modelling was performed using

the km function, with the linear fit plotted using ggplot2, comparing the actual versus predicted 16 week ΔDAS-28. Unless stated, the cut off for significance was $p<0.05$ for all tests. This part of the analysis was conducted by Kevin Blighe, biostatistician at the William Harvey institute, Queen Mary University.

4.4 Results

4.4.1 Patient Demographics

22 samples were used for baseline gene expression data, clinical characteristics of these patients are described in the previous chapter. Clinical characteristics of 20 patients for whom paired biopsies were available with sufficient quality RNA are described in Table 4.1. As in chapter 3, there were no significant differences between responders and non-responders at baseline. Of these 20 patients with paired samples, 8/20 were EULAR good / moderate responders.

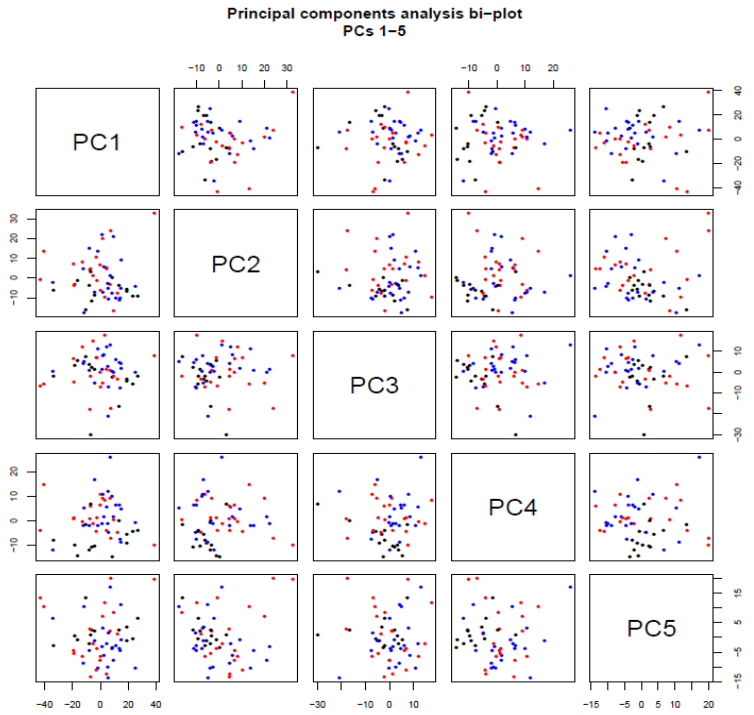
Table 4.1 Demographics and clinical characteristics of patients selected for molecular analysis

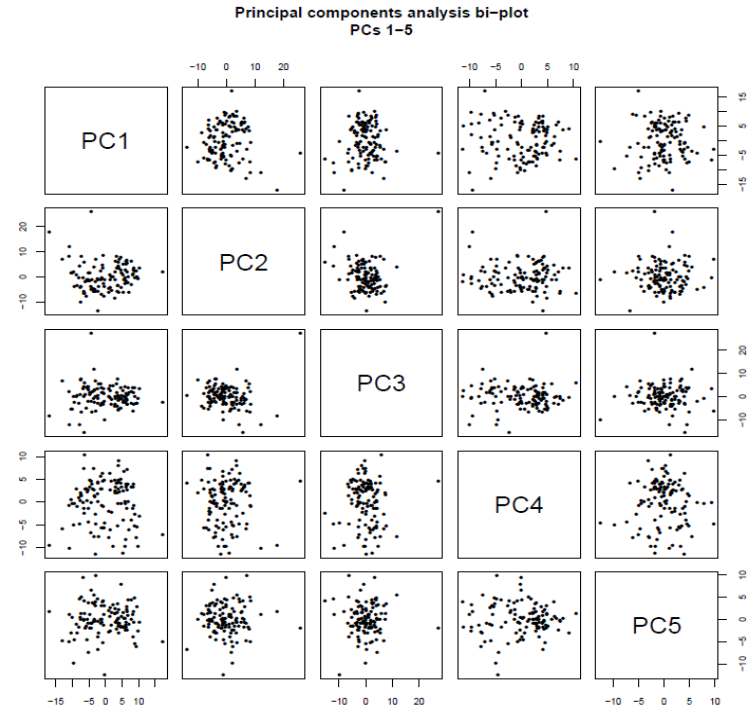
Age (yrs) mean ± sd	52.4±14.36
Female, n, (%)	16 (80)
Median disease duration months (IQR)	99 (24 -120)
RF positive, n, (%)	17 (85)
ACPA positive, n, (%)	17 (85)
ESR mm/hr median (IQR)	29 (9-59)
CRP mg/l mean ± sd	9 (0-18.75)
DAS-28 mean ± sd	5.64 (1)
SJ median (IQR)	6 (3-8.75)
TJ median (IQR)	11 (7.25-20.5)
VAS mean ± sd	63.45 ± 23.88
HAQ mean ± sd	2.07 ± 0.75
Erosive≥1 joint n, (%)	16 (80)
ST, median (IQR)	26 (23-30)
PD, median (IQR)	3 (2-6)
Median number of previous anti-TNF drugs, IQR	1 (0-4)
No of patients receiving Rituximab as first line biologic therapy, n (%)	6 (30)
No of patients on methotrexate n (%)	11 (55)
Methotrexate dose, median (IQR)	5 (0-17.5)
No of patients on prednisolone, n	10 (50)
Prednisolone dose, median (IQR)	2.5 (0-10)

Abbreviations RF=rheumatoid factor, ACPA= anti-citrullinated protein antibody, ESR = erythrocyte sedimentation rate, CRP = c-reactive protein, DAS-28= 28 joint count disease activity score, HAQ = Health assessment questionnaire, SJ = swollen joints, TJ = tender joints, VAS = visual analogue score, ST = synovial thickening, PD = power Doppler, EULAR = European union league of action against rheumatism, TNF = tumour necrosis factor, IQR interquartile range

4.4.2 Principal component analysis

Principal component analysis is used to explore large datasets by reducing dimensions to a few principal components that explain patterns[439]. Transcriptomic data is high dimensional, with a large number of genes in proportion to sample size, with only a few genes expected to correlate with the scientific question; PCA therefore reduces noise making the data more suitable for analysis[440]. The PCA plots in Figure 4.1 (A) show that there was little variation between samples – i.e. no outliers. Figure 4.1 (B) also demonstrates little variance between genes, with no outliers identified.





A B

Figure 4.1 Principal components analysis of (A) samples and (B) genes. No outliers were identified

4.4.3 The global molecular effects of rituximab

Following treatment, there was a significant change in 14 genes pre and post rituximab therapy ($p<0.05$), summarised in tables 4.2 and 4.3. Overall, there was significant downregulation of the B-cell associated gene *FCGR1* as well as a significant number associated with macrophage and dendritic cell signalling and activation (*CD163, CLEC4E, CD36*). *IL-2RA*, *IL-15* and *IL-1*β were also downregulated. Rituximab also significantly decreased expression of the T-cell associated chemokine *CCR5* and *CD96*, a specific T cell receptor protein. *CXCL10* expression, secreted in response to IFN-γ was also reduced. Decreased synovial expression of the angiogenesis related genes *MMP9, VEGFC* and *CD36* was also noted post Rituximab treatment. The fibroblast growth factor 1 gene, *FGF1* was also significantly upregulated following treatment. These results support previous observations documenting that rituximab downregulates genes involved in B and T cell immune responses and chemotaxis whilst inducing fibroblast genes involved in reparative processes[426, 429].

Table 4.2 Downregulated genes following rituximab treatment

Gene	Chromosome	p-value	Function
MMP9	20q 13.12	0.00365	Matrix metalloproteinase
CD163	12p13.31	0.00422	Macrophage marker, inducer of local inflammation
CCR5	3p21.31	0.00558	C-C chemokine receptor 5 Expressed by T-cells and macrophages
CD96	3q13.13-q13.2	0.00558	Member of immunoglobulin superfamily Antigen presentation, T and NK immune responses
CD11c	16p11.2	0.00831	Neutrophil and monocyte adherence, complement phagocytosis pathways
CLEC4E	12p13.31	0.0121	Cell adhesion, cell signalling, glycoprotein turnover
CD36	7q21.11	0.0136	Platelet surface receptor, cell adhesion
IL-15	4q31.21	0.0136	Cytokine, T-cell and NK cell proliferation
VEGFC	4q34.3	0.0327	Vascular endothelial growth factor
CXCL10	4q21.1	0.0362	CXCR3 receptor ligand Stimulates monocytes, NK and T cell migration and modulation
IL-1B	2q14.1	0.0262	Cytokine produced by activated macrophages
FCGR1A	1q21.2	0.0399	Fc gamma receptor
IL-2RA	10p15.1	0.0484	Subunit if IL-2 receptor

Table 4.3 Upregulated genes following rituximab treatment

Gene	Chromosome	p-value	Function
FGF1	5q31.3	0.044	Fibroblast growth factor 1 Cell survival and tissue repair

4.4.4 Response is associated with a reduction in macrophage associated genes and upregulation of reparative genes

Samples were then segregated into EULAR responders and non-responders at 16 weeks. In the responder group there was a significant reduction in relative expression of *CD163* and *CCL4*, macrophage associated genes. There was a significant upregulation of *FGF1, VEGFR3, PAD2* and *RORγT* in this group. *FGF1* and *VEGFR3* are genes associated with angiogenesis and wound healing, and it may therefore be the latter properties that contribute to clinical improvement. The relevance of *PAD2* and *RORγT* are discussed later on in this chapter.

Table 4.4 Upregulated genes in responders following Rituximab treatment

Gene	Chromosome	p-value	Function
FGF1	5q31.3	0.0078	Fibroblast growth factor 1 Cell survival and tissue repair
VEGFR3	5q35.3	0.016	Cell surface receptor tyrosine kinase associated with fibroblast growth factors
PAD2	1p36.13	0.023	Post-translational citrullination of proteins
RORγT	1q21.3	0.023	Important role in lymphoid organogenesis, Th17 differentiation

Table 4.5 Downregulated genes in responders following Rituximab treatment

Gene	Chromosome	p-value	Function
CD163		0.023	Fibroblast growth factor 1 Cell survival and tissue repair
CCL4		0.039	

4.4.5 High baseline expression of genes regulating B-cell differentiation, T-cell and TH17 responses and cell signalling significantly associate with a diminished delta DAS-28 response.

I went on to examine whether baseline synovial gene expression could identify subsequent EULAR responders vs non-responders at 16 weeks. I searched for significant correlations between the genes examined at baseline and changes in DAS-28 between baseline and 16 weeks. Figure 4.2 displays the 11 genes identified at baseline with significant correlations with Δ DAS-28.

High baseline expression of genes regulating B-cell differentiation *(CD19, LTA, CXCR5, IL-15, PAX5, IL-21*), T-cell and Th17 responses (*TBX21, CD96, IL-23A, IL-21, IL-15*) and cell signalling (*CLEC4E, VEGFR3*) were associated with a diminished Δ DAS-28 response.

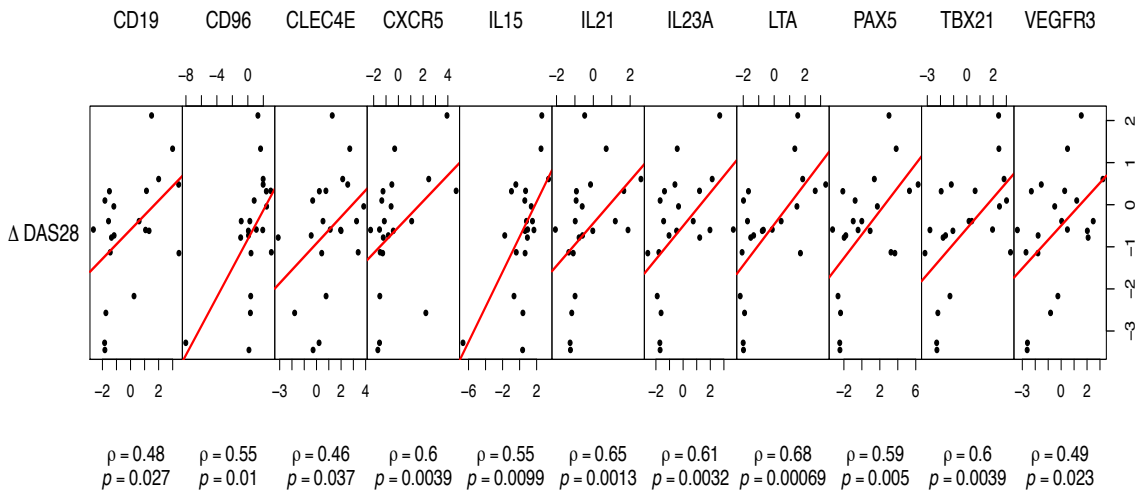


Figure 4.2 Baseline predictors of response to rituximab

High expression of 11 genes (*CD19, CD96, CLEC4E, CXCR5, IL15, IL21, IL23A, LTA, PAX5, TBX21, VEGFR3*) predicted 16-week Δ DAS-28 response. Δ DAS-28 scores are calculated as change from baseline; a fall in DAS-28 correlates with response to therapy. Gene expression is calculated using the mean of the tissue and expressed as relative change from the mean. P-values for each gene are derived using Spearman rank correlation, significance p<0.05.

4.4.6 Histological B-cell rich classification correlates with *MS4A1* expression

To add further validity to the B-cell scoring methods described in the previous chapter, I investigated whether the histological CD20 classification correlated with *MS4A1* expression at baseline. *MS4A1* expression was higher in the histological B-cell rich group than at baseline. In the B-cell poor group, *MS4A1* relative expression was 0, or the same as the average of all genes. At 16 weeks, *MS4A1* expression was similar in both B-cell rich and poor, and reduced overall from baseline (figure 4.3).

Unexpectedly, the reduction of *MS4A1* expression was reduced more in the B-cell poor group which may be in part explained by the greater variation observed in that group as a whole, or

the inference that synovial B-cell depletion is incomplete and that those with B-cells in germinal centres are resistant to depletion at the histological and molecular level.

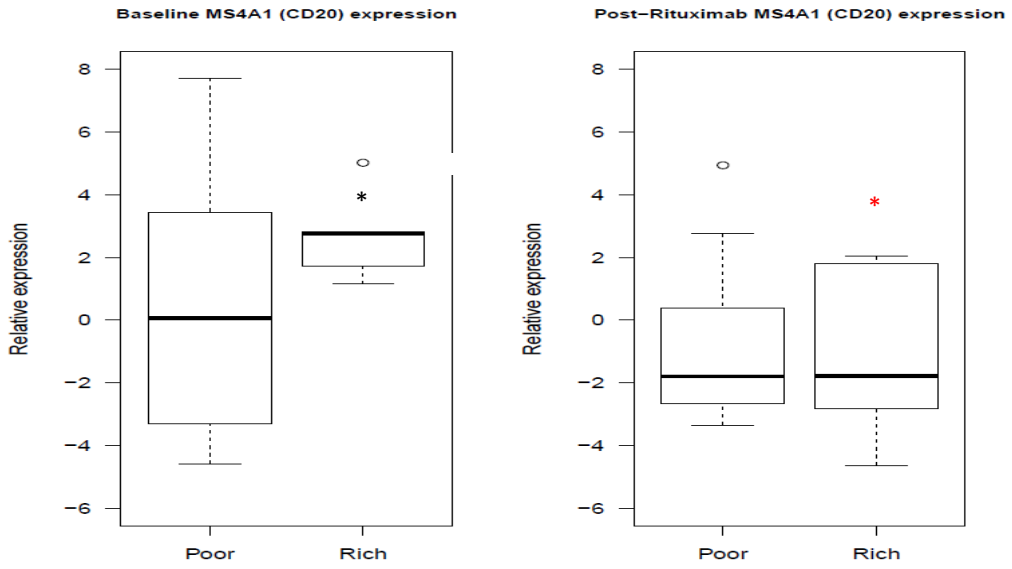


Figure 4.3 MS4A1 (CD20) expression pre and post rituximab therapy in samples classified as B-cell rich or poor by histology

Solid black lines represent the median expression of *MS4A1* in synovial tissue, boxes represent the upper and lower quartiles and lines represent the range. *p<0.05 when comparing the mean *MS4A1* expression in the histological B-cell rich group, *p<0.05 when comparing the change in *MS4A1* expression from baseline. ° represents outliers.

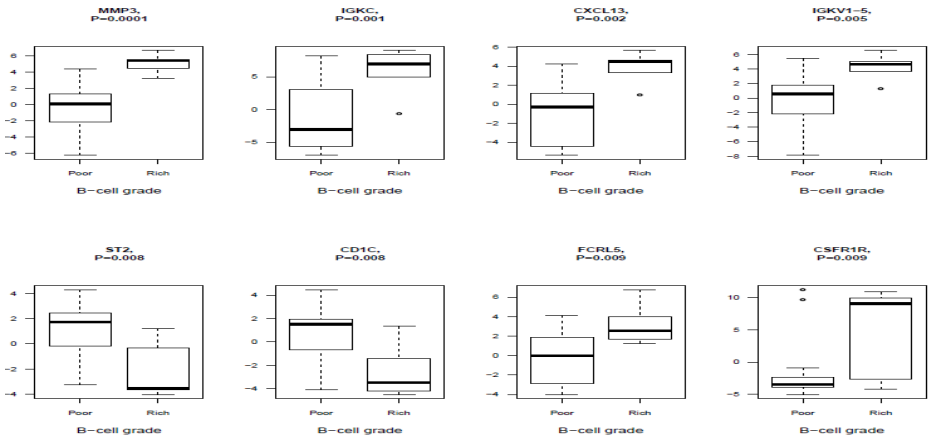
4.4.7 There is a high level of concordance between histological staining and CD20 gene expression

There was a significant association between the molecular and histological grading (p=0.0003, Fishers' exact test). Eight patients were deemed to be B-cell rich according to the CD20 histological score. All of these samples were categorised into tertiles and had high or intermediate levels of *MS4A1* expression. Only one patient sample was deemed to be *MS4A1*^{hi} but had been graded as B cell poor on histology. For the last sample that was graded as B-cell rich as per molecular classification the RNA was of adequate quality but the fixed synovial tissue was ungradable for IHC staining and therefore no comparison could be made. The high level of concordance between molecular CD20 expression and that seen on histology suggests that tissue collected via US guided biopsies is reflective of molecular processes.

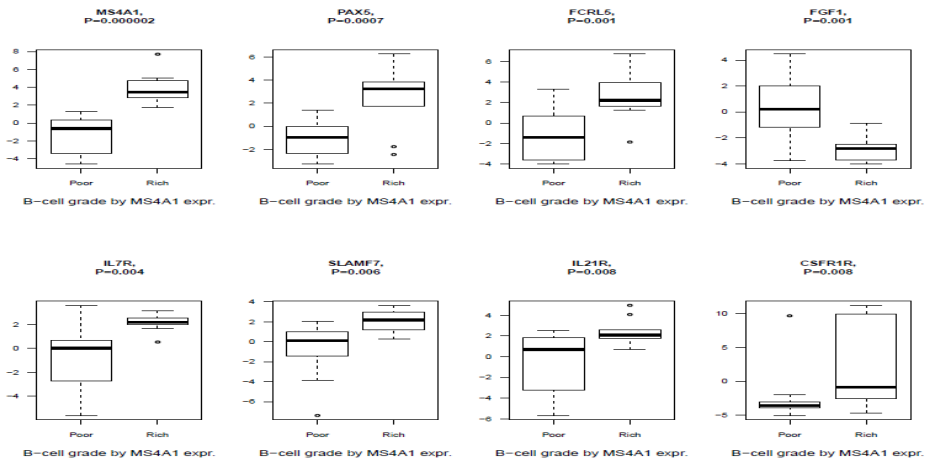
4.4.8 Genes involved in B-cell maturation, signalling and ELS formation are differentially expressed between *MS4A1*^{hi} and *MS4A1*^{lo} groups

Patients were first stratified according to the mean tissue expression of the CD20 molecular marker *MS4A1,* into *MS4A1*^{hi} or *MS4A1*^{lo} groups and significant differences in gene expression between the two groups examined. 18 genes were identified (table 4.7) as being significantly differentially expressed between the *MS4A1*^{hi} and *Ms4A1*^{lo} groups. A significant proportion of these genes (Benjamini *P*=0.0000034) were directly involved in ectopic lymphoid structure formation, B-cell maturation and signalling. Figure 4.4 illustrates the differential expression of the top 8 genes in each group, figure 4.5 demonstrates the classification via a heatmap, illustrating expression between the *MS4A1*^{hi} and *MS4A1*^{lo} groups.

Figure 4.4 Differential expression of genes between histological and molecular B-cell rich groups



A The top 8 genes segregating with CD20 (*MS4A1*) expression in the 'B-cell rich' group based on histological CD20 score. Solid black lines represent the median with boxes indicate the upper and lower quartiles, lines represent the range; °signifies outliers



B The top 8 genes segregating with CD20 (*MS4A1*) expression in the 'B-cell rich' group based on the molecular CD20 score. Solid black lines represent the median with boxes indicate the upper and lower quartiles, lines represent the range; °signifies outliers

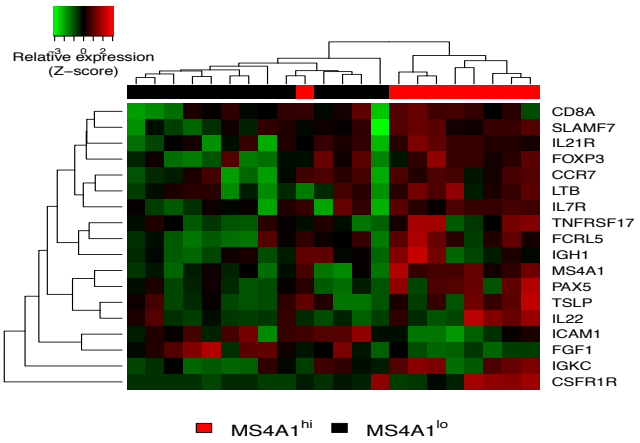


Figure 4.5 Heatmap of significant baseline genes associated with MS4A1 expression, classifying patients into MS4A1^{hi} and MS4A1^{lo}

18 genes were significantly associated with *MS4A1* expression at baseline through a linear model fig using a p-value cut-off of 0.05 for clustering and heatmap generation.

Table 4.6 18 genes segregate with MS4A1 expression in the molecular B-cell rich group

Gene	Chromosome	p-value	Function
PAX5	9p13.2	0.00073	B-cell specific activator protein
FCRL5	1q23.1	0.0013	Fc receptor like protein, involved in B-cell development and differentiation
FGF1	5q31.3	0.0013	Fibroblast growth factor 1 Cell survival and tissue repair
IL7R	5p13.2	0.0039	Receptor for IL-7, involved in lymphopoeisis
SLAMF7	1q23.3	0.0063	Th1 specific transcription factor that regulates IFNγ production
IL21R	16p12.1	0.0082	Receptor for IL-21
CSFR1R	1q.32	0.0084	Macrophage and monocyte specific growth factor
CCR7	17q21.2	0.0098	Homing of T-cells in peripheral lymphoid organs
IGKC	2p11.2	0.015	Immunoglobulin kappa constant, encodes the constant domain for kappa light chains
TNFRSF17	16p13.13	0.015	B-cell maturation factor
FOXP3	Xp11.23	0.026	Development and function of Tregs and expressed by some Bregs
IGH1	14q32.33	0.031	IgG heavy chain locus
ICAM1	19p13.2	0.036	Inducible glycloprotein of immunoglobulin family
CD8A	2p11.2	0.037	T-cell antigen
LTb	6p21.33	0.038	Produced by lymphocytes, involved in cell priming and ELS formation
IL22	12q15	0.044	Activates signal transduction molecules in immune processes
TSLP	5q22.1	0.007	Activates maturation of dendritic cells and T-cell cytokines

4.4.9 In the *MS4A1^{hi}* group, genes involved in ELS formation are associated with a diminished response to rituximab

I then focused only on the *MS4A1^{hi}* group and determined whether any genes were significantly associated with clinical response to Rituximab. Figure 4.6 demonstrates a heatmap of the top 6 genes (p<0.02) (*MS4A1, CXCR5, AIDCA, IL21, IL23A, VEGFR3*) which segregate between EULAR responders and non-responders. A p-value of 0.02 was chosen in this case to demonstrate the most strongly correlated genes. Figure 4.7 shows the top 8 genes (p<0.05) and the correlation of Δ DAS-28; higher expression of these genes (*AICDA, BLK, CXCR5, IL21, MS4A1, VEGFR3, WNT10AI*) results in a diminished response to Rituximab therapy.

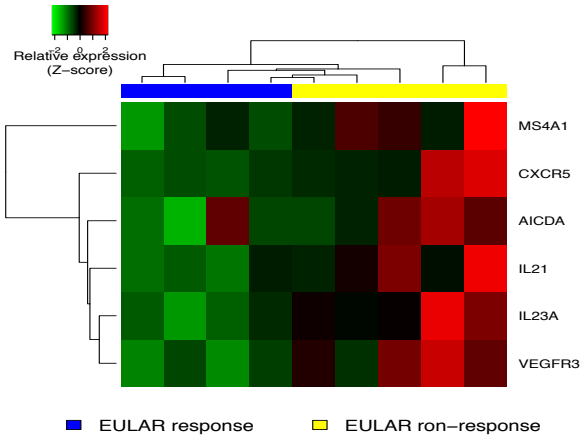


Figure 4.6 Heatmap demonstrating the top six significant baseline genes associated with EULAR response (p=value cutoff 0.02) in the focused *MS4A1^{hi}* group: *MS4A1. CXCR5, AICDA, IL21, IL23A, VEGFR3*

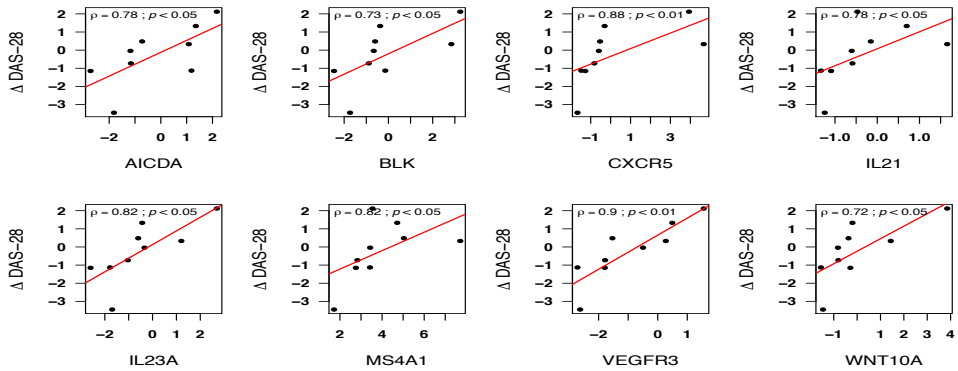


Figure 4.7 Baseline predictors of response to therapy in the MS4A1^{hi} group

High expression of 8 genes involved in B-cell maturation and ELS formation (*AICDA, BLK, CXCR5, IL21, IL23A MS4A1, VEGFR3, WNT104*) is associated with non-response to rituximab at 16 weeks in the MS4A1^{hi} subgroup. Δ DAS-28 scores are calculated as change from baseline; a fall in DAS-28 correlates with response to therapy. Gene expression is calculated using the mean of the tissue and expressed as a relative change from the mean. P-values for each gene are derived using Spearman rank correlation.

4.4.10 A baseline *MS4A^{hi}AICDA^{hi}* synovial gene signature predicts non-response to rituximab therapy

Given previous data identifying AID as a critical mediator of synovial B cell aggregate functionality and furthermore the significant association of AICDA with non-response to rituximab identified in this cohort I went on to investigate whether synovial baseline expression of AICDA could be applied to discriminate responders vs non responders to rituximab. Patients were divided at baseline into three groups: 1) *MS4A1^{hi}AICDA^{hi},* 2) *MS4A1^{hi}AICDA^{lo}* and *MS4A1^{lo}* (figure 4.8). We then identified significantly differentially expressed genes between these 3 groups and found that this provided a natural separation through clustering. For each group, correlating their baseline expression against their

respective 16 week Δ DAS-28 and taking significant correlates, it was possible to segregate 16-week responders and non-responders through groups, although this was not possible for the *MS4A1^{lo}* group.

The *MS4A1^{hi}AICDA^{hi}* correlated signature genes were then applied to the whole cohort of patients at baseline, irrespective of grade or level of expression of any gene. A ROC analysis of clinical response at 16 weeks based on this signature achieved an AUC of 0.86 suggesting that response to rituximab therapy can also be segregated by the *MS4A1^{hi}AICDA^{hi}* gene signature across the whole cohort.

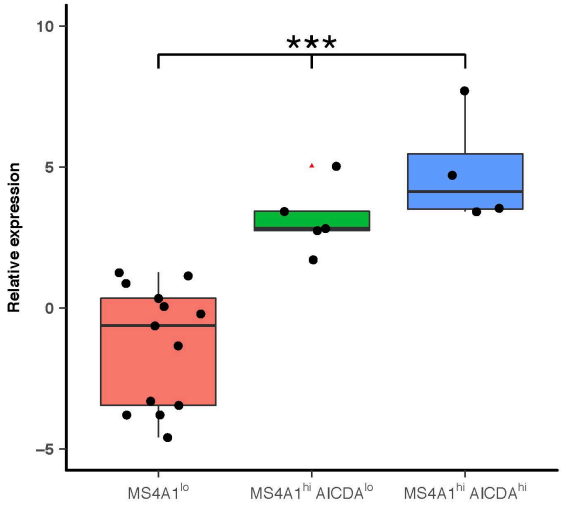


Figure 4.8 Segregation of cohort into MS4A1^{hi}AICDA^{hi}, MS4A1^{hi}AICDA^{lo} and MS4A1^{lo} groups

Scatter plots with box plot overlays (median with upper and lower quartiles) showing the segregation of groups by MS4A1 / AICDA expression ***p<0.001

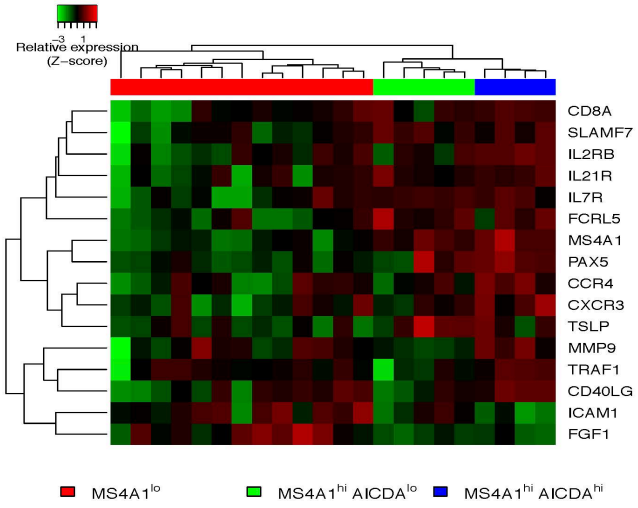


Figure 4.9 Clustering and heatmap showing the natural segregation of groups into three, with the top 16 genes by likelihood ratio test segregating each group.

Heatmap colour key: red indicates high gene expression when compared to the overall mean of the tissue; green correlates with decreased expression from mean score

4.4.11 A prediction model can be constructed using a *MS4A1^{hi}AICDA^{hi}* gene signature

Finally, we created a linear model predicting 16 week Δ DAS-28 and found that using the MS4A1^{hi}AICDA^{hi} gene signature it is possible to predict response at 16 weeks (adjusted R 0.865).

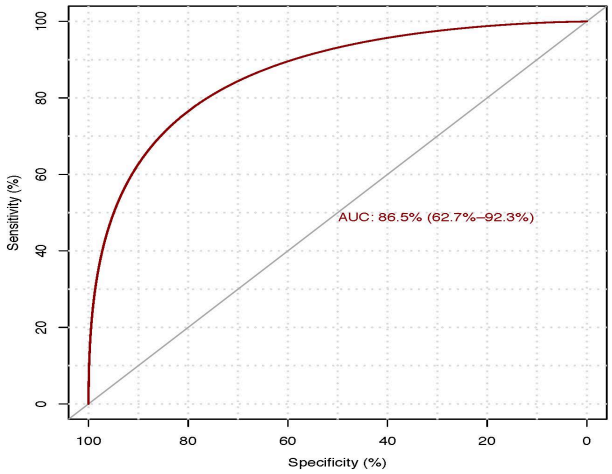


Figure 4.10 Receiver operating characteristic analysis to predict EULAR response.

Using a subset of genes(*MS4A1, SLAMF7, IL-2RB, FGF1, PAX5, FCRL5, IGKV1, TRAF1, IL-17R, IL-21R, MMP9 and CD8A*) that segregated MS4A1 / AICDA grouping through clustering an AUC of 86.5 (62.7-92.3) was achieved.

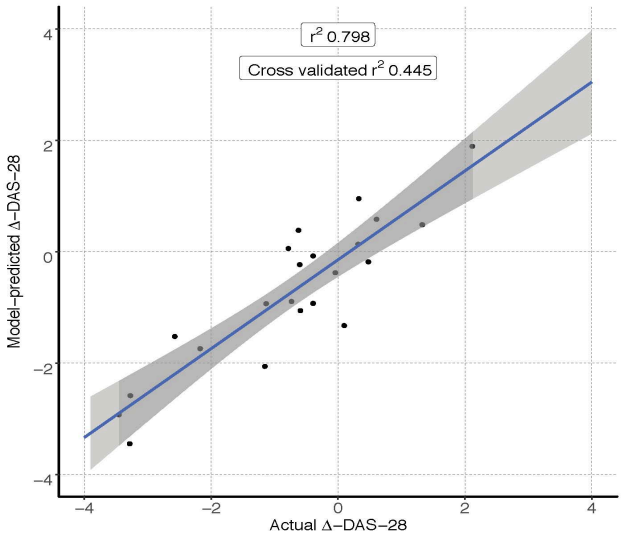


Figure 4.11 Prediction model of Δ DAS-28

Only genes that were significant baseline correlates of Δ DAS-28 from the *MSA41^{hi}AICDA$^{hi/lo}$* group were chosen. The final model achieved R2 0.798 and included *CD96, FCER2, IL1B, MMP25, CCL22, CD29*. The final predictive model of Δ DAS-28 was tested for auto-correlated errors using the Durbin Watson test using durbinWatsonTest (car package), and also non-linearity, skewness, kurtosis, and heteroscedasticity using gvmodel (gvlma) package). Model outliers were detected using outlierTest (car package). Finally, robustness of the model was

tested by shrinking r2 through 10-fold cross validation (CV) using crossval (bootstrap package) and comparing the fitted vesus predicted coefficients after each CV cycle.

4.5 Discussion

In the previous chapter I reported that the degree of baseline B cell infiltration does not predict subsequent response to therapy. Gene expression data shows rituximab differentially down regulates a number of genes involved in B and T cell activation and differentially upregulates fibroblast genes involved in reparative processes. Through this work I provide novel insights into mechanisms of resistance to rituximab identifying that high baseline expression of genes regulating B cell differentiation, T-cell and Th17 responses and cell signalling significantly associate with a diminished delta-DAS 28 response. Finally, I also identified a baseline B-cell signature that in this cohort reliably predicts clinical response to rituximab.

This data supports the notion that molecular analysis maybe a more sensitive biomarker of response [355, 356, 397, 441, 442]. Consistent with previous gene expression studies, there was a significant decrease in expression of the CD20 gene *MS4A1* following rituximab therapy although this did not vary between responders and non-responders. Surprisingly, the level of B-cell depletion appeared to be higher in the B-cell poor group both by histology and molecular analysis, suggesting that organised cellular infiltrates are somehow resistant to depletion.

There was significant modulation of T cell associated pathways following treatment with Rituximab. Firstly, there was a global reduction in the expression of T-cell associated chemokine receptor *CCR5* in responders. CCR5 is a functional receptor for several pro-

inflammatory cytokines, including CCL4 (macrophage inflammatory protein-1β), which was also downregulated in responders. Its other ligands include CCL3 (macrophage inflammatory protein 1α) and CCL5 (RANTES 'regulated upon activation, normal T cell expressed and secreted')[443, 444]. The role of CCR5 in inflammation has been highlighted through the identification of a potentially 'protective' polymorphism [445], although results from varying populations were conflicting. Furthermore, CCR5 blockade results in amelioration in mouse models of disease[446]. Downregulation of this gene and its ligand provides further in vivo evidence that this chemokine has a key role in RA and propagation of synovial inflammation.

Modulation of T-cell pathways was further evidenced by a global reduction in *IL-2A* and CD96 expression. One study has previously reported higher levels of IL-2A in patients who responded to Rituximab therapy [447]. Low serum levels of IL-2 have also been suggested as a prognostic marker of remission through a systematic review [448]. CD96 is a receptor expressed on both T-cells and natural killer cells and is involved in cell adhesion pathways [449]. Its exact role in RA remains undefined, however it is likely to aid cell migration after exposure to a selected antigen.

Rituximab has previously been shown to deplete T-cells, mainly of the CD4+ subtype [277]. Thurlings and colleagues also demonstrated a reduction in T-cells in the synovium follow B-cell depletion therapy [347]. There may be several mechanisms underpinning these observations. Some circulating T-cells express CD20 and therefore it is plausible that Rituximab may have a direct effect on depletion of these cells. Arguably however, the small proportion of these cells in peripheral blood, found by one study to be 3.2% [319], means that translation into a significant clinical response is unlikely. B-cells are known to stimulate CD4+

cells in the presence of activated dendritic cells [242, 450, 451] and Rituximab may indirectly result in the alteration of downstream T-cell signalling pathways. Furthermore, B-cells significantly contribute to the inflammatory cytokine milieu which could result in an alteration in T-cell activation and differentiation. Lastly, a reduction in T-cells may be expected as a response to disease amelioration and reduction of chronic inflammation which may not be directly related to the synovium.

Downregulation of *CD163, CLEC4E and CD36* lends support to previous findings that B-cell depletion also plays a role in the modulation of macrophage activation and correlates with the histological findings described in the previous chapter. CD163 is a cell surface marker of M2 macrophages. CLEC4E (MINCLE) is a transmembrane protein expressed mainly on macrophages in the innate immune response and can induce the production of TNFα, IL-6 and other proinflammatory cytokines through the Syk signalling cascade[452]. CD36, found on macrophages and dendritic cells, is less well studied and appears to have a range of biological functions, including negative regulation of angiogenesis and fatty acid oxidation[453] making its role here more difficult to define. The observation that a reduction in sublining macrophages correlates with response regardless of the DMARD used suggests a common end macrophage mediated pathway of inflammation. Lower numbers of circulating autoantibody producing B-cells and immune complexes could result in reduced activation of macrophages which carry both Fcγ receptors and C5a receptors for complement mediated activation[454]. In vitro, human monocyte derived macrophages showed decreased TNFα production after B-cell depletion therapy and increased expression of BAFF and IL-10mRNA further suggesting that Rituximab is able to modulate macrophage function[455].

Only one gene was significantly upregulated following the administration of Rituximab. FGF-1 is a heparin binding growth factor that stimulates proliferation and migration of cells and is abundantly expressed in the inflamed synovium. FGF-1 has been associated with repair and cell proliferation in hepatocytes and renal tubular cells, however its role in the synovium is thought to be pro-inflammatory[456]. FGF-1 and 2 have previously been shown to be present in the synovium of patients with high levels of inflammation and promote angiogenesis[456, 457], with the latter being more potent. What is not clear, however, is whether FGF-1 is produced in an attempt to heal the inflamed synovium or whether it forms an inherent, destructive part of the inflammatory process leading to joint damage. If the former part of the statement is true, then upregulation of this gene following rituximab may be reflective of its role in cell repair and regeneration, triggered by a decrease in inflammatory cytokines. The witnessed downregulation of other angiogenic (*VEGFC, CD36*) and matrix metalloproteinase-9 genes would support an overall reparative role of Rituximab in synovial healing.

I have commented briefly on the cytokine producing role of B-cells. In this cohort, there was a reduction in the expression of IL-15, IL-1β and IL-2RA. Reduced levels of serum and surface Il-15 were seen in conjunction with changes in T-cell populations in the peripheral blood of 33 patients who received Rituximab therapy [458]. Additionally, IL-15 induces growth and proliferation of B-cells [459, 460] and therefore downregulation of the gene could correlate with decreased circulating numbers. Furthermore, IL-15 levels are linked to IL-17 production and Treg numbers and are critical to memory CD8 T cell proliferation and production[461, 462]. The reduction in IL-15 could therefore be linked to Rituximab induced alteration of T-cell subsets, with a positive feedback loop, or directly due to B-cell depletion and the reduced need for ongoing proliferation. IL-1 is produced by a multitude of cells, including synovial

macrophages and dendritic cells. IL-1β is capable of inducing both IL-6 and TNF which act synergistically to promote inflammation and joint damage[463]. Downregulation of expression following Rituximab therapy may be related to the reduction or alteration of macrophage function. Finally, downregulation of the IL2R following treatment may be related directly to receptor depletion by Rituximab, as it acts to proliferate both growth and differentiation of B-cells[464].

The increased modulation of macrophage associated genes witnessed in the responder subset indicates that this may be one of the predominant mechanisms of clinical response. CCL4 is heavily involved in the recruitment of macrophages [393, 465] and a reduction of synovial macrophages has been shown to correlate with response to therapy[159, 160]. FGF1 and VEGFR3 are involved in reparative processes in tissue, however within the synovium result in neoangiogenesis, pannus formation and correlate with disease activity [466, 467]. PAD2 is key to citrullination of proteins[468] within the synovium whereas RORγT is a key regulator of Th17 differentiation[469]. Upregulation of these four genes in the responder group may result in response to synovial depletion of proteins; or may simply correlate to a group of patients in which antibody production and lymphoneogenesis play a more pivotal role in the disease process, perhaps resulting in a group that are more likely to response to CD20 depletion therapy.

Critically, I have reported on novel synovial pathways identified as mediating resistance to rituximab. Firstly, I demonstrated that differential upregulation of the TH17 associated genes: *TBX21, CD96, IL-23A, IL-21* and *IL-15* at baseline were associated with a diminished response to therapy. If we consider the function of each of these genes, it is possible to understand

how a subset of patients have disease that is predominantly driven by the Th17 pathway and therefore do not respond to depletion by Rituximab therapy. Modulation of the TH17 response by rituximab therapy has been shown to correlate with a decrease of local IL-17 production in the synovium, for which the presence of B-cells is required, resulting in amelioration of symptoms [278].

TBX21 (Tbet) is a critical T cell transcription factor that controls Th1 cell differentiation and the production of IFN-γ, a known predictor of non-response[420]. It also is a negative regulator of commitment to the Th17 cell lineage. T-cells co-express both IL-17 and IFN- γ and the balance between IFN-γ and IL-17 produced by Th1 and Th17 cells plays a pivotal role in inflammation, particularly through the activation of fibroblast like synoviocytes[470]. High expression of IL21 drives towards the production of IL-17, whereas TBX 21 favours IFN-γ production[471]. The observation that high expression of both IL21 and TBX 21 correlates with worse outcomes is of importance, suggesting that the original IFN-γ^+ T_H17 cell associated cytokines and transcription factors play a pivotal role in this subtype of disease, regardless of the downstream mechanism of inflammation.

Reduction of IL-15 following treatment with rituximab therapy has been shown to correlate with both TH17 depletion and improvement in clinical response [458]. A similar phenomenon is seen in Sjögren's syndrome, where treatment with rituximab results in a reduced glandular IL-17 expression. [472] High expression of IL-15 can be taken as a surrogate marker of Th17 and IL17 expression. I have already discussed the role of IL-21 in the differentiation of T cells to the Th17 subtype. Il-21 also promotes the migration of cells towards GC and aids in situ B-cell growth, differentiation to plasma cells and class switching[473-476], lending further

evidence to the hypothesis that GC's are functionally active and may continue to propagate inflammation despite peripheral blood B-cell depletion. Finally, IL-23 induces Th17 cells and is necessary for maintained pathogenicity in this lineage[477, 478]. The IL23R is found on memory T-cells and is an important stimulator of IL-17 production.

High levels of TH17 related genes may be associated with a reduced response to Rituximab therapy through a multitude of mechanisms. Following antigen presentation, these cells may play a key role in orchestrating immune infiltration and therefore 'one hit' or depletion of B-cells may not be able to ameliorate disease. Taken together, these results suggest that disruption of the Th17 response at a certain threshold, which resets the Th17/Treg ratio, is necessary to obtain a clinical response in individuals expressing this molecular pattern. Furthermore, we know that Th17 and IL-21 are also key orchestrators of ELS formation, highlighted in a recent study by Kelvey and colleagues who describe subtypes of RA synovia which can be segregated according to IL-17A and CD21L (the long isoform of CD21) gene expression, which are associated with tertiary lymphoid formation and B-lymphocyte pathways[479].

Stratifying patients according to *MS4A1* expression demonstrated that genes involved in B-cell regulation and functioning segregate with CD20 expression. The high level of concordance between the molecular MS4A1 and histological B-cell score provides both validity to the scoring methods from the first chapter and further evidence for the presence of a 'B-cell rich' molecular signature. *IGKC, FCRL5, LTβ, CSF1R and FGF1* were present in both the histological and molecular group. *IGKC*, or immunoglobulin kappa constant was identified to be a key gene differentially expressed between OA and RA samples [480], and was

identified as a key gene in a microarray based study defining a plasma cell signature[481]. The human Fc receptor-like 5 (FCRL5) is an IgG receptor expressed on B-cells which regulates the cellular response to antigens. A recent study has identified cell lines which co-express FCRL5 and CD21, resulting in enhanced B-cell activity capable of responding to immune complexes[482]. Furthermore, FCRL5(+) tissue-like memory B cells in peripheral blood are more likely to accumulate somatic mutations[483] and are found in greater numbers in autoimmune disease. Extrapolating these findings to synovial tissue, it is possible to speculate that the high co-expression of FCRL5 in the molecular B-cell rich group is suggestive of ongoing antigen presentation and B-cell activation. LTβ has been extensively studied for its pivotal role in lymphoid follicle development [276] and production of pro-inflammatory cytokines, matrix metalloproteinases and cell adhesion molecules[484]. Other pivotal genes expressed in the molecular B-cell rich group include *PAX5, FOXP3, CCR7* and the *IL-7R*, all involved in B-cell maturation and priming.

In this cohort, high expression of genes specifically involved in B-cell activation and the formation of ectopic lymphoid structures (*LTA, CXCR5, IL15, PAX5)* significantly associated with a diminished response to rituximab therapy. This observation was seen in the group as a whole, but also in the *MS4A1*^{hi} group at baseline, where high expression of *CXCR5, MS4A1, AICDA, BLK,* and *WNT107* enriched for non-response. AID is essential for somatic hypermutation and class switch DNA recombination and may result in in situ autoantibody production. Activated B-cell CD40 signalling induces AID expression, enhanced by interleukin 4 and toll like receptors [485]. CXCR5 is a chemokine which enhances trafficking of Tfh to germinal centres, binding to its ligand CXCL13, a chemoattractant for B-cells[486]. *BLK* or B-lymphoid kinase is crucial for B and T cell interactions, B cell receptor development and

certain haplotypes have been shown to confer risk of developing autoimmune diseases such as RA and SLE[487]. The Finally, the WNT family has been associated with synoviocyte like fibroblasts[488] which promote plasma cell survival and retention in ELS[489]. High expression of all five of these genes suggest that despite peripheral blood B-cell depletion, predominant overexpression of ELS genes within the synovium confers resistance to therapy.

Reclassifying individuals by *AICDA* expression revealed three distinct groups: those with little or no B-cell gene expression, those with high expression but no active germinal centres and finally those with high MS4A1 and active germinal centres, characterised by expression of AICDA. The latter group was least likely to respond to therapy. This may seem somewhat counterintuitive; but supports previous work suggesting that synovial lymphoid aggregates may act as reservoirs for autoreactive B cells[346, 490]. Thus, specifically targeting the generation of these autoreactive B-cells or administering repeat cycles of rituximab in a bid to disrupt B-cell trafficking and subsequent maturation and organization into ELS may result in a more favourable response in this subset of patients.

Finally, I then created a prediction algorithm based upon *MS4A1/AICDA* expression, demonstrating an AUC of 86.5%. Furthermore, by modelling the *MS4A1*^{hi}*AICDA*^{hi} group's baseline gene expression to delta DAS28, ten genes were identified whose expression could be modelled for response in all patients at baseline to successfully predict the delta DAS-28 for the group as a whole, irrespective of histology and clinical parameters at baseline. This needs to be confirmed using more samples but appears promising based upon the functionally linked activities of the genes described. Further changes in gene expression and response after one cycle of rituximab would also enhance a prediction model and may be able

to segregate those who require further cycles of Rituximab to disrupt T/B cell interaction from those in whom Th17 / IL23 pathways appear to be the major driver and are unlikely to respond.

In summary, it is possible to create a predictive algorithm based on individual synovial gene expression pre and post Rituximab. B-cell depletion is necessary but may not necessarily predict response to therapy, particularly at the synovial level. The biology of the genes within the response signature suggests that disruption of B-cell trafficking genes / ELS may be the key to response. Additionally, Th17 pathways may contribute substantially to the inflammatory process; disruption of these, either through repeated B-cell depletion or alternate therapy may be the key to tailoring response in targeted individuals. These findings need to be validated in a larger cohort of patients, but provide a step towards stratification based on molecular heterogeneity.

Chapter 5:

Imaging predictors of response to Rituximab therapy

Arguably the most important clinical outcome in the efficacious treatment of RA is to halt or reduce progression of joint damage. Several studies have confirmed through the use of plain radiographs or magnetic resonance imaging that Rituximab effectively prevents and slows down erosion progression[288, 289, 291, 298, 491-493] either as monotherapy or in combination with methotrexate. To date, few studies have focused on established RA imaging and tissue responses to biological therapy, particularly in the context of B-cell depletion.

Musculoskeletal US is a widely utilised tool used by rheumatologists in the diagnosis and assessment of RA. The pathogenesis of RA involves neovascularity and vasodilation in synovial tissue; angiogenesis plays a crucial role in both the development and perpetuation of inflammation within the joint[494]. US detects both synovial thickening and vascularisation of tissue, measured through grey scale synovial thickening (ST) and power doppler signal (PD). Ultrasound has been shown to be more accurate than clinical examination in the evaluation of joint synovitis[61, 495, 496] and correlates with changes at the tissue level but does not yet feature in treatment or prognostic algorithms.

Valor and colleagues undertook a study to explore whether the presence of B-cells in peripheral blood may influence subclinical synovitis[497]. All of the patients in this study had received two or more cycles of Rituximab and only two were classed as having active disease (DAS-28 >5.1). Interestingly, all of the patients had US synovial thickening and 41% had PD signal after treatment. The group found no correlation between US indices, DAS-28, SDAI or last Rituximab cycle and the amount of circulating peripheral blood B-cells.

Another study followed up patients who were deemed to need retreatment at 6-9 months after the first cycle of Rituximab and a second group of patients where a further cycle was not felt necessary based upon clinical disease activity[498]. They utilised a grey scale US score and also formulated a novel US7 score based upon the most commonly affected joints: wrist, MCP and PIP 2 and 3, and MTP 2 and 5. There was a significant reduction in GSUS, US7 and PD scores following treatment with Rituximab, with correlations noted between a reduction in tender joints, DAS-28 and GSUS. Additionally, the group observed a reduction in the size and number of erosions following Rituximab therapy. Patients requiring retreatment showed an increase in PD score in the months leading to therapy, indicating that this is a sensitive measure even in those with established disease.

Only a few studies have explored the relationship between US and synovial pathology and therefore direct evidence linking PD signals to active inflammation remains limited. Furthermore, to my knowledge, no studies have examined the modulatory effects of rituximab on synovial histopathology and its relationship to ultrasonographic outcomes. Results from other synovial based studies have so far been conflicting, although there is more consistent reporting of an association of PD with histological inflammation.

A recent study of 26 knee biopsies from a range of inflammatory conditions, including crystal arthropathies, found strong correlations between US synovitis grade and histological synovitis scores, US doppler grade and vascularity, with a moderate correlation with CD68 macrophages[499]. Andersen and colleagues conducted a study where synovial explants were removed from patients about to initiate biological therapy. Explants were cultured in

vitro and production of IL-6, monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1-beta were measured using a multiplex assay. The authors report that colour doppler US in vivo were correlated with IL-6 production in vitro[500]. They did not, however, directly measure correlations with histological synovitis. The same group had conducted an earlier study in 81 arthroscopic needle biopsies where they demonstrated PD correlations with the synovitis score, CD68, CD3, Ki67 and von Willebrand factor[501]. Similar findings were demonstrated by Takase et al in biopsies obtained prior to knee arthroplasty in patients with long-standing disease where PDUS and GSUS correlated with histological grade of synovitis[502]. Conversely, an older study by Koski and colleagues in which synovial tissue samples obtained from a range of large and small joints, bursae and tendon sheaths using the US guided biopsy technique showed no correlation with synovial thickening or power doppler scores, although there was an association between absence or presence of PD signal[503]. Interpretation of all of these studies is complicated, however, by different scoring methods and varying techniques of tissue acquisition.

There are multiple lines of evidence to suggest that B-cells play a role in bone damage, leading to the hallmark erosions seen in RA. Rituximab therapy inhibits the formation of erosions by altering the ratio of bone resorption and formation, inducing changes in the RANKL / osteoprotegrin ratio[491, 504, 505]. B-cell rich lymphoid aggregates are present at the junction of synovium and subchondral bone, with the ability to recruit osteoclast progenitor cells resulting in an increased number of osteoclasts and bone erosion[274]. The antibody producing role of B-cells may also be involved in the formation of erosions as CCP positive synovitis is associated with more infiltrating cells, cartilage degradation and bone destruction[186, 187, 506]. Furthermore, memory B-cells are capable of producing RANK-L

both in vivo and in vitro[507]. Despite ample evidence that B-cells are inherently involved in destructive processes in RA, how much they contribute to the initial processes of neovascularisation and synovial thickening remains to be elucidated.

Angiogenic factors and pro-inflammatory cytokines play a key role in the formation of new vessels within the synovium. In normal joints, there is a modest correlation between vascular endothelial growth factor (VEGF) and PDUS score, p=0.045 [508]. An ultrasound guided biopsy study of 12 patients with early arthritis showed significant correlations of PDUS with expression of angiogenic factors VEGF-A, angiopoetin-2 and Tie-2, with further correlations between US synovitis and VEGF-C and VEGF-R3. This study also demonstrated a correlation between PDUS and sublining macrophage infiltrates[509]. A previous study also identified high levels of angiogenesis associated gene expression within the synovial tissue and fluid of 8 RA patients with established disease who had a mean disease duration of 16.3 years[510], indicating that upregulation of proliferative growth factors can be witnessed at all stages of the disease.

Further studies exploring gene expression associated with angioneogenesis in humans are lacking in the literature. Increased mRNA expression of VEGF receptors, hepatocyte growth factor and insulin-like growth factor-1 and angiopoietin-1 has been observed in the collagen induced arthritis (CIA) mouse model of RA[511]. In further support of these findings, the use of an anti angiogenic and antiproliferative compound delayed disease onset, reduced severity and resulted in suppression of gene expression of bFGF and VEGF in the synovium with an overall reduction of synovial blood vessel formation[512]. Further animal studies have

proposed mechanisms to halt or reduce neoangiogenesis [513, 514] but are yet to be replicated in large human studies.

The studies described above raise a number of issues which lead to the hypothesis and aims of this chapter. Firstly, there is paucity of data regarding synovial cell infiltration and US scoring in established or longstanding RA. Indeed, it remains unclear whether findings from early RA can be extrapolated to a cohort of patients with long disease duration who have failed multiple therapies. Although there are studies assessing changes following bDMARD therapy with anti-TNF, only two studies were found that specifically assess ultrasound changes before and after B-cell depletion therapy. One of the studies hypothesised a correlation with peripheral blood B-cells, but did not address whether there may be a pro-inflammatory role for B-cells or lymphoid aggregates within the synovium. Finally, previous gene expression studies exploring changes before and after Rituximab therapy have not commented specifically on changes in expression of growth factors and genes involved in neoangiogenesis. All of these factors play an important role in assessing whether US imaging can potentially serve as a biomarker of response after B-cell depletion therapy, which forms the experimental objective of this chapter.

5.2 Hypothesis and Aims

The main hypothesis of this work was that B-cell rich patients will display higher levels of synovial thickening and ultrasound PD and that this can serve as a biomarker to guide response to therapy. The specific aims were to:

1. Ascertain if clinical parameters and histology correlate with US and PD at baseline
2. Determine if those with a B-cell rich pathotype have higher grades of ST and PDUS
3. Determine the modulatory effects of Rituximab of US imaging and whether this correlates with changes in histological infiltration
4. Explore whether higher levels of genes associated with angiogenesis associate significantly with ST and PD at baseline and at 16 weeks

5.3 Methods

5.3.1 Study Population

22 patients (18 males and 4 females) all fulfilling the revised 1987 criteria for RA enrolled as part of the R4RA pilot study at Barts Health NHS Trust were studied as described in the methods chapter. 21 patients had ultrasound data available at baseline, paired ultrasound data was only available for 14 patients.

5.3.2 Patient Assessment

Clinical parameters including number of swollen and tender joints, health assessment questionnaires, c-reactive protein and erythrocyte sedimentation rate were recorded at baseline and at 12 weeks follow up. Clinical response to rituximab were assessed using the EULAR response criteria.

5.3.3 US Assessment

US images were acquired by myself and two other researchers trained in musculoskeletal ultrasound, Nora Ng and Maria DiCicco. We used a GE Logic 9 ultrasound machine with a 14 MHz two dimensional M12 transducer. Longitudinal images of MCP joints 1-5 and three (midline, radial and ulnar) views of both wrists were acquired to form a 12 joint synovitis score. Additionally, details were recorded of the biopsied joint. Scans were performed at room temperature and images which conform to recommended standards of acquisition with at least 1mm of gel between the probe and overlying skin were obtained. Settings are as described in the methods chapter.

5.3.4 US guided biopsy

Synovial samples were taken using ultrasound guided synovial biopsy from all patients with a clinically involved joint at baseline and at 16 weeks. A minimum of 6 synovial samples were also retrieved and immediately immersed in RNALater (AMBION life technologies, UK) for subsequent RNA extraction as previously described.

5.4.4 High throughput quantitative real-time PCR

High throughput quantitative real-time PCR for 190 genes, pre-selected according to known roles in RA / B cell biology was performed using the Fluidigm© platform (Fluidigm Corporation, South San Francisco, California, USA) as described in the methods in chapter 4.

5.5.5 Statistical Analysis

Continuous variables are expressed as mean $\pm$ standard deviations. Comparison of pre-treatment and post-treatment clinical data, histological and US analysis was performed using the Wilcoxon signed rank test for paired data. For unpaired data, the Mann-Whitney U test was used to analyse for differences in median and rank. Associations were performed by deriving the Spearman rank correlation coefficient.

Gene correlations with clinical variables and synovial thickening and power doppler signals were calculated using Spearman rank correlation coefficients with $p<0.05$ deemed to be significant.

All statistical analysis and graphs were derived using Prism (version 7, compatible for Macintosh systems) or R Programming Language (R) The cut-off value for significance was <0.05 for all tests.

5.4 Results

5.4.1 Study population

US data was available for 21 patients at baseline and 14 patients at the 16-week timepoint due to loss of follow-up or an incomplete dataset.

Table 5.1 Demographics and clinical characteristics of patients with data available at baseline for analysis of ultrasound scores

Age (yrs) mean ± sd	53±13.78
Female, n, (%)	17 (81)
Median disease duration months (IQR)	99 (24 -120)
RF positive, n, (%)	16 (76)
ACPA positive, n, (%)	19 (90)
ESR mm/hr median (IQR)	22 (9-55)
CRP mg/l median (IQR)	9 (0-19.5)
DAS-28 mean ± (sd)	5.73 ± (1.07)
SJ median (IQR)	6 (3-8.5)
TJ median (IQR)	11 (7.5-19)
VAS mean ± sd	65.33 ±24.49
HAQ mean ± sd	2.08 ± 0.73
Erosive≥1 joint n, (%)	17 (81)
ST, median (IQR)	26 (23-30.5)
PD, median (IQR)	3 (2-5.5)
Median number of previous anti-TNF drugs, IQR	1 (0-1.5)
No of patients receiving Rituximab as first line biologic therapy, n (%)	6 (29)
No of patients on methotrexate n (%)	12 (57)
Methotrexate dose, median (IQR)	5 (0-17.5)
No of patients on prednisolone, n	10 (48)
Prednisolone dose, median (IQR)	0 (0-8.75)

Abbreviations

RF=rheumatoid factor, ACPA= anti-citrullinated protein antibody, ESR = erythrocyte sedimentation rate, CRP = c-reactive protein, DAS-28= 28 joint count disease activity score, HAQ = Health assessment questionnaire, SJ = swollen joints, TJ = tender joints, VAS = visual analogue score, ST = synovial thickening, PD = power Doppler, EULAR = European union

5.4.2 Treatment with rituximab did not result in a change in ST and PD US scores

There was no significant change in ST before and after Rituximab treatment in this cohort as a whole, despite the observation that there was a significant improvement in DAS-28. The median pre-treatment was 26 (21.74-31.25) and post treatment 25.5 (21.75-29.75), p=0.72. I then divided the groups into responders and non-responders, and witnessed no significant decrease (p=0.99, p=0.59, respectively) in ST or PD in these groups regardless of differences in clinical outcomes. Although there was a reduction in median power doppler score pre and post Rituximab treatment, pre 3 (1.75-6.25), post 2 (1-4.5) this reduction was not significant (p=0.29). Furthermore, no differences were detected pre and post treatment between responders and non-responders (p=0.99, p=0.13, respectively). At 16 weeks there were no differences in ST (p=0.47) and PD scores (p= 0.88) between responders and non-responders. I then redid the analysis with single joint scores, with no significant differences found within the groups. This was due to two reasons - the algorithm used to choose the affected joint to biopsy means that all samples had a ST score of at least 2; the second reason was because many of the joints biopsied were knees or elbows, which were not included in the overall joint score and therefore individual values had not been recorded.

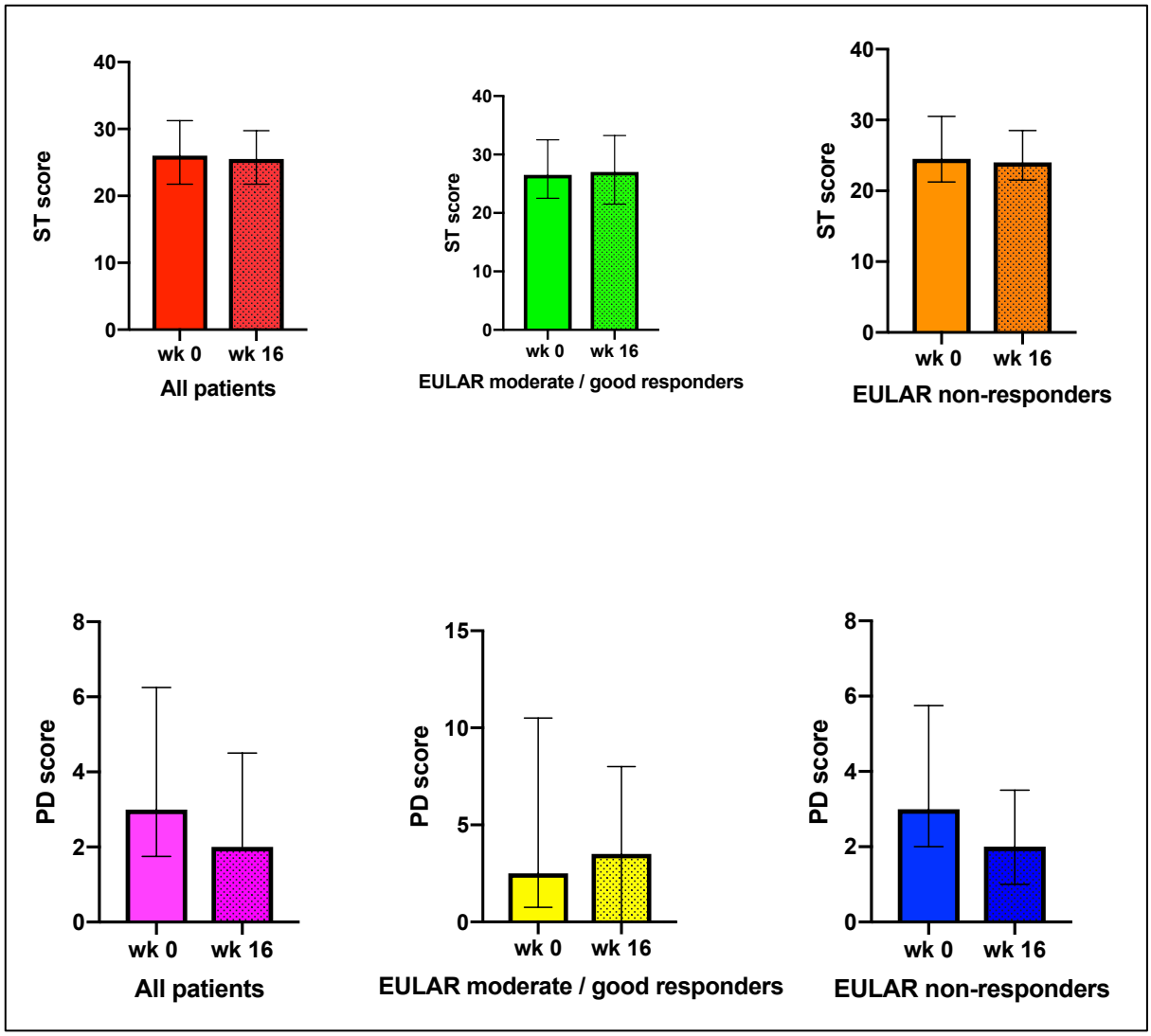


Figure 5.1 No differences were observed between synovial thickening (ST) and power doppler (PD) scores before and after treatment with Rituximab

Bars represent median, lines represent interquartile ranges. No values were significant using Wilcoxon ranked pairs test. Abbreviation ST = synovial thickening, PD = power dopppler

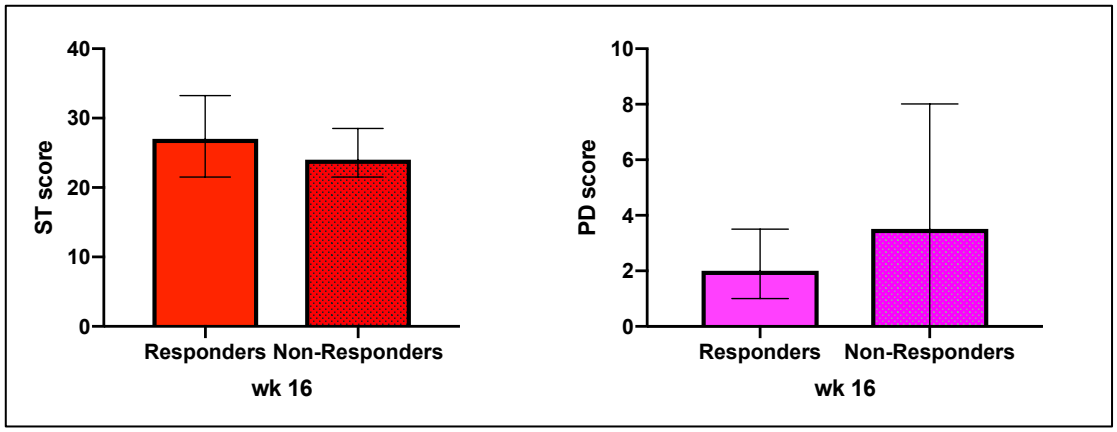


Figure 5.2 At 16 weeks there was no difference in synovial thickening (ST) or power doppler scores between responders and non-responders

Bars represent median, lines represent interquartile range

Abbreviations ST = synovial thickening, PD = power doppler

5.4.3 There is a significant correlation between US ST and PD at baseline

At baseline, there is a significant correlation between US ST scores and PD scores (p=0.02). This is lost at 16 weeks, although there a trend towards correlation remains (p=0.06).

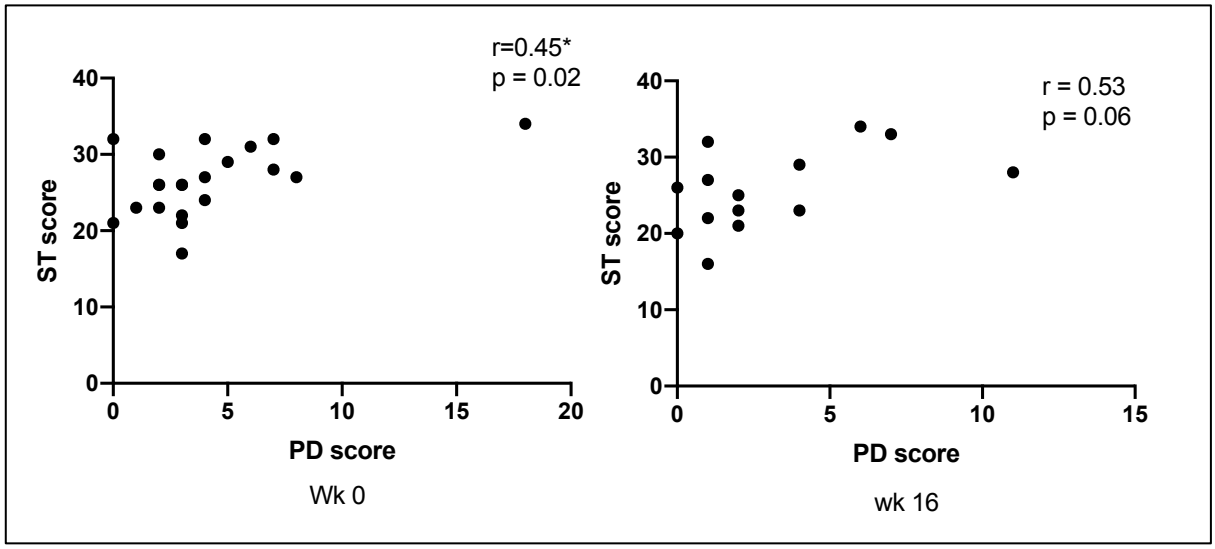


Figure 5.3 There was significant correlation between synovial thickening and power doppler at baseline but not at 16 weeks

Spearman's rank correlation coefficient. Abbreviations ST = synovial thickening, PD = power doppler, wk = week

*p<0.05, Spearman's correlation co-efficient

5.4.4 Serum levels of IgM with US ST baseline

Next, I went on to explore whether US ST and PD correlated with any clinical variables at baseline and at 16 weeks. At baseline, the only significant clinical or laboratory correlations found were serum levels of IgM (g/l) and ST (r = 0.56, p=0.010). The significance of this association is unclear and will be discussed further. At the 16-week timepoint, no associations were detected with clinical parameters, including the number of tender and swollen joints.

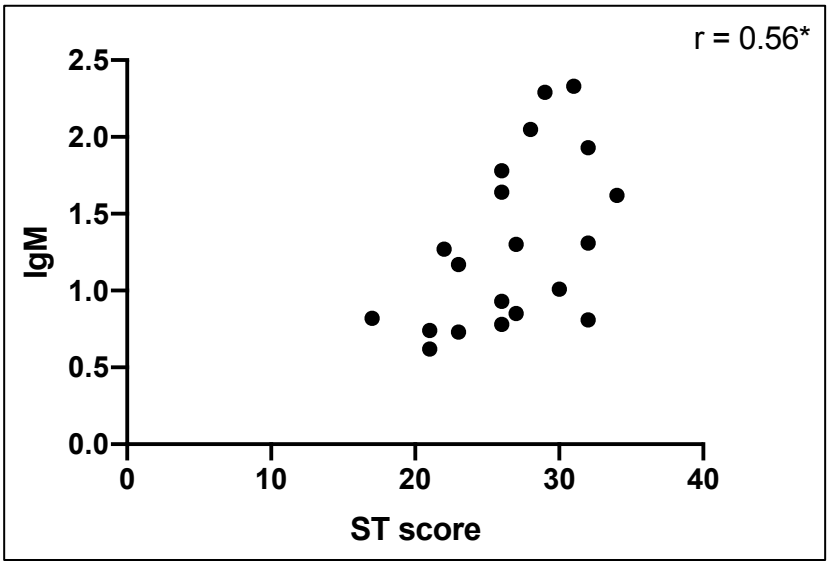


Figure 5.4 There is a positive correlation between serum IgM levels, g/l and synovial thickness scores

5.4.5 Patients in Imaging remission have a lower CRP and SJC

Next, I took patients in "imaging remission", with a PDUS <1 at 16 weeks and conducted analyses to ascertain whether there were differences between the two groups. Of the 14 samples where US data was available at the 16 week timepoint, 6/14 (42.9%) patients had a PD score of $\leq$1. 3/6 (50%) had a moderate or good EULAR response to Rituximab therapy. A similar proportion, 3/8 (37.5%) in the group with active synovitis defined by US criteria, however, displayed a good DAS-28 response and were deemed to be responders. CRP was the only discriminatory laboratory variable amongst those in PD remission and those with active synovitis on US (p=0.05). There was a trend towards a lower swollen joint count in those patients in US remission (p=0.09) but this did not reach significance. For all other clinical variables, no significant differences were found between the groups.

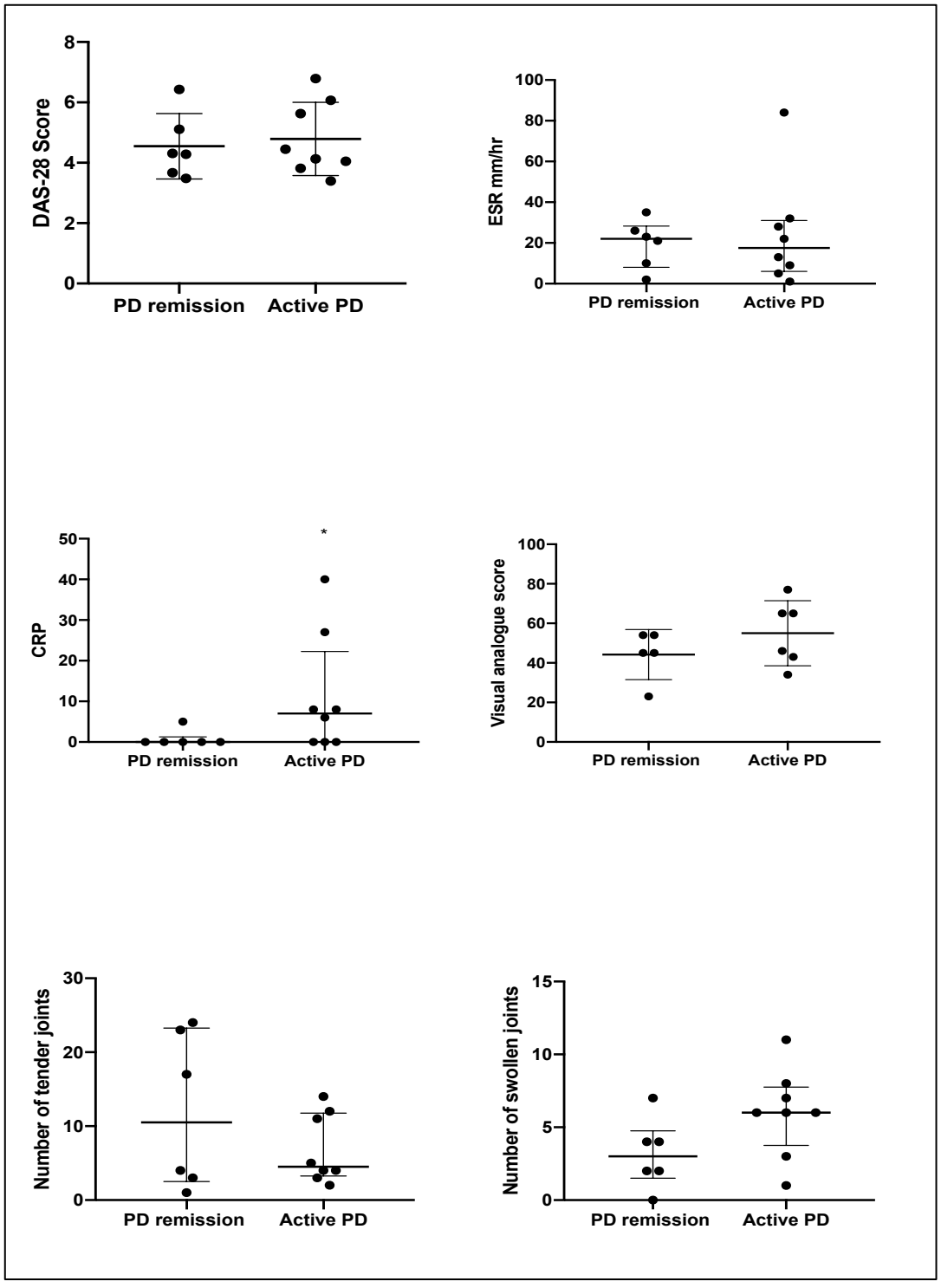


Figure 5.5 There is no difference in clinical variables between patients in PD remission compared with those with active PD on ultrasound

PD remission is defined as a score ≤1. There were no significant differences between groups using the Mann-Whitney test.

5.4.6 B-cell rich patients do not have higher ST and PD US scores

Contrary to the proposed hypothesis, I found that in this cohort of patients, B-cell poor patients had more ST at baseline than B-cell rich patients; median B-cell rich 23 (21-26) vs median B-cell poor, 26 (22-27.5), p=0.02. No significant differences were seen in PD signal between B-cell rich and poor patients. Unexpectedly, negative correlations were found between CD20 (r=-0.5, p = 0.031), CD68SL (r=-0.46, p = 0.049) and CD138 (r=-0.49, p = 0.035) and ST. No significant correlations were found between PD and synovial cell infiltrate.

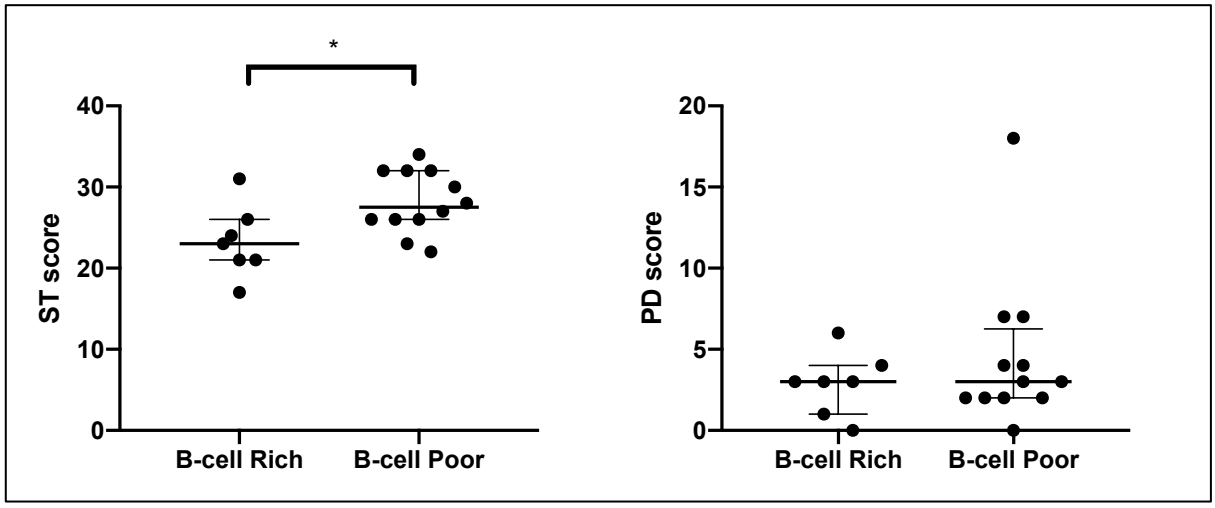


Figure 5.6 A B-cell poor synovial pathotype correlates with higher US synovial thickening at baseline

Solid lines represent the mean, boxes represent the standard deviation and lines represent the range, * p<0.05 using Mann Whitney test.

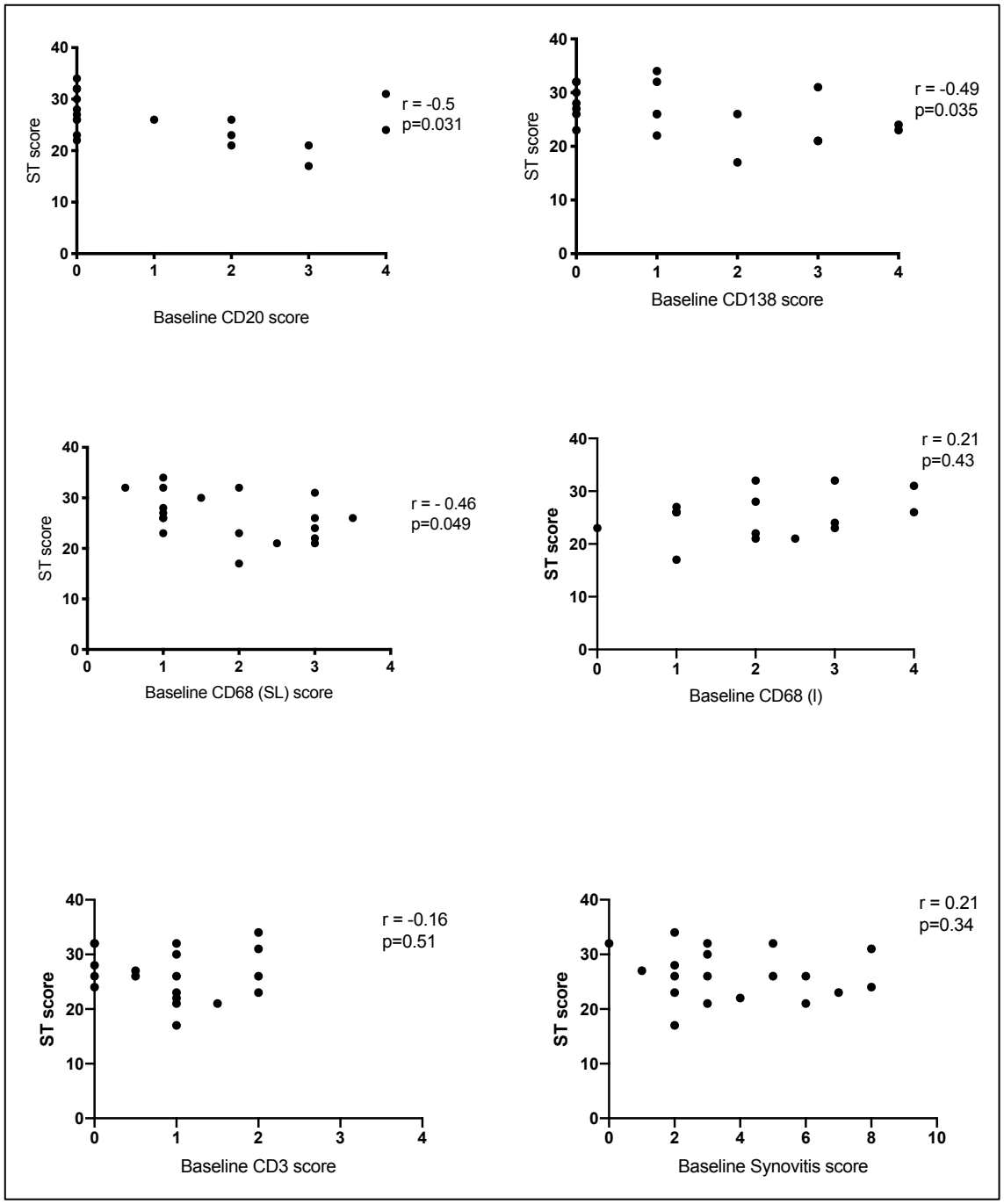


Figure 5.7 Correlation plots of A) ST and baseline SQ CD20 score, B) ST and SQ CD138 score, C ST and SQ CD68 sublining macrophage scores

Significant negative correlations were observed with ST scores, CD20, CD138 and CD68 (SL) infiltrations using Spearman's rank correlation coefficient.

Abbreviations SQ = semi-quantitative score, ST = synovial thickening

5.4.7 Genes involved in T-cell effector responses correlate with ST and PD scores

Using the Fluidigm data, I explored the function of genes with significant correlation with US synovial thickening, PD scores and erosions at baseline. The top ten genes which correlated with each variable are listed in table 4.2. *CD163, CD206* and *MS4A4A* showed negative correlations with ST, the latter two genes also associated with PD signal. CD163 is a macrophage specific protein; high levels of expression are characteristic of tissues responding to inflammation [515]. CD206 is a receptor expressed by macrophages and dendritic cells, with a role in collagen internalisation and degradation[516]. MS4A4A is a member of the membrane spanning, four domain family which includes CD20, FcRβ and HTm4 and has been demonstrated in immature dendritic cells, M2 macrophages and plasma cells[517]. These findings support the results of the histological analysis which showed an unexpected negative correlation with B-cells, macrophages and plasma cells.

Positive correlations were seen between synovial thickening, power doppler and IL-17A and IL-17F, members of the IL-17 family which share 50% homology[518]. *TNFRSF4* (TNF receptor superfamily, member 4), also known as CD134 or OX40, which plays a role in the activation of TH2 cells and subsequent cytokine production[519], also positively correlated with ST and PD. *OSM*, or oncostatin-M, a gp-130 signalling cytokine found in RA synovium was present in both groups. Of importance, IL18R and the IL18RAP (Il-18 receptor associated protein) which bind to IL-18, a key mediator in leucocyte extravasation and subsequent pro-angiogenic cytoking production[520], were found to have significant associations with US PD scores.

The expression of 34 genes correlated with the presence or absence of erosions in this cohort. *IL17F, IL17A, Il18RAP, IL18R1, TNFRSF4* and *OSM* expression also appeared in this group.

Again, there was a negative correlations of the genes *CD206, CD163* and *MS4A4A* with the presence of erosions.

At 16 weeks, levels of *ICOSLG, CTGF, CXCR6* and *IL18R1* correlated with both ST and PD.

Genes correlating with ST at baseline			**Genes correlating with ST at 16 weeks post Rituximab treatment**		
Gene	**r**	**p**	**Gene**	**r**	**p**
IL17F	0.65	1.7774E-05	***CTGF***	0.71	0.00045
TNFRSF4	0.62	5.3298E-05	***ICOSLG***	0.62	0.0033
PDCD1	0.61	7.9428E-05	***CXCR6***	0.52	0.020
CD206	-0.61	8.0163E-05	***FCER1A***	0.51	0.023
MSA4A4A	-0.61	8.0532E-05	***IL18R1***	0.45	0.044
FCRL4	0.61	9.6196E-05			
SMAD7	-0.59	0.00014			
CD163	-0.59	0.00015			
IL17A	0.58	0.00021			
OSM	0.58	0.00023			

Table 5.2 Genes correlating with ST at baseline and at 16 weeks post rituximab treatment

Genes correlating with PD at baseline			Genes correlating with PD at 16 weeks post Rituximab treatment		
Gene	r	p	Gene	r	p
CD206	-0.68	5.4814E-06	***ICOSLG***	0.84	4.3686E-06
IL18RAP	0.62	5.7255E-05	***CTGF***	0.69	0.00085
MSA4A4A	-0.58	0.00020	***MAF***	0.62	0.0034
TNFSF3	-0.58	0.00024	***FCER2***	0.57	0.0083
IL13RA2	-0.57	0.00026	***IL18R1***	0.56	0.0096
IL17F	0.56	0.00037	***PAD2***	0.56	0.011
LYVE1	-0.56	0.00041	***CXCR6***	0.55	0.013
OSM	0.55	0.00045	***TNFRSF17***	0.54	0.014
TNFRSF4	0.54	0.00059	***LTA***	0.54	0.014
IL18R1	0.54	0.00062	***VEGFR3***	0.53	0.017

Table 5.3 Genes correlating with PD at baseline and at 16 weeks post rituximab treatment

5.5 Discussion

Musculoskeletal ultrasound is a widely used by rheumatologists in the diagnosis, management and detection of relapse in RA. The ultimate goal of RA treatment is to prevent structural, long-lasting joint damage by inducing remission. The definition of remission, however, remains unclear. Moreover, whether changes in US ST and PD accurately reflect underlying inflammation at the tissue level has not been fully elucidated, particularly in longstanding disease.

The aim of this chapter was to ascertain whether treatment with Rituximab therapy resulted in changes in ST and PD signal and if this correlated with the presence of synovial infiltrates, in particular B-cells, and associated gene expression. The results differed from the original hypothesis, as I was unable to demonstrate significant changes in ST and PD following B-cell depletion. Secondly and unexpectedly, contrary to previous studies, I found a negative association between histological synovitis and ST of PD grading which also manifest itself at the gene expression level. Finally, despite a recognised role for B-cell biology in the formation of erosions, genes associated with ST and PD were largely had T-cell associated function.

In patients with established RA, disparities have been reported between the clinical assessment of tender and swollen joints and the presence of PD signal. Despite a significant reduction in DAS-28, the patients in this cohort did not undergo a significant reduction in ST and PD signal. There was also no association with tender or swollen joints. US data was available for six patients with moderate or good EULAR responses – 50% of these still had synovitis on ultrasound. This is in keeping with findings by Valor and colleagues [497],

although conflicts with reported findings from Reiche et al[498]. The main explanations for these findings come from investigating differences in patients with early RA and those with long standing RA, which all show varying levels of persisting GSUS and PD signals. A study designed to compare early and longstanding cohorts showed that 65.2% of patients with longstanding disease had grey scale thickening, or 'inactive synovitis', and at 12 months only 17.2% showed evidence of US remission[521]. Brown and colleagues recruited a cohort of patients who had a median disease duration of 7 years and found that despite being clinical remission, 87% had evidence of GSUS thickening and 63% had power doppler signal; 92% had synovitis on MRI which correlated with structural damage progression[522]. A further study showed that disease duration correlated with grey scale synovitis ($p=0.011$) and approximately 20% displayed PD signal in the absence of any tender or swollen joints[523]. This study also highlights limitations of the DAS-28 as a scoring system for active inflammatory disease, influenced by the weighting of the different components and the need to evaluate the utility of imaging in longstanding disease[524].

These findings lend support to the observation that in this cohort there was ongoing synovitis despite improvements in clinical parameters. It is known that patients with established RA have chronic inflammatory changes and at the simplest level, the absence of changes in ST scores may be due to the detection of chronic fibrous tissue rather than active inflammation or that the 16 week time point is too early for meaningful change. This notion is conflicted by a recent study suggesting that GS synovial hypertrophy without active PD is also sensitive to change, independent of the grade and duration of disease[525]. Changes in the synovial vascular bed, neoangiogenesis and capillary blood flow, however, have not yet clearly been described in patients with longstanding RA, although certainly studies with other biologic

therapies and long disease duration would suggest that it is possible to achieve a significant reduction in PD scores[102, 498, 526-529].

I demonstrate in this cohort that low PD activity correlates with low CRP levels and that there is a trend towards a lower swollen joint count which suggests that in a proportion of patients, PD is a sensitive marker of response to Rituximab therapy. Significant correlations were also seen between serum IgM levels and ST. Since I did not specifically test for RF IgM it is difficult to draw any sound scientific conclusions from this association, although it would be interesting to explore if specific RF fractions coupled with greater degrees of synovitis.

Further disparities between current ultrasound studies and my findings may also be explained by the "Treat to Target" or "Window of opportunity" approach that has been adopted by physicians, particularly in the last decade. This tight, targeted approach to therapy is temporally related to the rise in musculoskeletal ultrasound-based studies in the literature. This raises the possibility that plasticity of the synovium is greater in early disease, with tight control reflecting changes in ST and PD more accurately than in this cohort with longstanding disease activity. In the context of this study, several bDMARDs have been licensed after the onset of RA, inferring that physicians treating early in the disease had restricted therapeutic options to 'treat to target', resulting in high levels of ST and PD at baseline.

Another critical area of research highlighted by these findings is the need to define "true" remission and response in RA. Clinical remission does not invariably halt progression of structural damage and therefore whether true remission should be characterised by the

absence of PD signal is a question which remains to be answered. Despite improvements in DAS-28, none of the patients in this cohort achieved clinical (DAS-28 <2.6) or ultrasound remission (PD score of 0), yet did show response to therapy. The coordinators of the recent ARCTIC and TASER trials conclude that the use of ultrasound may lead to overtreatment and inefficient use of resources, as no significant differences in outcome were seen in the US tight control arm vs the conventional tight control arm[65, 66]. The answer may ultimately rest with the availability of resources and the ease at which US can be used as an adjunct to guide treatment.

Given previous work showing that B-cells may have a multifactorial role in the development of erosions and that ELS are associated with higher grades of synovitis, I was expecting to demonstrate that B-cell rich patients displayed more ST and PD. Surprisingly, patients who did not have large numbers of inflammatory cells within the synovium displayed higher degrees of imaging synovitis, with negative correlations of CD20, CD138 and CD168(SL) cells. One explanation for this may be that synovial proliferation is driven by activated synovial fibroblasts, which I did not test for within this work. Proliferation of synovial fibroblasts is a key feature of the diseased synovium, resulting in the production of matrix metalloproteinases and pro-inflammatory cytokines[530]. The subsequent creation of an inflammatory environment results in an increase in leucocyte infiltration which is coupled with neovascularisation through tissue hypoxia and the subsequent production of pro-angiogenic factors[531]. Another possibility is that the majority of the patients in this cohort displayed a 'low-inflammatory' or 'pauci-immune' subtype[369] or had predominantly fibroid driven disease. These are distinct molecular subsets of synovitis recently described by Dennis et al [356], which, despite molecular heterogeneity display similar clinical phenotypes to

lymphoid or macrophage rich disease. It is also worth questioning whether synovial cellularity is truly reflective of different disease processes or whether it presents a continuum of the same disease, leading to the speculation that the processes which lead to the initiation of synovial proliferation are not predominantly B-cell driven. Finally, it is important to note the therapeutic heterogeneity of this sample, as I have not explored the effects of concomitant prednisolone, methotrexate, varying number of cDMARDS and prior anti-TNF therapy on the synovium.

Analysis of gene expression profiles associated with synovial thickening and power doppler signal also revealed some novel and interesting findings. In keeping with the histological data, levels of macrophage and B-cell associated genes MS4A4A, CD163 and CD206 negatively correlated with ST and PD. In this cohort of patients, ST and PD appear to be more dependent on T-cell associated genes.

IL-17 plays a key role in RA pathogenesis. Most studies have focused on IL-17A, which shares 50% homology with IL17F suggesting overlapping biological roles. In keeping with my findings, serum IL-17 levels have previously been shown to correlate with US ST and PD, with significantly higher PD signals in patients with detectable levels of IL-17 [532]. Another study found that the percentage of IL-17 positive T-cells in synovial fluid correlated with PDUS, active disease and both in and ex vivo levels of VEGFA[533]. Additionally, IL-17 has been identified as part of a synovial cytokine expression profile associated with joint damage progression[378]. Several in vitro and mouse model experiments demonstrate a key role for IL-17 in angiogenesis. In vitro, IL-17 stimulates fibroblast like synoviocytes to secrete VEGF[534] and also results in an increased production of matrix metalloproteinases in

synovial tissue explants through the actions of TNF and OSM[535]. In this cohort, OSM expression was also upregulated in patients with higher PD and ST at baseline, suggesting that these genes may be acting synergistically to drive synovial proliferation. IL-17 can also promote osteoclastogenesis by directly stimulating osteoclast differentiation in vitro and through the production of RANK-L[536, 537]. Furthermore, several mouse models of arthritis demonstrated IL-17 mediated cartilage and bone erosion with amelioration of these findings after IL-17 blockade[206, 538, 539].

I also found that expression of both IL-18 and IL-18 RAP significantly correlated with ST and PD, suggesting a pivotal role for IL-18 in joint swelling. IL18 is significantly elevated in the joints and sera of patients with RA[540, 541] and is produced by a multitude of cells, including macrophages and synovial fibroblasts, the latter mediated by a TNFα dependent pathway[520, 542]. In vitro, synovial fibroblasts stimulated by IL-18 produce a number of adhesion molecules and angiogenic factors, including intercellular adhesion molecule 1 (ICAM-1) vascular cell adhesion molecular 1 (VCAM-1), VEGF, stromal derived factor as well as a number of leucocyte chemotactic factors[543, 544]. Murine models also demonstrate a pathogenic role of IL-18; administration of this cytokine worsens the severity of CIA, whereas blockade ameliorates disease[545-548]. Chew and colleagues[549] used IL-18 as marker of synovial inflammation in a study designed to utilise MRI findings to identify subclinical inflammation. mRNA levels of IL-18, however were undetectable in several patients, perhaps due to the sensitivity of the assay, and therefore no firm associations between imaging and cytokine levels were made. To my knowledge, no previous studies have assessed the relationship of IL-18 or its associated receptor or protein with US ST and PD, making this an important and novel finding, highlighting it as a potential biomarker of angiogenesis.

Analysis of genes associated with the presence or absence of erosions had marked similarity to those associated with ST and PD signal. The lack of B-cell associated genes in this subgroup suggests that similar mechanisms contribute to erosions as the end product of synovial inflammation, which in this cohort appear to be driven predominantly by cytokines derived from T-cells, macrophages and synovial fibroblasts.

Drawing findings from genes correlated with PD and ST at 16 weeks post Rituximab therapy is more challenging. I have therefore concentrated on the functions of genes appearing in both groups. Upregulation of the IL18R remains present in both groups at 16 weeks, indicating that despite B-cell depletion, the cytokine and its receptor may be associated with ongoing inflammation. The other two genes of note that appear to correlate with both ST and PD at ICOS-L and CTGF. Inducible costimulator (ICOS) and its ligand (ICOSL) regulate adaptive B and T cell processes by providing costimulatory signals following antigen exposure[550]. ICOS is associated with an increase in the suppressive activity of Tregs, which have been shown to have a higher frequency in inactive RA and negatively correlated with DAS-28 scores[551]. Although largely speculative, the restoration of Treg: TH17 ratios could explain the absence of IL17A and IL17F at the 16-week time point, mirrored by an increase in ICOS and its ligand.

CTGF, or connective tissue growth factor, is a heparin binding protein involved in inflammation, angiogenesis, wound healing, fibrosis and tumorigenesis[552] and has recently been identified as a potential biomarker of RA[553]. Without functional studies it is difficult to ascertain whether the witnessed association is due to the angiogenic or fibrosis inducing

properties of the protein. It may be possible that synovial reparative processes are upregulated following B-cell depletion resulting in resolution of inflammation and increased fibrosis which would correlate to a GSUS score. The association with power doppler, however, would suggest an alternate mechanism whereby the angiogenic properties of the growth factor play a more important role.

The work presented in this chapter raises several findings which differ from the original hypothesis but highlight critical pathways in the processes of synovial proliferation and joint swelling. The data described here does, however, have several limitations. Firstly, the small sample size inherently reduces the reliability and validity of any significant results. The semi-quantitative analysis of both synovial histology and US scores, although straightforward for the scoring physician, has limited sensitivity to detect real changes, and would have been improved by quantitative scoring methods. The results could also be influenced by the composite score rather than that of just the biopsied joint, although this in itself would be biased based upon the minimal ST score of 2 when selecting a joint to biopsy. Assessing US data at later timepoints such as six months or 12 months may also have yielded different results. Furthermore, formal scoring of erosions was not assessed within this work and would have provided a more reliable measure of structural change. Finally, correlation does not imply causality and therefore much of this discussion remains speculative based upon known biological pathways. Most of this data is observational but could form the starting point of several in vitro or vivo studies which would lend support to causality.

Nevertheless, this chapter presents some novel ideas and posing questions. I have demonstrated that ST and PD scores may not be as sensitive to change in long standing

disease and appear to remain unchanged at 16 weeks after Rituximab therapy. Despite previous data suggesting that synovial histology correlates with ST and PD scores, this data suggests that this correlation may be lost in long standing disease and that further studies should be undertaken in larger numbers of patients at different stages of the disease process. Finally, I have demonstrated that Th17 related pathways and IL-18 may play a key role in synovial neovascularisation and proliferation and that these may be altered by Rituximab therapy.

Chapter 6: General Discussion

RA is heterogenous disease with variation at clinical, biological and molecular levels. Rheumatologists now have a wealth of treatment options to treat RA, yet a sizeable proportion of patients remain resistant to certain therapies. Ideally, treatment in RA should be personalised in order to minimise disease progression and optimise quality of life, with utopian goals of disease remission, minimal side effects and a good quality of life. Current biomarkers are not sensitive enough to predict response to therapy. The synovium remains the key site of insult and injury in RA, yet the order of events leading from an initial break in tolerance to disease perpetuation remains unclear. The main focus of this thesis was to evaluate whether heterogeneity at the synovial level, particularly in the context of B-cell rich enriched tissue, could predict imaging and clinical response to rituximab, a drug that is already widely used in the treatment of RA.

I have examined a cohort of 24 patients with established RA who had failed or had a contraindication to anti-TNF therapy and assessed whether histological and molecular signatures in the synovium correlated with outcomes and imaging. I have reported a number of novel observations:

- Despite a reduction in CD20 cells and CD68SL macrophages after rituximab therapy, synovial histology did not correlate with clinical phenotypes and EULAR response
- High baseline expression of genes regulating ELN and Th17 responses are associated with a diminished DAS-28 response to rituximab, indicating that the synovial tissue may be resistant to B-cell depletion therapy, potentially through the production of autoantibodies and short-lived plasma cells
- Histological CD20 staining correlates with *MS4A1* expression, indicating that tissue collected through US guided synovial biopsies is reflective of molecular processes

- A *MS4A1*^{hi}*AICDA*^{hi} signature can predict non-response to the first cycle of rituximab, with an AUC of 0.86
- Upregulation of Th17 associated pathways is associated with non-response to Rituximab therapy
- Expression of genes involved in T-cell effector responses, in particular Th17 and expression of IL-18 correlate with US ST and PD scores, leading to the speculation that T-cells and fibroblasts may also play a key role in the expansion of the synovial cell layer
- Overarching findings suggest that modulation of Th17 pathways by Rituximab therapy may play a role in treatment stratification and response
- Finally, this work supports a role for further exploration of synovial tissue as a potential biomarker of response to therapies.

Currently, arthroscopy remains the gold standard of synovial tissue acquisition but remains an invasive procedure within a highly specialised setting. Minimally-invasive ultrasound guided biopsies are tolerable and safe and provide a good yield of synovial tissue[361]. Furthermore, they allow sampling of both large and small joints, the latter of which are not amenable to arthroscopy. Validation of US guided biopsies has been performed and been shown to comparable to arthoscopy[390] and forms part of the OMERACT research agenda[554]. More recently, the technique has been shown to yield a higher proportion of gradable synovial tissue than blind needle biopsies and comparable tissue for RNA extraction to surgical and portal and forceps techniques[364]. I have demonstrated herein that ultrasound guided synovial biopsies can be utilised to unravel and provide novel insights into disease pathogenesis and response to therapy. It also lends plausibility to the idea that in the

future, akin to several oncology models, molecular tissue markers may be integrated with clinical decision making to guide therapeutic choices.

The notion that biomarkers can aid prediction of response to therapies has been explored in several different studies. Alluded to previously, seropositivity for RF and ACPA has been shown to enhance response to Rituximab [313, 336, 555]. Despite these observations, a significant variability in response is seen within seropositive patients, indicating that alternate immune and pain pathways may mediate disease. Additionally, the type I interferon signature has been identified as a marker of non-response[420] but has not been validated in further large independent cohorts. The pursuit for alternate markers in the peripheral blood has so far lacked sensitivity or specificity for predicting response to biologic therapies and has therefore driven the search for biomarkers within synovial tissue.

Studies exploring synovial tissue and response however have to date provided conflicting data, highlighting the difficulty in extrapolating findings to larger cohorts due to methodological differences. In the context of anti-TNF therapy, one group demonstrated that synovial lymphoneogenesis was a negative predictor of response to therapy[411], whereas another group reported that aggregates were associated with a positive outcome [412]. In Rituximab based studies, a reduction of B-cells, immunoglobulin synthesis and plasma cells have been reported as predictors of response to therapy [347, 348] suggesting that modulation of B-cell associated pathways in the synovium play a role in response to therapy. Applying this data to larger cohorts remains problematic due to limited sample sizes and heterogeneity in treatment. The effect of previous DMARDs on the synovium and the optimal time to perform a biopsy following treatment to detect meaningful changes remains unknown, leading to study designs that are largely based upon times (usually 12 or 16 weeks)

in which physicians determine response to therapy in the clinical setting. I have demonstrated that in this cohort, although Rituximab resulted in a decrease in CD20 B-cells, plasma cells and synovial macrophages, this did not correlate with clinical response. I was also unable to demonstrate a direct relationship between ectopic lymphoid structures identified on histology and clinical outcome.

Molecular analysis of the synovium, however has demonstrated novel findings of significant biological importance. First and foremost, upregulation of genes involved in the formation of ELS were associated with non-response to therapy. It has previously been shown that the presence of plasma cell precursors, presumed to originate from solid tissue, in the peripheral blood is associated with poor response to therapy[320]. Autoreactive dominant B-cell and plasma cell clones have been identified in the diseased synovium in multiple joints of the same individual[351, 416], along with the presence of potentially pathogenic memory B-cells. Moreover, these clones were not identified in the peripheral blood. Increased frequency of the IGHV4-34, which is associated with autoreactivity in SLE, was also found within the RA synovium. B-cells from tissues which did not have lymphoid aggregates showed little intraclonal variability[351], in keeping with findings in animal models that fewer somatic mutations in the absence of germinal centres[556]. Further evidence for ongoing somatic hypermutation and class-switching within the synovium has been described by our group in an elegant SCID mouse model[346]. Taken together, this is suggestive that inflamed joint tissue forms a niche for autoreactive, dominant B-cells which act as antigenic targets and are capable of perpetuating inflammation both independently and through the migration or antigen dependent generation of plasmablasts. My findings that non-response is associated with genes that promote B-cell migration and ELS formation in the synovium adds further

evidence to the functional role of germinal centres. The lack of response to Rituximab may be due to two factors – 1) that peripheral blood B-cell depletion does not effectively target the synovial compartment in these patients or that 2) upregulation of these genes identifies a subset of patients who would benefit from further cycles of Rituximab in a bid to disrupt ELS formation.

The second key finding I have presented is the modulation of Th17 related genes following Rituximab therapy. The discovery of IL-17 producing CD4 T-cells, that required co-stimulation by CD28 and ICOS, in 2005 led to increased interest in the role of T-cells in RA pathogenesis[557, 558]. Several lines of evidence in RA patients and mouse models of arthritis implicate Th17 and their effector molecules in the pathogenesis of RA mainly through the production of the pleiotropic cytokine IL-17A [559]. This includes the activation of fibroblast-like synoviocytes, oscteoclast maturation and macrophage activation and function[560]. Th17 cells display a degree of plasticity and have also been shown to have a regulatory function, associated with IL-10 production[561]. It is therefore plausible that clinical response is dependent on the ratio of pathogenic and regulatory T cells.

The observation that a subset of patients display high IL-17A and CD21L expression in the synovium[479] is of particular importance in the context of my findings. FDCs, characterised by the CD21L expression, have been identified as key orchestrators in the development of ELS[562]. In line with my findings, McKelvey and colleagues found low numbers of histological GC+ELS within the synovium of 54 patients with RA, suggesting that expression of CD21L is reflective of an environment capable of producing ELS. It is therefore possible to speculate that in this cohort of patients, the expression of AID in the absence of histologically identified germinal centres mirrors a similar microenvironment which is proficient in the production of

lymphoid neogenesis, somatic hypermutation and ongoing antibody production. Th17 cells have been found adjacent to ELS[563] and therefore play a potential role in the induction and maintenance of these structures. This group found that patients with IL17+/CD21L+ had larger CD20 containing aggregates but did not observe increased numbers of ELS. Data from mouse models identify IL-17A are necessary to maintain lymphoid aggregate structure and function [564] which suggests that IL-17 may be involved in the stability of germinal centres and that this is linked to IL-23 expression[565]. As mentioned previously, whether germinal centres are representative of a specific synovial pathotype or whether are temporally related to stages of inflammation remains unknown. High expression of IL-17 may therefore be indicative of a distinct synovial pathotype with unique drivers of inflammation, or may simply vary at different timepoints in disease. The witnessed resistance to Rituximab therapy may therefore be due to several mechanisms: 1) in this subgroup of patients, it may be the Th17 pathway which drives inflammation and therefore B-cell depletion is of limited clinical benefit; 2) that the presence of IL-17 supports ongoing ELS formation which is not sufficiently disrupted by Rituximab therapy and supports the production of pathogenic B-cell autoantibodies in the synovium; and finally 3) that it is the ratio of Tregs to Th17 cells which is of critical importance in this subgroup and the manipulation of key regulatory cytokines may result in disease amelioration in this subset. Although Rituximab therapy has been shown to modulate T-cell function, in these patients this may not be enough to switch off inflammation.

Incorporating imaging as a clinical predictor of response is important, particularly in the context of early RA. MRI has been used as a predictive tool in undifferentiated arthritis[566] but remains a relatively expensive and time consuming modality. By contrast, US can be

performed quickly, cheaply and often in real-time in the setting of rheumatology clinics. Again, incorporating US in the assessment of early RA appears to increase the sensitivity of previous prediction models[567] but further data in routine use has not conferred any benefits over clinical assessment[65]. I have shown through this work that in established RA, synovial pathotype does not appear to correlate with ST and PD findings. Additionally, the absence or presence of synovitis on US did not correlate with EULAR response outcomes which questions our understanding of true remission in established disease. I unexpectedly found a negative correlation with CD20, CD138 and CD68 cells at both the histological and molecular level but did find a correlation between IL-17 associated genes. Again, this and previous findings confirming that IL-17 is associated with US changes, highlights the importance of Th17 cells and their associated cytokines in the pathogenesis of RA. It also suggests fibroblasts may play a crucial role in the processes contributing to synovial thickening and that Rituximab therapy does not result in an alteration of these pathways. Finally, upregulation of the ICOS ligand following rituximab therapy lends support to the notion of increased activity of Tregs resulting in resolution of inflammation.

The original hypothesis of this work was formulated on B-cell biology and associated pathways. I was expecting to demonstrate that B-cell rich patients had an enriched response to rituximab therapy and that the presence of associated B-cell gene signatures would predict response to therapy. I did not find any histological, clinical or ultrasound markers of response, but successfully demonstrated that the presence of an ELS / IL-17 rich molecular signature confers non-response to the first cycle Rituximab therapy and thus can be incorporated into a prediction model. I have also shown that in addition to B-cell depletion, rituximab modulates T-cell pathways which highlights the need for further understanding of changes

which occur secondary to B-cell depletion. US changes did not correlate with synovial histology in this cohort, which may in part be due to longstanding disease, or again due to the upregulation of alternate pathways. Additionally, there is no consensus on how many or which joints should be incorporated to formulate an US score which is truly reflective of disease activity – which adds a level of difficulty to the interpretation of US-based studies. Finally, I have shown that the US guided synovial biopsy can be used as a tool to aid our understanding of pathogenesis and treatment response, providing sufficient tissue for both histological analysis and RNA extraction.

The strengths of this work were that the tissue sampling and histological analysis methods were robust. Scoring was based on previously described methods, and the formation of the B-cell rich score was made in conjunction with expert opinion from a pathologist. Analysis using the Fluidigm© platform meant that a large number expression of a large number of genes could be explored, and only samples with good quality RNA were analysed. Conversely there are also several limitations to this data. The sample size was relatively small, but still comparable with previous studies. Patients enrolled in this pilot trial had longstanding disease and there were no stringent criteria on the number of previous conventional or biological DMARDs. Furthermore, the permissible use of steroid therapy may have resulted in changes which cannot be wholly attributed to modulation by Rituximab therapy. Fibroblast staining was not undertaken during the histological analysis – and could have been explored further to delineate the low-inflammatory or pauci-immune group. One could also argue that the genes in this dataset were pre-selected - although using known biological pathways reduces genetic noise and allow us to draw more robust conclusions. A larger sample size with further US and biopsy timepoints may have resulted in different results. Also, this is a

single-centre study and further work would be required across sites. Lastly, this was a non-blinded observational study with several investigators involved, which could theoretically result in bias and inconsistencies.

Nevertheless, this work leads to a number of critical unanswered questions and ideas for further work. Firstly, this data adds to the field of interest in the structure and function of ELS. Although conflicting results have arisen from TNF blockade, presence of germinal centres appears to confer resistance to Rituximab therapy indicating a role in disease pathogenesis. A question that remains unanswered in my work is whether disruption of these follicles would result in disease suppression and could be further explored by sequential biopsies in larger numbers of patients, with molecular analysis after second or third cycles. The increased use of next generation sequencing and sequential biopsies would allow us to answer whether the synovium provides a niche for pathogenic clones which are resistant to B-cell depletion therapy or whether repopulation occurs from autoreactive cells arising from other sources such a lymph nodes or bone marrow. This could enhance our understanding of Rituximab therapy and crucially explain both relapse and variations in response. Another key criticism of this work is that I have not incorporated any FACS data as this is currently forming part of another thesis, currently in progress. The ideal study would compare different B-cell subpopulations in the peripheral blood and synovium at different timepoints, with paired RNA analysis from each compartment in order to elucidate differences at the level of synovial tissue.

The imaging data presented here challenges our definitions of true remission and the relevance of subclinical synovitis in long-standing disease. More longitudinal studies are needed on radiographic progression in such patients. I have also highlighted the

controversies surrounding the true definition of remission in a 'treat to target' era, as data in the clinical setting remains conflicting. The utility of integrating US into a clinical prediction model is well described in early RA, but more studies are needed in patients with established disease with more robust clinical data sets.

Lastly, Th17 cells and their associated cytokines have recently been placed under the spotlight. Clinical trials with IL-17A blockade only resulted in modest clinical improvements in comparison to anti-TNF agents, IL-6 blockade and T-cell co-stimulation[568-570] in patients with RA and failed to reach primary endpoints. Longer term data, however, suggests that in those who did respond, clinical responses were sustained at one year[571]. A recent meta-analysis of IL-17 trials suggested that blockade was effective in achieving ACR20 and ACR 50 compared to placebo[572] but comment that the heterogeneity and short duration of the studies included make extrapolation of results difficult. Initial results have led to no further large trials but the observation that a subset of patients respond of critical importance. Large, randomised, double-blinded studies with adequate power to detect differences remain the gold standard of clinical trials. The data that I have presented and the trials described above pose the question of whether these results are truly applicable to a heterogenous disease. Using the synovial biopsy as a stratification tool, it is possible to divide patients into specific molecular subgroups – and perhaps blockade of the most upregulated pathways at the individual level would result in more promising results.

In an era of personalised medicine, it may be therefore be time to rethink scientific tradition. This is currently being addressed by our group through two biopsy based clinical trials: 1. 'R4RA' trial which is an Randomised open label study in anti-TNFα non-responders to investigate mechanisms for response – resistance to rituximab versus Tocilizumab in RA and

2. STRAP: Stratification of biologic therapies for Rheumatoid Arthritis by Pathobiology. Both trials have currently completed recruitment and therefore results are imminent.

In summary, this present thesis provides novel insights into both pathogenesis of disease and the complex role of alternative inflammatory pathways that result in resistance to Rituximab therapy. For both patients and clinicians, the landscape of RA is changing. The advent of biosimilars has resulted in choices that are less driven by economic responsibility. With a growing armamentarium of drugs, physicians should be compelled to think about therapeutic options based upon specific biological pathways. Current biomarkers are based upon clinical and laboratory parameters and do not sensitively discriminate those who will respond to treatment. In an era of advanced genomic and proteomic analysis, synovial pathobiology has the potential to unlock the future of personalised healthcare in RA through the utilisation of individual molecular signatures to guide clinical decision-making – leading to the right drug for the right patient at the right time.

References

1. Firestein, G.S., *Evolving concepts of rheumatoid arthritis.* Nature, 2003. **423**(6937): p. 356-61.
2. Spector, T.D., *Rheumatoid arthritis.* Rheum Dis Clin North Am, 1990. **16**(3): p. 513-37.
3. McInnes, I.B. and G. Schett, *The Pathogenesis of Rheumatoid Arthritis.* New England Journal of Medicine, 2011. **365**(23): p. 2205-2219.
4. Alamanos, Y. and A.A. Drosos, *Epidemiology of adult rheumatoid arthritis.* Autoimmunity Reviews, 2005. **4**(3): p. 130-136.
5. Carmona, L., et al., *The prevalence of rheumatoid arthritis in the general population of Spain.* Rheumatology, 2002. **41**(1): p. 88-95.

6. Drosos, A.A., et al., *Epidemiology of adult rheumatoid arthritis in northwest Greece 1987-1995.* J Rheumatol, 1997. **24**(11): p. 2129-33.
7. Rossini, M., et al., *Prevalence and incidence of rheumatoid arthritis in Italy.* Rheumatol Int, 2014. **34**(5): p. 659-64.
8. Brighton, S.W., et al., *The prevalence of rheumatoid arthritis in a rural African population.* J Rheumatol, 1988. **15**(3): p. 405-8.
9. Silman, A.J., et al., *Absence of rheumatoid arthritis in a rural Nigerian population.* J Rheumatol, 1993. **20**(4): p. 618-22.
10. Silman, A.J. and J.E. Pearson, *Epidemiology and genetics of rheumatoid arthritis.* Arthritis Res, 2002. **4 Suppl 3**: p. S265-72.
11. Del Puente, A., et al., *High incidence and prevalence of rheumatoid arthritis in Pima Indians.* Am J Epidemiol, 1989. **129**(6): p. 1170-8.
12. Hirsch, R., et al., *Rheumatoid arthritis in the Pima Indians: the intersection of epidemiologic, demographic, and genealogic data.* Arthritis Rheum, 1998. **41**(8): p. 1464-9.
13. Humphreys, J.H., et al., *The incidence of rheumatoid arthritis in the UK: comparisons using the 2010 ACR/EULAR classification criteria and the 1987 ACR classification criteria. Results from the Norfolk Arthritis Register.* Ann Rheum Dis, 2013. **72**(8): p. 1315-20.
14. Di, W.T., et al., *Incidence and Prevalence of Rheumatoid Arthritis in a Health Management Organization in Argentina: A 15-year Study.* The Journal of Rheumatology, 2016. **43**(7): p. 1306.
15. Symmons, D., et al., *The prevalence of rheumatoid arthritis in the United Kingdom: new estimates for a new century.* Rheumatology, 2002. **41**(7): p. 793-800.
16. Doran, M.F., et al., *Trends in incidence and mortality in rheumatoid arthritis in Rochester, Minnesota, over a forty-year period.* Arthritis Rheum, 2002. **46**(3): p. 625-31.
17. Hochberg, M.C., *Changes in the incidence and prevalence of rheumatoid arthritis in England and Wales, 1970-1982.* Semin Arthritis Rheum, 1990. **19**(5): p. 294-302.
18. Jacobsson, L.T., et al., *Decreasing incidence and prevalence of rheumatoid arthritis in Pima Indians over a twenty-five-year period.* Arthritis Rheum, 1994. **37**(8): p. 1158-65.
19. Shichikawa, K., et al., *Changes in the incidence and prevalence of rheumatoid arthritis in Kamitonda, Wakayama, Japan, 1965–1996.* Annals of the Rheumatic Diseases, 1999. **58**(12): p. 751-756.
20. Kaipiainen-Seppänen, O., et al., *Incidence of rheumatoid arthritis in Finland during 1980-1990.* Annals of the Rheumatic Diseases, 1996. **55**(9): p. 608-611.
21. Uhlig, T. and T.K. Kvien, *Is rheumatoid arthritis disappearing?* Annals of the Rheumatic Diseases, 2005. **64**(1): p. 7-10.
22. Grassi, W., et al., *The clinical features of rheumatoid arthritis.* Eur J Radiol, 1998. **27 Suppl 1**: p. S18-24.
23. Gallucci, M., *Facets pathology and treatment.* CardioVascular and Interventional Radiology, 2010. **33**: p. 144-145.
24. Caporali, R., et al., *Presenting features of polymyalgia rheumatica (PMR) and rheumatoid arthritis with PMR-like onset: a prospective study.* Annals of the Rheumatic Diseases, 2001. **60**(11): p. 1021.

25. Healey, L.A., *Polymyalgia rheumatica and seronegative rheumatoid arthritis may be the same entity.* J Rheumatol, 1992. **19**(2): p. 270-2.
26. Fleming, A., et al., *Early rheumatoid disease. II. Patterns of joint involvement.* Annals of the Rheumatic Diseases, 1976. **35**(4): p. 361-364.
27. Khurana, R. and S.M. Berney, *Clinical aspects of rheumatoid arthritis.* Pathophysiology, 2005. **12**(3): p. 153-165.
28. Turesson, C., et al., *Occurrence of extraarticular disease manifestations is associated with excess mortality in a community based cohort of patients with rheumatoid arthritis.* J Rheumatol, 2002. **29**(1): p. 62-7.
29. Gabriel, S.E., et al., *Survival in rheumatoid arthritis: a population-based analysis of trends over 40 years.* Arthritis Rheum, 2003. **48**(1): p. 54-8.
30. Nyhall-Wahlin, B.M., et al., *Smoking is a strong risk factor for rheumatoid nodules in early rheumatoid arthritis.* Ann Rheum Dis, 2006. **65**(5): p. 601-6.
31. Kochbati, S., et al., *[Bronchiectasis in rheumatoid arthritis. High resolution computed pulmonary tomography].* Tunis Med, 2003. **81**(10): p. 768-73.
32. Yunt, Z.X. and J.J. Solomon, *Lung Disease in Rheumatoid Arthritis.* Rheumatic diseases clinics of North America, 2015. **41**(2): p. 225-236.
33. Avnon, L.S., et al., *Pleural effusion associated with rheumatoid arthritis: what cell predominance to anticipate?* Rheumatol Int, 2007. **27**(10): p. 919-25.
34. Hetland, M.L., et al. *Radiographic progression and remission rates in early rheumatoid arthritis - MRI bone oedema and anti-CCP predicted radiographic progression in the 5-year extension of the double-blind randomised CIMESTRA trial*. Annals of the rheumatic diseases, 2010. **69**, 1789-95 DOI: 10.1136/ard.2009.125534.
35. Meune, C., et al., *High risk of clinical cardiovascular events in rheumatoid arthritis: Levels of associations of myocardial infarction and stroke through a systematic review and meta-analysis.* Arch Cardiovasc Dis, 2010. **103**(4): p. 253-61.
36. Meune, C., et al., *Trends in cardiovascular mortality in patients with rheumatoid arthritis over 50 years: a systematic review and meta-analysis of cohort studies.* Rheumatology (Oxford), 2009. **48**(10): p. 1309-13.
37. Solomon, D.H., et al., *Cardiovascular morbidity and mortality in women diagnosed with rheumatoid arthritis.* Circulation, 2003. **107**(9): p. 1303-7.
38. Avina-Zubieta, J.A., et al., *Risk of cardiovascular mortality in patients with rheumatoid arthritis: a meta-analysis of observational studies.* Arthritis Rheum, 2008. **59**(12): p. 1690-7.
39. Book, C., T. Saxne, and L.T. Jacobsson, *Prediction of mortality in rheumatoid arthritis based on disease activity markers.* J Rheumatol, 2005. **32**(3): p. 430-4.
40. Innala, L., et al., *Cardiovascular events in early RA are a result of inflammatory burden and traditional risk factors: a five year prospective study.* Arthritis Res Ther, 2011. **13**(4): p. R131.
41. Libby, P., *Role of inflammation in atherosclerosis associated with rheumatoid arthritis.* Am J Med, 2008. **121**(10 Suppl 1): p. S21-31.
42. Peters, M.J., et al., *EULAR evidence-based recommendations for cardiovascular risk management in patients with rheumatoid arthritis and other forms of inflammatory arthritis.* Ann Rheum Dis, 2010. **69**(2): p. 325-31.
43. Ropes, M.W., et al., *1958 Revision of diagnostic criteria for rheumatoid arthritis.* Bull Rheum Dis, 1958. **9**(4): p. 175-6.

44. Arnett, F., S. Edworthy, and D. Bloch, *The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis.* Arthritis Rheum, 1988. **31**: p. 315 - 324.
45. Sokolove, J. and V. Strand, *Rheumatoid arthritis classification criteria - It's finally time to move on!* Bull NYU Hosp Jt Dis, 2010. **68**(3): p. 232-8.
46. Banal, F., et al., *Sensitivity and specificity of the American College of Rheumatology 1987 criteria for the diagnosis of rheumatoid arthritis according to disease duration: a systematic literature review and meta-analysis.* Annals of the Rheumatic Diseases, 2009. **68**(7): p. 1184-1191.
47. Aletaha, D., et al., *2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative.* Arthritis Rheum, 2010. **62**(9): p. 2569-81.
48. Radner, H., et al., *Performance of the 2010 ACR/EULAR classification criteria for rheumatoid arthritis: a systematic literature review.* Ann Rheum Dis, 2014. **73**(1): p. 114-23.
49. Smolen, J.S., et al., *Treating rheumatoid arthritis to target: recommendations of an international task force.* Ann Rheum Dis, 2010. **69**(4): p. 631-7.
50. Anderson, J.K., et al., *Measures of rheumatoid arthritis disease activity: Patient (PtGA) and Provider (PrGA) Global Assessment of Disease Activity, Disease Activity Score (DAS) and Disease Activity Score With 28-Joint Counts (DAS28), Simplified Disease Activity Index (SDAI), Clinical Disease Activity Index (CDAI), Patient Activity Score (PAS) and Patient Activity Score-II (PASII), Routine Assessment of Patient Index Data (RAPID), Rheumatoid Arthritis Disease Activity Index (RADAI) and Rheumatoid Arthritis Disease Activity Index-5 (RADAI-5), Chronic Arthritis Systemic Index (CASI), Patient-Based Disease Activity Score With ESR (PDAS1) and Patient-Based Disease Activity Score Without ESR (PDAS2), and Mean Overall Index for Rheumatoid Arthritis (MOI-RA).* Arthritis Care & Research, 2011. **63**(S11): p. S14-S36.
51. Aletaha, D., et al., *Reporting disease activity in clinical trials of patients with rheumatoid arthritis: EULAR/ACR collaborative recommendations.* Ann Rheum Dis, 2008. **67**(10): p. 1360-4.
52. Fuchs, H., et al., *A simplified twenty-eight-joint quantitative articular index in rheumatoid arthritis.* Arthritis Rheum, 1989. **32**: p. 531 - 537.
53. van Riel, P.L. and L. Renskers, *The Disease Activity Score (DAS) and the Disease Activity Score using 28 joint counts (DAS28) in the management of rheumatoid arthritis.* Clin Exp Rheumatol, 2016. **34**(5 Suppl 101): p. S40-s44.
54. Gilek-Seibert, K., K. Prescott, and S. Kazi, *Outcome Assessments in Rheumatoid Arthritis.* Current Rheumatology Reports, 2013. **15**(11): p. 1-7.
55. van Gestel, A.M., et al., *Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis. Comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism Criteria.* Arthritis Rheum, 1996. **39**(1): p. 34-40.
56. Smolen, J.S., et al., *A simplified disease activity index for rheumatoid arthritis for use in clinical practice.* Rheumatology, 2003. **42**(2): p. 244-257.
57. Aletaha, D., et al., *Acute phase reactants add little to composite disease activity indices for rheumatoid arthritis: validation of a clinical activity score.* Arthritis Res Ther, 2005. **7**(4): p. R796-806.

58. Felson, D.T., et al., *The American College of Rheumatology preliminary core set of disease activity measures for rheumatoid arthritis clinical trials. The Committee on Outcome Measures in Rheumatoid Arthritis Clinical Trials.* Arthritis Rheum, 1993. **36**(6): p. 729-40.
59. Felson, D., *Defining remission in rheumatoid arthritis.* Annals of the rheumatic diseases, 2012. **71**(0 2): p. i86-i88.
60. Colebatch, A.N., et al., *EULAR recommendations for the use of imaging of the joints in the clinical management of rheumatoid arthritis.* Annals of the Rheumatic Diseases, 2013.
61. Naredo, E., *Ultrasound in rheumatoid arthritis.* Rheumatology, 2012. **51**.
62. Ogishima, H., et al., *Analysis of subclinical synovitis detected by ultrasonography and low-field magnetic resonance imaging in patients with rheumatoid arthritis.* Modern Rheumatology, 2014. **24**(1): p. 60-68.
63. Raffeiner, B., et al., *Use of power doppler to identify subclinical synovitis and risk of radiological progression in rheumatoid arthritis despite TNFalpha blocker remission.* Clinical Drug Investigation, 2013. **33**(SUPPL.2): p. S130-S132.
64. Ramirez, J., et al., *Sonographic characterization of subclinical synovitis in patients with rheumatoid arthritis in clinical remission. Preliminary results.* Annals of the Rheumatic Diseases, 2013. **72**.
65. Haavardsholm, E.A., et al., *Ultrasound in management of rheumatoid arthritis: ARCTIC randomised controlled strategy trial.* 2016. **354**.
66. Dale, J., et al., *Targeting ultrasound remission in early rheumatoid arthritis: the results of the TaSER study, a randomised clinical trial.* 2016. **75**(6): p. 1043-1050.
67. Smolen, J.S., et al., *Treating rheumatoid arthritis to target: 2014 update of the recommendations of an international task force.* Ann Rheum Dis, 2016. **75**(1): p. 3-15.
68. Stoffer, M.A., et al., *Evidence for treating rheumatoid arthritis to target: results of a systematic literature search update.* Annals of the Rheumatic Diseases, 2016. **75**(1): p. 16.
69. Crofford, L.J., *Use of NSAIDs in treating patients with arthritis.* Arthritis Res Ther, 2013. **15 Suppl 3**(Suppl 3): p. S2.
70. Smolen, J.S., et al., *EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2016 update.* Annals of the Rheumatic Diseases, 2017. **76**(6): p. 960.
71. Landewe, R.B., et al., *COBRA combination therapy in patients with early rheumatoid arthritis: long-term structural benefits of a brief intervention.* Arthritis Rheum, 2002. **46**(2): p. 347-56.
72. Cutolo, M., et al., *The supplementary therapeutic DMARD role of low-dose glucocorticoids in rheumatoid arthritis.* Arthritis Res Ther, 2014. **16 Suppl 2**: p. S1.
73. Kiely, P., et al., *Outcome in rheumatoid arthritis patients with continued conventional therapy for moderate disease activity--the early RA network (ERAN).* Rheumatology (Oxford), 2011. **50**(5): p. 926-31.
74. Aletaha, D. and J.S. Smolen, *Diagnosis and Management of Rheumatoid Arthritis: A Review.* Jama, 2018. **320**(13): p. 1360-1372.
75. Taylor, P.C., et al., *Baricitinib versus Placebo or Adalimumab in Rheumatoid Arthritis.* N Engl J Med, 2017. **376**(7): p. 652-662.

76. Fleischmann, R., et al., *Efficacy and safety of tofacitinib monotherapy, tofacitinib with methotrexate, and adalimumab with methotrexate in patients with rheumatoid arthritis (ORAL Strategy): a phase 3b/4, double-blind, head-to-head, randomised controlled trial.* Lancet, 2017. **390**(10093): p. 457-468.
77. Smolen, J.S., et al., *Rheumatoid arthritis.* Nature Reviews Disease Primers, 2018. **4**: p. 18001.
78. Viatte, S., D. Plant, and S. Raychaudhuri, *Genetics and epigenetics of rheumatoid arthritis.* Nat Rev Rheumatol, 2013. **9**(3): p. 141-53.
79. Yarwood, A., T.W.J. Huizinga, and J. Worthington, *The genetics of rheumatoid arthritis: risk and protection in different stages of the evolution of RA.* Rheumatology, 2014.
80. Jiang, X., et al., *To what extent is the familial risk of rheumatoid arthritis explained by established rheumatoid arthritis risk factors?* Arthritis Rheumatol, 2015. **67**(2): p. 352-62.
81. Frisell, T., et al., *Familial aggregation of arthritis-related diseases in seropositive and seronegative rheumatoid arthritis: a register-based case-control study in Sweden.* Ann Rheum Dis, 2016. **75**(1): p. 183-9.
82. Okada, Y., et al., *Genetics of rheumatoid arthritis contributes to biology and drug discovery.* Nature, 2014. **506**(7488): p. 376-81.
83. Stastny, P., *Association of the B-cell alloantigen DRw4 with rheumatoid arthritis.* N Engl J Med, 1978. **298**(16): p. 869-71.
84. Gregersen, P.K., J. Silver, and R.J. Winchester, *The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis.* Arthritis Rheum, 1987. **30**(11): p. 1205-13.
85. Viatte, S., et al., *Association of HLA-DRB1 haplotypes with rheumatoid arthritis severity, mortality, and treatment response.* JAMA, 2015. **313**(16): p. 1645-56.
86. MacGregor, A., et al., *HLA-DRB1*0401/0404 genotype and rheumatoid arthritis: increased association in men, young age at onset, and disease severity.* J Rheumatol, 1995. **22**(6): p. 1032-6.
87. Wordsworth, P., et al., *HLA heterozygosity contributes to susceptibility to rheumatoid arthritis.* Am J Hum Genet, 1992. **51**(3): p. 585-91.
88. du Montcel, S.T., et al., *New classification of HLA-DRB1 alleles supports the shared epitope hypothesis of rheumatoid arthritis susceptibility.* Arthritis Rheum, 2005. **52**(4): p. 1063-8.
89. Gyetvai, A., et al., *New classification of the shared epitope in rheumatoid arthritis: impact on the production of various anti-citrullinated protein antibodies.* Rheumatology (Oxford), 2010. **49**(1): p. 25-33.
90. Deighton, C.M., et al., *The contribution of HLA to rheumatoid arthritis.* Clin Genet, 1989. **36**(3): p. 178-82.
91. Eyre, S., et al., *High-density genetic mapping identifies new susceptibility loci for rheumatoid arthritis.* Nat Genet, 2012. **44**(12): p. 1336-40.
92. Kurko, J., et al., *Genetics of rheumatoid arthritis - a comprehensive review.* Clin Rev Allergy Immunol, 2013. **45**(2): p. 170-9.
93. Chatzikyriakidou, A., et al., *Genetics in rheumatoid arthritis beyond HLA genes: What meta-analyses have shown?* Seminars in Arthritis and Rheumatism, 2013. **43**(1): p. 29-38.

94. Farago, B., et al., *Protein tyrosine phosphatase gene C1858T allele confers risk for rheumatoid arthritis in Hungarian subjects.* Rheumatol Int, 2009. **29**(7): p. 793-6.
95. Goeb, V., et al., *Contribution of PTPN22 1858T, TNFRII 196R and HLA-shared epitope alleles with rheumatoid factor and anti-citrullinated protein antibodies to very early rheumatoid arthritis diagnosis.* Rheumatology (Oxford), 2008. **47**(8): p. 1208-12.
96. Menard, L., et al., *The PTPN22 allele encoding an R620W variant interferes with the removal of developing autoreactive B cells in humans.* J Clin Invest, 2011. **121**(9): p. 3635-44.
97. Metzler, G., et al., *The Autoimmune Risk Variant PTPN22 C1858T Alters B Cell Tolerance at Discrete Checkpoints and Differentially Shapes the Naive Repertoire.* J Immunol, 2017. **199**(7): p. 2249-2260.
98. Dai, X., et al., *A disease-associated PTPN22 variant promotes systemic autoimmunity in murine models.* The Journal of Clinical Investigation, 2013. **123**(5): p. 2024-2036.
99. Suzuki, A., et al., *Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis.* Nat Genet, 2003. **34**(4): p. 395-402.
100. Tamiya, G., et al., *Whole genome association study of rheumatoid arthritis using 27 039 microsatellites.* Hum Mol Genet, 2005. **14**(16): p. 2305-21.
101. Brachi, S., et al., *Dry synovitis: A unique entity? A multicenter study.* Annals of the Rheumatic Diseases, 2013. **72**.
102. Bowen, C.J., et al., *Improvement in symptoms and signs in the forefoot of patients with rheumatoid arthritis treated with anti-TNF therapy.* J Foot Ankle Res, 2010. **3**: p. 10.
103. Plenge, R.M., et al., *Two independent alleles at 6q23 associated with risk of rheumatoid arthritis.* Nat Genet, 2007. **39**(12): p. 1477-82.
104. Messemaker, T.C., et al., *A novel long non-coding RNA in the rheumatoid arthritis risk locus TRAF1-C5 influences C5 mRNA levels.* Genes Immun, 2016. **17**(2): p. 85-92.
105. Abdul-Sater, A.A., et al., *The signaling adaptor TRAF1 negatively regulates Toll-like receptor signaling and this underlies its role in rheumatic disease.* Nat Immunol, 2017. **18**(1): p. 26-35.
106. Plenge, R.M., et al., *TRAF1-C5 as a risk locus for rheumatoid arthritis--a genomewide study.* N Engl J Med, 2007. **357**(12): p. 1199-209.
107. Raychaudhuri, S., et al., *Common variants at CD40 and other loci confer risk of rheumatoid arthritis.* Nature Genetics, 2008. **40**(10): p. 1216-1223.
108. Bowes, J., et al., *Rare variation at the TNFAIP3 locus and susceptibility to rheumatoid arthritis.* Human Genetics, 2010. **128**(6): p. 627-633.
109. Meng, W., et al., *DNA methylation mediates genotype and smoking interaction in the development of anti-citrullinated peptide antibody-positive rheumatoid arthritis.* 2017. **19**(1): p. 71.
110. Richardson, B., et al., *Evidence for impaired t cell dna methylation in systemic lupus erythematosus and rheumatoid arthritis.* Arthritis & Rheumatism, 1990. **33**(11): p. 1665-1673.
111. Karouzakis, E., et al., *DNA hypomethylation in rheumatoid arthritis synovial fibroblasts.* Arthritis & Rheumatism, 2009. **60**(12): p. 3613-3622.
112. Nakano, K., et al., *DNA methylome signature in rheumatoid arthritis.* Ann Rheum Dis, 2013. **72**(1): p. 110-7.

113. Nelly, Z. and B. Rafic, *New insights into the classification of juvenile idiopathic arthritis.* International Journal of Rheumatic Diseases, 2012. **15**.
114. Niimoto, T., et al., *MicroRNA-146a expresses in interleukin-17 producing T cells in rheumatoid arthritis patients.* BMC Musculoskeletal Disorders, 2010. **11**(1): p. 209.
115. Miao, C.-g., et al., *New advances of DNA methylation and histone modifications in rheumatoid arthritis, with special emphasis on MeCP2.* Cellular Signalling, 2013. **25**(4): p. 875-882.
116. Vessey, M.P., L. Villard-Mackintosh, and D. Yeates, *Oral contraceptives, cigarette smoking and other factors in relation to arthritis.* Contraception, 1987. **35**(5): p. 457-64.
117. Sugiyama, D., et al., *Impact of smoking as a risk factor for developing rheumatoid arthritis: A meta-analysis of observational studies.* Annals of the Rheumatic Diseases, 2010. **69**(1): p. 70-81.
118. Cimmino, M.A., et al., *Dynamic contrast-enhanced, extremity-dedicated MRI identifies synovitis changes in the follow-up of rheumatoid arthritis patients treated with rituximab.* Clin Exp Rheumatol, 2014. **32**(5): p. 647-52.
119. van der Helm-van Mil, A.H., et al., *The HLA-DRB1 shared epitope alleles differ in the interaction with smoking and predisposition to antibodies to cyclic citrullinated peptide.* Arthritis Rheum, 2007. **56**(2): p. 425-32.
120. Makrygiannakis, D., et al., *Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells.* Ann Rheum Dis, 2008. **67**(10): p. 1488-92.
121. Lugli, E., et al., *Expression of citrulline and homocitrulline residues in the lungs of non-smokers and smokers: implications for autoimmunity in rheumatoid arthritis.* Arthritis Research & Therapy, 2015. **17**(1): p. 9.
122. O'Leary, S.M., et al., *Cigarette smoking impairs human pulmonary immunity to Mycobacterium tuberculosis.* Am J Respir Crit Care Med, 2014. **190**(12): p. 1430-6.
123. Ortega, E., C. Barriga, and A.B. Rodriguez, *Decline in the phagocytic function of alveolar macrophages from mice exposed to cigarette smoke.* Comp Immunol Microbiol Infect Dis, 1994. **17**(1): p. 77-84.
124. McLean, M.H., et al., *Does the microbiota play a role in the pathogenesis of autoimmune diseases?* Gut, 2015. **64**(2): p. 332-341.
125. Paget, S.A., *The Microbiome, Autoimmunity, and Arthritis: Cause and Effect: An Historical Perspective.* Trans Am Clin Climatol Assoc, 2012. **123**: p. 257-67.
126. Tsuchiya, N. and R.C. Williams, *Molecular mimicry--hypothesis or reality?* West J Med, 1992. **157**(2): p. 133-8.
127. Socransky, S.S., et al., *Microbial complexes in subgingival plaque.* J Clin Periodontol, 1998. **25**(2): p. 134-44.
128. Hajishengallis, G., *Periodontitis: from microbial immune subversion to systemic inflammation.* Nat Rev Immunol, 2015. **15**(1): p. 30-44.
129. Koziel, J., P. Mydel, and J. Potempa, *The Link Between Periodontal Disease and Rheumatoid Arthritis: An Updated Review.* Curr Rheumatol Rep, 2014. **16**(3).
130. Berthelot, J.M. and B. Le Goff, *Rheumatoid arthritis and periodontal disease.* Joint Bone Spine, 2010. **77**(6): p. 537-41.
131. Detert, J., et al., *The association between rheumatoid arthritis and periodontal disease.* Arthritis Research & Therapy, 2010. **12**(5): p. 218.

132. Mikuls, T.R., et al., *Periodontitis and Porphyromonas gingivalis in patients with rheumatoid arthritis.* Arthritis Rheumatol, 2014. **66**(5): p. 1090-100.
133. Marchesan, J.T., et al., *Porphyromonas gingivalis oral infection exacerbates the development and severity of collagen-induced arthritis.* Arthritis Res Ther, 2013. **15**(6): p. R186.
134. Moen, K., et al., *Synovial inflammation in active rheumatoid arthritis and psoriatic arthritis facilitates trapping of a variety of oral bacterial DNAs.* Clin Exp Rheumatol, 2006. **24**(6): p. 656-63.
135. Wegner, N., et al., *Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis.* Arthritis Rheum, 2010. **62**(9): p. 2662-72.
136. Wu, H.J., et al., *Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells.* Immunity, 2010. **32**(6): p. 815-27.
137. Dorozynska, I., et al., *Partial depletion of natural gut flora by antibiotic aggravates collagen induced arthritis (CIA) in mice.* Pharmacol Rep, 2014. **66**(2): p. 250-5.
138. Turnbaugh, P.J., et al., *The human microbiome project.* Nature, 2007. **449**(7164): p. 804-10.
139. Vaahtovuo, J., et al., *Fecal microbiota in early rheumatoid arthritis.* J Rheumatol, 2008. **35**(8): p. 1500-5.
140. Buchbender, C., et al., *Synovitis and bone inflammation in early rheumatoid arthritis: High-resolution multi-pinhole SPECT versus MRI.* Diagnostic and Interventional Radiology, 2013. **19**(1): p. 20-24.
141. Choi, I.Y., et al., *MRP8/14 serum levels as a strong predictor of response to biological treatments in patients with rheumatoid arthritis.* Ann Rheum Dis, 2015. **74**(3): p. 499-505.
142. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-98.
143. Cutolo, M., et al., *Evidence for the presence of androgen receptors in the synovial tissue of rheumatoid arthritis patients and healthy controls.* Arthritis & Rheumatism, 1992. **35**(9): p. 1007-1015.
144. Cutolo, M., et al., *Presence of estrogen-binding sites on macrophage-like synoviocytes and cd8+, cd29+, cd45ro+ t lymphocytes in normal and rheumatoid synovium.* Arthritis & Rheumatism, 1993. **36**(8): p. 1087-1097.
145. Tedeschi, S.K., B. Bermas, and K.H. Costenbader, *Sexual disparities in the incidence and course of SLE and RA.* Clin Immunol, 2013. **149**(2): p. 211-8.
146. Hughes, G.C., *Progesterone and autoimmune disease.* Autoimmunity Reviews, 2012. **11**(6–7): p. A502-A514.
147. Grimaldi, C.M., et al., *Estrogen alters thresholds for B cell apoptosis and activation.* J Clin Invest, 2002. **109**(12): p. 1625-33.
148. Grimaldi, C.M., V. Jeganathan, and B. Diamond, *Hormonal regulation of B cell development: 17 beta-estradiol impairs negative selection of high-affinity DNA-reactive B cells at more than one developmental checkpoint.* J Immunol, 2006. **176**(5): p. 2703-10.
149. Forslind, K., et al., *Sex: a major predictor of remission in early rheumatoid arthritis?* Ann Rheum Dis, 2007. **66**(1): p. 46-52.

150. Straub, R.H., et al., *Tumor necrosis factor-neutralizing therapies improve altered hormone axes: an alternative mode of antiinflammatory action.* Arthritis Rheum, 2006. **54**(7): p. 2039-46.
151. Cutolo, M., *SEX HORMONE ADJUVANT THERAPY IN RHEUMATOID ARTHRITIS.* Rheumatic Disease Clinics of North America, 2000. **26**(4): p. 881-895.
152. Huang, Q.Q. and R.M. Pope, *ROLE OF TOLL LIKE RECEPTORS IN RHEUMATOID ARTHRITIS.* Curr Rheumatol Rep, 2009. **11**(5): p. 357-64.
153. Ospelt, C., et al., *Overexpression of toll-like receptors 3 and 4 in synovial tissue from patients with early rheumatoid arthritis: toll-like receptor expression in early and longstanding arthritis.* Arthritis Rheum, 2008. **58**(12): p. 3684-92.
154. Kim, K.W., et al., *Up-regulation of stromal cell-derived factor 1 (CXCL12) production in rheumatoid synovial fibroblasts through interactions with T lymphocytes: role of interleukin-17 and CD40L-CD40 interaction.* Arthritis Rheum, 2007. **56**(4): p. 1076-86.
155. Sacre, S.M., et al., *Inhibitors of TLR8 reduce TNF production from human rheumatoid synovial membrane cultures.* J Immunol, 2008. **181**(11): p. 8002-9.
156. Khandpur, R., et al., *NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis.* Sci Transl Med, 2013. **5**(178): p. 178ra40.
157. Lin, A.M., et al., *Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis.* J Immunol, 2011. **187**(1): p. 490-500.
158. Wijbrandts, C.A., et al., *Absence of changes in the number of synovial sublining macrophages after ineffective treatment for rheumatoid arthritis: Implications for use of synovial sublining macrophages as a biomarker.* Arthritis Rheum, 2007. **56**(11): p. 3869-71.
159. Haringman, J., et al., *Synovial tissue macrophages: a sensitive biomarker for response to treatment in patients with rheumatoid arthritis.* Ann Rheum Dis, 2005. **64**(6): p. 834-8.
160. Bresnihan, B., et al., *Synovial tissue sublining CD68 expression is a biomarker of therapeutic response in rheumatoid arthritis clinical trials: consistency across centers.* J Rheumatol, 2009. **36**.
161. Lebre, M.C., et al., *Rheumatoid arthritis synovium contains two subsets of CD83-DC-LAMP- dendritic cells with distinct cytokine profiles.* Am J Pathol, 2008. **172**(4): p. 940-50.
162. Miossec, P., *Dynamic interactions between T cells and dendritic cells and their derived cytokines/chemokines in the rheumatoid synovium.* Arthritis Res Ther, 2008. **10 Suppl 1**: p. S2.
163. Jongbloed, S.L., et al., *Plasmacytoid Dendritic Cells Regulate Breach of Self-Tolerance in Autoimmune Arthritis.* The Journal of Immunology, 2009. **182**(2): p. 963.
164. Kiener, H.P., et al., *Synovial fibroblasts self-direct multicellular lining architecture and synthetic function in three-dimensional organ culture.* Arthritis Rheum, 2010. **62**(3): p. 742-52.
165. Bustamante, M.F., et al., *Fibroblast-like synoviocyte metabolism in the pathogenesis of rheumatoid arthritis.* 2017. **19**(1): p. 110.
166. Muller-Ladner, U., et al., *Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice.* Am J Pathol, 1996. **149**.

167. Frey, O., et al., *Induction of chronic destructive arthritis in SCID mice by arthritogenic fibroblast-like synoviocytes derived from mice with antigen-induced arthritis.* Arthritis Res Ther, 2018. **20**(1): p. 261.
168. Rivellese, F., et al., *Mast cells in rheumatoid arthritis: friends or foes?* Autoimmun Rev, 2017. **16**(6): p. 557-563.
169. Rivellese, F., et al., *Mast cells in early rheumatoid arthritis associate with disease severity and support B cell autoantibody production.* Annals of the Rheumatic Diseases, 2018. **77**(12): p. 1773.
170. Gotis-Graham, I., et al., *Synovial mast cell responses during clinical improvement in early rheumatoid arthritis.* Ann Rheum Dis, 1998. **57**(11): p. 664-71.
171. Gotis-Graham, I. and H.P. McNeil, *Mast cell responses in rheumatoid synovium. Association of the MCTC subset with matrix turnover and clinical progression.* Arthritis Rheum, 1997. **40**(3): p. 479-89.
172. St. John, A.L. and S.N. Abraham, *Innate Immunity and Its Regulation by Mast Cells.* The Journal of Immunology, 2013. **190**(9): p. 4458.
173. Deane, K.D., J.M. Norris, and V.M. Holers, *Preclinical rheumatoid arthritis: identification, evaluation, and future directions for investigation.* Rheum Dis Clin North Am, 2010. **36**(2): p. 213-41.
174. E, W., *On the occurrence of a factor in human serum activating the specific agglutination of sheep red corpuscles.* Acta Pathol Microbiol Scan, 1940. **17**: p. 172-88.
175. Ingegnoli, F., R. Castelli, and R. Gualtierotti, *Rheumatoid factors: clinical applications.* Disease markers, 2013. **35**(6): p. 727-734.
176. Nell, V.P.K., et al., *Autoantibody profiling as early diagnostic and prognostic tool for rheumatoid arthritis.* Annals of the Rheumatic Diseases, 2005. **64**(12): p. 1731-1736.
177. Borretzen, M., et al., *Differences in mutational patterns between rheumatoid factors in health and disease are related to variable heavy chain family and germ-line gene usage.* Eur J Immunol, 1997. **27**: p. 735 - 741.
178. Laurent, L., et al., *IgM rheumatoid factor amplifies the inflammatory response of macrophages induced by the rheumatoid arthritis-specific immune complexes containing anticitrullinated protein antibodies.* Ann Rheum Dis, 2015. **74**(7): p. 1425-31.
179. Jonsson, T., et al., *Raised IgA rheumatoid factor (RF) but not IgM RF or IgG RF is associated with extra-articular manifestations in rheumatoid arthritis.* Scand J Rheumatol, 1995. **24**(6): p. 372-5.
180. Nienhuis, R.L. and E. Mandema, *A NEW SERUM FACTOR IN PATIENTS WITH RHEUMATOID ARTHRITIS; THE ANTIPERINUCLEAR FACTOR.* Ann Rheum Dis, 1964. **23**: p. 302-5.
181. Hoet, R.M., R.A. Voorsmit, and W.J. Van Venrooij, *The perinuclear factor, a rheumatoid arthritis-specific autoantigen, is not present in keratohyalin granules of cultured buccal mucosa cells.* Clin Exp Immunol, 1991. **84**(1): p. 59-65.
182. Girbal-Neuhauser, E., et al., *The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deimination of arginine residues.* J Immunol, 1999. **162**(1): p. 585-94.

183. Pruijn, G.J., A. Wiik, and W.J. van Venrooij, *The use of citrullinated peptides and proteins for the diagnosis of rheumatoid arthritis.* Arthritis Res Ther, 2010. **12**(1): p. 203.
184. van Venrooij, W.J., J.J.B.C. van Beers, and G.J.M. Pruijn, *Anti-CCP antibodies: the past, the present and the future.* Nat Rev Rheumatol, 2011. **7**(7): p. 391-398.
185. van der Woude, D., et al., *Epitope spreading of the anti-citrullinated protein antibody response occurs before disease onset and is associated with the disease course of early arthritis.* Ann Rheum Dis, 2010. **69**(8): p. 1554-61.
186. Jilani, A.A. and C.G. Mackworth-Young, *The role of citrullinated protein antibodies in predicting erosive disease in rheumatoid arthritis: a systematic literature review and meta-analysis.* Int J Rheumatol, 2015. **2015**: p. 728610.
187. van Oosterhout, M., et al., *Differences in synovial tissue infiltrates between anti-cyclic citrullinated peptide-positive rheumatoid arthritis and anti-cyclic citrullinated peptide-negative rheumatoid arthritis.* Arthritis Rheum, 2008. **58**: p. 53 - 60.
188. Clavel, C., et al., *Induction of macrophage secretion of tumor necrosis factor alpha through Fcgamma receptor IIa engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen.* Arthritis Rheum, 2008. **58**(3): p. 678-88.
189. Adamopoulos, I.E., et al., *Interleukin-17A upregulates receptor activator of NF-kappaB on osteoclast precursors.* Arthritis Res Ther, 2010. **12**(1): p. R29.
190. Kuhn, K.A., et al., *Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis.* J Clin Invest, 2006. **116**(4): p. 961-73.
191. Sokolove, J., et al., *Immune complexes containing citrullinated fibrinogen costimulate macrophages via Toll-like receptor 4 and Fcγ receptor.* Arthritis & Rheumatism, 2011. **63**(1): p. 53-62.
192. Pruijn, G.J.M., *Citrullination and Carbamylation in the Pathophysiology of Rheumatoid Arthritis.* Front Immunol, 2015. **6**.
193. Kalim, S., et al., *Protein carbamylation in kidney disease: pathogenesis and clinical implications.* Am J Kidney Dis, 2014. **64**(5): p. 793-803.
194. Jin, J., et al., *Proteomic, functional, and domain-based analysis of in vivo 14-3-3 binding proteins involved in cytoskeletal regulation and cellular organization.* Curr Biol, 2004. **14**(16): p. 1436-50.
195. Yang, X., et al., *Structural basis for protein-protein interactions in the 14-3-3 protein family.* Proc Natl Acad Sci U S A, 2006. **103**(46): p. 17237-42.
196. Kilani, R.T., et al., *Detection of high levels of 2 specific isoforms of 14-3-3 proteins in synovial fluid from patients with joint inflammation.* J Rheumatol, 2007. **34**(8): p. 1650-7.
197. Maksymowych, W.P., et al., *Serum 14-3-3eta is a novel marker that complements current serological measurements to enhance detection of patients with rheumatoid arthritis.* J Rheumatol, 2014. **41**(11): p. 2104-13.
198. Maksymowych, W.P., et al., *14-3-3eta is a novel mediator associated with the pathogenesis of rheumatoid arthritis and joint damage.* Arthritis Res Ther, 2014. **16**(2): p. R99.
199. Hassfeld, W., et al., *Demonstration of a new antinuclear antibody (anti-RA33) that is highly specific for rheumatoid arthritis.* Arthritis Rheum, 1989. **32**(12): p. 1515-20.

200. Hassfeld, W., et al., *Autoimmune response to the spliceosome. An immunologic link between rheumatoid arthritis, mixed connective tissue disease, and systemic lupus erythematosus.* Arthritis Rheum, 1995. **38**(6): p. 777-85.
201. Blass, S., et al., *The stress protein BiP is overexpressed and is a major B and T cell target in rheumatoid arthritis.* Arthritis Rheum, 2001. **44**(4): p. 761-71.
202. Goldbach-Mansky, R., et al., *Rheumatoid arthritis associated autoantibodies in patients with synovitis of recent onset.* Arthritis Res, 2000. **2**(3): p. 236-43.
203. Cope, A.P., H. Schulze-Koops, and M. Aringer, *The central role of T cells in rheumatoid arthritis.* Clin Exp Rheumatol, 2007. **25**(5 Suppl 46): p. S4-11.
204. Alzabin, S. and R.O. Williams, *Effector T cells in rheumatoid arthritis: Lessons from animal models.* FEBS Letters, 2011. **585**(23): p. 3649-3659.
205. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annu Rev Immunol, 1989. **7**: p. 145-73.
206. Lubberts, E., et al., *IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis.* J Immunol, 2001. **167**(2): p. 1004-13.
207. Langrish, C.L., et al., *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation.* J Exp Med, 2005. **201**(2): p. 233-40.
208. Wilson, N.J., et al., *Development, cytokine profile and function of human interleukin 17-producing helper T cells.* Nat Immunol, 2007. **8**(9): p. 950-7.
209. Bystrom, J., et al., *Harnessing the Therapeutic Potential of Th17 Cells.* Mediators Inflamm, 2015. **2015**.
210. Azizi, G., F. Jadidi-Niaragh, and A. Mirshafiey, *Th17 Cells in Immunopathogenesis and treatment of rheumatoid arthritis.* Int J Rheum Dis, 2013. **16**(3): p. 243-53.
211. Cooles, F.A., J.D. Isaacs, and A.E. Anderson, *Treg cells in rheumatoid arthritis: an update.* Curr Rheumatol Rep, 2013. **15**(9): p. 352.
212. Broere, F., et al., *A2 T cell subsets and T cell-mediated immunity*, in *Principles of Immunopharmacology*, F.P. Nijkamp and M.J. Parnham, Editors. 2011, Birkhäuser Basel. p. 15-27.
213. Liu, M.F., et al., *The presence of cytokine-suppressive CD4+CD25+ T cells in the peripheral blood and synovial fluid of patients with rheumatoid arthritis.* Scand J Immunol, 2005. **62**(3): p. 312-7.
214. Mottonen, M., et al., *CD4+ CD25+ T cells with the phenotypic and functional characteristics of regulatory T cells are enriched in the synovial fluid of patients with rheumatoid arthritis.* Clin Exp Immunol, 2005. **140**(2): p. 360-7.
215. Ehrenstein, M.R., et al., *Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy.* J Exp Med, 2004. **200**(3): p. 277-85.
216. Lawson, C.A., et al., *Early rheumatoid arthritis is associated with a deficit in the CD4+CD25high regulatory T cell population in peripheral blood.* Rheumatology, 2006. **45**(10): p. 1210-1217.
217. van Amelsfort, J.M., et al., *CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid.* Arthritis Rheum, 2004. **50**(9): p. 2775-85.
218. Haufe, S., et al., *Impaired suppression of synovial fluid CD4+CD25- T cells from patients with juvenile idiopathic arthritis by CD4+CD25+ Treg cells.* Arthritis Rheum, 2011. **63**(10): p. 3153-62.

219. Noack, M. and P. Miossec, *Th17 and regulatory T cell balance in autoimmune and inflammatory diseases.* Autoimmun Rev, 2014. **13**(6): p. 668-77.
220. Yu, Z.-x., et al., *The ratio of Th17/Treg cells as a risk indicator in early acute respiratory distress syndrome.* Critical Care, 2015. **19**(1): p. 82.
221. Vasanthi, P., G. Nalini, and G. Rajasekhar, *Role of tumor necrosis factor-alpha in rheumatoid arthritis: a review.* APLAR Journal of Rheumatology, 2007. **10**(4): p. 270-274.
222. Choy, E.H. and G.S. Panayi, *Cytokine pathways and joint inflammation in rheumatoid arthritis.* N Engl J Med, 2001. **344**(12): p. 907-16.
223. Koch, A.E., S.L. Kunkel, and R.M. Strieter, *Cytokines in rheumatoid arthritis.* J Investig Med, 1995. **43**(1): p. 28-38.
224. Scott, I.C., et al., *A randomised trial evaluating anakinra in early active rheumatoid arthritis.* Clin Exp Rheumatol, 2016. **34**(1): p. 88-93.
225. Choy, E., *Understanding the dynamics: pathways involved in the pathogenesis of rheumatoid arthritis.* Rheumatology (Oxford), 2012. **51 Suppl 5**: p. v3-11.
226. Mima, T. and N. Nishimoto, *Clinical value of blocking IL-6 receptor.* Curr Opin Rheumatol, 2009. **21**(3): p. 224-30.
227. Burmester, G.R., et al., *Efficacy and safety of sarilumab monotherapy versus adalimumab monotherapy for the treatment of patients with active rheumatoid arthritis (MONARCH): a randomised, double-blind, parallel-group phase III trial.* Ann Rheum Dis, 2017. **76**(5): p. 840-847.
228. Wright, J.F., et al., *Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells.* J Biol Chem, 2007. **282**(18): p. 13447-55.
229. Gaffen, S.L., *Role of IL-17 in the Pathogenesis of Rheumatoid Arthritis.* Curr Rheumatol Rep, 2009. **11**(5): p. 365-70.
230. Tahir, H., et al., *Secukinumab in Active Rheumatoid Arthritis after Anti-TNFalpha Therapy: A Randomized, Double-Blind Placebo-Controlled Phase 3 Study.* Rheumatol Ther, 2017. **4**(2): p. 475-488.
231. Smolen, J.S., et al., *A randomised phase II study evaluating the efficacy and safety of subcutaneously administered ustekinumab and guselkumab in patients with active rheumatoid arthritis despite treatment with methotrexate.* Annals of the Rheumatic Diseases, 2017. **76**(5): p. 831.
232. Genovese, M.C., et al., *Efficacy and safety of secukinumab in patients with rheumatoid arthritis: a phase II, dose-finding, double-blind, randomised, placebo controlled study.* Annals of the Rheumatic Diseases, 2013. **72**(6): p. 863.
233. Szekanecz, Z., et al., *Chemokines and chemokine receptors in arthritis.* Front Biosci (Schol Ed). **2**: p. 153-67.
234. Koch, A.E., *Chemokines and their receptors in rheumatoid arthritis: Future targets?* Arthritis & Rheumatism, 2005. **52**(3): p. 710-721.
235. Koch, A.E., et al., *Synovial tissue macrophage as a source of the chemotactic cytokine IL-8.* J Immunol, 1991. **147**(7): p. 2187-95.
236. Koch, A.E., et al., *Epithelial neutrophil activating peptide-78: a novel chemotactic cytokine for neutrophils in arthritis.* J Clin Invest, 1994. **94**(3): p. 1012-8.
237. Szekanecz, Z., G. Szegedi, and A.E. Koch, *Angiogenesis in rheumatoid arthritis: pathogenic and clinical significance.* J Investig Med, 1998. **46**(2): p. 27-41.

238. Blades, M.C., et al., *Stromal cell-derived factor 1 (CXCL12) induces monocyte migration into human synovium transplanted onto SCID Mice.* Arthritis Rheum, 2002. **46**(3): p. 824-36.
239. Gronthos, S. and A.C. Zannettino, *The role of the chemokine CXCL12 in osteoclastogenesis.* Trends Endocrinol Metab, 2007. **18**(3): p. 108-13.
240. Manzo, A., et al., *Systematic microanatomical analysis of CXCL13 and CCL21 in situ production and progressive lymphoid organization in rheumatoid synovitis.* Eur J Immunol, 2005. **35**(5): p. 1347-59.
241. Vergunst, C.E., et al., *Modulation of CCR2 in rheumatoid arthritis: a double-blind, randomized, placebo-controlled clinical trial.* Arthritis Rheum, 2008. **58**(7): p. 1931-9.
242. Bugatti, S., et al., *B Cells in Rheumatoid Arthritis: From Pathogenic Players to Disease Biomarkers.* Biomed Res Int, 2014. **2014**.
243. LeBien, T.W., *Fates of human B-cell precursors.* Blood, 2000. **96**(1): p. 9-23.
244. Pieper, K., B. Grimbacher, and H. Eibel, *B-cell biology and development.* J Allergy Clin Immunol, 2013. **131**(4): p. 959-71.
245. Shapiro-Shelef, M. and K. Calame, *Regulation of plasma-cell development.* Nat Rev Immunol, 2005. **5**(3): p. 230-42.
246. Giltiay, N., C. Chappell, and E. Clark, *B-cell selection and the development of autoantibodies.* Arthritis Research & Therapy, 2012. **14**(Suppl 4): p. S1.
247. Allen, C.D. and J.G. Cyster, *Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function.* Semin Immunol, 2008. **20**(1): p. 14-25.
248. MacLennan, I.C., *Germinal centers.* Annu Rev Immunol, 1994. **12**: p. 117-39.
249. Gatto, D. and R. Brink, *The germinal center reaction.* J Allergy Clin Immunol, 2010. **126**(5): p. 898-907; quiz 908-9.
250. Garside, P., et al., *Visualization of specific B and T lymphocyte interactions in the lymph node.* Science, 1998. **281**(5373): p. 96-9.
251. Reif, K., et al., *Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position.* Nature, 2002. **416**(6876): p. 94-9.
252. Foy, T.M., et al., *gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory.* J Exp Med, 1994. **180**(1): p. 157-63.
253. Stavnezer, J., J.E.J. Guikema, and C.E. Schrader, *Mechanism and Regulation of Class Switch Recombination.* Annu Rev Immunol, 2008. **26**: p. 261-92.
254. Gearhart, P.J. and R.D. Wood, *Emerging links between hypermutation of antibody genes and DNA polymerases.* Nat Rev Immunol, 2001. **1**(3): p. 187-92.
255. Luo, Z., D. Ronai, and M.D. Scharff, *The role of activation-induced cytidine deaminase in antibody diversification, immunodeficiency, and B-cell malignancies.* J Allergy Clin Immunol, 2004. **114**(4): p. 726-35; quiz 736.
256. Berek, C., A. Berger, and M. Apel, *Maturation of the immune response in germinal centers.* Cell, 1991. **67**(6): p. 1121-9.
257. Jacob, J., et al., *Intraclonal generation of antibody mutants in germinal centres.* Nature, 1991. **354**(6352): p. 389-92.
258. Schroeder, H.W., Jr. and L. Cavacini, *Structure and function of immunoglobulins.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S41-52.
259. Dunnick, W., et al., *DNA sequences at immunoglobulin switch region recombination sites.* Nucleic Acids Res, 1993. **21**(3): p. 365-72.
260. Christmann, M., et al., *Mechanisms of human DNA repair: an update.* Toxicology, 2003. **193**(1-2): p. 3-34.

261. Oracki, S.A., et al., *Plasma cell development and survival.* Immunol Rev, 2010. **237**(1): p. 140-59.
262. Lopes-Carvalho, T. and J.F. Kearney, *Development and selection of marginal zone B cells.* Immunol Rev, 2004. **197**: p. 192-205.
263. Smith, D., G. Creadon, and P. Jena, *Di- and trinucleotide target preferences of somatic mutagenesis in normal and autoreactive B cells.* J Immunol, 1996. **156**: p. 2642 - 2652.
264. Tiburzy, B., et al., *Plasma cells in immunopathology: concepts and therapeutic strategies.* Semin Immunopathol, 2014. **36**(3): p. 277-88.
265. Moser, K., et al., *Stromal niches, plasma cell differentiation and survival.* Curr Opin Immunol, 2006. **18**(3): p. 265-70.
266. Lightman, S.M., A. Utley, and K.P. Lee, *Survival of Long-Lived Plasma Cells (LLPC): Piecing Together the Puzzle.* 2019. **10**(965).
267. Landsverk, O.J., et al., *Antibody-secreting plasma cells persist for decades in human intestine.* J Exp Med, 2017. **214**(2): p. 309-317.
268. Schneider, P., *The role of APRIL and BAFF in lymphocyte activation.* Current Opinion in Immunology, 2005. **17**(3): p. 282-289.
269. Allen, C.D., et al., *Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5.* Nat Immunol, 2004. **5**(9): p. 943-52.
270. Thien, M., et al., *Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches.* Immunity, 2004. **20**(6): p. 785-798.
271. Ramanujam, M., et al., *Mechanism of action of transmembrane activator and calcium modulator ligand interactor-Ig in murine systemic lupus erythematosus.* Journal of Immunology, 2004. **173**(5): p. 3524-3534.
272. Samuels, J., et al., *Impaired early B cell tolerance in patients with rheumatoid arthritis.* J Exp Med, 2005. **201**(10): p. 1659-67.
273. Menard, L., et al., *Inflammation-independent defective early B cell tolerance checkpoints in rheumatoid arthritis.* Arthritis Rheum, 2011. **63**(5): p. 1237-45.
274. Bugatti, S., et al., *Inflammatory lesions in the bone marrow of rheumatoid arthritis patients: a morphological perspective.* Arthritis research & therapy, 2012. **14**(6): p. 229.
275. Rapetti, L., et al., *B cell resistance to Fas-mediated apoptosis contributes to their ineffective control by regulatory T cells in rheumatoid arthritis.* Ann Rheum Dis, 2015. **74**(1): p. 294-302.
276. Takemura, S., et al., *Lymphoid neogenesis in rheumatoid synovitis.* J Immunol, 2001. **167**(2): p. 1072-80.
277. Melet, J., et al., *Rituximab-induced T cell depletion in patients with rheumatoid arthritis: association with clinical response.* Arthritis Rheum, 2013. **65**(11): p. 2783-90.
278. van de Veerdonk, F.L., et al., *The anti-CD20 antibody rituximab reduces the Th17 cell response.* Arthritis Rheum, 2011. **63**(6): p. 1507-16.
279. Kocijan, R., et al., *Differences in bone structure between rheumatoid arthritis and psoriatic arthritis patients relative to autoantibody positivity.* Ann Rheum Dis, 2014. **73**(11): p. 2022-8.
280. Pescovitz, M.D., *Rituximab, an anti-cd20 monoclonal antibody: history and mechanism of action.* Am J Transplant, 2006. **6**(5 Pt 1): p. 859-66.

281. Cohen, M.D. and E. Keystone, *Rituximab for Rheumatoid Arthritis.* Rheumatol Ther, 2015. **2**(2): p. 99-111.
282. Protheroe, A., et al., *Remission of inflammatory arthropathy in association with anti-CD20 therapy for non-Hodgkin's lymphoma.* Rheumatology, 1999. **38**(11): p. 1150-1152.
283. Edwards, J.C. and G. Cambridge, *Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes.* Rheumatology (Oxford), 2001. **40**(2): p. 205-11.
284. Edwards, J.C., et al., *Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis.* N Engl J Med, 2004. **350**(25): p. 2572-81.
285. Emery, P., et al., *The efficacy and safety of rituximab in patients with active rheumatoid arthritis despite methotrexate treatment: results of a phase IIB randomized, double-blind, placebo-controlled, dose-ranging trial.* Arthritis Rheum, 2006. **54**(5): p. 1390-400.
286. Rubbert-Roth, A., et al., *Efficacy and safety of various repeat treatment dosing regimens of rituximab in patients with active rheumatoid arthritis: results of a Phase III randomized study (MIRROR).* Rheumatology (Oxford), 2010. **49**(9): p. 1683-93.
287. Emery, P., et al., *Efficacy and safety of different doses and retreatment of rituximab: a randomised, placebo-controlled trial in patients who are biological naive with active rheumatoid arthritis and an inadequate response to methotrexate (Study Evaluating Rituximab's Efficacy in MTX iNadequate rEsponders (SERENE)).* Ann Rheum Dis, 2010. **69**(9): p. 1629-35.
288. Tak, P.P., et al., *Sustained inhibition of progressive joint damage with rituximab plus methotrexate in early active rheumatoid arthritis: 2-year results from the randomised controlled trial IMAGE.* Ann Rheum Dis, 2012. **71**(3): p. 351-7.
289. Peterfy, C., et al., *MRI assessment of suppression of structural damage in patients with rheumatoid arthritis receiving rituximab: results from the randomised, placebo-controlled, double-blind RA-SCORE study.* Ann Rheum Dis, 2016. **75**(1): p. 170-7.
290. Cohen, S.B., et al., *Rituximab for rheumatoid arthritis refractory to anti-tumor necrosis factor therapy: Results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial evaluating primary efficacy and safety at twenty-four weeks.* Arthritis Rheum, 2006. **54**(9): p. 2793-806.
291. Cohen, S.B., et al., *Continued inhibition of structural damage over 2 years in patients with rheumatoid arthritis treated with rituximab in combination with methotrexate.* Ann Rheum Dis, 2010. **69**(6): p. 1158-61.
292. Mease, P.J., et al., *Efficacy and safety of retreatment in patients with rheumatoid arthritis with previous inadequate response to tumor necrosis factor inhibitors: results from the SUNRISE trial.* J Rheumatol, 2010. **37**(5): p. 917-27.
293. Gomez-Reino, J.J., et al., *Comparative effectiveness of switching to alternative tumour necrosis factor (TNF) antagonists versus switching to rituximab in patients with rheumatoid arthritis who failed previous TNF antagonists: the MIRAR Study.* Ann Rheum Dis, 2012. **71**(11): p. 1861-4.
294. Haraoui, B., et al., *Safety and effectiveness of rituximab in patients with rheumatoid arthritis following an inadequate response to 1 prior tumor necrosis factor inhibitor: the RESET Trial.* J Rheumatol, 2011. **38**(12): p. 2548-56.
295. Assous, N., et al., *Rituximab therapy in rheumatoid arthritis in daily practice.* J Rheumatol, 2008. **35**(1): p. 31-4.

296. Bokarewa, M., et al., *Efficacy of anti-CD20 treatment in patients with rheumatoid arthritis resistant to a combination of methotrexate/anti-TNF therapy.* Scand J Immunol, 2007. **66**(4): p. 476-83.
297. Keystone, E., et al., *Safety and efficacy of additional courses of rituximab in patients with active rheumatoid arthritis: an open-label extension analysis.* Arthritis Rheum, 2007. **56**(12): p. 3896-908.
298. Keystone, E.C., et al., *Multiple courses of rituximab produce sustained clinical and radiographic efficacy and safety in patients with rheumatoid arthritis and an inadequate response to 1 or more tumor necrosis factor inhibitors: 5-year data from the REFLEX study.* J Rheumatol, 2012. **39**(12): p. 2238-46.
299. Harrold, L.R., et al., *Effectiveness of Rituximab for the Treatment of Rheumatoid Arthritis in Patients with Prior Exposure to Anti-TNF: Results from the CORRONA Registry.* The Journal of Rheumatology, 2015.
300. Lopez-Olivo, M.A., et al., *Rituximab for rheumatoid arthritis.* Cochrane Database Syst Rev, 2015. **1**: p. CD007356.
301. Edwards, J.C. and G. Cambridge, *Rheumatoid arthritis: the predictable effect of small immune complexes in which antibody is also antigen.* Br J Rheumatol, 1998. **37**(2): p. 126-30.
302. Leandro, M.J., J.C. Edwards, and G. Cambridge, *Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion.* Ann Rheum Dis, 2002. **61**(10): p. 883-8.
303. De La Torre, I., et al., *Total serum immunoglobulin levels in patients with RA after multiple B-cell depletion cycles based on rituximab: relationship with B-cell kinetics.* Rheumatology (Oxford), 2012. **51**(5): p. 833-40.
304. Popa, C., et al., *Repeated B lymphocyte depletion with rituximab in rheumatoid arthritis over 7 yrs.* Rheumatology (Oxford), 2007. **46**(4): p. 626-30.
305. Bredemeier, M., G.G. Campos, and F.K. de Oliveira, *Updated systematic review and meta-analysis of randomized controlled trials comparing low- versus high-dose rituximab for rheumatoid arthritis.* Clin Rheumatol, 2015. **34**(10): p. 1801-5.
306. Mariette, X., et al., *Evaluation of low-dose rituximab for the retreatment of patients with active rheumatoid arthritis: a non-inferiority randomised controlled trial.* Ann Rheum Dis, 2014. **73**(8): p. 1508-14.
307. Batticciotto, A., et al., *AB0297 Dose reduction in patients with rheumatoid arthritis responding to the standard rituximab regimen.* Annals of the Rheumatic Diseases, 2013. **72**(Suppl 3): p. A877.
308. Bavaliya, M.K. and P. Shenoy, *AB0470 Efficacy of Very low Dose (100MG) Rituximab in Active Rheumatoid Arthritis Despite Combinations Dmards.* Annals of the Rheumatic Diseases, 2015. **74**(Suppl 2): p. 1054.
309. Bruzzese, V., *Therapeutic effectiveness of minimal doses of rituximab in a patient with rheumatoid arthritis.* Int J Immunopathol Pharmacol, 2011. **24**(1): p. 265-7.
310. Bruzzese, V., et al., *Prompt efficacy of very low-dose rituximab on monoclonal B lymphocytosis in a rheumatoid arthritis patient.* Int J Rheum Dis, 2013. **16**(6): p. 764-5.
311. Bruzzese, V. and J. Pepe, *Unexpected and persistent depletion of B lymphocytes CD20 following a minimum dose of anti-CD20 antibody (Rituximab).* Reumatismo, 2009. **61**(4): p. 306-8.

312. den Broeder, A.A., et al., *Ultra-low dose of rituximab in rheumatoid arthritis: study protocol for a randomised controlled trial.* Trials, 2017. **18**(1): p. 403.
313. Buch, M.H., et al., *Updated consensus statement on the use of rituximab in patients with rheumatoid arthritis.* Ann Rheum Dis, 2011. **70**(6): p. 909-20.
314. van Vollenhoven, R.F., et al., *Long-term safety of rituximab in rheumatoid arthritis: 9.5-year follow-up of the global clinical trial programme with a focus on adverse events of interest in RA patients.* Ann Rheum Dis, 2013. **72**(9): p. 1496-502.
315. Marco, H., et al., *The effect of rituximab therapy on immunoglobulin levels in patients with multisystem autoimmune disease.* BMC Musculoskelet Disord, 2014. **15**: p. 178.
316. Roll, P., et al., *Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis.* Arthritis Rheum, 2006. **54**(8): p. 2377-86.
317. Teng, Y.K., et al., *Induction of long-term B-cell depletion in refractory rheumatoid arthritis patients preferentially affects autoreactive more than protective humoral immunity.* Arthritis Res Ther, 2012. **14**(2): p. R57.
318. Vital, E.M., et al., *Re-treatment of rheumatoid arthritis patients who were initial nonresponders to rituximab: comment on the article by Thurlings et al.* Arthritis Rheum, 2009. **60**(6): p. 1867; author reply 1867-8.
319. Leandro, M.J., et al., *Reconstitution of peripheral blood B cells after depletion with rituximab in patients with rheumatoid arthritis.* Arthritis Rheum, 2006. **54**(2): p. 613-20.
320. Dass, S., et al., *Highly sensitive B cell analysis predicts response to rituximab therapy in rheumatoid arthritis.* Arthritis Rheum, 2008. **58**(10): p. 2993-9.
321. Leandro, M.J., *B-cell subpopulations in humans and their differential susceptibility to depletion with anti-CD20 monoclonal antibodies.* Arthritis Res Ther, 2013. **15 Suppl 1**: p. S3.
322. Lim, S.H., et al., *Fc gamma receptor IIb on target B cells promotes rituximab internalization and reduces clinical efficacy.* Blood, 2011. **118**(9): p. 2530-40.
323. Reddy, V., et al., *Internalization of rituximab and the efficiency of B Cell depletion in rheumatoid arthritis and systemic lupus erythematosus.* Arthritis Rheumatol, 2015. **67**(8): p. 2046-55.
324. Pal, I., et al., *Effect of Fcgamma-receptor 3a (FCGR3A) gene polymorphisms on rituximab therapy in Hungarian patients with rheumatoid arthritis.* RMD Open, 2017. **3**(2): p. e000485.
325. Ajeganova, S., et al., *Effect of FCGR polymorphism on the occurrence of late-onset neutropenia and flare-free survival in rheumatic patients treated with rituximab.* Arthritis Res Ther, 2017. **19**(1): p. 44.
326. Viecceli, D., et al., *Correlation between cellular expression of complement regulatory proteins with depletion and repopulation of B-lymphocytes in peripheral blood of patients with rheumatoid arthritis treated with rituximab.* Rev Bras Reumatol Engl Ed, 2017. **57**(5): p. 385-391.
327. Rogers, L.M., et al., *Complement-Regulatory Proteins CFHR1 and CFHR3 and Patient Response to Anti-CD20 Monoclonal Antibody Therapy.* Clin Cancer Res, 2017. **23**(4): p. 954-961.
328. Winiarska, M., et al., *Statins impair antitumor effects of rituximab by inducing conformational changes of CD20.* PLoS Med, 2008. **5**(3): p. e64.

329. Arts, E.E., et al., *Statins inhibit the antirheumatic effects of rituximab in rheumatoid arthritis: results from the Dutch Rheumatoid Arthritis Monitoring (DREAM) registry.* Ann Rheum Dis, 2011. **70**(5): p. 877-8.
330. Das, S., et al., *Statins do not influence clinical response and B cell depletion after rituximab treatment in rheumatoid arthritis.* Ann Rheum Dis, 2013. **72**(3): p. 463-4.
331. Lehane, P.B., et al., *Effect of concomitant statins on rituximab efficacy in patients with rheumatoid arthritis.* Annals of the Rheumatic Diseases, 2014. **73**(10): p. 1906.
332. Chatzidionysiou, K., et al., *Highest clinical effectiveness of rituximab in autoantibody-positive patients with rheumatoid arthritis and in those for whom no more than one previous TNF antagonist has failed: pooled data from 10 European registries.* Annals of the Rheumatic Diseases, 2011. **70**(9): p. 1575-1580.
333. Quartuccio, L., et al., *Rheumatoid factor positivity rather than anti-CCP positivity, a lower disability and a lower number of anti-TNF agents failed are associated with response to rituximab in rheumatoid arthritis.* Rheumatology (Oxford), 2009. **48**(12): p. 1557-9.
334. Sellam, J., et al., *B cell activation biomarkers as predictive factors for the response to rituximab in rheumatoid arthritis: a six-month, national, multicenter, open-label study.* Arthritis Rheum, 2011. **63**(4): p. 933-8.
335. Gardette, A., et al., *High anti-CCP antibody titres predict good response to rituximab in patients with active rheumatoid arthritis.* Joint Bone Spine, 2014. **81**(5): p. 416-20.
336. Isaacs, J.D., et al., *Effect of baseline rheumatoid factor and anticitrullinated peptide antibody serotype on rituximab clinical response: a meta-analysis.* Ann Rheum Dis, 2013. **72**(3): p. 329-36.
337. Maneiro, R.J., et al., *Rheumatoid factor as predictor of response to abatacept, rituximab and tocilizumab in rheumatoid arthritis: Systematic review and meta-analysis.* Semin Arthritis Rheum, 2013. **43**(1): p. 9-17.
338. Couderc, M., et al., *Influence of gender on response to rituximab in patients with rheumatoid arthritis: results from the Autoimmunity and Rituximab registry.* Rheumatology (Oxford), 2014. **53**(10): p. 1788-93.
339. Ottaviani, S., et al., *Body Mass Index and response to rituximab in rheumatoid arthritis.* Joint Bone Spine, 2015. **82**(6): p. 432-6.
340. Lindenberg, L., et al., *Restrictive IgG antibody response against mutated citrullinated vimentin predicts response to rituximab in patients with rheumatoid arthritis.* Arthritis Res Ther, 2015. **17**: p. 206.
341. Adlowitz, D.G., et al., *Expansion of Activated Peripheral Blood Memory B Cells in Rheumatoid Arthritis, Impact of B Cell Depletion Therapy, and Biomarkers of Response.* PLoS One, 2015. **10**(6): p. e0128269.
342. Sellam, J., et al., *Blood memory B cells are disturbed and predict the response to rituximab in patients with rheumatoid arthritis.* Arthritis Rheum, 2011. **63**(12): p. 3692-701.
343. Owczarczyk, K., et al., *A plasmablast biomarker for nonresponse to antibody therapy to CD20 in rheumatoid arthritis.* Sci Transl Med, 2011. **3**(101): p. 101ra92.
344. Sellam, J., et al., *CCL19, a B cell chemokine, is related to the decrease of blood memory B cells and predicts the clinical response to rituximab in patients with rheumatoid arthritis.* Arthritis Rheum, 2013. **65**(9): p. 2253-61.
345. Jimenez-Boj, E., et al., *Interaction between synovial inflammatory tissue and bone marrow in rheumatoid arthritis.* J Immunol, 2005. **175**(4): p. 2579-88.

346. Humby, F., et al., *Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium.* PLoS Med, 2009. **6**(1): p. e1.
347. Thurlings, R.M., et al., *Synovial tissue response to rituximab: mechanism of action and identification of biomarkers of response.* Ann Rheum Dis, 2008. **67**(7): p. 917-25.
348. Kavanaugh, A., et al., *Assessment of rituximab's immunomodulatory synovial effects (ARISE trial). 1: clinical and synovial biomarker results.* Ann Rheum Dis, 2008. **67**(3): p. 402-8.
349. Mahévas, M., et al., *Long-Lived Plasma Cells in Autoimmunity: Lessons from B-Cell Depleting Therapy.* Frontiers in Immunology, 2013. **4**: p. 494.
350. Vital, E.M., et al., *Management of nonresponse to rituximab in rheumatoid arthritis: predictors and outcome of re-treatment.* Arthritis Rheum, 2010. **62**(5): p. 1273-9.
351. Scheel, T., et al., *V-region gene analysis of locally defined synovial B and plasma cells reveals selected B cell expansion and accumulation of plasma cell clones in rheumatoid arthritis.* Arthritis Rheum, 2011. **63**(1): p. 63-72.
352. Vos, K., et al., *Early effects of rituximab on the synovial cell infiltrate in patients with rheumatoid arthritis.* Arthritis Rheum, 2007. **56**(3): p. 772-8.
353. Teng, Y.K., et al., *Immunohistochemical analysis as a means to predict responsiveness to rituximab treatment.* Arthritis Rheum, 2007. **56**(12): p. 3909-18.
354. Teng, Y.K., et al., *Residual inflammation after rituximab treatment is associated with sustained synovial plasma cell infiltration and enhanced B cell repopulation.* Ann Rheum Dis, 2009. **68**(6): p. 1011-6.
355. Lindberg, J., et al., *The gene expression profile in the synovium as a predictor of the clinical response to infliximab treatment in rheumatoid arthritis.* PLoS One, 2010. **5**: p. e11310.
356. Dennis, G., et al., *Synovial phenotypes in rheumatoid arthritis correlate with response to biologic therapeutics.* Arthritis Research & Therapy, 2014. **16**(2): p. R90.
357. van de Sande, M.G., et al., *Evaluating antirheumatic treatments using synovial biopsy: a recommendation for standardisation to be used in clinical trials.* Ann Rheum Dis, 2011. **70**(3): p. 423-7.
358. Kane, D., et al., *Survey of arthroscopy performed by rheumatologists.* Rheumatology (Oxford), 2002. **41**(2): p. 210-5.
359. Humby, F., et al., *The synovial membrane as a prognostic tool in rheumatoid arthritis.* Autoimmun Rev, 2007. **6**(4): p. 248-52.
360. Koski, J.M. and M. Helle, *Ultrasound guided synovial biopsy using portal and forceps.* Ann Rheum Dis, 2005. **64**(6): p. 926-9.
361. Kelly, S., et al., *Ultrasound-guided synovial biopsy: a safe, well-tolerated and reliable technique for obtaining high-quality synovial tissue from both large and small joints in early arthritis patients.* Annals of the Rheumatic Diseases, 2013.
362. Mandelin, A.M., 2nd, et al., *Transcriptional Profiling of Synovial Macrophages Using Minimally Invasive Ultrasound-Guided Synovial Biopsies in Rheumatoid Arthritis.* Arthritis Rheumatol, 2018. **70**(6): p. 841-854.
363. Lazarou, I., et al., *Ultrasound-guided synovial biopsy of the wrist does not alter subsequent clinical or ultrasound disease activity assessments: a prospective study for incorporation of imaging in clinical trials.* Clin Exp Rheumatol, 2016.
364. Humby, F., et al., *A Multicenter Retrospective Analysis Evaluating Performance of Synovial Biopsy Techniques in Patients With Inflammatory Arthritis: Arthroscopic*

Versus Ultrasound-Guided Versus Blind Needle Biopsy. Arthritis Rheumatol, 2018. **70**(5): p. 702-710.
365. Smith, M.D., *The Normal Synovium.* Open Rheumatol J, 2011. **5**: p. 100-6.
366. Smith, M., et al., *Microarchitecture and protective mechanisms in synovial tissue from clinically and arthroscopically normal knee joints.* Ann Rheum Dis, 2003. **62**(4): p. 303-7.
367. Koch, A.E., *The role of angiogenesis in rheumatoid arthritis: recent developments.* Ann Rheum Dis, 2000. **59**(Suppl 1): p. i65-71.
368. Pitzalis, C., et al., *Ectopic lymphoid-like structures in infection, cancer and autoimmunity.* Nat Rev Immunol, 2014. **14**(7): p. 447-62.
369. Pitzalis, C., S. Kelly, and F. Humby, *New learnings on the pathophysiology of RA from synovial biopsies.* Curr Opin Rheumatol, 2013. **25**(3): p. 334-44.
370. Corsiero, E., et al., *Role of lymphoid chemokines in the development of functional ectopic lymphoid structures in rheumatic autoimmune diseases.* Immunol Lett, 2012. **145**(1-2): p. 62-7.
371. D'Agostino, M.-A., et al., *Scoring ultrasound synovitis in rheumatoid arthritis: a EULAR-OMERACT ultrasound taskforce—Part 1: definition and development of a standardised, consensus-based scoring system.* RMD Open, 2017. **3**(1).
372. Krenn, V., et al., *Grading of chronic synovitis--a histopathological grading system for molecular and diagnostic pathology.* Pathol Res Pract, 2002. **198**(5): p. 317-25.
373. Tak, P.P., et al., *Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity.* Arthritis Rheum, 1997. **40**.
374. Bugatti, S., et al., *Synovial tissue heterogeneity and peripheral blood biomarkers.* Curr Rheumatol Rep, 2011. **13**(5): p. 440-8.
375. Humby, F.C., et al., *Can Synovial Pathobiology Integrate with Current Clinical and Imaging Prediction Models to Achieve Personalized Health Care in Rheumatoid Arthritis?* Front Med (Lausanne), 2017. **4**: p. 41.
376. Yates, D.B. and J.T. Scott, *Rheumatoid synovitis and joint disease. Relationship between arthroscopic and histological changes.* Ann Rheum Dis, 1975. **34**(1): p. 1-6.
377. Rooney, M., et al., *Analysis of the histologic variation of synovitis in rheumatoid arthritis.* 1988. **31**(8): p. 956-963.
378. Kirkham, B.W., et al., *Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort).* Arthritis Rheum, 2006. **54**(4): p. 1122-31.
379. Koizumi, F., et al., *Synovitis in rheumatoid arthritis: Scoring of characteristic histopathological features.* Pathol Int, 1999. **49**.
380. Matsuno, H., et al., *Relationship between histological findings and clinical findings in rheumatoid arthritis.* Pathol Int, 2002. **52**(8): p. 527-33.
381. Tak, P.P., et al., *Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity.* Arthritis Rheum, 1997. **40**(2): p. 217-25.
382. Tak, P.P., et al., *Reduction of synovial inflammation after anti-CD4 monoclonal antibody treatment in early rheumatoid arthritis.* Arthritis Rheum, 1995. **38**(10): p. 1457-65.
383. Kraan, M.C., et al., *Immunohistological analysis of synovial tissue for differential diagnosis in early arthritis.* Rheumatology, 1999. **38**(11): p. 1074-1080.

384. Youssef, P.P., et al., *Microscopic measurement of cellular infiltration in the rheumatoid arthritis synovial membrane: a comparison of semiquantitative and quantitative analysis.* Br J Rheumatol, 1998. **37**(9): p. 1003-7.
385. Bresnihan, B., et al., *Microscopic measurement of synovial membrane inflammation in rheumatoid arthritis: Proposals for the evaluation of tissue samples by quantitative analysis.* British Journal of Rheumatology, 1998. **37**(6): p. 636-642.
386. Kraan, M.C., et al., *Quantification of the cell infiltrate in synovial tissue by digital image analysis.* Rheumatology (Oxford), 2000. **39**(1): p. 43-9.
387. Singh, J.A., et al., *Immunohistochemistry of normal human knee synovium: a quantitative study.* Annals of the Rheumatic Diseases, 2004. **63**(7): p. 785.
388. Lindblad, S. and E. Hedfors, *The synovial membrane of healthy individuals--immunohistochemical overlap with synovitis.* Clin Exp Immunol, 1987. **69**(1): p. 41-7.
389. Manzo, A., et al., *Secondary and ectopic lymphoid tissue responses in rheumatoid arthritis: from inflammation to autoimmunity and tissue damage/remodeling.* Immunological Reviews, 2010. **233**(1): p. 267-285.
390. Scire, C.A., et al., *Immunohistological assessment of the synovial tissue in small joints in rheumatoid arthritis: validation of a minimally invasive ultrasound-guided synovial biopsy procedure.* Arthritis Res Ther, 2007. **9**(5): p. R101.
391. Kirkham, B., et al., *Intraarticular variability of synovial membrane histology, immunohistology, and cytokine mRNA expression in patients with rheumatoid arthritis.* J Rheumatol, 1999. **26**(4): p. 777-84.
392. Dolhain, R.J., et al., *Distribution of T cells and signs of T-cell activation in the rheumatoid joint: implications for semiquantitative comparative histology.* Br J Rheumatol, 1998. **37**(3): p. 324-30.
393. Kraan, M.C., et al., *Comparison of synovial tissues from the knee joints and the small joints of rheumatoid arthritis patients: Implications for pathogenesis and evaluation of treatment.* Arthritis Rheum, 2002. **46**(8): p. 2034-8.
394. Soden, M., et al., *Immunohistological features in the synovium obtained from clinically uninvolved knee joints of patients with rheumatoid arthritis.* Br J Rheumatol, 1989. **28**(4): p. 287-92.
395. Kraan, M.C., et al., *Asymptomatic synovitis precedes clinically manifest arthritis.* Arthritis Rheum, 1998. **41**.
396. Rooney, M., et al., *Changes in lymphocyte infiltration of the synovial membrane and the clinical course of rheumatoid arthritis.* Arthritis Rheum, 1989. **32**(4): p. 361-9.
397. van Baarsen, L., et al., *Synovial tissue heterogeneity in rheumatoid arthritis in relation to disease activity and biomarkers in peripheral blood.* Arthritis Rheum, 2010. **62**: p. 1602 - 1607.
398. Orr, C.K., et al., *The Utility and Limitations of CRP, ESR and DAS28-CRP in Appraising Disease Activity in Rheumatoid Arthritis.* Front Med (Lausanne), 2018. **5**: p. 185.
399. Bugatti, S., et al., *B cell distribution and activation-induced cytidine deaminase expression in rheumatoid synovitis: clinical and bio-molecular correlates.* Annals of the Rheumatic Diseases, 2011. **70**(Suppl 2): p. A55.
400. Oldroyd, A.G.S., et al., *Long-term persistence with rituximab in patients with rheumatoid arthritis.* Rheumatology (Oxford), 2018. **57**(6): p. 1089-1096.
401. Kilcher, G., et al., *Rheumatoid arthritis patients treated in trial and real world settings: comparison of randomized trials with registries.* Rheumatology (Oxford), 2018. **57**(2): p. 354-369.

402. Wendler, J., et al., *Rituximab in patients with rheumatoid arthritis in routine practice (GERINIS): six-year results from a prospective, multicentre, non-interventional study in 2,484 patients.* Arthritis Res Ther, 2014. **16**(2): p. R80.
403. Owczarczyk, K., et al., *Clinical outcome and B cell depletion in patients with rheumatoid arthritis receiving rituximab monotherapy in comparison with patients receiving concomitant methotrexate.* Ann Rheum Dis, 2008. **67**(11): p. 1648-9.
404. Solau-Gervais, E., et al., *Efficacy of rituximab in the treatment of rheumatoid arthritis. Influence of serologic status, coprescription of methotrexate and prior TNF-alpha inhibitors exposure.* Joint Bone Spine, 2012. **79**(3): p. 281-4.
405. Wells, G.A., et al., *Minimum important difference between patients with rheumatoid arthritis: the patient's perspective.* J Rheumatol, 1993. **20**(3): p. 557-60.
406. Pope, J.E., et al., *The minimally important difference for the health assessment questionnaire in rheumatoid arthritis clinical practice is smaller than in randomized controlled trials.* J Rheumatol, 2009. **36**(2): p. 254-9.
407. Wolfe, F., et al., *Predicting mortality in patients with rheumatoid arthritis.* Arthritis Rheum, 2003. **48**(6): p. 1530-42.
408. Soliman, M.M., et al., *Effectiveness of rituximab in patients with rheumatoid arthritis: observational study from the British Society for Rheumatology Biologics Register.* J Rheumatol, 2012. **39**(2): p. 240-6.
409. Keystone, E., et al., *Improvement in patient-reported outcomes in a rituximab trial in patients with severe rheumatoid arthritis refractory to anti-tumor necrosis factor therapy.* Arthritis Rheum, 2008. **59**(6): p. 785-93.
410. Randen, I., et al., *The identification of germinal centers and follicular dendritic cell networks in rheumatoid synovial tissue.* Scand J Immunol, 1995. **41**: p. 481 - 486.
411. Canete, J., et al., *Clinical significance of synovial lymphoid neogenesis and its reversal after anti-tumour necrosis factor alpha therapy in rheumatoid arthritis.* Ann Rheum Dis, 2009. **68**: p. 751 - 756.
412. Klaasen, R., et al., *The relationship between synovial lymphocyte aggregates and the clinical response to infliximab in rheumatoid arthritis: a prospective study.* Arthritis Rheum, 2009. **60**: p. 3217 - 3224.
413. van de Sande, M.G., et al., *Different stages of rheumatoid arthritis: features of the synovium in the preclinical phase.* Ann Rheum Dis, 2011. **70**(5): p. 772-7.
414. Thurlings, R.M., et al., *Synovial lymphoid neogenesis does not define a specific clinical rheumatoid arthritis phenotype.* Arthritis Rheum, 2008. **58**(6): p. 1582-9.
415. Canete, J.D., et al., *Ectopic lymphoid neogenesis in psoriatic arthritis.* Ann Rheum Dis, 2007. **66**(6): p. 720-6.
416. Doorenspleet, M.E., et al., *Rheumatoid arthritis synovial tissue harbours dominant B-cell and plasma-cell clones associated with autoreactivity.* Ann Rheum Dis, 2014. **73**(4): p. 756-62.
417. Yanni, G., et al., *Intramuscular gold decreases cytokine expression and macrophage numbers in the rheumatoid synovial membrane.* Ann Rheum Dis, 1994. **53**(5): p. 315-22.
418. Dolhain, R.J., et al., *Methotrexate reduces inflammatory cell numbers, expression of monokines and of adhesion molecules in synovial tissue of patients with rheumatoid arthritis.* Br J Rheumatol, 1998. **37**(5): p. 502-8.

419. Julia, A., et al., *Identification of candidate genes for rituximab response in rheumatoid arthritis patients by microarray expression profiling in blood cells.* Pharmacogenomics, 2009. **10**(10): p. 1697-708.
420. Thurlings, R.M., et al., *Relationship between the type I interferon signature and the response to rituximab in rheumatoid arthritis patients.* Arthritis Rheum, 2010. **62**(12): p. 3607-14.
421. Raterman, H.G., et al., *The interferon type I signature towards prediction of non-response to rituximab in rheumatoid arthritis patients.* Arthritis Res Ther, 2012. **14**(2): p. R95.
422. Vosslamber, S., et al., *Pharmacological induction of interferon type I activity following treatment with rituximab determines clinical response in rheumatoid arthritis.* Ann Rheum Dis, 2011. **70**(6): p. 1153-9.
423. de Jong, T.D., et al., *A multi-parameter response prediction model for rituximab in rheumatoid arthritis.* Joint Bone Spine, 2018. **85**(2): p. 219-226.
424. Sellam, J., et al., *Serum IL-33, a new marker predicting response to rituximab in rheumatoid arthritis.* Arthritis Res Ther, 2016. **18**.
425. Tchetina, E.V., et al., *Rituximab Downregulates Gene Expression Associated with Cell Proliferation, Survival, and Proteolysis in the Peripheral Blood from Rheumatoid Arthritis Patients: A Link between High Baseline Autophagy-Related ULK1 Expression and Improved Pain Control.* Arthritis, 2016. **2016**: p. 4963950.
426. Gutierrez-Roelens, I., et al., *Rituximab treatment induces the expression of genes involved in healing processes in the rheumatoid arthritis synovium.* Arthritis Rheum, 2011. **63**(5): p. 1246-54.
427. Ducreux, J., et al., *Global molecular effects of tocilizumab therapy in rheumatoid arthritis synovium.* Arthritis Rheumatol, 2014. **66**(1): p. 15-23.
428. Badot, V., et al., *Gene expression profiling in the synovium identifies a predictive signature of absence of response to adalimumab therapy in rheumatoid arthritis.* Arthritis Res Ther, 2009. **11**: p. R57.
429. Hogan, V., et al., *Pretreatment synovial transcriptional profile is associated with early and late clinical response in rheumatoid arthritis patients treated with rituximab.* Ann Rheum Dis, 1888. **2012**: p. 71.
430. van der Pouw Kraan, T.C.T.M., et al., *Discovery of distinctive gene expression profiles in rheumatoid synovium using cDNA microarray technology: evidence for the existence of multiple pathways of tissue destruction and repair.* Genes And Immunity, 2003. **4**: p. 187.
431. Townsend, M.J., *Molecular and cellular heterogeneity in the Rheumatoid Arthritis synovium: clinical correlates of synovitis.* Best Pract Res Clin Rheumatol, 2014. **28**(4): p. 539-49.
432. Muramatsu, M., et al., *Class Switch Recombination and Hypermutation Require Activation-Induced Cytidine Deaminase (AID), a Potential RNA Editing Enzyme.* Cell, 2000. **102**(5): p. 553-563.
433. Corsiero, E., et al., *Single cell cloning and recombinant monoclonal antibodies generation from RA synovial B cells reveal frequent targeting of citrullinated histones of NETs.* Ann Rheum Dis, 2016. **75**(10): p. 1866-75.
434. Xu, X., et al., *Increased expression of activation-induced cytidine deaminase is associated with anti-CCP and rheumatoid factor in rheumatoid arthritis.* Scand J Immunol, 2009. **70**(3): p. 309-16.

435. Hsu, H.C., et al., *Overexpression of activation-induced cytidine deaminase in B cells is associated with production of highly pathogenic autoantibodies.* J Immunol, 2007. **178**(8): p. 5357-65.
436. Hsu, H.C., et al., *Inhibition of the catalytic function of activation-induced cytidine deaminase promotes apoptosis of germinal center B cells in BXD2 mice.* Arthritis Rheum, 2011. **63**(7): p. 2038-48.
437. Cantaert, T., et al., *B lymphocyte autoimmunity in rheumatoid synovitis is independent of ectopic lymphoid neogenesis.* J Immunol, 2008. **181**(1): p. 785-94.
438. Kim, H., et al., *Plasma cell development in synovial germinal centers in patients with rheumatoid and reactive arthritis.* J Immunol, 1999. **162**: p. 3053 - 3062.
439. Reich, D., A.L. Price, and N. Patterson, *Principal component analysis of genetic data.* Nature Genetics, 2008. **40**: p. 491.
440. Ma, S. and Y. Dai, *Principal component analysis based methods in bioinformatics studies.* Brief Bioinform, 2011. **12**(6): p. 714-22.
441. Lindberg, J., et al., *Variability in synovial inflammation in rheumatoid arthritis investigated by microarray technology.* Arthritis Res Ther, 2006. **8**(2): p. R47.
442. Tsubaki, T., et al., *Characterization of histopathology and gene-expression profiles of synovitis in early rheumatoid arthritis using targeted biopsy specimens.* Arthritis Research & Therapy, 2005. **7**(4): p. R825.
443. Brühl, H., et al., *Surface expression of CC- and CXC-chemokine receptors on leucocyte subsets in inflammatory joint diseases.* Clin Exp Immunol, 2001. **126**(3): p. 551-9.
444. Haringman, J.J., et al., *Chemokine and chemokine receptor expression in paired peripheral blood mononuclear cells and synovial tissue of patients with rheumatoid arthritis, osteoarthritis, and reactive arthritis.* Ann Rheum Dis, 2006. **65**(3): p. 294-300.
445. Prahalad, S., *Negative association between the chemokine receptor CCR5-Δ32 polymorphism and rheumatoid arthritis: A meta-analysis.* Genes Immun, 2006. **7**(3): p. 264-8.
446. Doodes, P.D., et al., *CCR5 is involved in resolution of inflammation in proteoglycan-induced arthritis.* Arthritis Rheum, 2009. **60**(10): p. 2945-53.
447. Avdeeva, A.S., et al., *[The immunologic predictors of effect of anti-B-cell therapy under rheumatoid arthritis].* Klin Lab Diagn, 2014(3): p. 48-52.
448. Katchamart, W., et al., *Predictors for remission in rheumatoid arthritis patients: A systematic review.* Arthritis Care Res (Hoboken), 2010. **62**(8): p. 1128-43.
449. Georgiev, H., et al., *Coming of Age: CD96 Emerges as Modulator of Immune Responses.* Front Immunol, 2018. **9**.
450. Bouaziz, J.-D., et al., *Therapeutic B cell depletion impairs adaptive and autoreactive CD4⁺ T cell activation in mice.* 2007. **104**(52): p. 20878-20883.
451. *<roadblock b cells.pdf>.*
452. Miyake, Y., et al., *Self and nonself recognition through C-type lectin receptor, Mincle.* Self Nonself, 2010. **1**(4): p. 310-3.
453. Silverstein, R.L. and M. Febbraio, *CD36, a Scavenger Receptor Involved in Immunity, Metabolism, Angiogenesis, and Behavior.* Sci Signal. **2**(72): p. re3.
454. Silverman, G.J. and D.L. Boyle, *Understanding the mechanistic basis in rheumatoid arthritis for clinical response to anti-CD20 therapy: the B-cell roadblock hypothesis.* 2008. **223**(1): p. 175-185.

455. Toubi, E., et al., *Changes in macrophage function after rituximab treatment in patients with rheumatoid arthritis.* Ann Rheum Dis, 2007. **66**(6): p. 818-20.
456. Byrd, V.M., et al., *Fibroblast growth factor-1 (FGF-1) enhances IL-2 production and nuclear translocation of NF-kappaB in FGF receptor-bearing Jurkat T cells.* J Immunol, 1999. **162**(10): p. 5853-9.
457. Sano, H., et al., *Detection of high levels of heparin binding growth factor-1 (acidic fibroblast growth factor) in inflammatory arthritic joints.* 1990. **110**(4): p. 1417-1426.
458. Diaz-Torne, C., et al., *Rituximab-induced interleukin-15 reduction associated with clinical improvement in rheumatoid arthritis.* Immunology, 2014. **142**(3): p. 354-62.
459. Budagian, V., et al., *IL-15/IL-15 receptor biology: a guided tour through an expanding universe.* Cytokine Growth Factor Rev, 2006. **17**(4): p. 259-80.
460. Armitage, R.J., et al., *IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation.* J Immunol, 1995. **154**(2): p. 483-90.
461. Halvorsen, E.H., et al., *Interleukin-15 induces interleukin-17 production by synovial T cell lines from patients with rheumatoid arthritis.* Scand J Immunol, 2011. **73**(3): p. 243-9.
462. Ku, C.C., et al., *Control of homeostasis of CD8+ memory T cells by opposing cytokines.* Science, 2000. **288**(5466): p. 675-8.
463. Schiff, M., *Role of interleukin 1 and interleukin 1 receptor antagonist in the mediation of rheumatoid arthritis.* Ann Rheum Dis, 2000. **59**(Suppl 1): p. i103-8.
464. Bo, M., et al., *Rheumatoid arthritis patient antibodies highly recognize IL-2 in the immune response pathway involving IRF5 and EBV antigens.* Scientific Reports, 2018. **8**(1): p. 1789.
465. Jiang, Y., et al., *Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes.* J Immunol, 1992. **148**(8): p. 2423-8.
466. Ballara, S., et al., *Raised serum vascular endothelial growth factor levels are associated with destructive change in inflammatory arthritis.* Arthritis Rheum, 2001. **44**(9): p. 2055-64.
467. Thomas, J.W., et al., *Acidic fibroblast growth factor in synovial cells.* Arthritis Rheum, 2000. **43**(10): p. 2152-9.
468. Foulquier, C., et al., *Peptidyl arginine deiminase type 2 (PAD-2) and PAD-4 but not PAD-1, PAD-3, and PAD-6 are expressed in rheumatoid arthritis synovium in close association with tissue inflammation.* Arthritis Rheum, 2007. **56**(11): p. 3541-53.
469. Ivanov, I.I., et al., *The Orphan Nuclear Receptor RORγt Directs the Differentiation Program of Proinflammatory IL-17⁺ T Helper Cells.* Cell. **126**(6): p. 1121-1133.
470. Kato, H., J. Endres, and D.A. Fox, *The roles of IFN-γ versus IL-17 in Pathogenic Effects of Human Th17 Cells on Synovial Fibroblasts.* Mod Rheumatol, 2013. **23**(6).
471. Abromson-Leeman, S., R.T. Bronson, and M.E. Dorf, *Encephalitogenic T cells that stably express both T-bet and ROR gamma t consistently produce IFNgamma but have a spectrum of IL-17 profiles.* J Neuroimmunol, 2009. **215**(1-2): p. 10-24.
472. Ciccia, F., et al., *Rituximab modulates IL-17 expression in the salivary glands of patients with primary Sjögren's syndrome.* Rheumatology, 2014. **53**(7): p. 1313-1320.
473. Pene, J., et al., *Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells.* J Immunol, 2004. **172**(9): p. 5154-7.

474. Good, K.L., V.L. Bryant, and S.G. Tangye, *Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21.* J Immunol, 2006. **177**(8): p. 5236-47.
475. Ettinger, R., et al., *IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells.* J Immunol, 2005. **175**(12): p. 7867-79.
476. Liu, R., et al., *A regulatory effect of IL-21 on T follicular helper-like cell and B cell in rheumatoid arthritis.* Arthritis Res Ther, 2012. **14**(6): p. R255.
477. McGeachy, M.J., et al., *TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology.* Nat Immunol, 2007. **8**(12): p. 1390-7.
478. Yago, T., et al., *IL-23 and Th17 Disease in Inflammatory Arthritis.* J Clin Med, 2017. **6**(9).
479. McKelvey, K.J., et al., *Co-expression of CD21L and IL17A defines a subset of rheumatoid synovia, characterised by large lymphoid aggregates and high inflammation.* PLoS One, 2018. **13**(8).
480. Li, W.C., et al., *Identification of differentially expressed genes in synovial tissue of rheumatoid arthritis and osteoarthritis in patients.* **0**(0).
481. Streicher, K., et al., *The plasma cell signature in autoimmune disease.* Arthritis Rheumatol, 2014. **66**(1): p. 173-84.
482. Franco, A., et al., *CD21 and FCRL5 form a receptor complex with robust B-cell activating capacity.* Int Immunol, 2018. **30**(12): p. 569-578.
483. Li, H., et al., *Fc Receptor-like 5 Expression Distinguishes Two Distinct Subsets of Human Circulating Tissue-like Memory B Cells.* J Immunol, 2016. **196**(10): p. 4064-74.
484. Braun, A., et al., *Lymphotoxin beta-mediated stimulation of synoviocytes in rheumatoid arthritis.* Arthritis Rheum, 2004. **50**(7): p. 2140-50.
485. Xu, Z., et al., *Regulation of aicda expression and AID activity: Relevance to somatic hypermutation and class switch DNA recombination.* Crit Rev Immunol, 2007. **27**(4): p. 367-97.
486. Rao, D.A., *T Cells That Help B Cells in Chronically Inflamed Tissues.* Front Immunol, 2018. **9**.
487. Simpfendorfer, K.R., et al., *Autoimmune disease-associated haplotypes of BLK exhibit lowered thresholds for B cell activation and expansion of Ig class-switched B cells.* Arthritis Rheumatol, 2015. **67**(11): p. 2866-76.
488. Sen, M., *Wnt signalling in rheumatoid arthritis.* Rheumatology, 2005. **44**(6): p. 708-713.
489. Bombardieri, M., et al., *A BAFF/APRIL-dependent TLR3-stimulated pathway enhances the capacity of rheumatoid synovial fibroblasts to induce AID expression and Ig class-switching in B cells.* Annals of the Rheumatic Diseases, 2011. **70**(10): p. 1857-1865.
490. Rosengren, S., et al., *Elevated autoantibody content in rheumatoid arthritis synovia with lymphoid aggregates and the effect of rituximab.* Arthritis Res Ther, 2008. **10**(5): p. R105.
491. Keystone, E., et al., *Rituximab inhibits structural joint damage in patients with rheumatoid arthritis with an inadequate response to tumour necrosis factor inhibitor therapies.* Ann Rheum Dis, 2009. **68**(2): p. 216-21.
492. Finckh, A., et al., *Evolution of radiographic joint damage in rituximab-treated versus TNF-treated rheumatoid arthritis cases with inadequate response to TNF antagonists.* Ann Rheum Dis, 2012. **71**(10): p. 1680-5.

493. Cimmino, M.A., et al., *Dynamic contrast-enhanced, extremity-dedicated MRI identifies synovitis changes in the follow-up of rheumatoid arthritis patients treated with rituximab.* Clinical and Experimental Rheumatology, 2014. **32**(5): p. 647-652.
494. Firestein, G.S., *Immunologic mechanisms in the pathogenesis of rheumatoid arthritis.* J Clin Rheumatol, 2005. **11**(3 Suppl): p. S39-44.
495. Matsos, M., et al., *Ultrasound of the hands and feet for rheumatological disorders: influence on clinical diagnostic confidence and patient management.* Skeletal Radiol, 2009. **38**(11): p. 1049-54.
496. Hammer, H.B. and L. Terslev, *Role of ultrasound in managing rheumatoid arthritis.* Curr Rheumatol Rep, 2012. **14**(5): p. 438-44.
497. Valor, L., et al., *Ultrasound-detected joint inflammation and B cell count: related variables for rituximab-treated RA patients?* Rheumatology International, 2016. **36**(6): p. 793-797.
498. Reiche, B.E., et al., *Usefulness of power Doppler ultrasound for prediction of re-therapy with rituximab in rheumatoid arthritis: a prospective study of longstanding rheumatoid arthritis patients.* Arthritis Care Res (Hoboken), 2014. **66**(2): p. 204-16.
499. Najm, A., et al., *Knee joint synovitis: study of correlations and diagnostic performances of ultrasonography compared with histopathology.* RMD Open, 2018. **4**(1): p. e000616.
500. Andersen, M., et al., *Association between IL-6 production in synovial explants from rheumatoid arthritis patients and clinical and imaging response to biologic treatment: A pilot study.* PLoS One, 2018. **13**(5): p. e0197001.
501. Andersen, M., et al., *Ultrasound colour Doppler is associated with synovial pathology in biopsies from hand joints in rheumatoid arthritis patients: a cross-sectional study.* Ann Rheum Dis, 2014. **73**(4): p. 678-83.
502. Takase, K., et al., *Simultaneous evaluation of long-lasting knee synovitis in patients undergoing arthroplasty by power Doppler ultrasonography and contrast-enhanced MRI in comparison with histopathology.* Clinical and experimental rheumatology, 2012. **30**(1): p. 85.
503. Koski, J.M., et al., *Power Doppler ultrasonography and synovitis: correlating ultrasound imaging with histopathological findings and evaluating the performance of ultrasound equipments.* Ann Rheum Dis, 2006. **65**(12): p. 1590-5.
504. Wheater, G., et al., *Suppression of bone turnover by B-cell depletion in patients with rheumatoid arthritis.* 2011. **22**(12): p. 3067-3072.
505. Boumans, M.J.H., et al., *Rituximab abrogates joint destruction in rheumatoid arthritis by inhibiting osteoclastogenesis.* 2012. **71**(1): p. 108-113.
506. Christensen, A.F., et al., *Cartilage oligomeric matrix protein associates differentially with erosions and synovitis and has a different temporal course in cyclic citrullinated peptide antibody (anti-CCP)-positive versus anti-CCP-negative early rheumatoid arthritis.* J Rheumatol, 2011. **38**(8): p. 1563-8.
507. Meednu, N., et al., *Production of RANKL by Memory B Cells: A Link Between B Cells and Bone Erosion in Rheumatoid Arthritis.* 2016. **68**(4): p. 805-816.
508. Kitchen, J. and D. Kane, *Greyscale and power Doppler ultrasonographic evaluation of normal synovial joints: correlation with pro- and anti-inflammatory cytokines and angiogenic factors.* Rheumatology (Oxford), 2015. **54**(3): p. 458-62.

509. Kelly, S., et al., *Angiogenic gene expression and vascular density are reflected in ultrasonographic features of synovitis in early Rheumatoid Arthritis: an observational study.* Arthritis Res Ther, 2015. **17**: p. 58.
510. Li, R.W., et al., *Dramatic regulation of heparanase activity and angiogenesis gene expression in synovium from patients with rheumatoid arthritis.* Arthritis Rheum, 2008. **58**(6): p. 1590-600.
511. Raatz, Y., et al., *Gene expression profiling and functional analysis of angiogenic markers in murine collagen-induced arthritis.* Arthritis Res Ther, 2012. **14**(4): p. R169.
512. Brahn, E., et al., *An angiogenesis inhibitor, 2-methoxyestradiol, involutes rat collagen-induced arthritis and suppresses gene expression of synovial vascular endothelial growth factor and basic fibroblast growth factor.* J Rheumatol, 2008. **35**(11): p. 2119-28.
513. Yin, G., et al., *Endostatin gene transfer inhibits joint angiogenesis and pannus formation in inflammatory arthritis.* Mol Ther, 2002. **5**(5 Pt 1): p. 547-54.
514. Rico, M.C., et al., *Amelioration of inflammation, angiogenesis and CTGF expression in an arthritis model by a TSP1-derived peptide treatment.* J Cell Physiol, 2007. **211**(2): p. 504-12.
515. Etzerodt, A. and S.K. Moestrup, *CD163 and Inflammation: Biological, Diagnostic, and Therapeutic Aspects.* Antioxid Redox Signal, 2013. **18**(17): p. 2352-63.
516. Heftdal, L.D., et al., *Soluble CD206 plasma levels in rheumatoid arthritis reflect decrease in disease activity.* Scand J Clin Lab Invest, 2017. **77**(5): p. 385-389.
517. Sanyal, R., et al., *MS4A4A: a novel cell surface marker for M2 macrophages and plasma cells.* Immunol Cell Biol, 2017. **95**(7): p. 611-619.
518. Kolls, J.K. and A. Linden, *Interleukin-17 family members and inflammation.* Immunity, 2004. **21**(4): p. 467-76.
519. Laustsen, J.K., et al., *Soluble OX40L is associated with presence of autoantibodies in early rheumatoid arthritis.* Arthritis Res Ther, 2014. **16**(5): p. 474.
520. Volin, M.V. and A.E. Koch, *Interleukin-18: a mediator of inflammation and angiogenesis in rheumatoid arthritis.* J Interferon Cytokine Res, 2011. **31**(10): p. 745-51.
521. Peluso, G., et al., *Clinical and ultrasonographic remission determines different chances of relapse in early and long standing rheumatoid arthritis.* Annals of the Rheumatic Diseases, 2011. **70**(1): p. 172.
522. Brown, A.K., et al., *Presence of significant synovitis in rheumatoid arthritis patients with disease-modifying antirheumatic drug–induced clinical remission: evidence from an imaging study may explain structural progression.* Arthritis Rheum, 2006. **54**.
523. Saleem, B., et al., *Disease remission state in patients treated with the combination of tumor necrosis factor blockade and methotrexate or with disease-modifying antirheumatic drugs: A clinical and imaging comparative study.* 2009. **60**(7): p. 1915-1922.
524. Barczyńska, T.A., et al., *DAS28 score vs. ultrasound examination for assessment of rheumatoid arthritis disease activity: comparison and discussion of pros and cons.* Reumatologia, 2015. **53**(4): p. 213-8.
525. Terslev, L., et al., *Is synovial hypertrophy without Doppler activity sensitive to change? Post-hoc analysis from a rheumatoid arthritis ultrasound study.* Arthritis Res Ther, 2018. **20**(1): p. 224.

526. Iagnocco, A., et al., *Power Doppler ultrasound monitoring of response to anti-tumour necrosis factor alpha treatment in patients with rheumatoid arthritis.* Rheumatology (Oxford), 2015. **54**(10): p. 1890-6.
527. Epis, O., et al., *Clinical and ultrasound evaluation of the response to tocilizumab treatment in patients with rheumatoid arthritis: a case series.* Rheumatol Int, 2014. **34**(5): p. 737-42.
528. Fisher, B.A., et al., *Decrease in articular hypoxia and synovial blood flow at early time points following infliximab and etanercept treatment in rheumatoid arthritis.* Clin Exp Rheumatol, 2016. **34**(6): p. 1072-1076.
529. Naredo, E., et al., *Power Doppler ultrasonographic monitoring of response to anti-tumor necrosis factor therapy in patients with rheumatoid arthritis.* Arthritis Rheum, 2008. **58**(8): p. 2248-56.
530. Bottini, N. and G.S. Firestein, *Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors.* Nat Rev Rheumatol, 2013. **9**(1): p. 24-33.
531. Szekanecz, Z., et al., *Angiogenesis in rheumatoid arthritis.* Autoimmunity, 2009. **42**(7): p. 563-573.
532. Fazaa, A., et al., *The level of interleukin-17 in serum is linked to synovial hypervascularisation in rheumatoid arthritis.* Joint Bone Spine, 2014. **81**(6): p. 550-1.
533. Gullick, N.J., et al., *Linking Power Doppler Ultrasound to the Presence of Th17 Cells in the Rheumatoid Arthritis Joint.* PLOS ONE, 2010. **5**(9): p. e12516.
534. Ryu, S., J.H. Lee, and S.I. Kim, *IL-17 increased the production of vascular endothelial growth factor in rheumatoid arthritis synoviocytes.* Clin Rheumatol, 2006. **25**(1): p. 16-20.
535. Moran, E.M., et al., *Human rheumatoid arthritis tissue production of IL-17A drives matrix and cartilage degradation: synergy with tumour necrosis factor-α, Oncostatin M and response to biologic therapies.* 2009. **11**(4): p. R113.
536. Kim, K.-W., et al., *Th17 Cytokines Regulate Osteoclastogenesis in Rheumatoid Arthritis.* The American Journal of Pathology, 2015. **185**(11): p. 3011-3024.
537. Kotake, S., et al., *IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis.* The Journal of Clinical Investigation, 1999. **103**(9): p. 1345-1352.
538. Pollinger, B., et al., *Th17 cells, not IL-17+ gammadelta T cells, drive arthritic bone destruction in mice and humans.* J Immunol, 2011. **186**(4): p. 2602-12.
539. Koenders, M.I., et al., *Blocking of Interleukin-17 during Reactivation of Experimental Arthritis Prevents Joint Inflammation and Bone Erosion by Decreasing RANKL and Interleukin-1.* The American Journal of Pathology, 2005. **167**(1): p. 141-149.
540. Bresnihan, B., et al., *Serum interleukin 18 and interleukin 18 binding protein in rheumatoid arthritis.* Ann Rheum Dis, 2002. **61**(8): p. 726-9.
541. Yamamura, M., et al., *Interferon-gamma-inducing activity of interleukin-18 in the joint with rheumatoid arthritis.* Arthritis Rheum, 2001. **44**(2): p. 275-85.
542. Marotte, H., et al., *Blocking ERK-1/2 reduces tumor necrosis factor alpha-induced interleukin-18 bioactivity in rheumatoid arthritis synovial fibroblasts by induction of interleukin-18 binding protein A.* Arthritis Rheum, 2010. **62**(3): p. 722-31.
543. Morel, J.C., et al., *A novel role for interleukin-18 in adhesion molecule induction through NF kappa B and phosphatidylinositol (PI) 3-kinase-dependent signal transduction pathways.* J Biol Chem, 2001. **276**(40): p. 37069-75.

544. Morel, J.C., et al., *Signal transduction pathways involved in rheumatoid arthritis synovial fibroblast interleukin-18-induced vascular cell adhesion molecule-1 expression.* J Biol Chem, 2002. **277**(38): p. 34679-91.
545. Smeets, R.L., et al., *Adenoviral delivery of IL-18 binding protein C ameliorates collagen-induced arthritis in mice.* Gene Ther, 2003. **10**(12): p. 1004-11.
546. Joosten, L.A.B., et al., *An IFN-γ-Independent Proinflammatory Role of IL-18 in Murine Streptococcal Cell Wall Arthritis.* 2000. **165**(11): p. 6553-6558.
547. Gracie, J.A., et al., *A proinflammatory role for IL-18 in rheumatoid arthritis.* J Clin Invest, 1999. **104**(10): p. 1393-401.
548. Amin, M.A., et al., *Interleukin 18 induces angiogenesis in vitro and in vivo via Src and Jnk kinases.* Ann Rheum Dis, 2010. **69**(12): p. 2204-12.
549. Chew, L.-C., et al., *Use of magnetic resonance imaging in detecting subclinical synovitis in rheumatoid arthritis and correlation of imaging findings with interleukin-18 levels.* 2016. **19**(8): p. 790-798.
550. Dong, C., et al., *ICOS co-stimulatory receptor is essential for T-cell activation and function.* Nature, 2001. **409**(6816): p. 97-101.
551. Wang, H.X., et al., *Dysregulated ICOS(+) proinflammatory and suppressive regulatory T cells in patients with rheumatoid arthritis.* Exp Ther Med, 2018. **16**(4): p. 3728-34.
552. Kubota, S. and M. Takigawa, *The role of CCN2 in cartilage and bone development.* J Cell Commun Signal, 2011. **5**(3): p. 209-17.
553. Yang, X., et al., *Serum connective tissue growth factor is a highly discriminatory biomarker for the diagnosis of rheumatoid arthritis.* Arthritis Res Ther, 2017. **19**(1): p. 257.
554. Humby, F., et al., *Evaluation of Minimally Invasive, Ultrasound-guided Synovial Biopsy Techniques by the OMERACT Filter-determining Validation Requirements.* J Rheumatol, 2015.
555. Lal, P., et al., *Inflammation and autoantibody markers identify rheumatoid arthritis patients with enhanced clinical benefit following rituximab treatment.* Arthritis Rheum, 2011. **63**: p. 3681 - 3691.
556. William, J., et al., *Evolution of autoantibody responses via somatic hypermutation outside of germinal centers.* Science, 2002. **297**(5589): p. 2066-70.
557. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nat Immunol, 2005. **6**(11): p. 1123-1132.
558. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nat Immunol, 2005. **6**(11): p. 1133-41.
559. Hashimoto, M., *Th17 in Animal Models of Rheumatoid Arthritis.* J Clin Med, 2017. **6**(7).
560. van Hamburg, J.P. and S.W. Tas, *Molecular mechanisms underpinning T helper 17 cell heterogeneity and functions in rheumatoid arthritis.* Journal of Autoimmunity, 2018. **87**: p. 69-81.
561. Stockinger, B. and S. Omenetti, *The dichotomous nature of T helper 17 cells.* Nature Reviews Immunology, 2017. **17**: p. 535.
562. Jones, G.W., D.G. Hill, and S.A. Jones, *Understanding Immune Cells in Tertiary Lymphoid Organ Development: It Is All Starting to Come Together.* Front Immunol, 2016. **7**: p. 401.

563. Chabaud, M., et al., *Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium.* Arthritis Rheum, 1999. **42**(5): p. 963-70.
564. Hsu, H.C., et al., *Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice.* Nat Immunol, 2008. **9**(2): p. 166-75.
565. Canete, J.D., et al., *Ectopic lymphoid neogenesis is strongly associated with activation of the IL-23 pathway in rheumatoid synovitis.* Arthritis Res Ther, 2015. **17**: p. 173.
566. Duer-Jensen, A., et al., *Bone edema on magnetic resonance imaging is an independent predictor of rheumatoid arthritis development in patients with early undifferentiated arthritis.* 2011. **63**(8): p. 2192-2202.
567. Filer, A., et al., *Utility of ultrasound joint counts in the prediction of rheumatoid arthritis in patients with very early synovitis.* Ann Rheum Dis, 2011. **70**(3): p. 500-7.
568. Genovese, M.C., et al., *A Phase II Randomized Study of Subcutaneous Ixekizumab, an Anti–Interleukin-17 Monoclonal Antibody, in Rheumatoid Arthritis Patients Who Were Naive to Biologic Agents or Had an Inadequate Response to Tumor Necrosis Factor Inhibitors.* 2014. **66**(7): p. 1693-1704.
569. Blanco, F.J., et al., *Secukinumab in Active Rheumatoid Arthritis: A Phase III Randomized, Double-Blind, Active Comparator– and Placebo-Controlled Study.* 2017. **69**(6): p. 1144-1153.
570. Fragoulis, G.E., S. Siebert, and I.B. McInnes, *Therapeutic Targeting of IL-17 and IL-23 Cytokines in Immune-Mediated Diseases.* 2016. **67**(1): p. 337-353.
571. Genovese, M.C., et al., *One-year efficacy and safety results of secukinumab in patients with rheumatoid arthritis: phase II, dose-finding, double-blind, randomized, placebo-controlled study.* The Journal of rheumatology, 2014. **41**(3): p. 414-421.
572. Kunwar, S., K. Dahal, and S. Sharma, *Anti-IL-17 therapy in treatment of rheumatoid arthritis: a systematic literature review and meta-analysis of randomized controlled trials.* Rheumatol Int, 2016. **36**(8): p. 1065-75.

Appendix A: Genes selected for Fluidigm © analysis

	IL20	CCL18	GSMB	CD19
CD40	CCL21	IL17RA	ICOSLG	TBX21
ID1	IGKV1-5	FOXP3	CLEC9A	IL15
IL15RA	TNFRSF4	LTA	FASLG	CXCR6
IL7	CD1C	TNFSF3	FCGRIIA	VEGFR3
CLEC6A	CD80	CCL17	KDR	FCGR1A
FCER2	IL6	CXCL9	TNFRSF17	ICAM1
HLADRB1	MMP3	FCGR2B	TRAF4	TRAF1
PAD2	AICDA	FLT1	VEGFC	MMP9
TIE2	CD274	IL18RAP	CCL3	CXCL8
CSFR1R	IL33	SLAMF7	IL1B	CD8A
CXCR3	LTB	TSLP	MAF	ST2
CD40LG	NOS2	BMP4	MMP25	IL2RB
CXCL11	BMP6	CD209	ANGPT2	CCL20
CXCL13	CCL13	DKK1	IL12A	CCR4
CXCR5	CD3E	IGHA1	CCR7	PAX5
FGF1	FCRL5	IGJ	CD11C	TLR6
IGKC	IGH1	PAD4	IL2RA	CCR5
CCL4	RSAD2	IL23A	RORGT	CLEC4E
IL21R	TNF	IL7R	WNT10A	MS4A1
RANKL	BLK	THEMIS	CCL22	FCGR1B
CD83	IL10	BMP5	CD11A	CD96
IL1A	IL22	CXCL10	CD163	
CD36	APO-C1	CCR 2	IL21	

Houskeeping Genes: GAPDH
UBC