Predictors of Drug-Free Remission in Rheumatoid Arthritis

Kenneth Frank Baker

Thesis submitted for the degree of

Doctor of Philosophy

Musculoskeletal Research Group,

Institute of Cellular Medicine,

Newcastle University.

First submission September 2017

Second submission following minor corrections March 2018

Abstract

Background

Rheumatoid arthritis (RA) is a common autoimmune disease characterised by joint inflammation and systemic manifestations. Remission is achievable with disease-modifying anti-rheumatic drugs (DMARDs) prescribed in modern treat-to-target strategies, albeit with potential side effects, and inconvenient and expensive safety monitoring. Half of patients can maintain remission following DMARD cessation, though this cannot be reliably predicted. Clinicians and patients thus face a dilemma – when is it appropriate to stop DMARDs in RA remission?

In this Thesis, I explore biomarkers of drug-free remission in RA in the setting of a prospective interventional cohort study of conventional synthetic DMARD (csDMARD) cessation.

Method

Patients with established RA satisfying clinical and ultrasound remission criteria discontinued all csDMARDs and were monitored for six months. The primary outcome was time-to-flare, defined as DAS28-CRP (disease activity score in 28 joints with C-reactive protein) ≥ 2.4. Baseline clinical and ultrasound measures, circulating cytokines, and peripheral $CD4^+$ T cell gene expression were assessed for their ability to predict time-to-flare and flare/remission status by Cox regression and receiver-operating characteristic (ROC) analysis.

Results

23/44 (52%) eligible patients experienced an arthritis flare at a median (IQR) of 48 (31.5 – 86.5) days following csDMARD cessation. A composite score incorporating five baseline variables (three genes, one cytokine, one clinical, no ultrasound) differentiated future flare and drug-free remission with an area under the ROC curve of 0.96 (95% CI 0.92-1.00), sensitivity 0.91 (0.78 – 1.00) and specificity 0.95 (0.84 – 1.00). Longitudinal analysis identified increased concentrations of circulating pro-inflammatory cytokines, and upregulation of proliferative genes by $CD4^+$ T cells at the onset of flare.

Conclusions

This study provides proof-of-concept evidence for the existence of biomarkers of drug-free remission in RA, and offers insights to the pathophysiology of arthritis flare. If validated, these biomarkers may help guide csDMARD withdrawal, with consequent minimisation of medication side effects and healthcare costs.

Dedication

To my wife, Ying Qiao, for her unwavering support, companionship and love.
To do this without you would have been impossible, thank you.

To our children, Emily and Timothy

Daddy has finished writing his ‘Big Book’ now, shall we play?

Acknowledgements

I would like to thank the following people for supporting this project:

1. My supervisors John Isaacs and Arthur Pratt for their advice, support, and mentorship throughout this project – from the early design phase through to the final analysis. Their support has been invaluable and a true inspiration – thank you.
2. Andrew Skelton (Experimental Scientific Officer, Bioinformatics Support Unit, Newcastle University) for writing the computer programming scripts, performing the bioinformatics analysis and generating figures for the RNAseq data presented in Chapter 7.
3. Adam Scadeng (Research Nurse) for performing the blinded joint counts, and for his help with coordinating the numerous patient study appointments.
4. Rafiqul Hussain and Jonathan Coxhead (Newcastle University Core Genomics Facility) for performing the library preparation and sequencing of RNA samples.
5. David Storey and Alison Page (Research Technicians, Newcastle University Central Biobank) for their assistance in cryopreservation storage and retrieval.
6. Ben Thompson (Consultant Rheumatologist, Freeman Hospital) for his enviable patience in re-scoring the many ultrasound images recorded during this study.
7. Oliver Eltherington (Research Technician) and Nicola Maney (Research Associate) for their assistance with the endless pipetting involved in the electrochemiluminescence assays (Chapter 6).
8. Arthur Pratt, Natasha West (PhD student), Laura Ridgley (PhD student) and Nicola Maney for providing cover for a single study visit during my absence.
9. Dennis Lendrem (Project Manager) and Deborah Stocken (Senior Lecturer in Biostatistics and Clinical Trials) for their guidance on statistical analysis workflows.
10. My funding bodies: the Wellcome Trust, via a Translational Medicine and Therapeutics Clinical PhD Fellowship (102595/Z/13/A); and the National Institute for Health Research, via an Infrastructure Doctoral Traineeship Award from the Newcastle NIHR Biomedical Research Centre (BH136167/PD0045).

Above all, I would like to thank all of the patients who have so generously donated their time and blood, and without whom none of this research would have been possible.

Table of Contents

List of Abbreviations

Abbreviation	Definition
ABT	abatacept
ACPA	anti-citrullinated peptide antibody
ACR	American College of Rheumatology
ACT-RAY study	Actemra (tocilizumab) radiographic study
ADA	adalimumab
AIC	Akaike information criterion
AMBP	α1-microglobulin/bikunin precursor
APC	antigen-presenting cell
ARCTIC study	Aiming for Remission in rheumatoid arthritis: a randomised trial examining the benefit of ultrasound in a Clinical TIght Control regimen study
AVERT study	Assessing Very Early Rheumatoid arthritis Treatment study
BATF	basic leucine zipper ATF-like transcription factor
bDMARD	biologic disease modifying anti-rheumatic drug
BeSt study	Behandal-Strategieën study
bFGF	basic fibroblast growth factor
Bio-FLARE study	Biological Factors that Limit sustAined Remission in rhEumatoid arthritis study
BioRRA study	Biomarkers of Remission in Rheumatoid Arthritis study
BME	bone marrow oedema
C/EBPβ	CCAAT/enhancer-binding protein beta
CAPN8	calpain 8
CCL	C-C motif chemokine ligand
CD	cluster of differentiation
CDAI	clinical disease activity index
CDCA7	cell division cycle associated 7
CI	confidence interval
CIC	ciclosporin
CIMESTRA study	Cyclosporine, Methotrexate, Steroid in Rheumatoid Arthritis study
CRP	C-reactive protein
csDMARD	conventional synthetic disease-modifying anti-rheumatic drug
CTLA-4	cytotoxic T lymphocyte associated protein 4
CV	coefficient of variation
CXCL	C-X-C motif ligand
DAS	disease activity score
DAS28	disease activity score including 28 joints
DC	dendritic cell
DEG	differentially expressed gene
DFR	drug-free remission
DGE	differential gene expression
DIP	distal interphalangeal
DMARD	disease-modifying anti-rheumatic drug
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DOSERA study	DOSE reduction or discontinuation of etanercept in methotrexate-treated rheumatoid arthritis subjects who have achieved a stable low disease activity-state study
DREAM study	Drug free REmission/low disease activity after cessation of tocilizumab (Actemra) Monotherapy study

Abbreviation	Definition
DRESS study	Dose REduction Strategy of Subcutaneous TNF inhibitors study
EBI3	Epstein-Barr virus-induced gene 3
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
EMPIRE study	Etanercept and Methotrexate in Patients to Induce Remission in Early Arthritis study
EMS	early morning stiffness
ERAS study	Early Rheumatoid Arthritis Study
ESR	erythrocyte sedimentation rate
ETN	etanercept
EULAR	European League Against Rheumatism
FACS	fluorescence-activated cell sorting
FAM102B	family with sequence similarity 102 member B
FC	fold change
FCS	foetal calf serum
FDR	false-discovery rate
FLARE-RA	flare in rheumatoid arthritis questionnaire
FLS	fibroblast-like synoviocytes
FSC-A	forward-scatter area
GLOESS	Global Outcome Measures in Rheumatology - European League Against Rheumatism Synovitis Score
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
GS	greyscale
GULP1	engulfment adaptor PTB domain containing 1
GWAS	genome-wide association study
HAQ-DI	health assessment questionnaire disability index
HBSS	Hanks balanced salt solution
HC	healthy control
HCQ	hydroxychloroquine
HGNC	Human Genome Organisation Gene Nomenclature Committee
HLA	human leukocyte antigen
HR	hazard ratio
HRA	(NHS) Health Research Authority
hsCRP	high-sensitivity C-reactive protein
IA	intra-articular
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
IM	intra-muscular
IMPROVED study	Induction therapy with Methotrexate and Prednisone in Rheumatoid Or Very Early arthritic Disease study
IP-10	interferon-γ induced protein 10kDa
IPA™	Ingenuity™ Pathway Analysis
IQR	interquartile range
IRC	inflammation-related cell
IRF	interferon regulatory factor
IU	international units

Abbreviation	Definition
JAK	Janus kinase
KIR2DS4	killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 4
LDA	low disease activity
LDLR	low-density lipoprotein receptor
LEAC cohort	Leiden Early Arthritis Clinic cohort
lincRNA	long intergenic non-coding ribonucleic acid
LLOD	lower limit of detection
logCPM	log counts per million
MAPK	p38 mitogen-activated protein kinase
MBDA	multibiomarker disease activity score
MCP	metacarpophalangeal
MCP	monocyte chemoattractant protein
MDC	macrophage-derived chemokine
MDHAQ	multidimentional health assessment questionnaire
MHAQ	modified health assessment questionnaire
MHC	major histocompatibility molecule
MIP	macrophage inhibitory protein
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTP	metatarsophalangeal
mTSS	modified total Sharp score
MTX	methotrexate
NAHBP	Newcastle Academic Health Partners Bioresource
NET	neutrophil extracellular trap
NHS	National Health Service
NICE	National Institute for Health and Care Excellence
NK	natural killer
NPV	negative predictive value
NSAID	non-steroidal anti-inflammatory drug
NTSJ	no tender or swollen joints
OA	osteoarthritis
OMERACT	Outcome Measures in Rheumatology
OR	odds ratio
ORM1	orosomucoid 1
PADI	peptidyl-arginase deiminase
PBMC	peripheral blood mononuclear cells
PBO	placebo
PD	power Doppler
PIP	proximal interphalangeal
POET study	Potential Optimisation of Expediency of TNFi study
PPV	positive predictive value
PRF	pulse-repetition frequency
PRIZE study	Productivity and Remission in a randomIZed controlled trial of Etanercept vs. standard of care in early rheumatoid arthritis
PROM	patient-reported outcome measure
PROMPT study	PRObable rheumatoid arthritis: Methotrexate versus Placebo Treatment study

Abbreviation	Definition
QoL	quality of life
QUALY	quality-adjusted life year
RA	rheumatoid arthritis
RADAI	rheumatoid arthritis disease activity index
RAI	Ritchie articular index
RAMIRIS	rheumatoid arthritis magnetic resonance image scoring system
RAPID3	routine assessment of patient index data 3
RAQoL	rheumatoid arthritis quality of life questionnaire
RCT	randomised controlled trial
REC	research ethics committee
REMINDRA study	REMission INDuction in Very Early Rheumatoid Arthritis study
RETRO study	Reduction of Therapy in patients with Rheumatoid arthritis in Ongoing remission study
RhF	rheumatoid factor
RINe	estimated ribonucleic acid integrity number
RNA	ribonucleic acid
RNAseq	ribonucleic acid sequencing
ROC	receiver-operating characteristic
ROC_{AUC}	area under the receiver-operating characteristic curve
RR	relative risk
rRNA	ribosomal ribonucleic acid
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SAA	serum amyloid A
SDAI	simple disease activity index
SF-36	36-item short form survey
SFZ	sulfasalazine
SH3RF3	SH3 domain containing really interesting new gene finger 3
SJC	swollen joint count
SNP	single nucleotide polymorphism
SPRI	solid phase reversible immobilisation
SSC-A	side-scatter area
SSC-W	side-scatter width
STAT	signal transducer and activation of transcription
SYTL4	synaptotagmin like 4
TARA study	TApering strategies in Rheumatoid Arthritis study
TARC	thymus and activation-regulated chemokine
TaSER study	Targeting Synovitis in Early Rheumatoid Arthritis study
TCR	T cell receptor
TFH	T follicular helper cell
TGFβ	transforming growth factor beta
TICORA study	Tight Control for Rheumatoid Arthritis study
TJC	tender joint count
TLR	Toll-like receptor
TMM	trimmed mean of M-values
TNF	tumour necrosis factor
TOC	tocilizumab
tREACH study	treatment in the Rotterdam Early Arthritis Cohort study

Abbreviation	Definition
Treg	regulatory T cell
TSHR	thyroid stimulating hormone receptor
UA	undifferentiated (inflammatory) arthritis
U-Act-Early study	a study of tocilizumab (ACTemra) and methotrexate in combination or as monotherapy in treatment-naïve patients with Early rheumatoid arthritis
ULN	upper limit of normal
ULOD	upper limit of detection
US	ultrasound
UV	ultraviolet
VAS	visual analogue scale
VCAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
voom	variance modelling at the observational level

List of Figures

List of Tables

Chapter 1. Introduction

1.1 General Introduction to Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by inflammation in the lining of joints termed synovitis (Smolen *et al.*, 2016a). Chronic synovitis causes joint pain, stiffness, swelling and ultimately erosions leading to irreversible joint deformity (McInnes and Schett, 2011). In addition to physical disability, additional extra-articular features such as accelerated atherosclerosis add to the excess morbidity and mortality of the disease (Turesson, 2013).

In this opening chapter, I will provide a brief clinical overview of the epidemiology, presentation, diagnosis and management of RA aimed at a general audience, in order to provide the necessary context to the subsequent background chapters in my thesis.

1.1.1 Prevalence and Incidence

RA is a common autoimmune disease, with an estimated worldwide prevalence of 0.24% (Cross *et al.*, 2014). Nevertheless, is recognised that this may under-represent the true prevalence of the disease due to low reporting in developing countries. In the UK and other developed Western countries, RA is estimated to affect between 0.5 and 1% of the adult population (Symmons *et al.*, 2002). Several worldwide retrospective cohort studies place the incidence of RA between 20 and 60 cases per 100,000 adults per year (Eriksson *et al.*, 2013; Humphreys *et al.*, 2013; Rossini *et al.*, 2014; Widdifield *et al.*, 2014).

RA has a female propensity with a female : male ratio of 3 : 1 (Scott *et al.*, 2010). The onset of RA can occur at any age, and peaks around the 45-75 years of age (Humphreys *et al.*, 2013). Thus with an increasingly ageing population, the cumulative lifetime risk of RA can be expected to increase over the next few decades (van Onna and Boonen, 2016).

1.1.2 Morbidity and mortality

RA inflicts a substantial burden of illness – in 2010 alone, it was the cause of an estimated 3.7 million years lived with disability worldwide (Cross *et al.*, 2014). The best-recognised morbidities associated with RA are physical disability and pain resulting from the progressive joint destruction associated with uncontrolled synovitis. Nevertheless, there is an equally

important wider spectrum of morbidity associated with this systemic autoimmune disease, ranging from accelerated atherosclerosis and ischaemic cardiovascular events, through to fatigue and depression (Scott *et al.*, 2010).

The causes of death in patients with RA are largely similar to the general population, namely cardiovascular disease, cancer, infection and respiratory disease (Radovits *et al.*, 2010). However, numerous studies have shown an excess age-standardised mortality ratio in RA of between 1.2 to 2 times that of the general population (Dadoun *et al.*, 2013). The reasons behind this so-called 'mortality gap' are complex, and likely reflect a combination of the deleterious effects of sustained immune dysregulation combined with other factors including medication side-effects and cigarette smoking, which is strongly implicated in the pathogenesis of RA (see Introduction 1.2.3). Crucially, whereas general mortality rates have significantly reduced over past decades, this rate of decline has been much slower in the RA population leading to a widening of this mortality gap (Gonzalez *et al.*, 2007; Humphreys *et al.*, 2014; Widdifield *et al.*, 2014).

1.1.3 Socioeconomic impact

The socioeconomic impact of RA is disproportionate to its prevalence, owing to its wide age range of onset and propensity to cause chronic disability and premature mortality. The direct healthcare costs of treating RA are substantial, and include inpatient and outpatient episodes, medication prescriptions and frequent drug monitoring. Furthermore, there is an enormous indirect economic impact of RA owing to the costs of lost work productivity, disability welfare allowances and social care. Indeed, the healthcare needs of RA sufferers, combined with societal consequences of long-term disability and lost work productivity, is estimated to cost the UK economy £4.75 billion per annum (National Institute for Health and Care Excellence, 2009).

1.1.4 Clinical Presentation

RA typically presents with an insidious onset of joint pain and swelling. Any synovial joint can be involved, but typically RA manifests symmetrically in the wrists and small joints of the hands and feet (Feist and Burmester, 2013). Several additional patterns of onset have also been described, including: acute systemic onset, migratory (palindromic) onset, and

polymyalgic RA, the latter of which is more commonly encountered in the elderly (Feist and Burmester, 2013).

The hallmark clinical features of active RA are pain, warmth, erythema and swelling of the affected joints (synovitis) (Maini, 2012). In addition, inflammation can occur in the tendon sheaths (tenosynovitis) resulting in pain, swelling and mechanical obstruction. If left untreated, irreversible joint and tendon damage can occur, leading to a stereotypical destructive pattern of arthritis (Figure 1.1). Furthermore, extra-articular inflammation leads to a constellation of associated pathology that can affect virtually any organ system (Table 1.1).

1.1.5 Diagnosis

There is no single investigation that is diagnostic of RA, which remains a clinical diagnosis based largely upon medical history and examination findings. Nevertheless, there are several biochemical, serological and radiological investigations that can provide evidence to support the diagnostic process (Table 1.2).

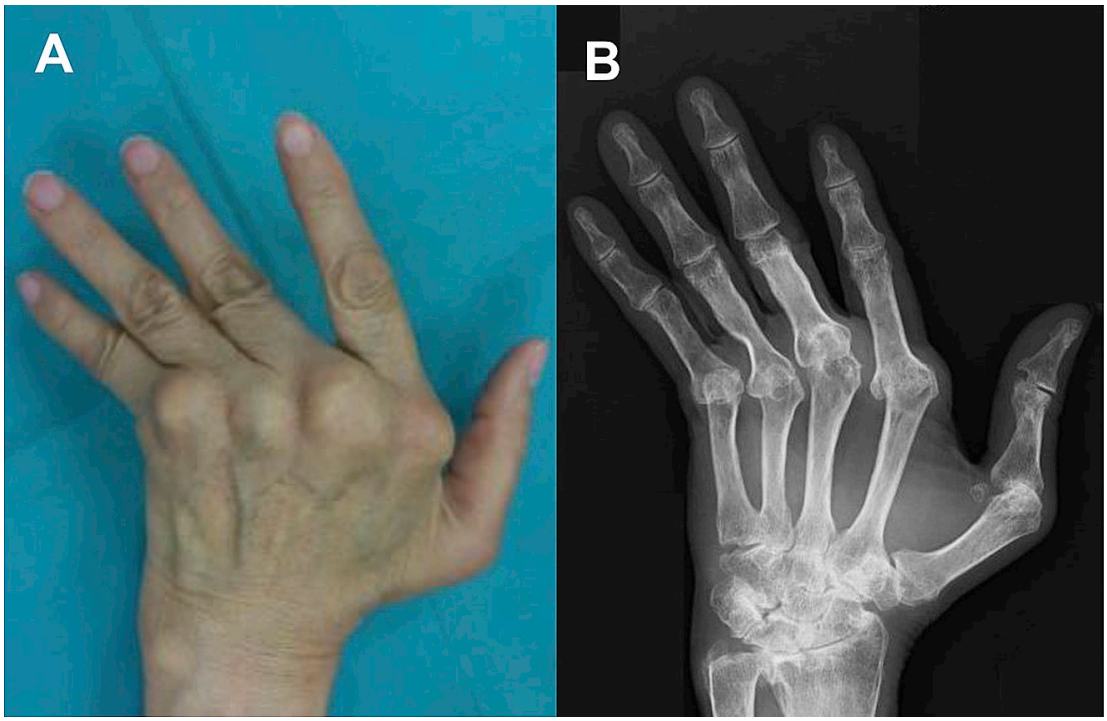


Figure 1.1 – Photograph (A) and plain radiograph (B) demonstrating the typical joint destruction seen in established uncontrolled rheumatoid arthritis. Severe ulnar deviation and palmar subluxation is evident at the metacarpophalangeal joints, together with radiographic evidence of bone erosions and periarticular osteopenia. Reproduced from Ishikawa (2017) under the terms of a CC BY-NC-ND 4.0 license (http://creativecommons.org/licenses/by-nc-nd/4.0/). © 2017 The Japanese Orthopaedic Association. Published by Elsevier B.V.

Table 1.1 – The extra-articular manifestations of rheumatoid arthritis. Adapted from Scott et al. (2010).

Organ system	Manifestation
Respiratory	Pulmonary fibrosis Pleural effusion Pulmonary nodules Caplan syndrome
Cardiovascular	Vasculitis Pericarditis Conduction defects
Neurological	Compression neuropathy Mononeuritis multiplex Cervical myelopathy
Skin	Rheumatoid nodules Neutrophilic dermatoses Vasculitic rash
Ocular	Scleritis Episcleritis Keratoconjunctivitis sicca
Systemic	Fatigue Depression Amyloidosis Osteoporosis

The diagnosis of RA has been formalised for research purposes by the creation of classification criteria endorsed by leading rheumatology societies. Until recently, the most widely used of these was the 1987 American College of Rheumatology (ACR) RA classification criteria (Table 1.3). Whilst providing a sensitivity and specificity of around 90% for the diagnosis of established RA versus non-RA rheumatic disease controls, the 1987 criteria relied heavily upon features of advanced disease such as radiographic joint damage. Consequently, the 1987 criteria were criticised for their insensitivity to early disease, which made them out-dated with a later evolution towards intervention at earlier stages of clinical presentation.

Table 1.2 – Key investigations that can aid the diagnosis of rheumatoid arthritis.

Category	Investigation
Laboratory measures of the acute-phase response	C-reactive protein Erythrocyte sedimentation rate
Serology	Rheumatoid factor Anti-citrullinated peptide autoantibodies
Imaging	Plain radiographs Musculoskeletal ultrasound

To address this shortfall, the ACR in collaboration with the European League Against Rheumatism (EULAR) released updated RA classification criteria in 2010 (Aletaha *et al.*, 2010). The 2010 criteria use a point-based system to define RA across four domains: joint involvement, serological status, biochemical inflammatory markers and symptom duration (Table 1.4). Whilst representing a significant advance on previous criteria, the 2010 criteria may still under-diagnose early RA, particularly in the approximately 25% of patients who are seronegative for rheumatoid factor (RhF) and anti-citrullinated peptide antibodies (ACPA).

1.1.6 Management

There are two principal aims in the management of RA, namely the alleviation of joint symptoms, and a reduction (ideally prevention) of joint damage and systemic disease manifestations. Long-term outcomes are more favourable when treatment is started early in the course of disease, and when there is comprehensive suppression of inflammation (discussed further in Introduction 1.3). Indeed, disease remission is now a realistic target of treatment for RA in the modern era (Smolen *et al.*, 2016b).

As a multi-faceted systemic autoimmune disease, RA is best managed in a multi-disciplinary approach incorporating rheumatologists, specialist nurses, physiotherapists, occupational therapists and podiatrists (National Institute for Health and Care Excellence, 2009). Up until the latter half of the 20th century, medical treatment was traditionally focussed on medications such as non-steroidal anti-inflammatory drugs (NSAIDs) which, although providing symptomatic relief, afford little if any protection against disease progression (Scott *et al.*, 2010). From the 1970s onwards, increasing emphasis was placed on the use of drugs that were able to modify the underlying disease process and hence prevent joint damage – the

Table 1.3 – The 1987 American College of Rheumatology (ACR) classification criteria for RA. MCP: metacarpophalangeal; MTP: metatarsophalangeal; PIP: proximal interphalangeal. Reproduced with permission from Arnett *et al.* (1988). Copyright © 1988 American College of Rheumatology.

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

Table 1.4 – The 2010 American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) classification criteria for RA. ACPA: anti-citrullinated peptide antibody; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RhF: rheumatoid factor. Table and footnotes are reproduced from '2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative', Aletaha *et al*., Annals of the Rheumatic Diseases, vol 69, pages 1580-1588, copyright 2010, with permission from BMJ Publishing Group Limited.

A total score of ≥ 6 is required for a diagnosis of rheumatoid arthritis	**Score**
A. Joint involvement §	
• 1 large joint ¶	0
• 2-10 large joints	1
• 1-3 small joints (with or without involvement of large joints) #	2
• 4-10 small joints (with or without involvement of large joints)	3
• >10 joints (at least 1 small joint) **	5
B. Serology †† (at least 1 test result is needed for classification)	
• Negative RhF *and* negative ACPA	0
• Low-positive RhF *or* low-positive ACPA	2
• High-positive RhF *or* high-positive ACPA	3
C. Acute phase reactants ‡‡ (at least 1 test result is needed for classification)	
• Normal CRP AND normal ESR	0
• Abnormal CRP OR abnormal ESR	1
D. Duration of symptoms §§	
• <6 weeks	0
• ≥6 weeks	1

"The criteria are aimed at classification of newly presenting patients. In addition, patients with erosive disease typical of rheumatoid arthritis (RA) with a history compatible with prior fulfilment of the 2010 criteria should be classified as having RA. Patients with longstanding disease, including those whose disease is inactive (with or without treatment) who, based on retrospectively available data, have previously fulfilled the 2010 criteria should be classified as having RA. Although patients with a score of <6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time. § Joint involvement refers to any swollen or tender joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are excluded from assessment. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based on the pattern of joint involvement. ¶ "Large joints" refers to shoulders, elbows, hips, knees, and ankles. # "Small joints" refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.** In this category, at least 1 of the involved joints must be a small joint; the other joints can include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (e.g., temporomandibular, acromioclavicular, sternoclavicular, etc.). †† Negative refers to IU values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay; low-positive refers to IU values that are higher than the ULN but ≤3 times the ULN for the laboratory and assay; high-positive refers to IU values that are >3 times the ULN for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF.‡‡ Normal/abnormal is determined by local laboratory standards.§§ Duration of symptoms refers to patient self-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status."

so-called disease modifying anti-rheumatic drugs (DMARDs) (Moreland *et al.*, 2001). Many of the early DMARDs including methotrexate, sulphasalazine and hydroxychloroquine are still in widespread use today, although their mechanisms of action remain largely obscure (Scott *et al.*, 2010). Whilst often effective in attenuating the progression of synovitis and joint damage, DMARDs carry a wide spectrum of potential adverse effects ranging from gastrointestinal upset to serious complications including hepatitis and bone marrow suppression (Moreland *et al.*, 2001; Ledingham *et al.*, 2017). For this reason, the majority of DMARDs require regular blood test safety monitoring (Ledingham *et al.*, 2017).

Over the past two decades, increasing knowledge of the molecular basis of inflammation in RA, coupled with technological advances in therapeutic antibody production, have led to the development of novel biopharmaceutical agents. The mechanisms of action of these so-called "biologics" are typified by the potent but selective blockade of an inflammatory mediator, usually by means of a specific antibody or receptor fusion protein (McInnes and Schett, 2017). The first of these agents to be licensed for use in RA was infliximab, a chimeric monoclonal antibody directed against tumour necrosis factor α (TNF-α) (Elliott *et al.*, 1994). The following years have seen an explosion in the number of biologic agents, and their potent action has revolutionised the treatment of RA resistant to conventional synthetic DMARD therapy. Although more specific in their action and hence more widely tolerated than their small-molecule DMARD counterparts, biologics still carry risks of serious adverse events, most notably an increased risk of severe infection (Ramiro *et al.*, 2014). More recently still, the development of novel small molecule inhibitors of cell signalling pathways involved in the inflammatory response, such as the Janus kinase inhibitors, offer the possibility of selective immunosuppression with orally-available drugs (Baker and Isaacs, 2018).

Surgery in the form of arthrodesis, arthroplasty, tendon reconstruction and nerve decompression are generally considered interventions of last resort when all options of medical therapy have been exhausted. Indeed, with increasing potency of immunosuppressive medications and their more aggressive and earlier use, rates of orthopaedic intervention in RA have declined significantly in the previous two decades (Kievit *et al.*, 2013).

1.1.7 Summary

RA is a common autoimmune disease with a potentially devastating impact upon the lives of individual patients, as well as posing a substantial burden to healthcare systems and the wider society. RA was historically a disease characterised by inexorable joint destruction and

disability with few effective treatments. However, early and aggressive immunosuppression has afforded unparalleled advances in disease control, such that disease remission is now a realistic and achievable target for the majority of patients. Nevertheless, such treatments are not without the risk of serious side effects and require close and frequent clinical monitoring.

1.2 Immunopathogenesis of Rheumatoid Arthritis

The pathological hallmark of RA is the rheumatoid pannus – a dense inflammatory cellular proliferation found at sites of synovial joint destruction. The RA pannus is an amorphous mix of both of joint stromal cells (e.g. synoviocytes, osteocytes etc.) as well as extra-articular cells recruited to the joint (e.g. lymphocytes, macrophages etc.) (Smolen *et al.*, 2016a). The temporal order, relative importance and redundancy of this plethora of autoimmune processes underlying the pathogenesis of RA has long provided a challenge to resolve in a unifying model of RA pathogenesis. Indeed, it is becoming increasingly apparent that RA is a dynamic and fluid process that is different both between individual patients and even within the same patient over time. In this respect, RA is perhaps best conceptualised as a clinical syndrome which encompasses multiple distinct immune pathologies under a unifying phenotypical umbrella (McInnes and Schett, 2011).

In this chapter, I present a concise review of the key players in RA immunopathogenesis, with a particular focus upon an increasing weight of evidence that supports a central orchestrating role for the $CD4^+$ T cell.

1.2.1 $CD4^+$ T cell and the initiation of adaptive immunity

$CD4^+$ T cells play a unique and crucial role in the initiation of the adaptive immune response. Antigen-presenting cells (APCs), including macrophages and dendritic cells, take up and process proteins which are presented as antigenic peptide fragments bound to surface-expressed major histocompatibility (MHC) class II molecules (Murphy and Weaver, 2016). Each clone of $CD4^+$ T cell expresses a unique T cell receptor (TCR), which binds to its cognate antigen:MHC-II complex. Once activated by further co-stimulatory surface interactions with the APC, $CD4^+$ T cells can release large amounts of immunomodulatory cytokines and provide further co-stimulation to activate other arms of the adaptive immune response including $CD8^+$ cytotoxic T cells, plasma cells and B lymphocytes (Murphy and Weaver, 2016). $CD4^+$ T cells are thus crucially placed both to initiate adaptive immunity and to direct or polarise the ensuing immune response.

1.2.2 The 'shared epitope' hypothesis

Early studies of twin discordance highlighted the association of RA with an abundance of haplotypes of the major histocompatibility (MHC) locus (Gregersen *et al.*, 1987). The MHC is located on the short arm of chromosome 6, and mainly encodes the human leukocyte antigen (HLA) molecules, classified as MHC class I (HLA-A, HLA-B and HLA-C in humans) and MHC class II (HLA-DP, HLA-DQ, HLA-DR in humans) (Fernando *et al.*, 2008). MHC-II are of particular importance in autoimmunity, given their critical role in the presentation of exogenous antigen by APCs to $CD4^+$ T cells, and thus the initiation of the adaptive immune response. MHC-II molecules comprise two chains – α and β – the latter of which contains a highly variable region known as the 'peptide-binding groove' (Brown *et al.*, 1993). Variability in this peptide-binding groove affects the affinity to which different antigenic peptide fragments can bind and so be presented to $CD4^+$ T cells, thus providing a mechanistic model to account for the genetic propensity towards autoimmunity associated with certain MHC haplotypes (Fernando *et al.*, 2008).

The so-called 'shared epitope' describes a common amino acid motif in residues 70 to 74 in various alleles of *HLA-DRB1* (e.g. **0101*, **0401* and **0404*) which have been strongly associated with the development of seropositive RA (Holoshitz, 2010). Although the exact nature of the autoantigens important in the initiation of RA are not clear, it has been demonstrated that citrullination can increase the binding affinity of peptides with the MHC shared-epitope (Hill *et al.*, 2003). This is an important observation given the abundance of anti-citrullinated peptide antibodies that can be detected in the sera of many patients with RA. Cigarette smoking, which is strongly associated with seropositive RA in epidemiological studies, is also known to promote peptide citrullination via peptidyl-arginase deiminase (PADI) enzymes, and smoking interacts with HLA-DRB1 status to promote risk of RA in a multiplicative manner (Klareskog *et al.*, 2006). Furthermore, the bacterium *Porphyromonas gingivalis*, which is also associated with seropositive RA, contains a PADI enzyme which can citrullinate human host peptides (Wegner *et al.*, 2010). The shared epitope thus provides an attractive unifying hypothesis to link the environmental agents such as cigarette smoke together with MHC genotype, albeit only in the case of seropositive RA.

1.2.3 CD4$^+$ T cells are key players in RA pathogenesis

1.2.3a Genetic and epigenetic evidence supports a central involvement of CD4$^+$ T cells in the development of RA

A growing body of evidence places CD4$^+$ T cells at the heart of RA pathogenesis. First, the shared-epitope hypothesis demonstrates the importance of antigen presentation via MHC-II in the pathogenesis of RA, and thus the importance of CD4$^+$ T cells in this process. Indeed, blockade of CD28:CD80/86 co-stimulation between antigen presenting cells and effector T cells has been exploited therapeutically by abatacept, a cytotoxic T lymphocyte associated protein 4 (CTLA-4):Ig Fc fusion protein licensed for the treatment of RA and juvenile idiopathic arthritis (Keating, 2013).

Second, single nucleotide polymorphisms (SNPs) found outside the MHC that are associated with RA in genome-wide association studies (GWAS) are disproportionately located within chromosome loci enriched for genes that are important in CD4$^+$ T cell function (Diogo *et al.*, 2014). For example, the strongest genetic association with RA outside of the MHC complex that has been identified to-date is an SNP (1858C to T) in the *PTPN22* gene. This leads to an amino acid substitution (R620W) in the enzyme lymphoid phosphatase, which is likely to play an important role in the regulation of T cell function (Begovich *et al.*, 2004). Furthermore, the R620W allele of *PTPN22* has been shown to interact with shared epitope alleles of HLA-DRB1 and cigarette smoking, such that the odds ratio of developing ACPA-positive RA in an individual with all three risk factors is over 20 times that of an individual with none (Kallberg *et al.*, 2007).

Third, many of the SNPs associated with RA overlap with regions of altered chromatin methylation, known as "chromatin marks", in CD4$^+$ T cells implicating their roles in cell-specific altered gene regulation (Trynka *et al.*, 2013). For example, demethylation of the promoter of *CD40LG*, a gene located on the X chromosome which encodes the co-stimulatory molecule CD40L, has been observed in the CD4$^+$ T cell subset of female RA patients (Liao *et al.*, 2012). As discussed by Zhang and Zhang (2015), this observation suggests a possible mechanistic link between over-expression of CD40L in CD4$^+$ T cells and increased susceptibility to RA in women. This is further supported by the link between CD40/CD40L interactions and Th17 differentiation in mice (Iezzi *et al.*, 2009) and the production of the pro-Th17 cytokine IL-23 by human DCs upon CD40 ligation *in vitro* (Sender *et al.*, 2010).

Fourth, patterns of gene expression by CD4$^+$ T cells have been associated with an increased risk of developing RA. Using a genome-wide microarray analysis of CD4$^+$ T cell gene

expression in cohort of 173 patients with undifferentiated arthritis, Pratt *et al.* (2012) identified a 12-gene signature that could predict the subsequent development of RA. This signature included several STAT3-inducible genes, and constitutive phosphorylation of STAT3 within $CD4^+$ T cells showed a strong correlation with circulating IL-6 levels (Anderson *et al.*, 2016). Interestingly, these observations were strongest for ACPA-negative individuals, and provide a potential mechanistic basis by which upregulated IL-6 signalling via $CD4^+$ T cells could promote the development of RA (Anderson *et al.*, 2016).

Fifth, several observations converge to suggest that $CD4^+$ T cell proliferation is a central feature of RA pathogenesis. $CD4^+$ T cells are found within RA synovium and produce pro-inflammatory cytokines (Steiner *et al.*, 1999). Furthermore, oligoclonal populations of $CD4^+$ T cells with reduced T cell receptor diversity have been observed in the circulation (Wagner *et al.*, 1998) and joints (Khazaei *et al.*, 1995) of patients with RA, in keeping antigen-driven clonal expansion as part the disease process. Indeed, chronic activation and proliferation is thought to underpin the premature immunosenescence seen in $CD4^+$ T cell populations in RA patients (Weyand *et al.*, 2014), thought in part to be due to defective telomerase activity within these cells (Fujii *et al.*, 2009). Whether immunosenescence contributes to the pathogenesis of RA or is merely a downstream consequence of the disease is unknown; nevertheless, both possibilities implicate $CD4^+$ T cells in the immunopathology of the disease.

1.2.3b Potential roles of $CD4^+$ T cell subsets in RA pathogenesis

Over past decades there has been much effort to categorise $CD4^+$ T cells into functional subsets defined by the nature of immunity they induce (Gizinski and Fox, 2014). Initial attempts to categorise $CD4^+$ T cells focussed on defining two subsets: Th_1 or Th_2, responsible for cell-mediated and humoral immunity respectively (Gizinski and Fox, 2014). This model later proved to be overly simplistic, and recent years have witnessed an expanding range of proposed $CD4^+$ cellular subsets, including Th17, Th9, T follicular helper (T_{FH}), and various subdivisions of regulatory T cells (T_{reg}) (Hirahara and Nakayama, 2016). The translation of murine and *in vitro* observations to human immunity is at times obtuse, and limited by uncertainty regarding the plasticity of such cellular subsets. Nevertheless, with such classifications it has been possible to demonstrate that certain subsets of $CD4^+$ T cell are strongly implicated in RA pathogenesis.

Arguably the most strongly associated with the development of RA is the Th_{17} cell, so-called because of their propensity to secrete the pro-inflammatory cytokine interleukin-17 (IL-17)

and further defined by their expression of the transcription factor retinoic acid-related orphan receptor γ (RORγ) (Gizinski and Fox, 2014). Elevated levels of IL-17 isoforms have been observed in the plasma and synovial fluid of RA patients (Jain *et al.*, 2015), and *in vitro* co-culture of human Th_{17} cells and synovial fibroblasts can induce the production of inflammatory cytokines and destructive matrix metalloproteinases (MMPs) in an IL-17 dependent manner (van Hamburg *et al.*, 2011). Blockade of IL-17 can ameliorate inflammatory arthritis in animal models (Lubberts *et al.*, 2001), and a reduction in plasma IL-17 levels has been observed in RA patients following drug therapy (Jain *et al.*, 2015). Although IL17 blockade has been therapeutically disappointing in RA, several specific anti-IL-17 therapeutics are either licensed or currently in clinical development for the treatment of other autoimmune diseases including psoriasis, psoriatic arthritis and ankylosing spondylitis (Baker and Isaacs, 2018).

More recently, a further $CD4^+$ T cell subset implicated in autoimmunity has been described – the Th_{22} cell (Azizi *et al.*, 2015). These cells are characterised by their secretion of IL-22, and absence of other $CD4^+$ subset markers. Circulating Th_{22} cells are increased in RA patients, and have been shown to correlate with the levels of IL-22 and clinical disease activity scores (Zhang *et al.*, 2011; Zhang *et al.*, 2012; Zhao *et al.*, 2013). Nevertheless, the prevalence of Th_{22} cells appears to correlate with Th_{17} cells, and the extent to which these different subsets are independently associated with the development of RA remains debateable.

Contrary to the pro-inflammatory action of subsets such as Th_{17} cells, it is now well established that a further subset of $CD4^+$ T cells known as regulatory T cells (T_{reg}) act to maintain immunological tolerance to autoantigens and play an important role in the prevention of autoimmune diseases such as RA. T_{regs} can develop centrally in the thymus (natural T_{regs}), or can develop peripherally (induced T_{regs}) under the influence of anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β) (Noack and Miossec, 2014). Human T_{regs} are characterised by the expression of the transcription factor forkhead box P3 (FoxP3), and can actively suppress the inflammatory response through production of cytokines such as IL-10 and TGF-β and by directly suppressing the action of effector T cells (Noack and Miossec, 2014). Indeed, the adoptive transfer of T_{regs}, or their differentiation *in vivo*, can protect against experimentally-induced inflammatory arthritis in animal models and holds promise as a potential avenue of future tolerogenic therapy in human rheumatic diseases (Miyara *et al.*, 2014). Nevertheless, the plasticity between effector and regulatory T cells and the often obscure nature of the autoantigens driving the immune

response in RA pose significant challenges to the clinical translation of T_{reg} therapy (Baker and Isaacs, 2014).

1.2.4 B lymphocytes and plasma cells

Despite substantive evidence to support the role of T cells in the pathogenesis of RA, they are by no means the only immune cell type to play a role in disease pathogenesis. B lymphocytes, and their derivative plasma cells, have long been known to contribute to the development of autoimmunity in RA. Indeed, one of the first insights in to the immune pathogenesis of RA came in the 1940s with the observation that sera from patients with RA could induce haemagglutination when mixed with sheep blood (Alexander, 1967). The precipitating agent was named rheumatoid factor, which was later identified to be a range of autoantibodies directed against the Fc portion of IgG (immunoglobulin G). More recently, autoantibodies directed against modified protein epitopes such as anti-citrullinated peptide antibodies (ACPA) and anti-carbamylated protein antibodies have also been identified, and assays of ACPA titres are now routinely used in clinical diagnosis of RA. Retrospective studies of stored serum samples have demonstrated that autoantibodies such as ACPA develop up to a decade before the onset of clinical disease, and undergo class-switching and affinity maturation in the few years prior to symptom onset (Rantapaa-Dahlqvist *et al.*, 2003; van der Woude *et al.*, 2010). Furthermore, there is now some evidence to support a direct pathogenic role of ACPA in the development of rheumatoid synovitis (Kocijan *et al.*, 2013). Nevertheless, up to a third of patients with RA are seronegative for both RhF and ACPA, highlighting that these autoantibodies are not necessary for the development of clinical disease.

The therapeutic depletion of B cells by use of the monoclonal antibody rituximab has now been routinely used for over a decade to control severe RA. Rituximab binds to CD20 which, interestingly, is only expressed on immature pro/pre-B lymphocytes and not by antibody-producing mature plasma cells (Cohen and Keystone, 2015). This highlights the pleiotropic effects of B cells beyond antibody production alone, which include antigen presentation and potent secretion of cytokines and chemokines (Cohen and Keystone, 2015). In addition to forming a major constituent of reactive lymph node follicles, B cells and plasma cells can often be found organised within ectopic lymphoid structures within inflamed RA synovium, the presence of which may hold promise as a biomarker of therapeutic response to B cell depletion (Humby *et al.*, 2017). Correspondingly, circulating levels of the B cell chemokine

C-X-C Motif Chemokine Ligand 13 (CXCL13) have been shown to correlate with clinical and imaging measures of synovitis in RA (Bugatti *et al.*, 2012), and the return of B cells following rituximab treatment (Rosengren *et al.*, 2011).

1.2.5 Innate immune cells

Although adaptive immune cells are believed to be the key initiators of autoimmunity in RA, they are hugely outnumbered in the rheumatoid pannus by their innate immune counterparts. Macrophages feature prominently in inflamed synovium, and secrete both tissue-damaging matrix metalloproteinases (MMPs) as well as pro-inflammatory chemokines and cytokines, such as tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), which serve to amplify the immune response (McInnes and Schett, 2017). Neutrophils are not constitutively present within the joint, but are recruited in large numbers to the synovial fluid during joint inflammation. As well as releasing pro-inflammatory reactive oxygen species, deranged necrosis and the formation of DNA-containing neutrophil extracellular traps (NETs) may play a role in RA pathogenesis as in other related connective tissue diseases such as systemic lupus erythematosus (Mitsios *et al.*, 2016). Further innate immune cells are found within the rheumatoid pannus and play a role in perpetuating inflammation and promoting tissue damage, including both mast cells and natural killer cells (McInnes and Schett, 2011).

Dendritic cells (DCs), either resident within the synovium or recruited to the joint, sample antigenic peptides and present these to $CD4^+$ T cells. Both the local environment in which antigen uptake occurs, together with the inherent pro- or anti-inflammatory propensity of the DC, are thought to be important to the priming and polarisation of the ensuing adaptive immune response (Ganguly *et al.*, 2013). Indeed, several groups are actively investigating the potential of ex-vivo tolerogenic conditioning of DCs as a future cellular therapy for RA (Raker *et al.*, 2015).

1.2.6 Fibroblast-like synoviocytes

The cells of the joint parenchyma, including synoviocytes, fibroblasts, chondrocytes, osteoblasts and osteoclasts, were historically viewed in RA as the passive victims of an externally mediated immune attack. However, emerging evidence particularly over the past decade implicates many of these cells within disease pathogenesis, not least the synoviocyte. Two types of synoviocyte have been characterised: type A and type B (Iwanaga *et al.*, 2000). Type-A synoviocytes bear macrophage-specific surface markers and are thought to represent

the tissue-resident macrophages of the joint, responsible for phagocytosis of cellular debris and capable of antigen presentation through MHC-II molecules. Type-B synoviocytes closely resemble fibroblasts, and in health function to secrete lubricating proteoglycans and glycosaminoglycans (Iwanaga *et al.*, 2000). However, in RA these fibroblast-like synoviocytes (FLS) can become grossly dysregulated and produce large amounts of pro-inflammatory cytokines and chemokines together with destructive MMPs (Bottini and Firestein, 2013). Furthermore, observations of epigenetic modifications in FLS isolated from RA pannus are thought to be important in maintaining their pro-inflammatory phenotype (Frank-Bertoncelj and Gay, 2014). Furthermore, human FLS can migrate between joints via the vasculature when experimentally grafted to mice (Lefevre *et al.*, 2009), although it is unknown whether this malignant-like behaviour occurs in human disease.

1.2.7 Environmental associations

Epidemiological studies have identified several environmental factors associated with the development of RA, especially seropositive disease. As already discussed (see Introduction 1.2.2), the most striking and well-characterised of these is tobacco smoking. Furthermore, exposure to other toxins including silica dust (Stolt *et al.*, 2005) and urban airborne pollutants (Jung *et al.*, 2017) have also been linked to an increase risk of RA.

Various lifestyle and dietary factors have also been associated with the development and persistence of RA. Several studies suggest a protective role for a Mediterranean diet (Forsyth *et al.*, 2017), perhaps due to higher levels of monounsaturated fatty acids (Matsumoto *et al.*, 2018) which may be protective against the development of RA (de Pablo *et al.*, 2018). Furthermore, modest alcohol intake has been shown to protect against the onset of RA in several case-control studies (Scott *et al.*, 2013b). Obesity has been associated with an increased risk of RA in several studies (Lu *et al.*, 2014; Ljung and Rantapaa-Dahlqvist, 2016; Turesson *et al.*, 2016).

Recent years have witnessed increasing interest in the role of human microflora in the pathogenesis of autoimmune disease, not least inflammatory arthritides such as RA. The huge number of microbes that colonise mucosal sites such as the oral cavity, intestines and lungs, together with their interactions with host immunity, provide a rich source of environmental modulators of the immune system (Clemente *et al.*, 2018). Proof-of-concept studies with mice reared in germ-free environments have demonstrated the importance of specific elements of the murine gut microbiome – for example, segmented filamentous bacteria – in the

development of experimental arthritis (Wu *et al.*, 2010). In humans, increased prevalence of *Prevotella copri* has been observed in faecal samples from patients with RA (Scher *et al.*, 2013), and *P.copri*-derived peptides can drive Th_1 responses *in vitro* by peripheral blood mononuclear cells of RA patients (Pianta *et al.*, 2017). Although still in its infancy, study of the diverse variation in the human microbiome seems likely to yield further insights to specific triggers and novel therapeutic avenues for the treatment of RA. Indeed, microbiome alterations may conceivably play a role in the mechanistic effects of many if not all of the dietary and lifestyle factors mentioned above (Clemente *et al.*, 2018).

1.2.8 Summary

Although the exact mechanisms by which autoimmunity is initiated and maintained in RA remain uncertain, several key players, including both immune and joint stromal factors, are known to be important. At the heart of the disease lies the $CD4^+$ T cell, which is firmly implicated in RA pathogenesis by evidence from human genetic and epigenetic studies. Autoimmunity is detectable in RA patients many years before the onset of clinical disease, and is a dynamic and fluid process that is different both between individual patients and even within the same patient over time. In this respect, RA is perhaps best conceptualised as a clinical syndrome which encompasses multiple distinct immune pathologies under a unifying phenotypical umbrella, in which the $CD4^+$ T cell likely plays a crucial orchestrating role.

1.3 Treatment approaches in rheumatoid arthritis

The past two decades have witnessed a remarkable transformation in RA prognosis, from a disease of inexorable joint destruction and disability to one where remission is now commonplace. This clinical revolution reflects the emergence of new therapies, including highly potent and selective biopharmaceutical agents, in tandem with paradigm shifts in regimens of drug initiation and dose escalation.

In this section, I will summarise the role of DMARDs in the treatment of RA and the evolution of RA treatment paradigms, to provide a conceptual context in which to frame this Thesis.

1.3.1 The "step-up" paradigm of DMARD therapy

The gradual acceptance of DMARDs in mainstream rheumatology practice in the latter decades of the 20th century led to the emergence of the "step-up" paradigm of RA treatment (Figure 1.2). In this model, medications are sequentially introduced to control joint inflammation, with the trigger for escalation of DMARD therapy being poor disease control (Moreland *et al.*, 2001). This treatment approach ensures that only those patients with severe disease receive the most costly treatment; cost in this sense defined in terms of the individual patient (i.e. side effect profile) and the healthcare provider (i.e. monetary cost of the medication).

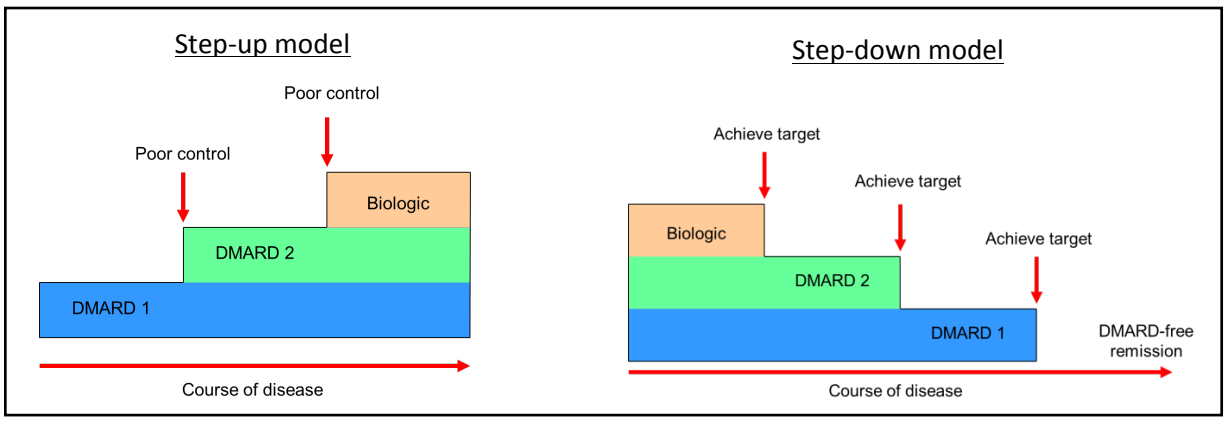


Figure 1.2 – The "step-up" and "step-down" models of disease-modifying anti-rheumatic drug (DMARD) therapy in rheumatoid arthritis. Adapted from Dale *et al.* (2007).

A step-up approach to DMARD therapy does nevertheless carry significant disadvantages. Perhaps the most notable of these is the time taken to escalate drug therapy, thus effectively delaying commencement of an effective DMARD regimen for those with the most severe disease. Indeed, strong evidence exists to support the concept of a so-called "window of opportunity" for the treatment of RA, whereby substantial improvements in clinical and patient-reported outcomes are observed when effective DMARD therapy is introduced early in the evolution of the disease (Breedveld, 2011). A meta-analysis of 12 studies (1133 patients) found that early DMARD commencement correlated with a 33% reduction (95% CI: 50% to 16%) in long-term radiographic progression compared to those who received DMARDs later, even though the average delay in DMARD commencement was just 9 months (Finckh *et al.*, 2006). In a more recent meta-analysis of data from three observational early RA cohorts (2079 patients), a significant decrease in sustained DMARD-free remission was observed with increasing delay to DMARD initiation (hazard ratio (HR) 0.989 [95% CI 0.983 to 0.995] per week increase in symptom duration) (van Nies *et al.*, 2014). Nevertheless, the validities of both meta-analyses are weakened somewhat by heterogeneity in patient characteristics and DMARD regimens, and by their inclusion of observational study data. As suggested by Raza and Filer (2015) it is also important to consider the potential confounding by disease subtype in that patients with an insidious onset of symptoms, and hence delayed presentation and commencement of DMARDs, may have an inherently worse prognosis that is independent of treatment delay. Despite the limitations outlined above, the evidence that early intervention can improve long-term outcomes in RA is compelling and as such features prominently in international treatment guidelines (Singh *et al.*, 2016; Smolen *et al.*, 2017).

1.3.2 Treatment-to-target in rheumatoid arthritis

The improved patient outcomes observed with early DMARD therapy has led to the emergence of the "treat-to-target" approach, whereby DMARDs are escalated until a pre-determined target of arthritis activity is achieved (Smolen *et al.*, 2016b). For the majority of patients, this target is remission – now a realistic aim with modern DMARD therapy (Smolen *et al.*, 2016b). Indeed, patient outcomes of target-based treatment approaches have been demonstrated to be superior to that guided by symptoms alone in many branches of medicine. This is perhaps most notable in the treatment of diabetes mellitus, where treatment to a target level of glycaemic control can significantly reduce microvascular complications of the disease (Fullerton *et al.*, 2014). It is perhaps therefore not surprising that emerging evidence supports improved RA outcomes with a treat-to-target approach. In the landmark Tight Control for

Rheumatoid Arthritis (TICORA) study, 111 patients with active established RA were randomised to receive either conventional treatment or intensive treatment to a target DAS44 < 2.4 by means of rapid escalation of DMARD therapy and intra-articular corticosteroid injection (Grigor *et al.*, 2004). Patients in both arms were reviewed every three months, with disease activity assessment measured by a metrologist blinded to study arm allocation. After 18 months, the mean fall in DAS44 was greater in the intensive treatment arm (-3.5 vs. -1.9, difference 1.6 [95% CI: 1.1 – 2.1, p<0.0001]) with lower rates of radiographic progression and no excess adverse effects (Grigor *et al.*, 2004). Other clinical trials have found similar benefits for target-based treatment (Schoels *et al.*, 2010) which have now been incorporated within the treat-to-target recommendations of an international task force (Smolen *et al.*, 2016b).

1.3.3 The "step-down" paradigm of DMARD therapy

Despite the apparent advantages of a treat-to-target model, the long half-lives of DMARDs ultimately result in several months before a change in DMARD therapy is reflected in a change in arthritis activity. The benefits of adopting a treat-to-target approach therefore remain limited by the constraints of a step-up model, with patients suffering from severe arthritis potentially taking many months to escalate to effective therapy (Dale and Porter, 2010). It is thus apparent that an alternative approach to the treatment of RA is required if the therapeutic benefit of early effective DMARD therapy is to be maximised.

One potential solution to this problem is offered by the paradigm of "step-down" DMARD therapy (Figure 1.2). In this model, potent combination DMARD therapy is commenced as early as possible in the evolution of the disease, ideally at the point of diagnosis and including biologic agents if necessary. Combination DMARD therapy is then continued until a treatment target is reached; upon achieving this, DMARD therapy is then reduced to the minimum level required to maintain the target (Dale *et al.*, 2007).

This step-down paradigm differs from the step-up model in two important regards. Firstly, patients are commenced on a potent combination of multiple DMARD therapies early in the course of their disease, precisely at the time when aggressive treatment is most likely to yield the best long-term outcomes. Secondly, the step-down model minimises the burden of DMARD therapy once the treatment target has been achieved, rather than perpetuating DMARD regimens indefinitely. Thus despite potentially greater adverse events and financial costs in the initial phase, the long-term cost-effectiveness of the step-down approach has been

calculated to be more favourable than alternative strategies (Tosh *et al.*, 2011). Nevertheless, combination DMARD therapy is not required in all cases and methotrexate monotherapy in a treat-to-target approach can be equally efficacious in selected patients (Gaujoux-Viala *et al.*, 2014).

In certain regards, the past decade has seen a gradual transition towards a partial step-down model. Early treatment with combination DMARDs is now advocated by current treatment guidelines, either within the first few months in the case of treatment failure (Singh *et al.*, 2016; Smolen *et al.*, 2017), or even at first presentation (National Institute for Health and Care Excellence, 2009). Systemic and/or intra-articular glucocorticoids are also recommended as induction therapy in the initial stages of disease to help maximise disease control in this window of opportunity (Singh *et al.*, 2016; Smolen *et al.*, 2017). Nevertheless, routine use of biologic therapy within the first three months of disease diagnosis is not currently recommended (Smolen *et al.*, 2017), even though some patients with severe disease will ultimately require them. Furthermore, although DMARD withdrawal is cautiously suggested in current guidelines (Smolen *et al.*, 2017), routine DMARD tapering to compete cessation – at least outside of the setting of paediatric and adolescent rheumatology – remains a controversial area with no clear consensus opinion (Kuijper *et al.*, 2017).

There are major obstacles to the widespread adoption of a *bona fide* step-down treatment approach as described above. Firstly, there is a current paucity of biomarkers that, when measured at first presentation, can both reliably predict those patients who are destined to develop severe RA and who will respond to specific DMARDs (Dale and Porter, 2010). Recent developments in the search for such biomarkers lies outside the scope of this report (for a review, see Mohan and Assassi (2015)), but their current absence nonetheless remains a crucial barrier to the effective use of a step-down treatment model in the clinic. This is especially true for biologics, which are expensive and thus difficult to justify economically as first or even second-line therapy in the absence of such predictors of disease severity and treatment response. For this reason, current UK guidelines mandate trial of at least two conventional synthetic DMARDs before biologic therapy can be introduced (National Institute for Health and Care Excellence, 2009). As will be discussed later, a further problem with the step-down model is that there is currently no reliable method to predict the risk of arthritis flare upon DMARD reduction, effectively stalling any attempts to wean DMARD therapy in an individualised manner.

1.3.4 Summary

The past few decades have witnessed a revolution in the treatment of RA, from the introduction of small molecular DMARDs to the impact of biologic agents and the emergence of treat-to-target approaches aiming for clinical remission. The importance of an early "therapeutic window of opportunity" has led to the intensification of DMARD regimens and the proposal of a new paradigm of step-down DMARD therapy. However, the adoption of a full-scale step-down model of DMARD therapy is limited by a paucity of biomarkers that are predictive of future severity of disease, response to DMARDs and risk of arthritis flare upon DMARD withdrawal. Current practice therefore relies heavily on a step-up approach to DMARD therapy, with consequent missed opportunities for better long-term outcomes.

1.4 Measuring RA disease activity

Accurate and reliable measures of disease severity are central to the effective management and research of RA. Although a simple concept, the objective measurement of RA severity can be challenging to achieve in practice. Factors complicating assessment include patient subjectivity, the fluctuating intermittent nature of synovitis and the wide range of different parameters that can be measured: from patient-reported symptoms such as joint pain, through to musculoskeletal imaging and the laboratory measurement of blood-borne inflammatory markers.

In general, there are two main groups of disease activity metrics in RA: those that directly measure the inflammatory response, and those that measure the clinical manifestations and consequences of joint inflammation. This latter group can be further differentiated into physician-observed and patient-experienced measures, although in practice there is overlap between them **(**Figure 1.3**)**. In this chapter, I will summarise the key available methods of measuring disease activity in RA as relevant to the latter chapters of this Thesis.

1.4.1 Composite clinical scores of disease activity

Composite clinical scores are popular methods of RA disease activity assessment that bring together complementary measures such as painful and swollen joint counts, global assessment scores and laboratory measures of inflammation (Table 1.5). One of the earliest examples is the Disease Activity Score (DAS), a composite clinical scoring system incorporating pain and swelling in 44 joints, a patient global assessment score and ESR (van der Heijde *et al.*, 1990). Whilst the DAS provided an objective measurement of disease activity, it included joints that are challenging to examine clinically such as in the feet. In response to this problem, an abbreviated version of the DAS including 28 joints was developed and became known as the DAS28-ESR (Prevoo *et al.*, 1995). Although the DAS28-ESR has been criticised for underestimating disease activity in those with predominant foot involvement (Bakker *et al.*, 2012), its ease of use and reproducibility have led widespread adoption within both research and clinical settings. Indeed, DAS28-ESR forms the basis of the EULAR criteria of RA treatment response (van Gestel *et al.*, 1996) and is central to guidelines published by the National Institute for Health and Care Excellence (NICE) which govern eligibility for biologic therapy in the UK (National Institute for Health and Care Excellence, 2007). More recently, the DAS28 has been further modified by the use of CRP (Fransen *et al.*, 2003),

considered by many to be a more specific and sensitive marker of inflammation in RA compared to the traditional ESR method (van Riel, 2014). However, DAS28-CRP can underestimate disease activity when used with DAS28-ESR thresholds, and alternative slightly lower thresholds for DAS28-CRP have been recently proposed to compensate for this (Fleischmann *et al.*, 2015; Fleischmann *et al.*, 2017).

Recent years have seen the development of further composite scoring systems such as the Simple Disease Activity Index (SDAI) (Smolen *et al.*, 2003) and Clinical Disease Activity Index (CDAI) (Aletaha *et al.*, 2005), both notable for their inclusion of an additional physician global assessment parameter. It has been argued that SDAI provides a more reproducible measure of RA activity by placing more emphasis on objective measures such as

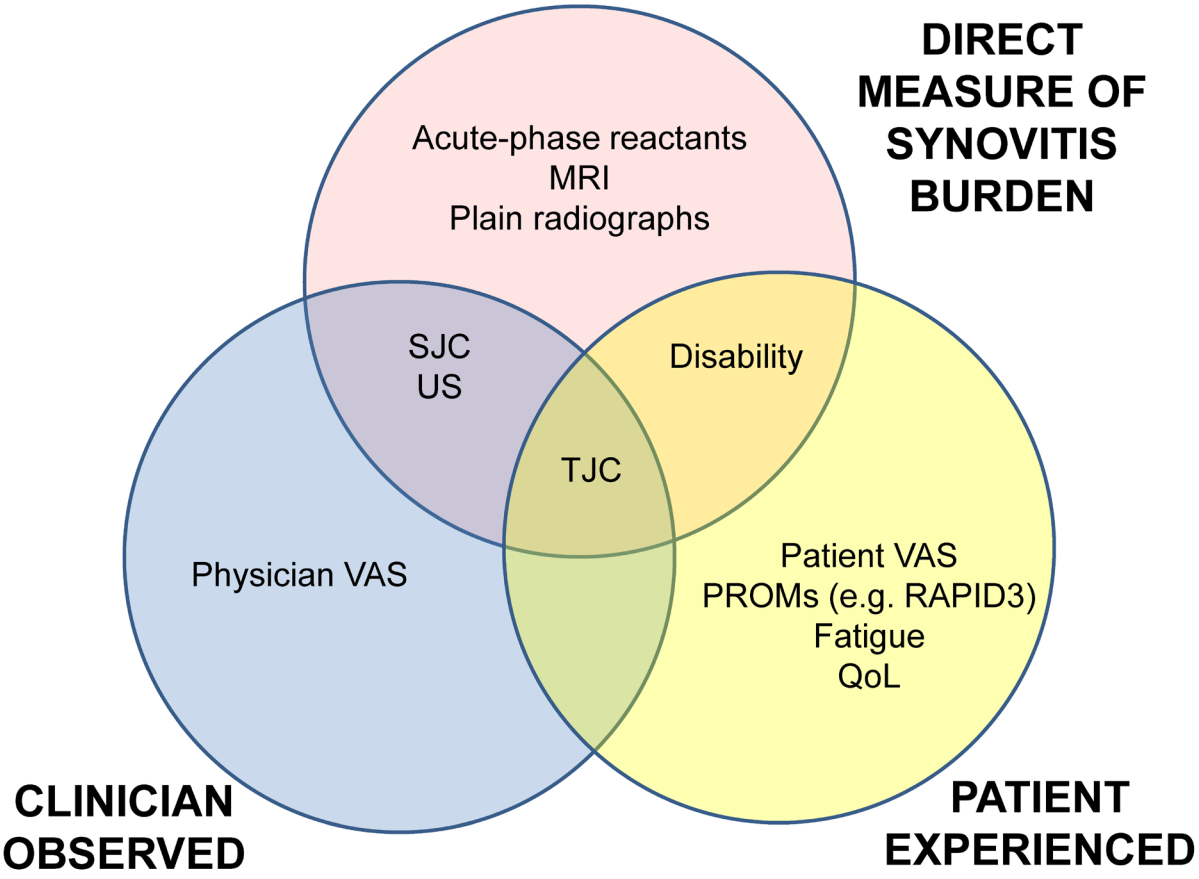


Figure 1.3 – Overview of current measures of disease activity in rheumatoid arthritis. The precise extent to which measures are directly reflective of synovitis burden versus clinician-observed and/or patient experienced phenomena is contentious, and is discussed further in this section. MRI: magnetic resonance imaging; PROM: patient-reported outcome measure; QoL: quality of life; SJC: swollen joint count; TJC: tender joint count; US: ultrasound; VAS: visual analogue scale.

Table 1.5 – Various composite clinical disease activity scores for measuring disease activity in RA, adapted from Klarenbeek *et al.* (2011a). Note that use of DAS28-ESR thresholds for DAS28-CRP assessment has been shown to underestimate disease activity. Shown in *italics* are alternative DAS28-CRP score thresholds validated against DAS28-ESR, as proposed by Fleischmann *et al.* (2015)* and Fleischmann *et al.* (2017)†. CDAI: clinical disease activity index; CRP: C-reactive protein; DAS: disease activity score; ESR: erythrocyte sedimentation rate; RAI: Ritchie articular index; SDAI: simple disease activity index; SJC: swollen joint count; TJC: tender joint count; VAS: visual analogue score (on a 0-10cm or 0-100mm scale).

Activity Score	**Formula**	**Remission**	**Low disease activity**	**Moderate disease activity**	**High disease activity**
DAS	$0.53938\sqrt{(RAI)} + 0.06465(SJC44) + 0.330\ln(ESR) + 0.00722(VAS_{patient}[mm])$	<1.6	$1.6 \leq score \leq 2.4$	$2.4 < score \leq 3.7$	> 3.7
DAS28-ESR	$0.56\sqrt{(TJC28)} + 0.28\sqrt{(SJC28)} + 0.70\ln(ESR) + 0.014(VAS_{patient}[mm])$	< 2.6	$2.6 \leq score \leq 3.2$	$3.2 < score \leq 5.1$	> 5.1
DAS28-CRP	$0.56\sqrt{(TJC28)} + 0.28\sqrt{(SJC28)} + 0.36\ln(CRP[mg/L]+1) + 0.014(VAS_{patient}[mm]) + 0.96$	< 2.6 *< 2.4 **	$2.6 \leq score \leq 3.2$ *$2.4 \leq score \leq 2.9$ **	$3.2 < score \leq 5.1$ *$2.9 < score \leq 4.6$ †*	> 5.1 *> 4.6 †*
SDAI	$TJC28 + SJC28 + VAS_{physician}$ [cm] + $VAS_{patient}$ [cm] + CRP [mg/dL]	≤ 3.3	$3.3 < score \leq 11$	$11 < score \leq 26$	> 26
CDAI	$TJC28 + SJC28 + VAS_{physician}$ [cm] + $VAS_{patient}$ [cm]	≤ 2.8	$2.8 < score \leq 10$	$10 < score \leq 22$	> 22

joint swelling and less emphasis on subjective measures such as patient global assessment in comparison to DAS28.

1.4.2 Patient-reported outcome measures

Recent years have witnessed a surge of interest in patient-reported outcome measures (PROMs) in RA. The development of PROMs such as the Rheumatoid Arthritis Disease Activity Index (RADAI) (Stucki *et al.*, 1995) and the routine assessment of patient index data 3 (RAPID3) score (Pincus *et al.*, 2008) provide validated methods of disease activity assessment without the requirement for physician assessment or laboratory tests. Furthermore, PROMs can also provide a measure of systemic symptoms such as fatigue and joint stiffness, which are not included in standard composite disease scores despite being frequently cited by patients as the most disabling aspects of the disease. However, whilst PROMs correlate well with composite clinical disease activity indices in active disease, their use as treatment targets remains controversial. Indeed, the RAPID3 score tends to correlate less robustly with composite clinical disease indices at remission levels (Kim *et al.*, 2014a), particularly in the absence of a swollen joint assessment (Castrejon *et al.*, 2013). Nevertheless, a study of 705 outpatients by Rintelen *et al.* (2013) found that although the sensitivity of RADAI-5 for identifying patients who satisfied SDAI remission was low (60%, 95% CI 53-68%) the specificity was impressively high (92%, 95% CI 89-94%). Furthermore, both RAPID3 and RADAI-5 have been demonstrated as successful tools for patient-administered screening of arthritis flares (Bossert *et al.*, 2012), which is arguably a more feasible clinical role compared to use as definitions of remission for treatment targets. Indeed, a PROM specifically designed for detection of RA flare – the flare in rheumatoid arthritis (FLARE-RA) questionnaire – has recently been validated against clinical measures of disease activity for patient self-assessment of disease flare, which could be employed between clinic visits (Fautrel *et al.*, 2017).

1.4.3 Disability and quality of life scoring systems

As with other chronic diseases, RA can have a profound effect upon physical function and quality of life. Questionnaire-based assessment methods can quantify physical disability and quality of life, many of which have been validated in RA (Table 1.6).

Table 1.6 – Selection of instruments that have been validated for the assessment of disability and quality of life in patients with rheumatoid arthritis. Adapted from Maska et al. (2011).

Instrument	Purpose	Timeframe
Health Assessment Questionnaire Disability Index (HAQ-DI)	Assesses physical function via impact on daily activities (41 items)	Past week
Modified Health Assessment Questionnaire (MHAQ)	Abbreviated version of HAQ-DI (8 items)	Past 3 months
Multidimensional Health Assessment Questionnaire (MDHAQ)	MHAQ plus two additional items regarding walking and sports/recreation activity (10 items).	Past week
Health Assessment Questionnaire II (HAQ-II)	Assesses physical function via impact on daily activities (10 items)	Past week
Improved Health Assessment Questionnaire (Improved HAQ)	Shortened alternative to HAQ-DI (24 items)	Present
Rheumatoid Arthritis Quality of Life (RAQoL)	Assesses quality of life and activities of daily living (30 items)	Past week
EuroQol EQ-5D	Assesses QoL and physical function across five domains (5 items)	Present
36-item Short Form Survey (SF-36)	Assesses QoL and physical function across eight domains (36 items)	Mostly 4 weeks (single general question for past year)

A major limitation with the use of such questionnaires in assessing disease activity in RA is that in established disease it can be difficult to separate disability secondary to permanent joint damage from disability secondary to reversible joint inflammation, or indeed disability secondary to another comorbid disease. Furthermore, capturing the impact that RA has on physical function and quality of life from such a 'snapshot' assessment is challenging given the highly fluctuating nature of disease activity in RA. Nevertheless, disability and quality of life assessments can provide complementary measures of disease impact in longitudinal research studies, and in the economic evaluation of therapeutic interventions.

1.4.4 The role of imaging in measuring RA disease activity

1.4.4.a Plain radiographs

For several decades, the gold standard approach to using imaging to assess disease activity in RA was to measure the accumulation of erosive joint damage on serial plan radiographs of the hands and feet. Plain radiographs provide an objective measure of the consequences of active

synovitis and hence remain widely used in clinical practice and as an outcome measure in clinical trials. Nevertheless, plain radiographs are limited by their inability to directly visualise synovitis and hence provide a necessarily retrospective measure of disease activity. A further limitation is that peri-articular structures such as tendons and ligaments are not identified by plain radiographs, and hence inflammation in these important areas can be easily overlooked. Finally and perhaps most importantly, the irreversible nature of joint erosions makes assessment of erosive progression unacceptable in the modern era where prevention of joint damage is a key treatment aim.

1.4.4.b Magnetic resonance imaging (MRI)

The advent of magnetic resonance imaging (MRI) has revolutionised musculoskeletal radiology through an ability to visualise both bone and the surrounding non-calcified joint structures. Furthermore, MRI can visualise the consequences of active arthritis including bone oedema and synovitis (McGonagle and Tan, 2008). It is now well-established that bone marrow oedema and synovitis visualised on MRI imaging correlate closely with future radiographic joint erosions (Ostergaard and Moller-Bisgaard, 2014). Thus, MRI can be used to detect early reversible signs of joint inflammation before irreversible joint damage occurs – indeed, MRI abnormalities can even precede the onset of clinical arthritis by several months (van Steenbergen *et al.*, 2016). Nevertheless, MRI carries significant limitations including the cost and size of scanning machinery, the need for specialist musculoskeletal radiologists to interpret scan images and the occasional need to administer intravenous gadolinium contrast agents (Tan *et al.*, 2012). Furthermore, the strong magnetic field precludes the use of MRI in patients with metallic foreign bodies such as many implantable cardiac defibrillators (Tan *et al.*, 2012). Taken in combination, these limitations place a restriction upon the widespread use of MRI in current rheumatology practice.

1.4.4.c Musculoskeletal ultrasound

Ultrasound (US) imaging relies upon ultra-high frequency sound waves and their reflection at the interfaces between tissues of different acoustic density to create a so-called “B-mode” greyscale visualisation of tissue structures (Hoskins *et al.*, 2010). Furthermore, by exploiting the Doppler shift of sound waves reflected from moving structures, the “power Doppler” US modality provides a direct visualisation of blood flow (Schmidt, 2004). Through this combination of greyscale and power Doppler imaging, it is possible to visualise both joint

erosions and active synovitis, the latter in the form of synovial thickening and hypervascularity. Furthermore, periarticular structures such as tendons and ligaments can also visualised together with the joint in real-time dynamic imaging. Such strengths, combined with the relatively low cost of equipment and the ability to record and interpret images at the bedside, make US an attractive imaging modality for a fluctuating disease such as RA (Grassi *et al.*, 2004).

Although not necessitated by current ACR classification criteria, musculoskeletal US plays an increasingly important role in the diagnosis and management of RA beyond clinical assessment (D'Agostino *et al.*, 2016b). This is particularly true for increased synovial vascularity visualised as power Doppler (PD) signal, the finding of which is widely regarded as signifying active synovitis (Fukae *et al.*, 2014). Indeed, the presence of PD has been shown to outperform clinical features of joint inflammation such as tender joint count in predicting future radiographic progression (Cheung *et al.*, 2016).

The presence of PD correlates with histological measures of synovitis in synovial tissue (Takase *et al.*, 2012; Andersen *et al.*, 2014b; Abe *et al.*, 2016), and has been shown to correlate with the presence of Th17 cells in synovial fluid (Gullick *et al.*, 2010). Increased colour Doppler flow has also been shown to correlate with the production of the pro-inflammatory chemokine monocyte chemoattractant protein 1 (MCP-1) in *ex vivo* culture of synovial biopsy tissue (Andersen *et al.*, 2014a). The presence of PD occurs early in the course of disease, and in the setting of undifferentiated inflammatory arthritis can add discriminatory diagnostic value for RA beyond standard clinical assessment alone, especially in seronegative patients (Freeston *et al.*, 2010; Nam *et al.*, 2016). Ultrasound parameters, in particular PD, are highly responsive to changes in disease activity (Naredo *et al.*, 2007) and have been shown to improve within as little as one week after initiation of biologic DMARD therapy (D'Agostino *et al.*, 2016c). Furthermore, the presence of PD has been shown to portend a poor prognosis in terms of both future radiographic progression and risk of arthritis flare, even in those patients who achieve clinical remission (Han *et al.*, 2016b). This raises the question of whether treat-to-target approaches based on US synovitis yields superior outcomes compared to clinical assessment and remains an area of current active research (Wakefield *et al.*, 2012).

Despite its advantages, US nevertheless has several limitations; of particular note, the variation in technique and subjectivity of image interpretation can lead to substantial inter-operator variability (Tan *et al.*, 2012). In addition, the increasing fidelity of modern US equipment brings increasing difficulty in ascribing diagnostic and prognostic significance to low-grade US synovitis. In study of 207 healthy individuals who underwent a 32-joint US

scan, 182/187 (97%) had at least one abnormal finding: 95 (46%) had synovial effusion alone (usually at the great toe), 79 (42%) had greyscale synovial hypertrophy (GS), and 8 (4%) had PD (Padovano *et al.*, 2016). In a recent case-control study using a 10-joint US protocol, 127 healthy individuals were age- and sex-matched to 127 patients from an early arthritis cohort of patients with RA or undifferentiated arthritis of diagnosis duration < 6 months (Millot *et al.*, 2011). Although the prevalence of US findings were significantly greater ($p<0.001$) in early arthritis patients (GS: 75%, PD: 50%, erosion 36%), there were considerable numbers of healthy controls with US abnormalities (GS: 9%, PD: 4%, erosion: 11%), albeit to a lesser degree than the study by Padovano *et al.* (2016) perhaps reflecting the fewer number of joints scanned. Nonetheless, the magnitude of GS in RA joints has been shown to negatively correlate with years since last clinical episode of joint inflammation, suggesting that GS may be a useful indicator of recent inflammatory burden (Gartner *et al.*, 2015).

The substantial levels of US abnormalities in healthy individuals make it challenging to define active synovitis in terms of ultrasonographic parameters. Whilst PD is generally accepted to be a marker of active disease, the clinical significance of low-grade PD signal is contentious, particularly in the absence of GS. Whereas some authors define active synovitis as the presence of any level of PD in any single joint (Grassi and Filippucci, 2003), others adopt more stringent definitions such as PD in ≥2 joints (Dale *et al.*, 2016), or PD in the presence of moderate GS (Ramirez *et al.*, 2014). This lack of consensus surrounding the 'minimum-acceptable' level of US findings that are consistent with absence of disease results in substantial heterogeneity between different studies and is a significant barrier to research.

1.4.5 Measurement of circulating inflammatory mediators

A further measure of RA activity is by the measurement of circulating levels of markers and mediators of the inflammatory response. Basic measures of inflammation including ESR and CRP have been in widespread clinical use for decades, and provide an objective and reliable measure of the degree of systemic inflammation. Unfortunately however, the limited nature of synovitis in RA, particularly in mild disease when inflammation can be restricted to a few small joints, often results in little if any detectable rise in these standard laboratory measures despite ongoing active disease.

Following technological advances in the detection of cytokines and chemokines in human-derived samples, several groups explored the association between the circulating levels of individual inflammatory mediators and disease activity in RA. Increased synovial and

circulating concentrations of many cytokines including TNF-α (Espersen *et al.*, 1991; Vreugdenhil *et al.*, 1992), IL-1β (Eastgate *et al.*, 1988), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Xu *et al.*, 1989), and IL-6 (Houssiau *et al.*, 1988) have been demonstrated to positively correlate with arthritis activity in RA. Indeed, such observations provided important early insights to the immune dysregulation underlying the disease. However, with increasing knowledge of the dysregulation of molecular mediators of inflammation in RA came an appreciation of the marked heterogeneity in the dynamic networks of cytokine imbalance between individual RA patients (Arend, 2001). It is thus apparent that the measurement of a single cytokine/chemokine in isolation is limited in its applicability as a biomarker of disease activity that can be reliably applied across all patients in a clinical context.

To address this shortcoming, attempts have been made to define composite laboratory biomarkers of disease activity in RA. Particularly notable in this regard is the multibiomarker disease activity (MBDA) score, which is commercially marketed as Vectra® DA (Crescendo Bioscience Inc., San Francisco, USA) (Centola *et al.*, 2013). The MBDA score was developed with the aim of providing a quantitative measure of disease activity to complement composite clinical disease activity scores such as DAS28. Using a systematic and statistically-driven approach, a signature of 12 different proteins were identified and their serum concentrations combined to create a score from 0 to 100 that correlated with disease activity scores in samples from several multinational clinical trials (Centola *et al.*, 2013). The MBDA score has been shown to track longitudinal changes in clinical disease activity (Hirata *et al.*, 2013) and predict future radiographic progression in several studies (Markusse *et al.*, 2014b; Hirata *et al.*, 2016; Li *et al.*, 2016). In a recent meta-analysis of three clinical studies of over 562 patients with RA, patients with a high MBDA score had a relative risk of radiographic progression of 5.1 (95% CI 2.5 – 10.1, $p<0.0001$) compared to those with a low score; for comparison, the relative risk of progression was 1.6 ($p = 0.01$) and 1.4 for categories of CRP and DAS28-CRP respectively (Curtis *et al.*, 2017). However, in a separate study MBDA did not predict progression to RA for 45 patients with undifferentiated arthritis (UA), though MBDA levels were higher in RA patients than controls (Maijer *et al.*, 2015). Furthermore, in a clinical trial of abatacept versus adalimumab in RA, MBDA samples from 524 patients failed to show an association with clinical measures of disease activity (Fleischmann *et al.*, 2016). Indeed, the utility of MBDA in the management of RA beyond standard laboratory and clinical measures of disease activity is uncertain, has been challenged by some commentators (Yazici and Swearingen, 2014; Pincus *et al.*, 2017).

1.4.6 Synovial joint biopsy

A major criticism of the aforementioned approaches to disease activity assessment are that they are all, to varying degrees, surrogate measures of synovial inflammation. However, by ultrasound or arthroscopic-guided biopsy it is possible to obtain samples of synovial tissue directly at the site of disease (Lazarou *et al.*, 2015). Histological analysis of synovial tissue can reveal the number and cellular subtype of infiltrating leukocytes and their organisation, thus providing additional information on the underlying immunopathological processes beyond solely quantifying joint inflammation (Filkova *et al.*, 2016). Importantly, synovial biopsies also allow for the study of stromal cells such as synovial fibroblasts, which likely play a role in the initiation and perpetuation of chronic synovitis (see Introduction 1.2.6). Advanced next-generation and single-cell sequencing technologies allow for detailed profiling of gene expression and epigenetic modifications within both infiltrating leukocytes and stromal cells, and is an intense area of current research.

Nevertheless, synovial biopsy has several disadvantages that have thus far limited its use in routine clinical practice. Although the procedure can be performed under local anaesthetic and is well-tolerated by patients, it is nevertheless an invasive procedure which requires additional resources and staff training to deliver on a wide scale (Lazarou *et al.*, 2015). Furthermore, the precise relationship between measures of synovial pathobiology and disease prognosis and treatment response remain to be fully elucidated. Technical challenges also exist, not least surrounding the range of joints amenable to biopsy and the targeting of biopsy to actively-inflamed synovial regions (Filkova *et al.*, 2016). Nevertheless, synovial biopsy does hold promise as a potential measure of disease activity that may in the future be used to help guide diagnosis, stratify patients for therapy, and assess the effectiveness of treatment (Orr *et al.*, 2017).

1.4.7 Summary

The measurement of disease activity in RA is not simply a pedantic exercise, but is crucial both in the clinic to assess response to therapy, and in the research and development of novel therapies. Nevertheless, the many varied manifestations of RA pose substantial challenges to the accurate and precise measurement of the activity of the disease. As is evident from the discussion above, no single measure of disease activity can adequately encompass all of the diverse domains of RA. Disease activity is thus best assessed by a combination of

complementary measures, which needs to be tailored to the specific clinical or research situation in which they are employed.

1.5 Defining Remission in Rheumatoid Arthritis

With the advent of modern DMARD therapy, the past two decades have witnessed a remarkable paradigm shift in the treatment of RA. Whereas previously the treatment aim was to retard joint damage, current treatment guidelines advocate treating to a target of disease remission – a target that is now achievable for many patients.

In this subsection, I will explore the various approaches to define RA remission in clinical practice, the frequency with which disease remission can be achieved, and the concept of remission 'depth'.

1.5.1 Clinical definitions of RA remission

Following the widespread introduction of DMARDs in the latter part of the 20th century, there was growing interest in the concept of "remission" as a therapeutic target in RA. In a seminal paper, Pinals *et al.* (1981) defined complete remission in RA as "the total absence of all articular and extra-articular inflammation and immunologic activity related to RA". The authors proposed clinical criteria that were adopted by the ACR as a working definition of RA remission (Table 1.7). However, very few patients were able to achieve these remission criteria in practice, and the inclusion of a time criterion demanded that remission could only be defined in retrospect; the criteria were thus of limited use in guiding treatment or prospective research studies (van Tuyl *et al.*, 2009).

With evidence emerging from clinical trials of the prognostic value of composite clinical scores, attention focussed on redefining RA remission in terms of disease activity. For example, DAS28-ESR < 2.6 can be defined as remission based upon correlation with the 1981 preliminary ACR remission criteria (Fransen *et al.*, 2004). However, disease activity thresholds defined by DAS28-ESR do not directly translate to the now widely used DAS28-CRP (see Introduction 1.4.1), and a lower remission threshold of DAS28-CRP<2.4 has been proposed (Fleischmann *et al.*, 2015).

Nevertheless, although high DAS28 scores correlate well with poor outcomes, it became apparent that both ESR- and CRP-based DAS28 remission correlates rather poorly with both absence of joint pain and swelling (Makinen *et al.*, 2005) and future progression of joint damage (Aletaha and Smolen, 2011). In 2011, the ACR and EULAR published joint remission criteria in an attempt to develop a definition of remission in RA that is "stringent but achievable" (Felson *et al.*, 2011). The ACR/EULAR 2011 criteria were developed through

Table 1.7 – The 1981 American College of Rheumatology preliminary criteria for clinical remission in rheumatoid arthritis. ESR, erythrocyte sedimentation rate. Reproduced with permission from Pinals *et al.* (1981). Copyright © 1981 American College of Rheumatology.

At least five of the following criteria must be fulfilled for at least 2 consecutive months:
1. Duration of early morning stiffness not exceeding 15 minutes
2. No fatigue
3. No joint pain (by history)
4. No joint tenderness or pain on motion
5. No soft tissue swelling in joints or tendon sheaths
6. ESR less than 30mm/hr for a female or 20mm/hr for a male

consensus expert opinion, and validated through their ability to predict favourable radiological outcomes using data from prospective clinical trials. Incorporated within the 2011 ACR/EULAR remission criteria are both a Boolean definition and an index definition based on SDAI score (Table 1.8).It was acknowledged that the exclusion of foot and ankle joints from the remission criteria has potential to limit its specificity, though in a validation exercise it was demonstrated that patients with active synovitis in these areas typically failed to meet the other remission criteria (Felson *et al.*, 2011).

Low rates of radiological progression and favourable long-term functional outcomes in patients achieving ACR/EULAR remission have now been demonstrated by several independent groups (Klarenbeek *et al.*, 2011a; Sakellariou *et al.*, 2013). Furthermore, cross-sectional studies have demonstrated that swollen and tender joint counts are on average lower when remission is defined using ACR/EULAR criteria versus DAS28 (Kuriya *et al.*, 2012; Thiele *et al.*, 2013). Nevertheless, the ACR/EULAR remission definition has been criticised by many authors for placing an overly strict threshold on the $VAS_{patient}$, which can be affected by osteoarthritis and other comorbidities, such that it may lack sensitivity for remission when used in clinical practice (Kuriya *et al.*, 2012; Masri *et al.*, 2012; Studenic *et al.*, 2012; Vermeer *et al.*, 2012; Svensson *et al.*, 2013; Thiele *et al.*, 2013; Baker *et al.*, 2017). For example, a cross-sectional analysis of 6864 RA patients found that non-RA factors, such as male gender and the presence of degenerative spinal disease, were significantly associated with failure to achieve ACR/EULAR remission despite satisfying DAS28-ESR remission (Thiele *et al.*, 2013). Furthermore, the effect of non-RA factors upon patient VAS may be disproportionately greater in patients with lowest disease activity; for example, a prospective

Table 1.8 – The American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) 2011 remission criteria for rheumatoid arthritis. Reproduced from Annals of the Rheumatic Diseases, Felson et al., volume 70, pages 404-413, copyright 2011, with permission from BMJ Publishing Group Ltd.

Boolean definition	At any time point, patient must satisfy all of the following: Tender joint count ≤ 1 Swollen joint count ≤ 1 C reactive protein ≤ 1mg/dL (10mg/L) Patient global assessment ≤ 1 (on a 0-10 scale)
Index definition	At any time point, patient must have a Simplified Disease Activity Index score of ≤3.3

longitudinal study of 260 RA patients demonstrated that patient VAS is positively associated with older age and health distress, though only in patients with a DAS < 4.2 (Ward *et al.*, 2017). Indeed, even in the original ACR/EULAR publication consensus opinion suggested a higher mean patient VAS of < 2.2 as a remission threshold if all other parameters were consistent with remission (Felson *et al.*, 2011).

In the original publication of the ACR/EULAR remission criteria, future radiographic progression was less frequently observed in those who satisfied ACR/EULAR Boolean remission (23%) and SDAI remission (23%) versus DAS28-ESR remission (40%) at baseline, which the authors present as evidence for the superiority the ACR/EULAR remission definitions (Felson *et al.*, 2011). Indeed, this is mirrored in a prospective cohort study of 535 RA patients, which demonstrated radiographic progression after 2 years in 20%, 24% and 30% of patients who were in remission at baseline as defined by ACR/EULAR Boolean, SDAI and DAS28-CRP<2.6 remission respectively (Lillegraven *et al.*, 2012). Despite the apparent enhanced specificity of ACR/EULAR remission to identify those patients who do not develop radiographic progression, it is nevertheless troubling that approximately 20-25% of patients can develop future joint damage despite satisfying ACR/EULAR remission at baseline. Whilst one could argue that this reflects an inherent instability of remission in RA, Lillegraven *et al.* (2012) even observed radiographic progression in 7/41 (17%) of patients who satisfied ACR/EULAR Boolean remission on two or more occasions, suggesting that joint damage can accumulate despite sustained clinical remission.

1.5.2 The role of imaging in defining RA remission

The progression of joint damage despite fulfilment of clinical remission criteria suggests the presence of substantial levels of subclinical synovitis that, although asymptomatic, can nevertheless have an adverse impact on long-term outcomes (Brown *et al.*, 2008). This has led many commentators to suggest that imaging to detect subclinical synovitis should be incorporated within definitions of remission in RA (Haavardsholm *et al.*, 2012; Wakefield *et al.*, 2012).

1.5.2.a Ultrasound abnormalities persist despite clinical remission

Ultrasonographic evidence of synovial pathology in the form of power Doppler (PD) and/or greyscale synovial hypertrophy (GS) persists in patients with RA in clinical remission regardless of how clinical remission is defined. In a cross-sectional study of 107 patients receiving DMARDs for RA (Brown *et al.*, 2006), synovitis was similarly detectable in patients who satisfied DAS28-ESR remission (GS: 84%, PD: 51%) and those who satisfied 1981 ACR remission (GS: 81%, PD: 55%). Indeed, a similar study by the same group showed that newer remission criteria were similarly insensitive to the presence of PD, which was present in 65/128 (51%), 15/29 (52%) and 34/74 (46%) of patients satisfying DAS28-ESR, ACR/EULAR Boolean and SDAI remission respectively (Saleem *et al.*, 2011). In a recent meta-analysis of 19 studies including 1369 RA patients in clinical remission, Nguyen *et al.* (2014) demonstrated an overall prevalence of combined GS/PD positivity of 44%, which was comparable across different clinical remission criteria (Figure 1.4). The prevalence of GS alone and combined GS/PD positivity was greater in patients with longstanding RA > 2 years duration (GS: 87% vs 64%, GS/PD: 45% vs 34%, $p<0.001$), perhaps reflecting the chronicity of synovitis in these patients (Nguyen *et al.*, 2014).

Despite the high prevalence of US abnormalities in RA remission, there is some evidence to suggest a degree of correlation, albeit modest, between low clinical disease activity scores and the absence of PD. In a cross-sectional study of 97 RA patients in clinical remission, a significantly lower prevalence of PD in a 42-joint US examination was observed in those in SDAI remission (though not in DAS28 remission); nevertheless, SDAI remission had only modest predictive value for the absence of PD (likelihood ratio 2.24, 95% CI 1.25 – 4.01) (Balsa *et al.*, 2010). In a further study, absence of PD in the MCP joints and wrists was associated with absence of MCP/wrist joint swelling (OR 6.60, $p=0.0039$), SDAI remission (OR 5.06, $p=0.045$) and low functional disability defined as Steinbrocker stage I or II

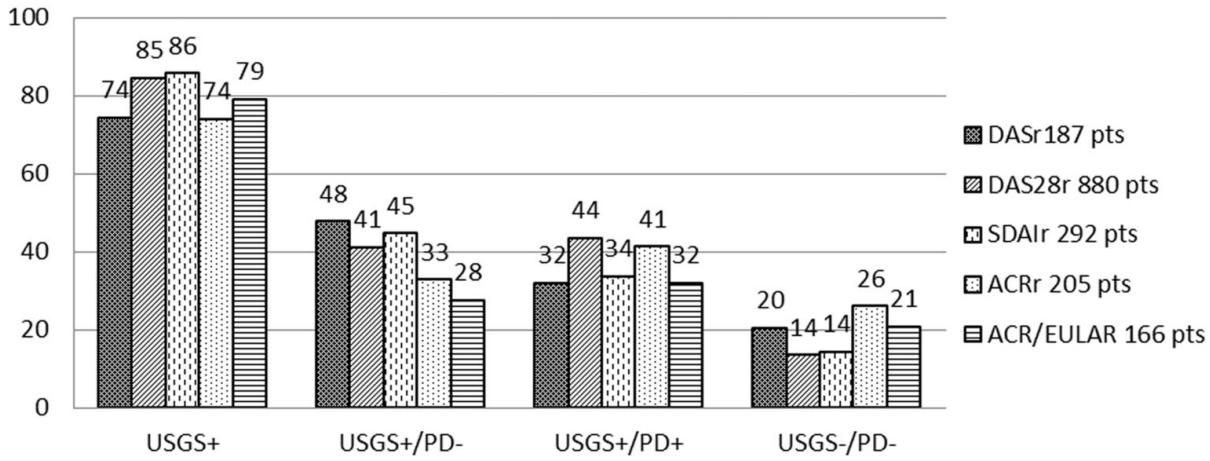


Figure 1.4 - Prevalence of ultrasound findings by different clinical definitions of remission in a meta-analysis by Nguyen *et al.* (2014). DASr: disease activity score (DAS) remission; DAS28r: either DAS28-ESR or DAS28-CRP remission; SDAI: simple disease activity index remission; ACRr: American College of Rheumatology 1981 ACR remission criteria; ACR/EULAR: ACR/European League Against Rheumatism (EULAR) Boolean remission criteria. Reproduced from Nguyen et al, 'Prevalence of ultrasound-detected residual synovitis and risk of relapse and structural progression in rheumatoid arthritis patients in clinical remission: a systematic review and meta-analysis', Rheumatology, 2014, volume 53, issue 11, pages 2110-8, by permission of Oxford University Press on behalf of the British Society for Rheumatology.

(OR 9.23, p=0.0049) (Nemoto *et al.*, 2014). Indeed, the combination of all three of these criteria had 100% predictive value for the absence of PD (Nemoto *et al.*, 2014), though no data has been published regarding replication in an independent cohort. In another study of 126 RA patients in clinical remission, lower DAS28-ESR scores (DAS28-ESR≤1.98 vs 1.98<DAS28-ESR≤2.6) were associated with lower GS positivity (44.7% vs. 78%, p<0.01) and PD positivity (32.9% vs. 72%, P<0.01), although the prevalence of PD even in the lowest disease activity score group remains substantial (Geng *et al.*, 2016). Nevertheless, the presence of GS and PD in healthy controls makes it difficult to confidently ascribe clinically relevant thresholds for low-grade ultrasonographic findings (see Introduction 1.4.4.c).

1.5.2.b In RA clinical remission, the presence of PD is associated with active synovitis

Mounting evidence suggests that the presence of PD indicates ongoing subclinical synovitis, even in those patients who have satisfied clinical remission criteria. A small study of RA patients in SDAI remission for ≥ 6 months showed that PD was present in the hands/MCPJs of 17/29 (59%) patients, with a significant correlation between presence of PD and bone erosions when analysed at the level of both the joint (p<0.001) and patient (p=0.032)

(Kawashiri *et al.*, 2014). However, no correlation was observed between the presence of PD and the levels of CRP, ESR or angiogenic biomarkers (Kawashiri *et al.*, 2014). In contrast, a study of 55 patients in DAS28-ESR remission for ≥ 6 months showed that 'active synovitis on US' (defined as GS≥2 and PD≥1) was significantly positively correlated with both clinical disease activity scores (DAS28 and SDAI) (Ramirez *et al.*, 2014). Furthermore, higher levels of circulating angiogenic biomarkers were observed in the active synovitis group, including amongst others vascular endothelial factor-D (VEGF-D), matrix metallopeptidase-2 (MMP-2) and basic fibroblast growth factor (bFGF) (Ramirez *et al.*, 2014).

Further studies suggest a link between the presence of PD in clinical remission and persistent subclinical inflammation in synovial biopsies. Ramirez *et al.* (2015) obtained synovial biopsies from 20 RA patients in remission (DAS28-ESR<2.6 for ≥ 6 months) with PD, and compared them to synovial tissue from 22 patients with clinically active RA and 10 non-inflammatory controls. Immunohistochemistry revealed less vascularity, bFGF and CXCL12 for PD-positive RA remission compared with active RA, though these were all significantly greater than non-inflammatory controls (Ramirez *et al.*, 2015). 8/20 (40%) of RA remission patients lost clinical remission over the 12 months following biopsy; these patients had significantly more synovial B cells and mast cells compared to those who maintained remission (Ramirez *et al.*, 2015).

In another biopsy study, knee synovium was compared between 25 PD-negative RA patients in clinical remission (DAS<1.6 for ≥ 6 months), 10 PD-negative RA patients with low disease activity (LDA: 1.6<DAS<2.4 for ≥ 6 months), and 50 PD-positive DMARD-naïve RA patients with moderate/high disease activity (mean DAS28 = 5.00) (Alivernini *et al.*, 2017). Both remission and LDA patients were receiving concurrent methotrexate and anti-TNFα therapy. Immunohistochemistry showed significantly less staining for CD68, CD20, CD3, CD31 and collagen deposition for PD-negative remission patients versus the PD-positive moderate/high disease activity group; furthermore, these levels were comparable for the remission vs. PD-negative LDA groups (Alivernini *et al.*, 2017). The authors therefore suggest that, compared with DAS, the absence of PD may be a better predictor of the absence of histological synovitis. However, the lack of a PD-positive remission group, and the influence of DMARD therapy upon ultrasonographic and histological measures, make it difficult to interpret these results further.

1.5.2.c In RA remission, the presence of PD is a poor prognostic factor

Emerging data from longitudinal studies demonstrate that PD is likely to be a negative prognostic factor in the setting of RA clinical remission. In a recent systematic review and meta-analysis, Han *et al.* (2016b) examined the prognostic value of baseline US abnormalities in terms of predicting the risk of future arthritis flare and radiographic progression across 13 longitudinal studies of RA patients in clinical remission with follow-up durations ranging from 3 to 24 months (Brown *et al.*, 2008; Scire *et al.*, 2009; Saleem *et al.*, 2010; Peluso *et al.*, 2011; Raffeiner *et al.*, 2011; Foltz *et al.*, 2012; Saleem *et al.*, 2012; Yoshimi *et al.*, 2013; Geng *et al.*, 2014; Iwamoto *et al.*, 2014; Ogishima *et al.*, 2014; Ramírez García *et al.*, 2014; van der Ven *et al.*, 2014). This meta-analysis demonstrated that the presence of PD was significantly associated with risk of future arthritis flare (odds ratio (OR) 4.52, 95% CI 2.61-7.84, P<0.00001) and future bone erosions both at the patient level (OR 12.80, 95% CI 1.29 – 126.81, p=0.03) and at the joint level (OR 11.85, 95% CI 5.01 – 28.03, p<0.00001) (Han *et al.*, 2016b). There was substantial heterogeneity between the included studies, but subgroup analyses did not show any significant differences in duration of remission, disease duration and DMARD therapy (Han *et al.*, 2016b). The studies with a follow-up period of less than 1 year showed a stronger association of PD with arthritis flare (OR 19.98 vs. 3.41), suggesting that inclusion of studies with a short duration of follow-up did not bias towards a lower risk of flare (Han *et al.*, 2016b). Furthermore, the presence of GS was also associated with risk of future arthritis flare (OR 3.69, 95% CI 1.71 – 7.93, p<0.0008), though the authors comment that this may be a reflection of the interaction between GS and PD positivity (Han *et al.*, 2016b). Following publication of this meta-analysis, a further longitudinal study of 126 RA patients in clinical remission have also demonstrated a significant association between PD positivity and arthritis flare (Geng *et al.*, 2016). Furthermore, a recent study of 472 RA patients in clinical remission demonstrated that the presence of PD tenosynovitis was significantly associated with risk of future patient-reported flare (OR 1.95, 95% CI 1.17 – 3.26), though not with radiographic erosion (Bellis *et al.*, 2016).

1.5.2.d Assessment of subclinical synovitis by MRI

A key advantage of MRI over US imaging is that the former can visualise bone marrow oedema (BME), which is believed to precede bone erosion, and thus has potential to be a more sensitive marker of subclinical synovitis. Several studies support a strong association between BME and future radiographic progression in patients with active RA (Ostergaard and

Moller-Bisgaard, 2014). Fewer studies have examined the role of MRI imaging in clinical remission, though persistent subclinical synovitis and BME appear to be common in this setting. In a prospective study of 56 early RA patients who had achieved sustained (≥6 months) clinical remission, MRI of the dominant hand revealed substantial levels of subclinical inflammation (94.6% synovitis, 46.4% BME and 58.9% tenosynovitis) (Lisbona *et al.*, 2014). No significant differences in MRI parameters were found at baseline (i.e. prior to the initiation of DMARD therapy) between patients who developed new bone erosions versus those who did not; however, BME scores at 12 months were greater for those patients who had developed new erosions (4.8 ± 5.6 vs. 1.4 ± 2.6, p=0.03) (Lisbona *et al.*, 2014). A further hand MRI study by the same group found that a trend towards a lower prevalence of BME in patients who satisfied SDAI or ACR/EULAR Boolean remission compared to those in DAS28-ESR remission (DAS28-ESR remission: 64/119 [53.8%]; SDAI remission (86 patients): 42/86 [48.8%]; Boolean remission: 40/82 [48.8%]), though this failed to reach statistical significance (Lisbona *et al.*, 2016).

Furthermore, emerging data from recent studies suggest that MRI-detected inflammation may be predictive of erosive progression. In a prospective MRI study of 85 patients with RA in remission/LDA (DAS28-CRP<3.2), synovitis and BME were seen at baseline in 87% and 23% respectively, and baseline BME was independently associated with radiographic progression at 12 months (OR 1.25, 95% CI 1.09 – 1.43, p=0.0013) (Gandjbakhch *et al.*, 2011). In a further multi-centre study by the same research group of 254 patients with RA in remission/LDA, multivariate analysis showed a significant association between baseline BME score >5 and radiographic progression after 6 or 12 months follow-up – an effect which was limited to RhF positive patients (entire cohort: OR 2.42 [95% CI 1.24 – 4.72]; RhF+: OR 4.41 [95% CI 1.72 – 11.35]; RhF-: OR 1.09 [95% CI 0.40 – 2.80]) (Gandjbakhch *et al.*, 2014). However, in another prospective study of 85 RA patients in remission or LDA (DAS<2.4), multivariate analysis failed to show a significant association between BME or synovitis on MRI and either arthritis relapse or radiographic progression, whereas PD on ultrasound imaging was significantly associated with both events (Foltz *et al.*, 2012).

1.5.2.e Defining an optimal imaging protocol for the detection of subclinical synovitis

The selection of an optimal joint set for imaging studies in RA remission is a balance between ensuring detection of subclinical synovitis where present, versus the cost of increased time, resources and training required to scan a large number of joints.

The optimal number and distribution of joints to include when assessing for US synovitis is uncertain and contentious (Ohrndorf *et al.*, 2013). There are a wide array of different musculoskeletal US routines in the published literature, ranging from comprehensive 78-joint scans (Hammer *et al.*, 2010) through to focussed scans of the dominant hand only (Saleem *et al.*, 2011). Nevertheless, it is important to note is that increasing number of joints scanned does not necessarily increase the yield of clinically relevant information. In the meta-analysis discussed previously, Nguyen *et al.* (2014) analysed US data from 1369 patients in clinical remission and found the proportion of patients with at least one joint abnormality, particularly PD, showed very little variation between studies using different scan protocols (Figure 1.5). Indeed, the US7 scan protocol proposed by Backhaus *et al.* (2009), which includes 7 joints in the dominant hand and foot, has been shown to offer comparable results to an extensive yet laborious 78-joint US scan (Hammer and Kvien, 2011) and a 12-joint US scan (Leng *et al.*, 2016). Similarly, a study of 47 patients with RA in clinical remission (DAS28-ESR<2.6) found that the presence of PD on both a 44-joint scan and a reduced 12-joint set were predictive of arthritis flare at 6 months with overlapping confidence intervals (44-joint: OR 8.21, 95% CI 1.49 – 45.1, p=0.016; 12-joints: 5.82, 95% CI 1.07 – 31.61, p=0.041) (Janta *et al.*, 2016).

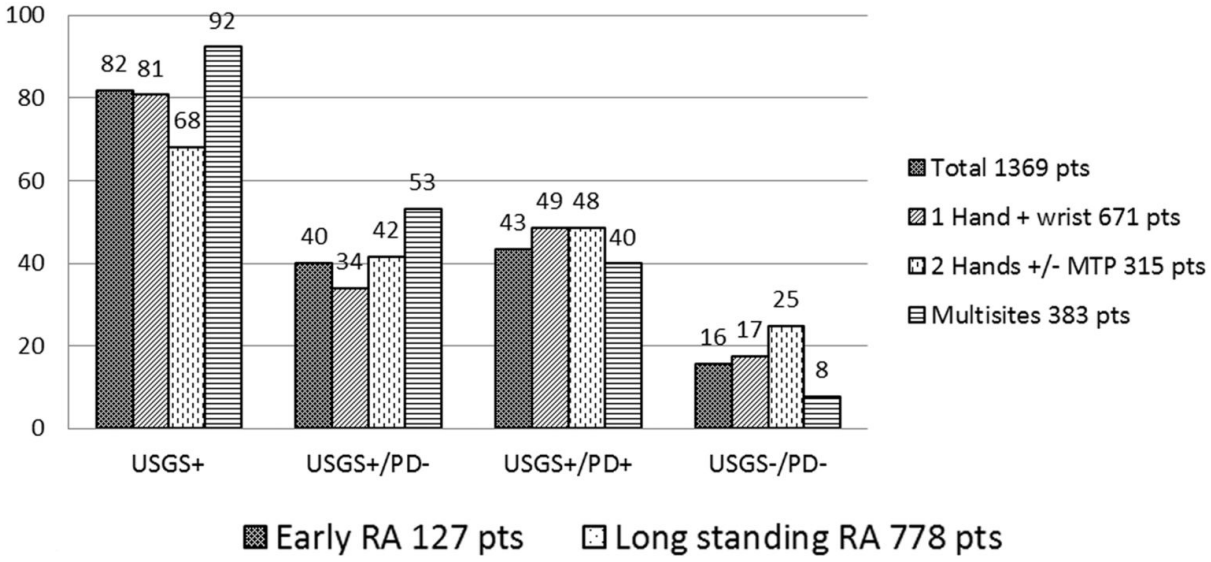


Figure 1.5 – Prevalence of findings by different ultrasound routines in a meta-analysis of studies of RA patients in clinical remission by Nguyen *et al.* (2014). Reproduced from Nguyen et al, 'Prevalence of ultrasound-detected residual synovitis and risk of relapse and structural progression in rheumatoid arthritis patients in clinical remission: a systematic review and meta-analysis', Rheumatology, 2014, volume 53, issue 11, pages 2110-8, by permission of Oxford University Press on behalf of the British Society for Rheumatology.

Recently, the Outcome Measures in Rheumatology-Ultrasound (OMERACT-US) Task Force proposed a EULAR-endorsed standardised scoring system for PD scoring known as the Global OMERACT-EULAR Synovitis Score (GLOESS). The GLOESS consists of the sum of PD scores across a standardised full set of 22 joints, or reduced set of 9 joints (Naredo *et al.*, 2011). However, in a validation study by the OMERACT-US group of 89 RA patients starting abatacept, change in GLOESS over 24 weeks follow-up failed to show a significant correlation between change in DAS28 or DAS28-defined outcomes after 24 weeks of follow-up, thus calling in to question the validity of GLOESS as a measure of treatment response (D'Agostino *et al.*, 2016a). An independent group analysed data from standardised US of 36 joints and 4 tendons in a cohort of 439 RA patients, and proposed two candidate reduced joint sets (7 joints/2 tendons, and 9 joints/2 tendons) that retained 78-85% of data in unilateral scans (Aga *et al.*, 2016). These joint sets remain to be validated in an independent cohort.

Whilst debate continues within the US imaging community regarding an optimal joint set, interest has grown in the use of whole-body MRI for the detection of subclinical synovitis in RA remission. In a feasibility study of 20 patients with active RA, a whole-body contrast-enhanced MRI protocol was developed to assess inflammation at 76 peripheral joints, 30 entheses and the spine (Axelsen *et al.*, 2014). Readability was ≥70% for most peripheral joints, although hand distal interphalangeal joints (DIPJs) and feet PIPJs/DIPJs were more difficult to assess (Axelsen *et al.*, 2014). Nevertheless, the requirement for a 60 minute scan using a 3 Tesla MRI machine are likely to be substantial barriers to its current widespread and routine use in clinical practice, though this may improve with future technological advances.

1.5.2.f Limitations of imaging in the assessment of RA remission

Despite the apparent prognostic value of modern imaging techniques in detecting subclinical synovitis in RA remission, there remain substantial barriers in the application of this to routine clinical practice. As discussed above, uncertainty surrounding the optimal number of joints to scan in the setting of remission leads to substantial heterogeneity between studies, making direct comparisons difficult. There are also technical and pragmatic limitations to both ultrasound and MRI modalities, as discussed in Introduction 1.4.4.

Despite these limitations, enthusiasm in the research community remains high for the future translation of imaging-defined remission as a target of disease treatment. Indeed, the development of ultrasound-defined targets of disease control is the central focus of the ongoing ‘Targeted Ultrasound Initiative’, an international collaborative research group

(Wakefield *et al.*, 2012). However, recent results from the Targeting Synovitis in Early Rheumatoid Arthritis (TaSER) study challenge this treatment paradigm (Dale *et al.*, 2016). In this open-label prospective study, 111 patients with newly diagnosed RA were randomised to treatment strategies targeting either DAS28-ESR<3.2 (control arm) or a combined DAS28-ESR/US construct (intervention arm). In the control arm, DMARD therapy was escalated if DAS28-ESR≥3.2. In the intervention arm, treatment was escalated if: DAS28-ESR>5.1, 3.2<DAS28-ESR≤5.1 plus ≥2 swollen joints, or DAS28-ESR≤5.1 plus PD in ≥2 joints. DMARD escalation was performed according to the same schedule in both arms (oral methotrexate followed by: triple therapy with oral methotrexate, triple therapy with subcutaneous methotrexate, and triple therapy with etanercept). After 18 months, patients in the intervention arm received significantly more intensive DMARD therapy, though with no significant benefits in terms of change in DAS-44, change in the area under DAS-44 curve or MRI measures (bone erosion or BME). More patients achieved DAS44 remission in the intervention arm though only at the 18 month time point (43% vs 66%, p=0.03), and no difference in achievement of ACR/Boolean remission was observed. Whilst it is possible that statistically significant differences in clinical endpoints may only become apparent after several years of follow-up, the authors argue that these are unlikely to be clinically significant nor justify the greater burden of DMARD therapy and expenditure of time and resources for US scanning in the intervention group (Dale *et al.*, 2016).

These observations are further corroborated by results from the ARCTIC study (Aiming for Remission in rheumatoid arthritis: a randomised trial examining the benefit of ultrasound in a Clinical TIght Control regimen) (Haavardsholm *et al.*, 2016). In this randomised controlled trial (RCT), 238 patients with newly diagnosed RA were randomised in a 1:1 ratio to conventional management (target of clinical remission defined as DAS44<2.6 and no swollen joints) or a 'tight control' (target of clinical remission plus absence of PD on 32-joint ultrasound scan). The primary endpoint was the proportion of patients who achieved a combination of clinical remission, no swollen joints and no radiographic progression after 16-24 months. Prescription of combination csDMARDs and bDMARDs was greater in the tight-control versus conventional therapy arms, though occurrence of adverse events was comparable. Despite this however, no significant difference was observed in the primary endpoint between the two groups (21/112 (19%) versus 16/118 (22%) patients in the conventional vs. tight-control groups respectively, p=0.54).

In summary, whereas current evidence suggests that the presence of PD is a poor prognostic factor even in the setting of clinical remission, it would appear that targeting treatment

approaches towards ultrasound-defined remission does not afford significant clinical benefits. Research in the area remains ongoing, including an ongoing study exploring a treat-to-target strategy in early RA defined by the absence of BME on MRI imaging (Moller-Bisgaard *et al.*, 2015). However, at present there is a lack of data to support the routine use of imaging to define treatment targets in RA.

1.5.3 The concept of 'depth' of remission in RA

The concept of disease remission in RA is largely borrowed from the field of oncology, where remission is defined in terms of duration of cancer-free survival with a period (often 5 years) defined as disease 'cure'. Disease relapse in this setting is relatively easy to define, being a recurrence of the original malignancy, with a relatively clear binary distinction between relapse and cure. Extrapolating this concept to the setting of a chronic autoimmune disease such as RA poses several challenges, not least a reconciliation with the stochastic nature of arthritis flares and the variety of different measures of disease activity.

Addressing this issue, Schett *et al.* (2016) propose a hierarchical 'shell model' of remission in RA encompassing three related yet distinct remission states: clinical, imaging/serological, and immunological remissions (Figure 1.6). In this model, clinical remission is conceptualised as an absence, or virtual absence, of clinical symptoms and signs of active arthritis such that there is no or very minimal impact upon physical function and quality of life. Within this clinical remission group exists a proportion of patients who have subclinical inflammation, which the authors propose could be detected by either imaging and/or sensitive serological analysis. Patients who have no detectable subclinical inflammation are defined as being in imaging/serological remission, though a proportion of these could still be expected to have a future episode of disease activity owing to an underlying persistent immune dysregulation. Only those patients who have regained a state of immunological tolerance are said to have achieved immunological remission, which Schett *et al.* (2016) propose could be assessed by RhF and ACPA status. This hierarchical model of RA remission is attractive in that it acknowledges the distinction between different levels of remission in a pragmatic clinical approach. However, it could be argued that the distinction between imaging/serological and immunological remission is rather arbitrarily defined by the sensitivity of currently available technologies to measure disease activity.

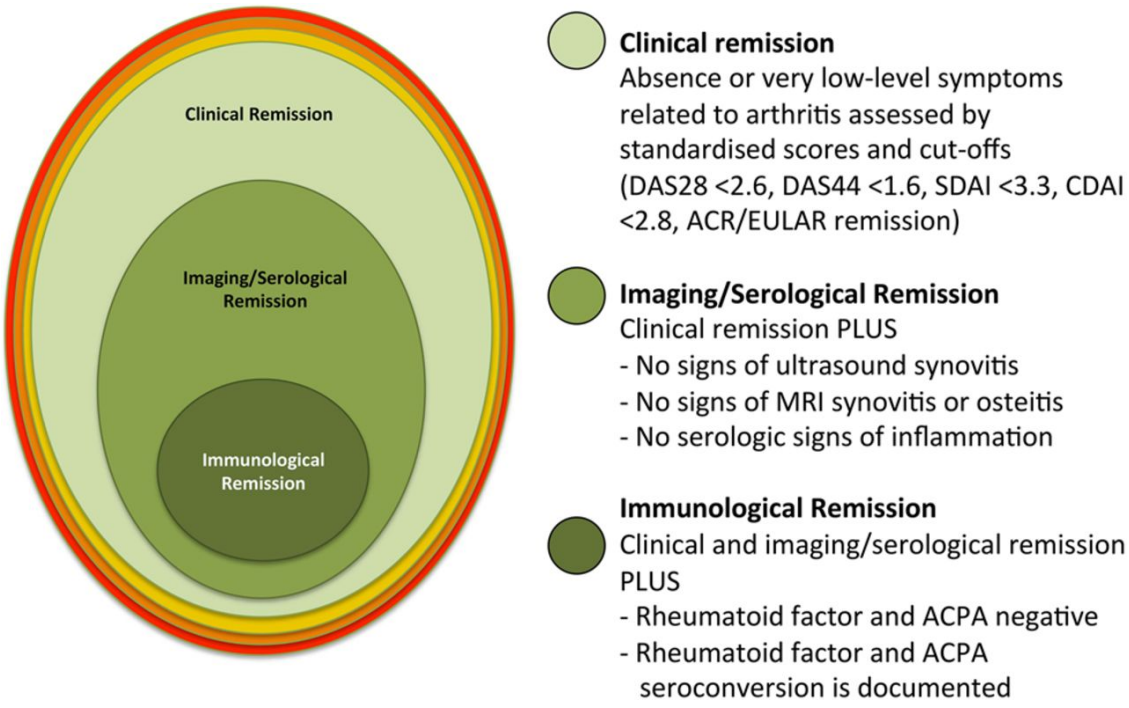


Figure 1.6 – The 'shell model' of RA remission, as proposed by Schett *et al.* (2016). Distinct hierarchical levels of remission can be conceptualised by the absence of clinical, imaging, serological and immunological measures of synovitis. Reproduced from Annals of the Rheumatic Diseases, Schett et al, volume 75, pages 1428-37, copyright 2016, with permission from BMJ Publishing Group Ltd.

An alternative conceptualisation of RA remission is to consider an 'iceberg model', whereby remission is considered in terms of a continuous spectrum of disease activity (Figure 1.7). In this model, disease activity can be visible clinically as symptoms or joint destruction, or invisible as subclinical immune dysregulation. The threshold at which this immune dysregulation manifests in clinically significant disease varies between individuals, and can be modified by DMARD therapy and, potentially, by environmental insults such as concurrent infection or smoking. Assessment of the extent of immune dysregulation becomes increasingly difficult with increasing depth below the clinical threshold, as available knowledge and technologies are stretched to their current limits. It is conceivable that with greater depth of RA remission comes improved long-term prognosis and potential for DMARD de-escalation, as will be discussed in the next subchapter.

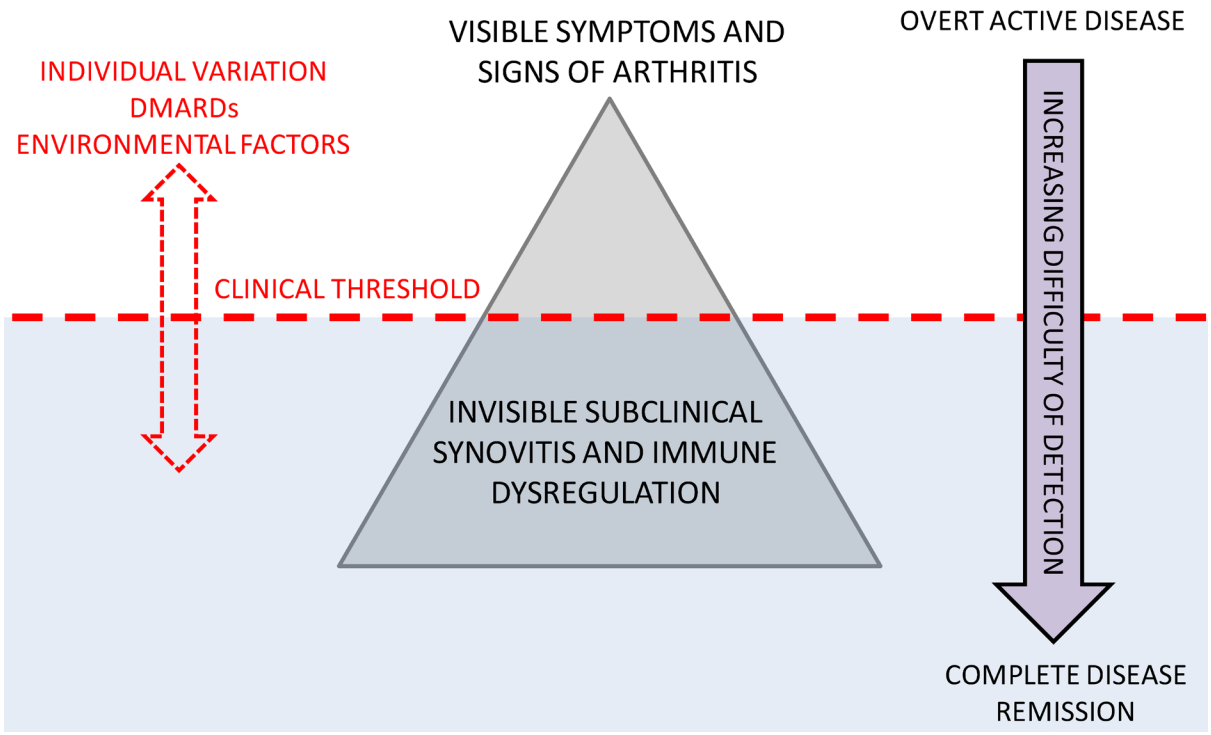


Figure 1.7 – The 'iceberg model' of RA remission. Autoimmune processes (represented by the grey triangle) can manifest as clinical disease or may be invisible as subclinical synovitis and immune dysregulation. The threshold (red) above which disease is manifest varies between individual patients, and can be influenced by DMARD therapy and environmental factors. With increasing depth of remission comes greater difficulty in detecting and measuring immune dysregulation (purple), which is limited by available technology and knowledge.

1.5.4 Summary

Advances in modern DMARD therapy have made disease remission an achievable target for many patients with RA. Nevertheless, the varied and often subjective nature of RA symptomatology, together with the stochastic nature of arthritis flares, pose significant challenges in defining clinically meaningful thresholds of disease activity that are consistent with remission. Current international consensus guidelines define remission solely in terms of clinical composite scores, and do demonstrate prognostic value in predicting risk of radiographic progression and arthritis flare. Nevertheless, strict thresholds on patient global assessment scores can disproportionally preclude patients with comorbidities such as osteoarthritis (OA), and even patients who satisfy the most stringent of clinical remission criteria still can develop further radiographic joint damage. Indeed, modern imaging modalities including US and MRI demonstrate substantial levels of subclinical inflammation across all definitions of clinical remission. Imaging measures of subclinical synovitis are

associated with histological and biochemical measures of inflammation, as well as risk of future arthritis flare and radiographic progression. However, imaging abnormalities are relatively common in healthy individuals and there is uncertainty surrounding the acceptable levels of imaging abnormalities that are consistent with stable remission.

Indeed, rather than viewing remission as a binary state, it may be more realistic to consider remission in terms of a continuous spectrum of subclinical disease activity, which can be assessed by clinical, imaging and immunopathological means. Stratification by depth of remission could thus help separate patients at high risk of arthritis flare and progression for whom close surveillance is warranted, versus those at low risk for whom DMARD tapering and withdrawal could be considered – a conjectural though nonetheless intriguing concept.

1.6 DMARD-free remission in RA

The introduction of modern DMARD therapy and its initiation earlier in the clinical course of disease have enabled an increasing proportion of patients to achieve disease remission. As noted previously, this has led to the suggestion of a 'step-down' paradigm of drug therapy, whereby DMARDs are tapered when clinical remission has been achieved (discussed in Introduction 1.3.3). This step-down approach raises the tantalising concept of drug-free remission (DFR) – a state whereby disease activity could be so low as to permit complete DMARD withdrawal without subsequent arthritis flare or progression. In this section, I will attempt to answer three important questions surrounding sustained DFR in RA based upon evidence from the current published literature, namely:

1. Can drug-free remission in RA be achieved?
2. What are the benefits and risks of DMARD withdrawal?
3. What is the optimal strategy for DMARD withdrawal?

1.6.1 Can drug-free remission in RA be achieved?

DFR in RA is more than a simple theoretical concept and its existence is well documented (van den Broek *et al.*, 2011; Scott *et al.*, 2013a). There have been many studies exploring the potential to taper biologic DMARD therapy once remission is achieved. However, the majority of these studies do not involve complete DMARD cessation and hence are less relevant to this discussion. In this subsection, I will focus exclusively upon evidence from clinical studies addressing complete DFR in RA.

1.6.1.a Evidence from observational cohort studies

Clinical remission is a frequent outcome of modern DMARD therapy in RA. In a large meta-analysis, Ma *et al.* (2010) explored the prevalence of clinical remission in 17 observational studies (4762 patients) and 20 RCTs (4290 patients). Within the observational studies, average prevalence of DAS-defined remission (DAS≤1.6 or DAS28-ESR≤2.6) in patients receiving DMARD therapy was 33% (9 studies). Within the RCTs, DAS-defined remission was observed in 26% with DMARD monotherapy and 42% with combination DMARDs (13 studies). Indeed, current studies suggest that increasing proportion of patients have achieved clinical remission with DMARD therapy over recent years. For example, an analysis of data

from a Norwegian early arthritis cohort of 2573 patients showed significant improvements in the rates of clinical remission by all measures from 2000 to 2010 (Figure 1.8), an effect which the authors attributed to earlier initiation of more aggressive DMARD therapy (Aga *et al.*, 2015).

With remission rates approaching 50% in modern practice, some longitudinal cohorts now include data on patients who have achieved remission and successfully discontinued DMARDs, providing observational data on the prevalence of DFR in authentic clinical settings. Van der Woude *et al*. (2009) examined the prevalence of DFR in two large RA cohorts: the British Early Rheumatoid Arthritis Study (ERAS) and Dutch Leiden Early Arthritis Clinic (LEAC). All patients had received non-biologic DMARDs, with DFR defined as no joint swelling and a clinical impression of remission in the absence of DMARD treatment. Substantial rates of DFR were observed in 15% (68/454 patients) and 9.4% (84/895 patients) in the LEAC and ERAS cohorts respectively (van der Woude *et al.*, 2009).

A more recent study of the LEAC cohort explored the prevalence of DFR in patients diagnosed with RA between 1993 and 2011 (Ajeganova *et al.*, 2016). Sustained DFR was defined as the absence of synovitis on clinical examination until the end of study follow-up and for at least 1 year after DMARD cessation, and was achieved by 155/1007 (15.4%) of patients. Functional ability in the sustained DFR group, as measured by the HAQ, was commensurate with the general population (median HAQ 0.13, IQR 0.63).

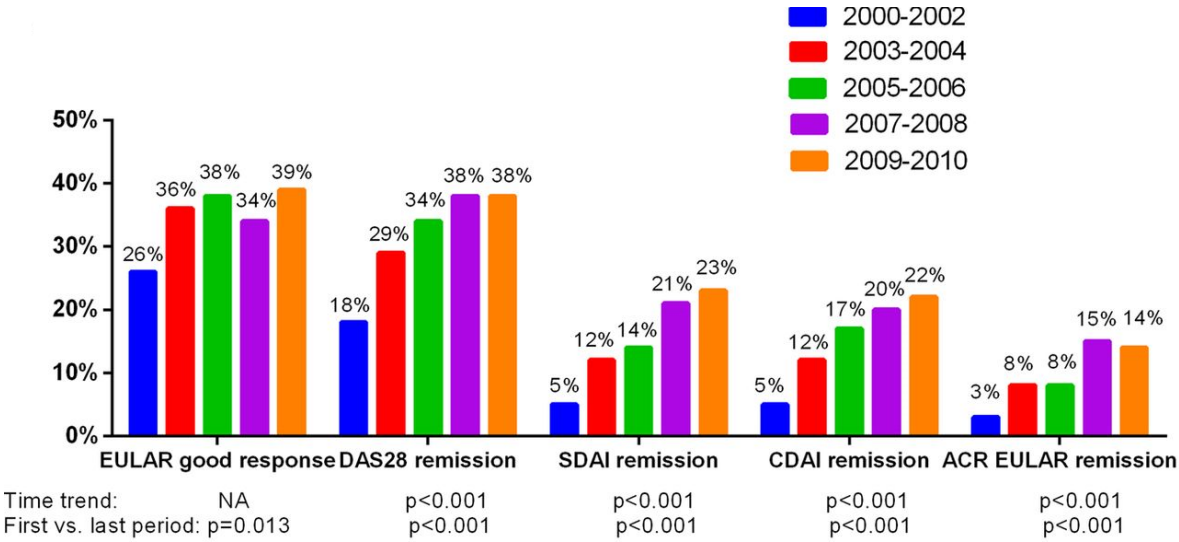


Figure 1.8 – Response and remission rates after six months of methotrexate monotherapy by year of treatment initiation. Reproduced from Annals of the Rheumatic Diseases, Aga *et al.*, volume 74, pages 381-88, copyright 2010, with permission from BMJ Publishing Group Ltd.

Long-term outcomes after 15 years of follow-up were explored in a smaller cohort of RA patients recruited between 1986 and 1989 in Finland (Tiippana-Kinnunen *et al.*, 2010). Of the 70 patients studied, 11 (16%) had permanently discontinued DMARD therapy, of which 9 (13% of total) satisfied the 1981 ACR remission criteria. Good long-term outcomes were observed in patients who were able to discontinue DMARDs with a lower rate of radiological progression and similar disability scores compared with those patients who continued DMARD therapy.

1.6.1.b Evidence from clinical trials of DMARD cessation in established RA

Longitudinal cohort data provide clear evidence of the existence of DFR, with approximately 10-15% of patients achieving this status. Nevertheless, the observational and uncontrolled nature of these studies make it difficult to draw firm conclusions as to the risk of arthritis flare attributable to DMARD cessation. There have been several clinical trials exploring the complete cessation of DMARD therapy in RA remission, many of which have been published in the last 5 years (Table 1.9). However, the majority of these studies assess DFR as a secondary or post-hoc outcome measure. Furthermore, the wide range of different DMARD treatment regimens and inconsistent definitions of remission makes comparison difficult. In the only published meta-analysis on the topic, O'Mahony *et al.* (2010) identified six randomised controlled trials (RCTs) of complete DMARD withdrawal incorporating a total of 503 patients (De Silva and Hazleman, 1981; Ahern *et al.*, 1984; Van der Leeden *et al.*, 1986; Kremer *et al.*, 1987; Gotzsche *et al.*, 1996; ten Wolde *et al.*, 1996). Pooled data from the RCTs showed a relative risk of flare or deterioration in RA with continued DMARD therapy versus treatment withdrawal of 0.31 (95% CI 0.16 to 0.57, $p<0.001$). Nevertheless, trials in the setting of early rheumatoid arthritis were excluded from this meta-analysis, and the included trials were published between 1981 and 1996 and hence all included historical DMARDs such as penicillamine and gold, which are now rarely used in modern rheumatology practice. Furthermore, several of the trials studied DMARD withdrawal in patients who were not in clinical remission.

One RCT included within the meta-analysis is noteworthy for its size and extended duration of follow-up. In this multi-centre study (ten Wolde *et al.*, 1996), 285 patients with established RA were randomised to continue their current DMARD monotherapy or switch to placebo in a double-blind design. All patients satisfied a remission definition at baseline adapted from the 1981 ACR remission criteria, and their progress was reviewed for 1 year. The cumulative

Table 1.9 – Summary of studies of disease-modifying anti-rheumatic drug (DMARD) withdrawal in rheumatoid arthritis that have investigated complete DMARD-free remission. Studies highlighted in **bold** investigated DMARD-free remission as the primary outcome. Where glucocorticoids were permitted, these were withdrawn at time of DMARD withdrawal unless otherwise stated. ABT, abatacept; ACR, American College of Rheumatology; ADA: adalimumab; CIC, ciclosporin; CRP, C-reactive protein; DAS, disease activity score, EMS, early morning stiffness; ESR, erythrocyte sedimentation rate; ETN, etanercept; HCQ, hydroxychloroquine; IA, intra-articular; IM: intra-muscular; IQR, inter-quartile range; MTX, methotrexate; NTSJ, no tender or swollen joints; PBO, placebo; RAI: Ritchie articular index; SDAI, Simple Disease Activity Index; SFZ: sulfasalazine; SJC, swollen joint count; TNFα: tumour necrosis factor alpha; TOC, tocilizumab. Notes: a: 2.6 < DAS28-ESR < 3.2 at ≤2 visits during the remission period was permitted; b: study also included further 122 patients with undifferentiated inflammatory arthritis (data not shown); c: 160 patients were recruited to the initial study, of which 139 completed the 5 year extension; d: DMARD-free remission maintained for 3 and 6 months in 11 and 7 patients respectively (data limited by incomplete follow-up); e: 94% of enrolled patients satisfied 2010 ACR/EULAR RA classification criteria. Adapted from Tanaka and Hirata (2014).

Study	DMARDs	Number of patients	Disease activity at enrolment	Disease duration	Intervention	Remission definition	Number of patients entering DMARD withdrawal phase	Proportion maintaining remission after DMARD withdrawal	Duration of follow-up
Ahern et al. (1984)	**D-penicillamine**	**38**	**NTSJ for > 6 months**	**Mean 11.6 years since diagnosis**	**Treatment continuation or PBO, randomised, single-blind**	**NTSJ**	**19**	**4/19 (21%) PBO arm** **(vs. 17/19 (89%) continuation arm)**	**12 months after DMARD withdrawal**
Bijlsma *et al.* (2016) U-Act-Early study	MTX, TOC, HCQ, TNFα inhibitor	317	Mean DAS28-ESR 5.2	Mean symptom duration 26 days	Randomised, double-blind: TOC+MTX, TOC+PBO, MTX+PBO. Escalation to HCQ & TNFα inhibitor if remission not achieved. DMARD withdrawal once sustained remission achieved.	DAS28-ESR < 2.6 and SJC≤4 for ≥24 weeks [a]	265	77/265 (29%), of which: 37 TOC+MTX 28 TOC+PBO 12 MTX+PBO	2 years after enrolment
El Miedany *et al.* (2016)	**Various biological and non-biological**	**157**	**DAS28-ESR < 2.6 for > 6 months**	**Disease duration ≥ 18 months**	**Randomised, open label:** **Arm 1: half dose bDMARDs** **Arm 2:Half dose bDMARDs and csDMARDs** **Arm 3: Stop bDMARDs, half dose csDMARDs** **Arm 4: Stop bDMARDs and csDMARDs** **Arm 5: Continue all DMARDs**	**DAS28-ESR < 3.2**	**157** **(of which 31 had complete DMARD cessation)**	**1: 13/31 (42%)** **2: 19/32 (59%)** **3: 21/31 (68%)** **4: 24/31 (77%)** **5: 2/32 (6%)**	**12 months after DMARD withdrawal**

Table 9.1 (continued)

Study	DMARDs	Number of patients	Disease activity at enrolment	Disease duration	Intervention	Remission definition	Number of patients entering DMARD withdrawal phase	Proportion maintaining remission after DMARD withdrawal	Duration of follow-up
Emery *et al.* (2014) PRIZE study	ETN, MTX, maintenance prednisolone permitted until week 26.	306	DAS28-ESR 5.8 mean	Mean symptom duration 6 months	All patients open label ETN+MTX for 1 year. Those in remission (n=193) entered randomised, double-blind phase: ETN+MTX, MTX+PBO or PBO+PBO for 39 weeks. Those with DAS28-ESR≤3.2 entered DMARD withdrawal.	DAS28-ESR < 2.6	131 (of which 110 in remission)	28/50 (56%) ETN+MTX 19/35 (54%) MTX+PBO 15/25 (60%) PBO+PBO	65 months after enrolment (total DMARD-free time varied between arms)
Emery *et al.* (2015) AVERT study	ABT, MTX, maintenance prednisolone permitted in first year	351	Mean DAS28-CRP 5.4	Mean symptom duration 0.56 years	Randomised, double-blind. ABT, MTX or ABT+MTX, DMARDs withdrawn after 12 months if DAS28-CRP<3.2.	DAS28-CRP < 2.6	223 (of which 176 in remission)	14/50 (28%) ABT 18/73 (25%) ABT+MTX 9/53 (17%) MTX	6 months after DMARD withdrawal
Haschka et al. (2016) **RETRO study**	**Various biological & non-biological, prednisolone ≤5mg/day**	**110**	**DAS28-ESR < 2.6 for >6 months**	**Median 5 years since diagnosis**	**Randomised, open-label. 3 arms: DMARD continuation; 50% reduction; 50% reduction for 6 months then complete DMARD withdrawal.**	**DAS28-ESR < 2.6**	**63** **(of which 27 had complete DMARD cessation)**	**13/27 (48%) withdrawal arm** **(versus 22/36 (61%) reduction arm and 36/38 (84%) continuation arm)**	**12 months after enrolment**
Heimans *et al.* (2016) IMPROVED study	MTX, HCQ, SFZ, ADA, prednisolone.	479[b]	Mean DAS44 3.3	Symptom duration 18 weeks (median)	Open-label. MTX + prednisolone for first 4 months. If remission not achieved, then randomised to MTX+HCQ+SFZ+prednisolone or MTX+ADA. DMARD withdrawal once remission achieved.	DAS44 < 1.6	Not stated	Not stated [89/479 (19%) of total recruited achieved DMARD-free remission]	2 years after study enrolment

Table 9.1 (continued)

Study	DMARDs	Number of patients	Disease activity at enrolment	Disease duration	Intervention	Remission definition	Number of patients entering DMARD withdrawal phase	Proportion maintaining remission after DMARD withdrawal	Duration of follow-up
Hetland *et al.* (2010) CIMESTRA study	MTX, CIC, HCQ, SFZ, IA corticosteroids	139[c]	Median DAS28-ESR 5.26	Symptom duration < 6 months	Randomised to CIC +/- MTX with later addition of HCQ (see text). DMARD withdrawal if remission for ≥12 months	1981 ACR remission criteria	Not stated	Not stated [17% of total recruited achieved DMARD-free remission]	5 years after study enrolment
Huizinga *et al.* (2015) ACT-RAY study	TOC +/- MTX, other non-biologic DMARDs and corticosteroids	553	Mean DAS28-ESR 6.34	Mean 8.2 years since diagnosis	0-6m: TOC+MTX or TOC-PBO, double blind. 6-12m: Addition of other DMARDs if required, open label. 12m: DMARD withdrawal	DAS28-ESR < 2.6	Not stated	Not stated [28/472 (6%) remaining in study after 12 months achieved DMARD-free remission at 2 years]	2 years after study enrolment
Kita et al. (2012)	**Various**	**13**	**SDAI 20.2 mean**	**Mean symptom duration 14 weeks**	**DMARD withdrawal at 1 year, open-label**	**SDAI ≤ 3.3 and ≥ 66% improvement in MRI bone marrow oedema**	**5**	**3/5 (60%)**	**12 months after DMARD withdrawal**
Klarenbeek *et al.* (2011b) BeSt study	Various	508	DAS44 4.4 mean	Median 23-26 weeks symptom duration	Randomised to open-label DMARD arms (see text). DMARD withdrawal if remission achieved for 6 months	DAS44 < 1.6	Not stated	Not stated [115/508 (23%) achieved DMARD-free remission, of which 59/115 (51%) maintained remission at 5 year time-point]	5 years after study enrolment [median (IQR) duration of remission of 23(15-25) months]

Table 9.1 (continued)

Study	DMARDs	Number of patients	Disease activity at enrolment	Disease duration	Intervention	Remission definition	Number of patients entering DMARD withdrawal phase	Proportion maintaining remission after DMARD withdrawal	Duration of follow-up
Kuijper *et al.* (2016) tREACH study	MTX, SFZ, HCQ, ETN, ADA, ABT, glucocorticoids	281	Mean DAS44 3.35	Mean 24 weeks symptom duration	Open label. Randomised to MTX+oral prednisolone or MTX+SFZ+HCQ+oral/IM steroid. Later escalation to biologic therapy if remission not achieved. Withdrawal of DMARDs if remission sustained over ≥3 months.	DAS44<1.6	141	34/141 (24%) [d]	2 years after enrolment
Nam *et al.* (2014) EMPIRE study	MTX +/- ETN	110[e]	DAS28-CRP 4.14 mean	Median symptom duration 6–8 months	Randomised to MTX+ETN or MTX+PBO, ETN withdrawn after 1 year or 26 weeks of remission, MTX withdrawn 12 weeks later if in remission.	NTSJ	110	4/110 (3.6%)	78 weeks after enrolment
Nishimoto *et al.* (2014a) DREAM study	TOC +/- glucocorticoids	187	DAS28-ESR ≤ 3.2	Median 7.8 years since diagnosis	Open-label TOC cessation, glucocorticoids remained unaltered	DAS28-ESR < 2.6	187	17/187 (9%)	12 months after DMARD cessation
ten Wolde et al. (1996)	**Various non-biological**	**285**	**Modified 1981 ACR remission for > 1 year**	**Median 9 years since diagnosis**	**Randomised, double-blind. Treatment continuation or PBO,**	**Absence of flare, defined as: SJC ≥ 3 and 2/3 of RAI > 9, EMS > 45 min or raised ESR**	**285**	**90/143 (63%) PBO arm** **(vs. 112/142 (79%) in continuation arm)**	**12 months after DMARD cessation**

incidence of arthritis flare was 38% in the placebo arm versus 22% for DMARD continuation, giving a relative risk of arthritis flare upon DMARD withdrawal in a multivariate analysis of 2.6 (95% CI 1.4 to 4.6). In an extension to the study, those patients whose arthritis flared were reviewed for a further 12 months after recommencement of DMARD therapy (ten Wolde *et al.*, 1997). Of the 51 patients included in this analysis, 24/51 (47%) demonstrated at least an ACR20 response by 3 months after DMARD recommencement, and at 12 months disease activity was judged to be in remission, mild or moderate/severe categories in 18 (35%), 22 (43%) and 11 (22%) patients respectively. Again, it is noteworthy that many of the DMARDs used were either antiquated or prescribed at low doses (e.g. 7.5mg/week methotrexate) compared to current clinical practice. The true efficacy of re-introduction of modern regimens DMARD therapy is therefore likely to be greater than that reported in this study.

In a publication of interim results from the RETRO[1] study (Haschka *et al.*, 2016), 101 patients with established RA of median 5 years duration and DAS28-ESR < 2.6 were randomised to either continue DMARD therapy, reduce DMARDs to 50% dose, or reduce to 50% for 6 months then stop. Patients in the study took a range of different biologic and non-biologic DMARDs at baseline, including corticosteroids. At 12 months, 67/101 (66%) patients remained in DAS28-ESR remission; significantly more patients maintained remission in the continuation arm (36/38 [84%]) versus the reduction (22/36 [61%], p=0.036) and complete DMARD cessation (13/27 [48%], p=0.003) arms. The majority of arthritis flares in the reduction and cessation arms occurred in the first 6 months after entry to the study.

In another recent study (El Miedany *et al.*, 2016), 157 patients with established RA (disease duration ≥ 18 months) and in clinical remission (DAS28-ESR<2.6 for > 6 months) were randomised to 5 arms: DMARD continuation; half-dose biologic DMARDs (bDMARDs); half dose bDMARDs and conventional synthetic DMARDs (csDMARDs); stop bDMARDs and half-dose csDMARDs; and stop all DMARDs. A range of DMARDs were permitted, though oral corticosteroid therapy was not allowed in the 12 months prior to study inclusion. All patients were reviewed monthly for one year with a clinical and 40-joint ultrasound scan assessments at each visit. Arthritis flare was defined as DAS28-ESR>3.2. Overall, 78/157 (50%) remained in DFR at 12 months, though proportionally more patients flared in arms that involved bDMARD cessation (Table 1.9).

[1] For the purposes of readability, study acronyms are not expanded in this chapter, but are listed in full in the abbreviations section at the beginning of this Thesis.

The recently published ACT-RAY study explored DFR, albeit as a secondary outcome in a rather complicated study design (Huizinga *et al.*, 2015). This trial recruited 553 patients with established RA resistant to methotrexate monotherapy. Patients were randomised to receive tocilizumab with either methotrexate or placebo in a double-blinded manner. After 6 months, open label non-biologic DMARDs could be added if required to control disease activity. After 1 year, DMARD therapy was tapered to complete cessation in patients achieving a DAS28-ESR < 2.6. Overall, 28/472 (6%) of patients remaining in the study at 1 year had achieved DFR at the 2 year time-point.

In the DREAM study (Nishimoto *et al.*, 2014a), 187 patients were recruited with RA of median duration 7.8 years, receiving tocilizumab monotherapy for a median of 4 years and with a DAS28-ESR ≤ 3.2. Patients did not receive any other concomitant DMARDs, though of note a third were taking low-dose glucocorticoids throughout the study. Tocilizumab was discontinued at enrolment and 17/187 (9%) achieved DFR (DAS28-ESR < 2.6) at 1 year after tocilizumab cessation.

1.6.1.c Evidence from clinical trials in the setting of early RA

There have been many clinical trials exploring the relative efficacy of different regimens of DMARDs in the setting of early RA, of which some have explored DFR as a secondary outcome measure. The largest and most comprehensive of these studies is the Dutch Behandal-Strategieën (BeSt) study, a multicentre open-label RCT (Goekoop-Ruiterman et al., 2005). In this study, 508 patients with RA of symptom duration < 2 years and a median of 2 weeks since diagnosis were randomised to four different treatment arms comprising: sequential DMARD monotherapy; step-up combination therapy; initial combination therapy with prednisolone; and initial combination therapy with infliximab. Importantly, DMARDs were tapered to complete cessation in all groups for patients who achieved DAS remission (DAS≤ 1.6) for at least 6 months. In a 5 year analysis, 115/508 (23%) achieved drug-free remission, of which 59/115 (51%) remained in sustained DFR at the 5 year follow-up point with a median (IQR) duration of remission of 23 (15-25) months (Klarenbeek *et al.*, 2011b). The rates of sustained remission were comparable across all four treatment arms with no statistically significant difference in this small post-hoc analysis. Patients whose arthritis flared were restarted on DMARD therapy, with 25/53 (47%) and 39/53 (74%) patients regaining remission at 3 and 6 months after DMARD recommencement respectively. Of the remaining patients, 11/53 (21%) achieved low-disease activity (DAS ≤ 2.4); only one patient

failed to achieve at least low disease activity at 6 months, with an additional 2 patients lost to follow-up. Importantly, no significant radiographic progression was observed in those patients who experienced a flare of arthritis versus those who remained in DFR, in part attributable to the close monitoring and rapid recommencement of DMARDs upon arthritis flare (Klarenbeek *et al.*, 2011b).

In the CIMESTRA study (Hetland *et al.*, 2006), 160 patients with RA of less than 6 months duration were treated with intra-articular steroid injections and randomised to receive either methotrexate/ciclosporin or methotrexate/placebo, with the later withdrawal of ciclosporin/placebo and addition of hydroxychloroquine. If patients then achieved 1981 ACR remission for at least 12 months, then DMARD therapy was gradually tapered to complete cessation. In a 5-year analysis of 139 patients, DFR was achieved in 19% and 14% of patients in the methotrexate/ciclosporin and methotrexate/placebo arms respectively, with no significant difference between arms (Hetland *et al.*, 2010).

In the PRIZE study (Emery *et al.*, 2014), 306 patients with early-onset RA of mean 6 months symptom duration were treated with open-label 50mg etanercept and methotrexate for 1 year. The 193 patients who achieved DAS28-ESR < 2.6 were then randomised to receive 25mg etanercept plus methotrexate (ETN+MTX), methotrexate plus placebo (MTX+PBO), or double placebo (PBO+PBO). After 39 weeks, the 131 patients with DAS28-ESR ≤ 3.2 (of which 110 had DAS28-ESR<2.6) then had all drugs withdrawn and were reviewed for a further 26 weeks. The proportion of patients achieving sustained DAS28 remission at 39 weeks was significantly different between groups: 40/63 (63%), 26/65 (40%) and 15/65 (23%) in the ETN+MTX, MTX+PBO and PBO+PBO arms respectively ($p=0.009$ for ETN+MTX vs. MTX+PBO, $p<0.001$ for ETN+MTX vs. PBO+PBO). The proportion of patients achieving DAS28 point remission at 65 weeks (i.e. after DMARD withdrawal) were 28/63 (44%), 19/65 (29%) and 15/65 (23%) in the ETN+MTX, MTX+PBO and PBO+PBO arms respectively ($p=0.02$ for ETN+MTX vs. PBO+PBO, other comparisons not statistically significant). In terms of the 110 patients who were in DAS28-ESR remission at the start of the DMARD withdrawal period, remission at week 65 was achieved by 28/50 (56%), 19/35 (54%) and 15/25 (60%) of patients in the ETN+MTX, MTX+PBO and PBO+PBO arms respectively (p values not presented in paper). Importantly, the total biologic-free follow-up period was much longer in the placebo arms compared to the etanercept plus methotrexate arm. Thus, whilst this study does highlight the high rate of arthritis flare upon etanercept cession, it is difficult to directly compare the groups with regards to the optimal strategy for etanercept withdrawal.

In the EMPIRE study (Nam *et al.*, 2014), 110 patients with inflammatory arthritis of median symptom duration approximately 7 months (of which 94% satisfied 2010 ACR/EULAR RA classification criteria) were randomized to either methotrexate plus etanercept or methotrexate plus placebo in a double-blinded fashion. Etanercept or placebo was stopped after 1 year or when patients achieved remission for 26 weeks, defined as no tender or swollen joints in a 44 joint count. Methotrexate was then withdrawn 12 weeks later if patients maintained remission. At 78 weeks after enrolment, 4/110 (3.6%) had achieved drug-free remission. Of note, the strict definition of remission used in this study, and the withdrawal of etanercept regardless of disease activity, may have resulted in a lower rate of observed DFR.

In the tREACH study (Kuijper *et al.*, 2016), 281 DMARD-naïve patients with early RA (mean symptom duration 24 weeks) were randomised to treatment with methotrexate monotherapy or triple therapy (methotrexate, sulfasalazine and hydroxychloroquine). Short course bridging glucocorticoid therapy was used in both groups at baseline, and if DAS>2.4 after initial therapy then treatment was escalated to methotrexate plus anti-TNFα biologic. If sustained remission was achieved (DAS<1.6 for ≥3 months), then DMARDs were tapered in a standardised protocol to complete cessation if remission maintained. After 2 years of follow-up, 159/281 (57%) patients achieved sustained remission with no significant difference between initial methotrexate monotherapy vs. triple therapy groups. Of 141 patients who entered DMARD withdrawal phase, DFR was achieved in 34/141 (24%). DFR was sustained for 3 and 6 months in 11 and 7 patients respectively, although this was partly limited by incomplete follow-up data in many patients (Kuijper *et al.*, 2016).

1.6.1.d Evidence from trials of DMARD therapy in very early RA

With strong evidence to support an early therapeutic window of opportunity in RA, there has been increasing interest in the treatment of very early stages of disease, with the rationale that a brief course of DMARDs may allow for complete resolution of arthritis. Recent studies have explored the possibility of DMARD treatment in patients with undifferentiated arthritis or even in pre-symptomatic individuals with the aim of preventing progression to RA – a rather distinct concept to DFR in RA that will not be discussed further here. Nevertheless, several studies in patients with a very early diagnosis of RA do shed light on the potential for DFR if therapy is initiated in the very early phase of disease.

In the U-Act-Early study (Bijlsma *et al.*, 2016), 317 DMARD-naïve patients with a median symptom duration of 26 days were randomised to receive tocilizumab and methotrexate (TOC+MTX), tocilizumab plus placebo (TOC+PBO), or methotrexate plus placebo

(MTX+PBO) in a double-blind trial design. Patients were then reviewed at monthly intervals for 104 weeks, with standardised escalation of DMARD therapy (including later use of tocilizumab in the MTX+PBO arm, and anti-TNFα biologics in all arms) if remission was not achieved. The primary endpoint was sustained remission, defined as DAS28-ESR < 2.6 and ≤ 4 swollen joints for ≥ 24 weeks (though 2.6<DAS28-ESR<3.2 on ≤2 visits during this period was allowed). Overall, 265/317 (84%) patients achieved sustained remission; significantly more patients achieved sustained remission on their initial DMARD regimen in the tocilizumab arms (relative risk (RR) 2.00, 95% CI 1.59 – 2.51, P<0.0001 for TOC+MTX vs, MTX+PBO; RR 1.86, 1.48 – 2.32, p<0.0001 for TOC+PBO vs. MTX+PBO). Patients who achieved sustained remission had gradual tapering of their DMARDs to eventual cessation if remission maintained, with 77/265 (29%) achieving DFR for ≥ 12 weeks. In a post-hoc analysis, the proportion of patients achieving DFR was significantly greater in those who initially received tocilizumab: 37/106 (35%), 28/103 (27%) and 12/108 (11%) patients achieved DFR in the MTX+TOC, TOC+PBO and MTX+PBO arms respectively (p<0.0001 for TOC+MTX vs. MTX+PBO, and p=0.0037 for TOC+PBO vs. MTX+PBO). This study therefore suggests that early initiation of biologic therapy in a step-down treatment paradigm may be beneficial in facilitating future drug-free remission. Although the minimum duration of sustained DFR defined in this study was quite short (12 weeks), an observational 3-year extension to explore longer-term outcomes is currently in progress (U-Act-After study, NCT01918267).

In a Japanese study, 13 patients with a diagnosis of RA satisfying 2010 ACR/EULAR RA classification criteria with a mean symptom duration of 13 weeks were treated with a range of non-biologic DMARDs including methotrexate, sulfasalazine, tacrolimus and glucocorticoids (Kita *et al.*, 2012). All patients were seropositive for either RhF and/or ACPA, and exhibited peri-articular bone oedema on hand MRI. After 1 year of treatment, DMARD therapy was stopped in patients who achieved remission, defined as an SDAI ≤ 3.3 and an improvement of ≥ 66% in bone oedema on MRI. Five patients discontinued DMARD therapy, of which three maintained DFR at 12 months – one patient restarted DMARD therapy after arthritis flare and one patient was lost to follow-up. No worsening of bone marrow oedema was observed in the three patients who maintained DFR.

In the IMPROVED study (Heimans *et al.*, 2016), 479 patients with RA and 122 patients with undifferentiated inflammatory arthritis (UA) (overall median symptom duration of 18 weeks) were treated with methotrexate and prednisolone. Patients in early remission (DAS < 1.6) at 4 months tapered prednisolone and DMARDs to cessation whilst remission was maintained.

Those who did not achieve remission at four months were randomised to methotrexate plus adalimumab or triple therapy (methotrexate, hydroxychloroquine, sulfasalazine and low dose prednisolone). DMARDs were tapered and stopped in the randomisation arms if remission was achieved. At 2 years after enrolment, significantly more UA patients achieved drug-free remission compared to RA patients (41/112 (34%) of UA patients vs. 89/479 (19%) of RA patients, $p<0.001$). Furthermore, those patients who achieved early remission on methotrexate monotherapy were significantly more likely to achieve drug-free remission that those who required combination DMARDs (111/387 (29%) in early remission vs. 13/161 (8%) in combination DMARD arms achieved drug-free remission after 2 years, p value not stated in paper), though further analysis of RA vs. UA patients in this subset was not provided.

In the PROMPT study (van Aken *et al.*, 2014), 110 patients were recruited with 'UA', defined as fulfilment of the 1958 ACR classification criteria for probable RA (Ropes *et al.*, 1958) and the absence of fulfilment of the 1987 ACR RA diagnostic criteria. Patients were randomised to receive methotrexate or placebo, with the aim of identifying whether methotrexate could prevent progression of UA to RA. After 12 months, methotrexate was withdrawn in those patients who had not developed RA according to the 1987 criteria. In retrospect however, many of the patients recruited were found to fulfil the subsequently published 2010 ACR/EULAR RA diagnostic criteria, and hence this study provides some data concerning the efficacy and withdrawal of methotrexate in very early RA. Of the 110 patients recruited, 43 satisfied 2010 ACR/EULAR RA diagnostic criteria at baseline, of which 5/19 (26%) and 6/24 (25%) achieved DFR ($DAS \leq 1.6$) after 5 years follow-up in the methotrexate and placebo arms respectively. Due to the design of the study, methotrexate was not withdrawn in those patients who satisfied 1987 RA diagnostic criteria even if remission had been achieved; DFR rates may thus have been higher than that observed if drug tapering had also been employed in this group. Nevertheless, the comparable rates of DFR in methotrexate vs. placebo arms suggest that a substantial proportion of patients with early disease who satisfy 2010 ACR/EULAR RA diagnostic criteria can experience spontaneous remission. The authors propose that this may represent the natural history of early RA, or perhaps reflect a lack of specificity in the diagnostic criteria (van Aken *et al.*, 2014).

The large multinational placebo-controlled AVERT study has explored the use of abatacept to induce drug-free remission in very early RA (Emery *et al.*, 2015). In this study, 351 patients with RA of mean symptom duration 0.56 years were randomised to receive abatacept plus methotrexate, abatacept monotherapy or methotrexate monotherapy. Glucocorticoids were permitted in the first year. Drug therapy was withdrawn after 12 months in the 223 patients

who achieved a good response (DAS28-CRP<3.2). Of the 176 patients who had a DAS28-CRP < 2.6 at DMARD withdrawal, remission was sustained for 6 months in 18/73 (25%), 14/50 (28%) and 9/53 (17%) of patients in the abatacept plus methotrexate, abatacept monotherapy and methotrexate monotherapy arms respectively, although the statistical significance of the differences between treatment arms in this sub-analysis was not presented. Furthermore, no significant increase in bone marrow oedema, synovitis or erosions on unilateral hand and wrist MRI were observed in a post-hoc analysis of those patients who remained in remission following DMARD cessation (Peterfy *et al.*, 2016).

1.6.2 What are the benefits and risks of DMARD withdrawal?

Tapering and withdrawal of DMARDs in RA remission clearly has the potential to afford many benefits, for both individual patients and the wider healthcare system. One obvious benefit is a reduced risk of medication-related adverse effects, which range from relatively trivial (though nevertheless intrusive) reversible side effects such as nausea to potentially life-threatening serious effects such as irreversible organ damage. Whereas some of these serious adverse effects are idiosyncratic such as methotrexate-induced pneumonitis (Saravanan and Kelly, 2004), some exhibit a dose-response relationship such as the correlation between lifetime methotrexate exposure and liver toxicity (Whiting-O'Keefe *et al.*, 1991). It is thus apparent that a sustained period without DMARD therapy, even if ultimately this therapy is restarted in the future, has the potential to afford long-term benefits. This is analogous to the now widely practised concept of drug holidays in bisphosphonate treatment for osteoporosis, in order to reduce the risk of medication-induced side effects (Adler *et al.*, 2016).

One particular adverse effect that is common to all DMARD therapy is an increased risk of infection. In a meta-analysis of seven clinical trials (732 patients) of methotrexate vs. placebo in RA (Lopez-Olivo *et al.*, 2014), infection was more likely in those patients who received methotrexate (RR 1.3, 95% CI 1.0 – 1.6). Infection risk is generally greater with bDMARDs compared to their synthetic counterparts; a recent systematic review including 15 clinical trials demonstrated hazard ratios ranging from 1.0 to 1.8 for serious infections in bDMARD vs. csDMARD groups (Ramiro *et al.*, 2017). Although population-level risks of infection usually drop with increasing duration of DMARD therapy owing to healthy survivor bias (i.e. early drop-out of those susceptible to infection) (Fautrel and den Broeder, 2015), it is still conceivable that infections could be reduced by DMARD tapering in those established on therapy. However, this theory is yet to be tested in a prospective trial setting,

In addition to reducing the risk of medication side effects, DMARD withdrawal may also afford economic benefits by reducing the financial costs of unnecessary treatment. This is particularly notable for bDMARDs, whose cost often runs in to tens of thousands of pounds per year per patient. Even a partial reduction in bDMARD dose can lead to considerable savings, as has recently been demonstrated by the DRESS study (van Herwaarden *et al.*, 2015). In this randomised open-label non-inferiority study, 180 patients treated with adalimumab or etanercept were randomised (2:1) to either taper or continue their bDMARD therapy. bDMARD tapering was non-inferior to bDMARD continuation, with a mean cost saving of €12 280 per patient per year (Kievit *et al.*, 2016). Impact on quality of life was minimal, with a substantial saving of €390,493 per quality-adjusted life year (QALY) lost (Kievit *et al.*, 2016).

The medication costs of csDMARDs are substantially lower than bDMARDs, with an estimated annual cost of £300 versus £10,000 per patient respectively (National Audit Office, 2009). Nevertheless, given the relatively high prevalence of RA, csDMARD withdrawal in even a fraction of patients could be expected to yield substantial cost savings. For example, in the UK an estimated 580,000 adults are living with RA (National Audit Office, 2009); csDMARD withdrawal in only 10% could thus be expected to save £1.74 million per year. Furthermore, the use of csDMARDs carries further substantial financial costs in terms of the monitoring systems that are required for their safe prescription. Potential adverse effects such as hepatitis and bone marrow suppression mandate regular blood monitoring at monthly or two-monthly intervals for the vast majority of csDMARDs (Ledingham *et al.*, 2017), and is estimated to cost the NHS £17 million per year for RA patients alone (National Audit Office, 2009).

In addition to the clear benefits of reduced medication-related adverse effects and financial savings, it has also been shown that RA patients value several less tangible though nevertheless important benefits of DMARD withdrawal. During my previous MRes degree, I performed a qualitative study of 13 patients with established RA in clinical remission or low disease activity (Baker *et al.*, 2015). Patients considered DMARD withdrawal in the context of their normal life, with issues such as the inconvenience of blood monitoring and even the physical act of taking medication viewed as significant barriers to a normal lifestyle. To these patients, DMARD withdrawal offered a potential mechanism by which to restore a degree of normality to their lives, which had otherwise been disrupted by living with a chronic illness such as RA (Baker *et al.*, 2015). Similar positive benefits of DMARD withdrawal were also

identified in a qualitative study of 20 RA patients, including hope, happiness and relief when considering DMARD cessation (Markusse *et al.*, 2014a).

The obvious disadvantage to DMARD withdrawal is the risk of arthritis flare, which, as discussed earlier, lies approximately in the region of 50%. The stochastic nature of RA flare creates further uncertainty for any patient considering DMARD withdrawal. Aside from pain and stiffness, the onset of flare can also herald a loss of function that can have important consequences for the patient. For example, patients vulnerable to loss of function by virtue of manual employment or through caring for others are less likely to consider DMARD withdrawal (Baker *et al.*, 2015).

A further concern is that arthritis activity may be subsequently more difficult to control, even if DMARD therapy is restarted. Only a handful of studies explore the long-term outcomes of patients who flare following DMARD withdrawal, though the small amount of published data is largely reassuring. Studies of complete or partial DMARD withdrawal have demonstrated that the vast majority of patients who experienced an arthritis flare regained remission by 6 months after resumption of their previous DMARD therapy (Table 1.10). A handful of studies of partial or complete DMARD withdrawal have also assessed radiographic progression, with the majority demonstrating no significant progression of joint erosions in those patients who experienced an arthritis flare versus those who remained in sustained remission (Table 1.11). It thus appears that swift reintroduction of DMARDs at the point of flare leads to a restoration of clinical remission in the majority of patients, with minimal risk of joint damage during the flare period – however, evidence from long-term follow-up studies are limited.

1.6.3 What is the optimal strategy for DMARD withdrawal?

Increasing evidence supports the feasibility and safety of DMARD withdrawal in RA remission, and recommendations to support consideration of the approach are included in national and international guidelines of RA management (Table 1.12). However, there currently remains very little evidence to inform the optimal strategy of DMARD withdrawal, not least due to a lack of consistency in DMARD withdrawal strategies employed in the published literature. Consequently, current guideline recommendations are based on consensus opinion only and largely defer to the discretion of the managing clinician. Indeed, where ACR guidelines recommend against complete DMARD cessation in RA remission, it is nonetheless acknowledged that this is based on clinical experience alone and that the available evidence is of very low quality (Singh *et al.*, 2016).

There are a range of possible questions to consider when designing a strategy of DMARD withdrawal (Table 1.13), the answers to which remain uncertain. A hierarchy of DMARD withdrawal has recently been proposed which recommends initial discontinuation of glucocorticoids, followed by bDMARDs and finally csDMARDs (Smolen *et al.*, 2017). This recommendation is based on consensus opinion and influenced by long-term medication side-effect profiles (hence the prioritisation of glucocorticoid withdrawal), the high financial cost of biologic agents, and concern surrounding the potential formation of neutralising anti-drug antibodies in bDMARD monotherapy. Whether the strategy of initial bDMARD tapering is superior to initial csDMARD tapering is the subject of two currently recruiting RCTs: the REMINDRA study (NCT02935387), and the TARA study (van der Ven *et al.*, 2014).

Table 1.10 – Summary of prospective studies of RA remission incorporating cessation of at least one DMARD and that include data regarding rates of return to clinical remission following resumption of DMARDs in those patients who experienced an arthritis flare. Studies highlighted in **bold** incorporated tapering strategies that culminated in complete DMARD cessation in at least one study arm. ABT: abatacept; ADA: adalimumab; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; ETN: etanercept; HCQ: hydroxychloroquine; LDA: low disease activity; MTX: methotrexate; PBO: placebo; SFZ: sulfasalazine; TNF: tumour necrosis factor; TOC: tocilizumab. *: exact number restarting ADA not stated in manuscript. Adapted from Kuijper *et al.* (2015) and Verhoef *et al.* (2017).

Study	**Intervention**	**Patients restarting previous DMARDs after arthritis flare**	**Outcome following DMARD resumption**
Ahern *et al.* (1984)	**D-penicillamine**	**15**	**13 (87%) regained clinical remission after 4 months**
Brocq *et al.* (2009)	TNF-inhibitor discontinuation (csDMARDs and glucocorticoids<5mg/day continued)	15	All patients regained remission (DAS28-ESR<2.6) within 20 weeks (mean 5.6 weeks)
Chatzidionysiou *et al.* (2016) ADMIRE study	ADA cessation (csDMARDs and glucocorticoids continued)	9	9/9 regained remission (DAS28-ESR<2.6) by 1 year after enrolment (8/9 within 12 weeks)
El Miedany *et al.* (2016)	**Various biologic and non-biologic DMARDs**	**79**	**All patients regained remission (DAS28-ESR<2.6) within 4 months.**
Ghiti Moghadam *et al.* (2016) POET study	Randomisation to anti-TNF bDMARD continuation or discontinuation. (csDMARDs and glucocorticoids continued)	195	After 6 months: 132 (68%) regained remission (DAS28-ESR<2.6) after median 14 weeks 33 (17%) achieved LDA (2.6≤DAS28-ESR<3.2) after median 12 weeks
Inui *et al.* (2014) RESUME study	ETN discontinuation (csDMARDs continued)	13	All patients regained LDA (DAS28-ESR<3.2) after mean 3.7 months.
Klarenbeek *et al.* (2011b) BeSt study	**Various biologic and non-biologic DMARDs**	**53**	**After 6 months: 39 (74%) regained remission (DAS<1.6) 11 (21%) achieved LDA (1.6≤DAS≤2.4)**
Kuijper *et al.* (2016) tREACH study	**MTX, SFZ, HCQ, ETN, ADA, ABT, glucocorticoids**	**45**	**By Kaplan-Meir analysis: 65% (95% CI: 50-79%) regained remission after 6 months**

Table 1.10 (continued)

Study	Intervention	Patients restarting previous DMARDs after arthritis flare	Outcome following DMARD resumption
Nishimoto *et al.* (2014b) RESTORE study	**TOC**	**157**	**After 3 months: 139 (89%) regained remission (DAS28-ESR<2.6)**
Smolen *et al.* (2015) CERTAIN study	Certolizumab pegol cessation (csDMARDs and glucocorticoids continued)	10	10/10 (100%) achieved CDAI-defined LDA/remission (CDAI) after 28 weeks
Tanaka *et al.* (2015) HONOR study	ADA cessation (MTX continued)	c. 15*	90% regained LDA (DAS28-ESR<3.2) within 6 months, all patients regained LDA by 9 months.
ten Wolde *et al.* (1997)	**Various csDMARDs**	**51**	**After 12 months, by physician global assessment: 18 (35%) regained clinical remission 22 (43%) regained mild disease activity**
van Vollenhoven *et al.* (2016) DOSERA study	Randomisation to continue ETN, half-dose ETN, or PBO (csDMARDs and glucocorticoids continued)	35	After 35 weeks: 19/20 (95%) in PBO arm and 13/15 (87%) in half-dose ETN arm achieved LDA/remission after median 3.9 and 5.9 weeks respectively

Table 1.11– Summary of studies of DMARD withdrawal that report rates of radiographic progression in those patients who experienced an arthritis flare. Studies highlighted in bold using tapering strategies that culminated in complete DMARD cessation in at least one study arm. ABT: abatacept; ADA: adalimumab; CRP: C-reactive protein; DFR: drug-free remission; ESR: erythrocyte sedimentation rate; ETN: etanercept; IFX: infliximab; LDA: low disease activity; ΔmTSS: change in modified total Sharp score; MTX: methotrexate; NS: not stated; PBO: placebo. *: DMARD tapering initiated in patients with low disease activity rather than remission. *data from exploratory analysis of subset of 115 patients who achieved DMARD-free remission. Adapted from Kuijper et al. (2015) and Verhoef et al. (2017).

Study	Intervention	Proportion patients flared after DMARD withdrawal (flare definition)	Radiographic progression
Chatzidionysiou *et al.* (2016) ADMIRE study	Randomisation to continued ADA+MTX (A) or MTX monotherapy (B)	A: 2/16 (13%) B: 13/15 (87%)	No significant progression after 28 weeks between study arms
Emery *et al.* (2014) PRIZE study	**Randomisation to continued ETN + MTX (A), PBO + MTX (B) or PBO + PBO (C)**	**A: 23/63 (37%) B: 39/65 (60%) C: 50/65 (23%) (DAS28-ESR>2.6 at week 24 or 29)**	**No significant progression after ≤39 weeks (mean ΔmTSS, A: 0.1, B: 0, C :0.4. p=0.79, 0.48 & 0.34 for A vs. B, A vs. C and B vs. C respectively)**
Fautrel *et al.* (2016) STRASS study	Randomisation to continued ETN/ADA (A) or progressive spacing to eventual discontinuation (B) (csDMARDs and glucocorticoids condtinued)	49/64 (77%) (DAS28-ESR>2.6)	No significant progression in A vs. B after 18 months (median ΔmTSS 0 vs. 0, p=0.7)
Klarenbeek *et al.* (2011b) BeSt study	**Tapering followed by complete cessation of DMARDs when DAS44<1.6 (various)**	**53/115 (46%) ** (DAS44≥1.6)**	**No significant progression in flare vs. DFR groups after 1 year (median ΔmTSS 0 vs. 0, p=0.44)**
Smolen *et al.* (2013) PRESERVE study	Randomisation to continued ETN (A), 50% dose ETN (B) or placebo (C) (MTX continued)	190 (DAS28-ESR>3.2)	Minimal but statistically significant progression in C vs. A after 1 year (ΔmTSS 0.6 vs. -0.06, p=0.03)
Smolen *et al.* (2014) OPTIMA study	Randomisation to continued ADA (A) or placebo (B) (MTX continued)	28 (DAS28-CRP≥3.2)	Non-significant trend towards progression in B vs. A after 18 months (ΔmTSS ≥0.5: 89% vs. 81%, p=0.06)

Table 1.11 (continued)

Study	Intervention	Proportion patients flared after DMARD withdrawal (flare definition)	Radiographic progression
Takeuchi *et al.* (2015)	Non-randomised withdrawal of ABT if DAS28-CRP<3.2 (csDMARDs and glucocorticoids continued)	12 (DAS28-CRP>2.7 at 2 consecutive visits)	No significant progression in ABT cessation versus continuation groups after 1 year (ΔmTSS 0.80 vs. 0.32, p=0.37)
Tanaka *et al.* (2010) RRR study	Discontinuation of IFX (MTX & glucocorticoids continued)	56/102 (55%) (DAS28-ESR≥3.2)	No significant progression in flare vs. sustained LDA after 1 year (median ΔmTSS 1.5 vs. 0, p=0.11)
Tanaka *et al.* (2015) HONOR study	Discontinuation of ADA (csDMARDs & glucocorticoids continued)	20/52 (38%) (DAS28-ESR≥3.2 at 1 year)	Statistically significant progression in both LDA and flare patients at 1 year (mean ΔmTSS 0.59, [p=0.02] and 1.59 [p=0.04] in remission vs flare respectively)
van Herwaarden *et al.* (2015) DRESS study	Randomisation to continued ADA/ETN (A) or progressive spacing to eventual discontinuation (B) (csDMARDs and glucocorticoids continued)	14/119 (12%) (rise in DAS28-CRP>1.2, or rise >0.6 and DAS28-CRP≥3.2)	Minimal but statistically significant progression in A vs. B after 18 months (mean ΔmTSS 0.75 vs. 0.15, p<0.05)
Yamanaka *et al.* (2016) ENCOURAGE study	Randomisation to ETN continuation (A) or ETN discontinuation (B) (csDMARDs +/- glucocorticoids continued)	A: 4/32 (13%) B: 13/28 (46%) (DAS28-ESR≥2.6)	No significant progression in A vs. B after 1 year (mean ΔmTSS 0.3 vs. 0.7, p>0.05)

Table 1.12 – Recommendations regarding DMARD withdrawal in current national and international RA clinical management guidelines.

Organisation	Year	Recommendation
National Institute for Health and Care Excellence (National Institute for Health and Care Excellence, 2009)	2009 (updated 2015)	"In people with established RA whose disease is stable, cautiously reduce dosages of disease-modifying or biological drugs. Return promptly to disease-controlling dosages at the first sign of a flare."
Scottish Intercollegiate Guidelines Network (Scottish Intercollegiate Guidelines Network, 2011)	2011	"Where parallel or step-down strategies are employed, DMARDs should be carefully and slowly withdrawn in patients who are in remission."
European League Against Rheumatism (Smolen *et al.*, 2017)	2016	"If a patient is in persistent remission after having tapered GC, one can consider tapering bDMARDs, especially if this treatment is combined with a csDMARD." "If a patient is in persistent remission, tapering the csDMARD could be considered."
American College of Rheumatology (Singh *et al.*, 2016)	2015	"If the patient is in remission: taper DMARD therapy, taper TNFi, non-TNF biologic, or tofacitinib" "If the patient's disease is in remission, do not discontinue all RA therapies"
Canadian Rheumatology Association (Bykerk *et al.*, 2012)	2011	"If a patient achieves sustained remission after discontinuation of NSAID and glucocorticoids, a reduction in traditional and biologic DMARD can be attempted with caution as a shared decision between the patient and physician."

Table 1.13 – Summary of the key considerations when designing a strategy of DMARD withdrawal.

Consideration	Issues
Eligibility criteria for withdrawal	Which clinical remission criteria to use? How long should a patient be in remission before attempting DMARD withdrawal? Should imaging measures of synovitis be included? Are there any biomarkers that can stratify patients by risk of arthritis flare?
Hierarchal order of withdrawal	In what order should combination DMARD therapy be withdrawn?
Rate of withdrawal	Should DMARDs be stopped abruptly or gradually tapered? If gradually tapered, what is the optimum speed of DMARD withdrawal?
Monitoring during withdrawal period	How frequently should patients be monitored during DMARD withdrawal? What form should such monitoring take? (e.g. patient-reported measures, clinician assessment, blood sampling, musculoskeletal imaging)
Criteria for DMARD resumption	Given the unpredictable nature of RA disease activity and its imprecise measurement, how tolerant should the clinician be to small increases in disease activity during and after the withdrawal period?

1.6.4 Summary

Evidence from observational cohorts and interventional trials suggest that DFR can be achieved in approximately 10 to 25% of all patients with RA. The majority of studies to-date have recruited patients with active disease at baseline, and hence the overall prevalence of DFR in these studies is a function of both the induction of remission and, if remission is achieved, the maintenance of remission after DMARD withdrawal. When focussing exclusively on those patients who have already achieved remission with DMARD therapy, limited data suggest that up to half may maintain this state for at least one year after drug cessation. It appears that those patients who experience an arthritis flare do not display significant radiological progression and generally have an excellent response to recommencement of DMARD therapy if required, although long-term follow-up data is limited. Rates of arthritis flare may be greater upon biologic DMARD cessation compared with non-biologic DMARDs, although higher drug-free remission rates may be achieved with the use of DMARDs at an early disease stage. Nevertheless, comparison between studies is very difficult owing to a wide array of different DMARD regimens, inconsistent definitions of remission and different protocols of DMARD withdrawal.

1.7 Predicting drug-free remission in RA

The proportion of patients who can both achieve and maintain DFR is small, and the risk of arthritis flare following DMARD withdrawal is considerable. This has led some authors to recommend against the cessation of DMARDs in RA remission (O'Mahony *et al.*, 2010). Such recommendations may be sagacious when applied to whole populations, but neglect the compelling evidence that some individual patients are able to achieve sustained DFR. Indeed, reliable biomarkers that are able to predict DFR at an individual patient level would revolutionise the approach to step-down therapy in RA (Isaacs, 2010). Such biomarkers would ideally need to be measurable before the cessation of DMARD therapy to be a useful and effective guide to treatment in the clinic.

In this section, I will summarise the current available evidence for biomarkers of DFR in studies of DMARD withdrawal. For simplicity, discussion is divided between clinical, imaging and laboratory biomarkers – however, in practice, these categories are unlikely to be mutually exclusive and considerable interaction between individual parameters is to be expected. Finally, I will explore the potential immunological mechanisms that may be permissive of DFR, and the extent to which this may inform the search for biomarkers to measure such processes.

1.7.1 Clinical biomarkers of DFR

Several recurring themes are emerging from studies of DMARD withdrawal that point towards clinical characteristics that may be helpful in predicting DFR (Table 1.14). In the previously mentioned observational study of ERAS and LEAC cohorts (van der Woude *et al.*, 2009), a multivariate Cox-regression analysis identified several baseline clinical factors that were independently associated with DFR. Many of these factors, although statistically significant, carried little predictive value. However, four factors were strongly associated with reduced levels of DFR: RhF positivity, the presence of ACPA, HLA-SE positivity and a protracted symptom onset (van der Woude *et al.*, 2009).

The rapidity and intensity of initial DMARD therapy is also likely to be an important factor influencing the likelihood of a patient achieving DFR. Evidence to suggest this comes from a later analysis of the LEAC cohort in which DFR rates were compared between patients treated in different decades in a Cox-regression model adjusted for baseline swollen joint

Table 1.14 – Baseline biomarkers predictive of flare or drug-free remission in studies of complete or partial DMARD withdrawal in RA remission. Studies highlighted in **bold** using tapering strategies that culminated in complete DMARD cessation in at least one study arm. See text and Table 1.9 for further details regarding study design. ACPA, anti-citrullinated peptide antibody; GS: greyscale; HAQ, Health Assessment Questionnaire; HLA, human leukocyte antigen; HR, hazard ratio; MTX, methotrexate; OR, odds ratio; PCS, prospective cohort study; PD: power Doppler; RAI, Ritchie articular index; RCT, randomised controlled trial; RhF, rheumatoid factor; RR, relative risk; SFZ, sulfasalazine. mTSS: modified total Sharp score. *: effect size not stated in manuscript.

Study	Type	Baseline predictors of flare after DMARD withdrawal	Effect in multivariate analysis (95% CI)
El Miedany *et al.* (2016)	**Egyptian RCT 157 patients**	**ACPA+**	**OR_{flare} 5.35 – 8.64 (dependent on study arm)**
Fautrel *et al.* (2016) STRASS study	French RCT 137 patients	Baseline HAQ RhF+	HR_{flare} 2.07 (1.23 – 3.49) HR_{flare} 1.99 (1.03 – 3.83)
Ghiti Moghadam *et al.* (2016) POET study	Dutch RCT 817 patients	Baseline DAS28 Disease duration > 10 years GS>1 and/or PDS>0 on ultrasound	HR_{flare} 1.39 (1.21 – 1.60) HR_{flare} 1.29 (1.03 – 1.61) HR_{flare} 1.69 (1.11 – 2.53)
Haschka *et al.* (2016) RETRO study	**German RCT 110 patients**	**ACPA +**	**OR_{flare} 5.2 (1.1-24.9, p=0.038)**
Iwamoto *et al.* (2014)	Japanese PCS 42 patients	Total ultrasound GS score Total ultrasound PDS score	p = 0.005* (univariate) p = 0.002* (univariate)
(Kawashiri *et al.*, 2017)	Japanese retrospective cohort 40 patients	Joint erosions on ultrasound	OR_{flare} 8.35 (1.78 – 53.2, p=0.006)
Klarenbeek *et al.* (2011b) BeSt study	**Dutch RCT 508 patients**	**ACPA+ High mean DAS-44 prior to remission Baseline HAQ SFZ vs. MTX as last DMARD**	**OR_{flare} 7.5 (2.9-19.4) OR_{flare} 4.7 (1.5-15.2) OR_{flare} 0.41 (0.19-0.88) OR_{flare} 3.5 (1.5-15.2)**
Kuijper *et al.* (2016) tREACH study	**Dutch RCT 281 patients**	**Female sex Paid employment Baseline DAS-44**	**$OR_{remission}$ 0.352 (p=0.01) $OR_{remission}$ 0.404 (p=0.03) $OR_{remission}$ 0.0587 (p=0.022)**
Naredo *et al.* (2015)	Spanish PCS 77 patients	Total ultrasound PDS score	OR_{flare} 29.92 (6.81 – 131.40, p < 0.0005)

Table 1.14 (continued)

Study	Type	Baseline predictors of flare after DMARD withdrawal	Effect in multivariate analysis (95% CI)
Tanaka *et al.* (2010) RRR study	Japanese RCT 114 patients	Disease duration RhF titre	p = 0.0019* p = 0.0128*
Tanaka *et al.* (2015) HONOR study	Japanese RCT 75 patients	Baseline DAS28-4ESR	$OR_{remission}$ 0.143 (0.029 – 0.143, p = 0.0174)
ten Wolde *et al.* (1996)	**Dutch RCT 285 patients**	**RhF+ High DMARD dose Painless swollen joints**	**RR_{flare} 1.9 (1.0-3.6) RR_{flare} 2.3 (1.3-4.2) RR_{flare} 1.8 (1.0-3.3)**
van der Woude *et al.* (2009)	**Observational Dutch LEAC cohort (454 patients) British ERAS cohort (895 patients)**	**<u>LEAC cohort</u>: ACPA+ <u>ERAS cohort</u>: Long symptom duration (months) Acute onset (protective against flare) High RAI score High modified HAQ score RhF+ HLA-SE+**	**$HR_{remission}$ 0.09 (0.04-0.20, p<0.001) $HR_{remission}$ 0.94 (0.89-0.99, p=0.029) $HR_{remission}$ 2.03 (1.15-3.59, p=0.015) $HR_{remission}$ 0.92 (0.88-0.97, p=0.001) $HR_{remission}$ 0.66 (0.44-0.99, p=0.044) $HR_{remission}$ 0.28 (0.16-0.49, p<0.001) $HR_{remission}$ 0.44 (0.26-0.73, P=0.002)**
van Vollenhoven *et al.* (2016) DOSERA study	Swedish RCT 73 patients	Patient pain VAS mTSS radiographic erosion score	OR_{flare} 1.08 (1.01 – 1.15, p=0.018) OR_{flare} 1.05 (1.02 – 1.09, p = 0.005)
Yamanaka *et al.* (2016) ENCOURAGE study	Japanese RCT 222 patients	Lower baseline SDAI	p = 0.015*

count, inflammatory markers and autoantibody status. In this analysis, rates of DFR were significantly greater in those patients who received target-driven intensive DMARD therapy versus initial low-dose DMARD monotherapy (HR for DFR 3.72 [95% CI: 1.60 to 8.62] versus 1.13 [0.48 to 2.64], $p < 0.001$) (Ajeganova *et al.*, 2016). Nevertheless, part of this difference may be attributable to the increased availability of newer DMARDs and biologic agents in the intensive DMARD group.

Only a handful of interventional studies of DMARD cessation have investigated predictors of DFR, and mainly as exploratory post-hoc analyses (Table 1.14). High DMARD dose, high mean DAS and greater disability scores prior to remission were predictive of flare upon DMARD-withdrawal (Klarenbeek *et al.*, 2011b), suggesting that patients with historically active disease are less likely to remain in remission following DMARD cessation. This is perhaps unsurprising given the well-established correlation between early disease activity and long-term outcomes. This is further supported by exploratory analyses of several studies that suggest at least a trend towards higher rates of sustained DFR with earlier instigation of biologic therapy, including tocilizumab (Bijlsma *et al.*, 2016), etanercept (Emery *et al.*, 2014) and abatacept (Emery *et al.*, 2015).

1.7.2 Imaging biomarkers of sustained remission

Musculoskeletal imaging, in particular musculoskeletal US, may be another potentially useful biomarker in predicting DFR. As has already been discussed, there is now increasing evidence to show that the presence of PD in patients in remission on DMARD therapy can predict future radiographic erosions and arthritis flare (see Introduction 1.5.2.b-c). Nevertheless, there remains a paucity of evidence as to whether the absence of imaging measures of subclinical synovitis offers a more favourable prognosis to those patients in remission wishing to withdraw from DMARD therapy.

Only a single published study exists that explores the value of ultrasound parameters in predicting arthritis flare following complete DMARD cessation. In this rather complex study, 157 patients with RA in remission (DAS28-ESR < 2.6) were randomised to one of 5 arms, including: treatment continuation; 50% bDMARD dose; 50% bDMARD and 50% csDMARD; bDMARD cessation and 50% csDMARDs; and cessation of both bDMARDs and csDMARDs (El Miedany *et al.*, 2016). No significant difference was found between those who remained in remission and those who experienced an arthritis flare in relation to either PD or GS in 40 joints at baseline, either across the study as a whole or in individual treatment

arms. No data was presented regarding the predictive utility of ultrasound-detected erosions in this study (El Miedany *et al.*, 2016).

Several studies have explored the role of ultrasound in predicting which patients with RA in remission can safely taper or stop bDMARDs. Baseline csDMARDs were invariably continued in these studies and hence they address a different scenario to complete DMARD cessation; nevertheless, such studies can still offer insights in to the role of ultrasound in the measurement of subclinical synovitis in RA remission. In a recent Japanese study (Iwamoto *et al.*, 2014), 42 patients with established RA of mean 8.2 years duration and a DAS28-ESR < 2.6 were withdrawn from various different biologic therapies. The doses of concurrent non-biological DMARDs including low-dose oral corticosteroids were not changed. After 6 months, 23 (55%) patients remained in remission; the levels of both PD and GS synovitis in 40 joints at baseline were significantly lower in the sustained remission group versus those whose arthritis flared. GS and PD synovitis were strongly correlated, though a multivariate analysis to identify their individual association with sustained remission was not possible in this small study (Iwamoto *et al.*, 2014).

In a similar study (Naredo *et al.*, 2015), 77 patients with established RA of mean 13.1 years duration and DAS28-ESR < 2.6 underwent tapering (though not complete withdrawal) of biologic therapy. Synthetic DMARDs and low-dose prednisolone (≤5mg/day) were permitted and not changed during the study. Arthritis flare occurred in 35 (45%) of patients, and multivariate analysis showed that the presence of PD was independently associated with risk of flare (OR 29.92, 95% CI 6.81 – 131.4, $p<0.0005$).

In the POET study (Ghiti Moghadam *et al.*, 2016), 871 patients with established RA in low disease activity (DAS28-ESR<3.2) and receiving anti-TNFα agents for ≥1 year were randomised 2:1 to stop or continue their biologic therapy. A 20-joint US scan was performed at baseline, with a positive scan defined as GS>1 and/or PD>0 at any joint; flare of arthritis was defined as DAS28>3.2 and >0.6 greater than at baseline. An interim analysis of 12-month follow-up data in 251 patients who stopped biologic therapy showed a statistically significant though modest association between a positive US scan and arthritis flare after biologic withdrawal (HR 1.69, 95% CI 1.11 – 2.53, $p<0.05$) (Lamers-Karnebeek *et al.*, 2016). Although it could be expected that PD may outperform GS in this setting, an analysis of the predictive value of PD independent of GS was not presented in this preliminary report.

In contrast, a study of anti-TNF withdrawal in 47 patients with established RA in DAS28-ESR remission found no significant correlation between baseline levels of PD or GS synovitis

and arthritis flare at 2 years of follow-up (Saleem *et al.*, 2010). Of note, this study used a limited US assessment of 11 joints in the dominant hand and wrist compared with the studies by Iwamoto *et al*. (2014) and Naredo *et al.* (2015).

Whereas a signal of increased flare following DMARD withdrawal is starting to emerge for PD, the prognostic value of GS is far more uncertain. In a study by Alivernini *et al.* (2016), 42 patients with established RA (mean 10.9 years duration) in sustained clinical remission (DAS<1.6 on 3 visits over 9 months) and no PD on a 16-joint US scan had tapering and withdrawal of TNFα inhibitors. Twenty-nine patients completely stopped their biologic, of which 16 experienced a flare (defined as rise in DAS >1.2). Baseline mean synovial thickness was significantly greater at the 2nd and 5th metatarsophalangeal joints in those patients who subsequently flared, though the absolute differences were small (<0.3mm) between groups in this univariate analysis without multiple test correction (Alivernini *et al.*, 2016).

In an observational study, Kawashiri *et al.* (2017) followed 40 patients in low disease activity (DAS28-ESR<3.2) with a total PD score ≤ 1 on 22 joint-scan who discontinued bDMARD therapy. No significant association between baseline GS and arthritis flare was observed, although the presence of ultrasound-detected erosions was a significant poor prognostic marker (OR_{flare} 8.35, 95% CI 1.78-53.2, p = 0.006). This is in keeping with the results of the DOSERA study (van Vollenhoven *et al.*, 2016), which found a statistically significant though modest association between baseline modified total Sharp radiographic erosion score (mTSS) and arthritis flare following dose reduction and cessation of etanercept (OR_{flare} 1.05, 95% CI 1.02 – 1.09, p=0.005).

Given the high sensitivity of MRI in the detection of subclinical synovitis and bone marrow oedema, this imaging modality has the potential to be a useful prognostic tool in the setting of DMARD withdrawal – however, this remains a largely under-researched area. This was explored in a sub-analysis of 55 patients in the RETRO study (dOliveira *et al.*, 2016); despite MRI abnormalities in over half of patients, baseline MRI scores did not significantly differ between groups (median RAMRIS score 9 vs. 8 in flare vs. remission groups respectively, p=0.27).

In summary, current evidence suggests that the presence of ultrasound-detected PD is likely to portend a greater risk of flare following predominantly biologic DMARD cessation. In contrast, there is insufficient evidence to assess the prognostic value of GS in this setting. In particular, a lack of consensus on fundamental parameters such as the number of joints to scan and thresholds of GS and PD consistent with remission, results in considerable differences in

ultrasound methodology making comparison between studies difficult. MRI does not suffer from the same methodological uncertainties as US, though limited data suggest that its prognostic value in the setting of DMARD withdrawal may be limited. More research in this area is needed before firm conclusions can be drawn as to the optimal use of US in guiding DMARD withdrawal (D'Agostino *et al.*, 2016b).

1.7.3 Laboratory biomarkers of DFR

As noted above, seropositivity for either RhF or ACPA was associated with lower rates of DFR in several studies (Table 1.13), mirroring data from observational cohorts. In particular, the aforementioned RETRO study is notable for its detailed analysis of serological predictors of DFR. In this study, ACPA positivity was significantly associated with risk of arthritis flare following DMARD withdrawal (OR_{flare} 5.2, 95% CI 1.1-24.9, p=0.038). Further testing for autoantibodies against a panel of 10 different citrullinated, carbamylated and acetylated proteins demonstrated a significant increase in risk of flare with increasing numbers of autoantibodies (18%, 34% and 55% risk of flare with 0-1, 2-5 and >5 autoantibodies respectively, p=0.011) (Figueiredo *et al.*, 2017). The authors suggest that a broader specificity of autoantibodies may reflect a greater dysregulation of the immune system, which could explain the greater risk of flare observed in these patients (Figueiredo *et al.*, 2017).

Levels of circulating pro-inflammatory cytokines may also play a role in predicting which patients can successfully achieve DFR – one commercially available method of measuring such cytokines is the MBDA assay (see Introduction 1.4.5). In the RETRO study, MBDA score was an independent predictor of flare following DMARD withdrawal (OR 8.5, 95% CI 2.0-36.4, p=0.004) (Rech *et al.*, 2016). Furthermore, MBDA score interacted with the presence of ACPA to predict flare following DMARD withdrawal (MBDA-/ACPA-: 13%, MBDA-/ACPA+: 32%, MBDA+/ACPA-: 33%, MBDA+/ACPA+: 76% risk of flare, p=0.0001 for double negative vs. double positive) (Rech *et al.*, 2016). These results therefore suggest the presence of subclinical inflammation in some patients, which may drive arthritis relapse upon DMARD withdrawal. This concept is further supported by the DREAM study (Nishimoto *et al.*, 2014a), in which low levels of serum IL-6 prior to withdrawal of tocilizumab were predictive of DFR – though this may simply be a surrogate marker of efficacy of this anti-IL-6 biologic agent.

Combining clinical, imaging and laboratory biomarkers of remission across a variety of disparate studies is challenging. A recent attempt to synthesise biomarkers of successful bDMARD tapering and withdrawal was complicated by extensive heterogeneity between different studies (Tweehuysen *et al.*, 2017). Although a meta-analysis was not possible, a systematic review and qualitative analysis was performed including results from 15 independent patient cohorts. 17 and 33 biomarkers were included in the settings of bDMARD tapering and cessation respectively. However, only three biomarkers were identified as consistent predictors of sustained remission, namely: high adalimumab trough level, low baseline mTSS score and short symptom duration. In particular, the authors commented that reporting bias in the studies was a major limitation in confidently excluding non-predictive biomarkers in the final analysis (Tweehuysen *et al.*, 2017).

1.7.4 What are the biological mechanisms permissive for DMARD-free remission?

The state of DMARD-free remission may reflect distinct immunological phenotypes. One possibility is that DMARD-free remission represents the natural progression of RA in certain individuals, with a "burn-out" of active inflammation secondary to a loss of inflammatory drive. The observation that the rates of DMARD-free remission remain relatively constant across different DMARD regimes has been cited by some authors as anecdotal evidence to support the hypothesis that DMARD-free remission is a part of the natural history of RA (Scott *et al.*, 2013a). If this is the case, then the state of DMARD-free remission may permitted by a lack of pro-inflammatory mediators. Evidence that potentially supports this view lies with the so-called "inflammation-related cell" (IRC) – a hyper-responsive subset of $CD4^+$ T cells ($CD45RB^{high}CD45RA^+CD45RO^{low}CD62L^-$) that are postulated to contribute to a systemic chronic inflammatory state, and have been observed in both RA (Ponchel *et al.*, 2002) and ACPA+ 'pre-RA' individuals (Hunt *et al.*, 2016). It has been shown that lower levels of circulating IRCs correlates with lower risk of flare in patients with RA in remission receiving DMARD therapy (Burgoyne *et al.*, 2008) and also with lower risk of flare upon withdrawal of anti-TNF treatment (Saleem *et al.*, 2010). Nevertheless, these observations remain to be confirmed in the setting of DMARD-free remission and by independent research groups.

An alternative hypothesis is that patients who achieve DMARD-free remission do so by virtue of an active pro-tolerogenic mechanism that shifts the immune system balance from autoimmunity back to self-tolerance. If this is the case, then elucidation of this mechanism

could provide novel insights in to potential new avenues of therapy for RA. Indeed, evidence does exist to support a dysregulation of regulatory T cell populations in RA, which may contribute to the pathogenesis and perpetuation of the disease (Sempere-Ortells *et al.*, 2009). Furthermore, the number of circulating T_{regs} (defined as $CD4^{+}CD25^{high}CD127^{low/-}$) have been shown to be low in patients with active RA compared to healthy controls, whereas T_{reg} numbers in patients in clinical remission are comparable to that observed in healthy individuals (Kawashiri *et al.*, 2011). Similarly, the number of T_{regs} (defined as $CD4^{+}CD25^{high}FoxP3^{+}CD62L^{+}$) were increased in patients able to achieve sustained remission following anti-TNF withdrawal (Saleem *et al.*, 2010). However, T_{regs} are notoriously difficult to distinguish from activated T cells without functional assays to confirm a regulatory phenotype, and the observed correlations with remission do not prove a causal link with disease pathogenesis. Nevertheless, such observations do provide some evidence to suggest that active pro-tolerogenic mechanisms such as T_{regs} may play a role in the maintenance of DMARD-free remission.

Valuable insights in to the mechanisms permissive for DMARD-free remission may also be gained from the study of immunosuppressive drug withdrawal in other conditions. Particularly relevant in this regard is the field of solid organ transplantation, where powerful immunosuppression is required to prevent allograft rejection. Sometimes this immunosuppression is stopped, either because of patient non-concordance or due to a life-threatening complication such as lymphoproliferative disease. The usual outcome of this is allograft rejection, but on rare occasions such patients demonstrate continued graft survival in the complete absence of immunosuppression despite a fully functional immune system – a phenomenon termed “operational tolerance” (Monaco, 2004). Several international collaborative studies have identified peripheral blood gene expression signatures unique to operational tolerance in renal (Brouard *et al.*, 2007; Sagoo *et al.*, 2010; Baron *et al.*, 2015) and liver (Martinez-Llordella *et al.*, 2008; Li *et al.*, 2012a) transplant recipients. Furthermore, a prospective clinical trial of immunosuppressant withdrawal in liver transplant recipients with stable allograft function has identified similar gene expression profiles unique to those patients who achieve operational tolerance (Bohne *et al.*, 2012). Intriguingly, distinct patterns of gene expression are seen in operational tolerance to different organ grafts; a signature enriched for B-cell genes is seen in operational tolerance to renal grafts whereas genes involved in natural killer cell function and iron homeostasis are seen in tolerant liver transplant recipients (Lozano *et al.*, 2011). This raises the possibility of multiple pathways to tolerance – in other words, the pathway to immune tolerance may be dictated by the original

immunological insult and/or by different mechanisms of immune homeostasis between different individuals and disease states (Massart *et al.*, 2017).

1.7.5 Summary

Unequivocal evidence demonstrates that DMARD-free remission in RA can be achieved, albeit in a small proportion of patients. Historically, the number of patients achieving disease remission was so small that the possibility of DMARD withdrawal was rarely encountered. However, an increasing proportion of patients are now able to achieve sustained remission with early DMARD initiation in modern treat-to-target regimens. Therefore, the conundrum of how to balance risks versus benefits of continued DMARD therapy versus medication withdrawal in such cases is increasingly encountered in clinical practice. Biomarkers that are predictive of sustained DMARD-free remission are crucial to guide effective step-down therapy, although very few studies have explored this area and fewer still investigate DFR as a primary outcome. Furthermore, the vast majority focus on bDMARD and neglect csDMARD withdrawal, thus disregarding biologic-naïve patients who have successfully achieved sustained remission by the treat-to-target paradigm.

Seropositivity for ACPA or RhF have both consistently been associated with lower rates of DMARD-free remission across multiple studies. Other clinical parameters, novel blood-borne biomarkers and musculoskeletal imaging may all potentially be useful in guiding DMARD withdrawal, although evidence is lacking and where present is often contradictory. DMARD-free remission may represent a lack of inflammatory drive or the net effect of active pro-tolerogenic mechanisms, the further characterisation of which may lead to novel avenues of future therapy. Further research in this area is urgently needed if the concept of step-down DMARD therapy is to become a clinical reality.

Chapter 2. Objectives

In the preceding Introduction, I discuss the evidence to support two key assertions, namely:

1. Approximately half of patients with RA who achieve clinical remission with DMARD therapy can expect to achieve sustained drug-free remission (DFR) following DMARD withdrawal
2. There is a lack of reliable biomarkers that, when measured prior to DMARD withdrawal, can predict which patients can achieve DFR.

To address this unmet clinical need, the primary objective of this study is:

<u>Primary Objective:</u> To identify baseline biomarkers prior to DMARD withdrawal that are predictive of DFR in RA, including:

a. **Clinical features**
b. **Ultrasound features**
c. **Differential gene expression in $CD4^+$ T cells isolated from peripheral blood by next-generation RNA sequencing**
d. **Differential cytokine levels in peripheral blood**

In order to satisfy this objective, this study takes the form of a prospective longitudinal interventional clinical trial of DMARD withdrawal in patients with RA in clinical and ultrasound remission - the Biomarkers of Remission in Rheumatoid Arthritis (BioRRA) Study (defined and justified further in the Methods chapter). In the process of conducting this study, I thus also address two secondary objectives:

<u>Secondary Objective 1</u>: To assess the correlation between ultrasound measures of joint inflammation and clinical measures of disease activity in the setting of RA remission

<u>Secondary Objective 2</u>: To assess the longitudinal changes in circulating cytokines/chemokines, and peripheral $CD4^+$ T cell transcriptomic profile, in order to gain insight in to the immunological events underlying sustained DFR and RA flare.

A specific focus of this project will be the exploration of global gene expression within $CD4^+$ T cells as a potential biomarker of DFR. A whole-genome approach was chosen based on the aforementioned success of peripheral blood microarray analysis in identifying biomarkers of operational tolerance to solid organ grafts (see Introduction 1.7.4). The decision to focus upon the transcriptome of $CD4^+$ T cells is based upon the central importance of this cell in both the pathogenesis of RA (see Introduction 1.2.3) as well as the potential immunological mechanisms underlying sustained remission in the disease (see Introduction 1.7.4). Furthermore, by analysing gene expression exclusively within the $CD4^+$ T cell subset I aim to minimise non-specific variations in gene expression that could otherwise mask a remission biomarker signature in whole blood. Finally, the feasibility of high purity isolation of $CD4^+$ T cells from peripheral blood samples and subsequent transcriptomic analysis has been demonstrated by previous work in the Newcastle University Musculoskeletal Research Group, which has identified the presence of a 12-gene signature in peripheral $CD4^+$ T cells that can predict future progression from undifferentiated arthritis to RA (Pratt *et al.*, 2012).

Nevertheless, it is recognised that by restricting gene analysis solely to the $CD4^+$ T cell subset there is a risk that potential biomarkers in other peripheral blood cells may be overlooked. To address this, additional samples will also be taken at study visits including whole blood samples for potential future transcriptomic analysis and peripheral blood mononuclear cells (PBMCs) for potential future flow cytometry analysis. Whilst not necessarily forming part of the aims of this study, the collection of these additional samples will provide opportunity for alternative future biomarker analyses and hence maximise the yield of data from this unique patient cohort.

Chapter 3. Methods

In this chapter, I present the methods used during the course of this PhD project. First, I summarise the key considerations in the design and conduct of the Biomarkers of Remission in Rheumatoid Arthritis (BioRRA) Study, together with the methods of ultrasound examination. Amendments to the study design are then discussed and justified. A detailed account of the laboratory procedures for sample handling and processing is described, followed by the methods of statistical analysis. Finally, the ethical and governance arrangements for oversight of the study are presented.

3.1 Inclusion and exclusion criteria

The inclusion, exclusion and DMARD-cessation criteria for the Biomarkers of Remission in Rheumatoid Arthritis (BioRRA) Study are summarised in Table 3.1.

3.1.1 Justification of inclusion criteria

Patients were only recruited if they had a clinical diagnosis of RA made at least 1 year prior to the baseline study visit. A duration of 12 months from diagnosis was mandated to permit a degree of certainty on the clinical diagnosis. Furthermore, after consultation with local rheumatologists, it was deemed unlikely that DMARD tapering would be considered in clinical practice at an earlier time point. The inclusion criteria did not require a formal diagnosis of RA according to published diagnostic criteria, because such formal assessment on behalf of the referring clinician was considered a possible disincentive to patient referral. Furthermore, clinical notes were not available to the research team at the baseline study visit for patients referred from centres outside of Newcastle. Thus, medical notes review by the research team (for the purposes of diagnostic criteria assessment) prior to study enrolment would have posed a substantial barrier to patient recruitment. A pragmatic compromise was thus reached whereby patients could only be enrolled to the study if they had a clinical diagnosis of RA, with fulfilment of diagnostic criteria assessed retrospectively by the research team after study enrolment (see Methods 3.6.3).

Clearly, it was necessary for patients to be both in clinical remission and willing to discontinue DMARD therapy before they could be enrolled in the study. However, it was felt

Table 3.1 – Inclusion, exclusion and DMARD cessation criteria for the BioRRA study. Patients who met the inclusion and exclusion criteria were enrolled for the baseline visit, but only those patients who met the DMARD-cessation criteria stopped DMARDs and continued in study follow-up. *: ACR/EULAR Boolean remission was initially used as the clinical remission definition, though this was subsequently changed to DAS28-CRP < 2.4 (see Methods 3.2).

Inclusion criteria	1. Clinical diagnosis of rheumatoid arthritis made by consultant rheumatologist at least 12 months previously 2. Current single or combination use of methotrexate, sulfasalazine and/or hydroxychloroquine 3. Arthritis currently in remission, as judged clinically by referring healthcare professional 4. Willing to consider DMARD withdrawal
Exclusion criteria	1. Use of biologic therapy within the past 6 months 2. Received steroids within past 3 months (enteral, parenteral or intra-articular) 3. Use of any DMARD other than methotrexate, sulfasalazine or hydroxychloroquine within the past 6 months (or past 12 months for leflunomide) 4. Current pregnancy, or pregnancy planned within next 6 months 5. Current participation within another clinical trial 6. Inability to provide informed consent
DMARD-cessation criteria	1. Clinical remission, defined as DAS28-CRP < 2.4 * 2. Absence of power Doppler signal on 7-joint ultrasound scan

that mandating a predetermined level of disease activity could have been a disincentive to patient referral, and thus judgement on this was deferred to the referring clinician for the purposes of study recruitment.

3.1.2 Justification of exclusion criteria

Patients who were receiving or who had recently received biologic therapy were excluded from the study for several reasons. First, there was concern that patients who were receiving bDMARDs would, by therapeutic indication, have more aggressive underlying disease and

thus be at higher risk of arthritis flare following DMARD cessation. Indeed, in comparison to csDMARD cessation, substantially higher rates of arthritis flare have been observed in some of the published studies of bDMARD withdrawal (see Introduction 1.6). Second, there was concern that arthritis flare would be more difficult to control in such patients, owing to both a more aggressive underlying disease pathophysiology and the potential for anti-drug antibody formation during the DMARD-free period. Third, there was a desire to minimise heterogeneity between individual patients at enrolment, and the potent selective nature of bDMARDs could have potentially skewed or masked any generic biomarkers of DFR. For example, the anti-IL-6R agent tocilizumab could be expected to mask any IL-6-related signatures in cytokine measurements and/or $CD4^+$ T cell transcriptional profile, as well as potentially influencing disease activity assessment by spurious suppression of CRP levels. Fourth, observations from qualitative interviews as part of my MRes project (Baker *et al.*, 2015) and from patient-public engagement suggested that patients who were currently receiving bDMARDs were generally not inclined to consider DMARD withdrawal. Finally, regulatory issues surrounding the prescription of bDMARDs in the NHS had the theoretical potential to make it difficult to restart bDMARD therapy in those patients who experienced an arthritis flare either during or after their participation in the study.

Only patients receiving methotrexate, sulfasalazine and/or hydroxychloroquine were permitted to enter the study, reflecting a balance between reducing baseline patient heterogeneity whilst maintaining sufficiently flexible eligibility criteria for the purposes of study recruitment. A longer exclusion period applied to previous use of leflunomide, reflecting the prolonged half-life of this drug secondary to entero-hepatic recirculation.

Patients who had received recent systemic glucocorticoids were excluded, as this was felt likely to interfere with sensitivity of ultrasound assessment and the detection of a remission biomarker signature. Routes of local glucocorticoid delivery – including topical, inhaled and intra-nasal – were permitted as the effect on systemic immunity was deemed negligible.

Patients who were, or were planning to become, pregnant were excluded as it was expected that the physiological changes that occur in pregnancy would likely influence any putative biomarker signature. Furthermore, the significant effect of pregnancy on RA activity could be expected to alter the balance between sustained remission and flare in pregnant participants.

Participation in another clinical trial was grounds for exclusion from the study on the basis that DMARD cessation could potentially influence the outcomes of such studies.

3.2 Remission criteria

In the original BioRRA study design, the 2011 ACR/EULAR remission criteria were used to define clinical remission. However, after the first 3 months of recruitment it became apparent that several patients recorded visual analogue scores (VAS) of just over 10/100, and were thus failing to achieve remission despite the absence of any tender or swollen joints, the absence of PD synovitis and undetectable CRP levels. This observation has been replicated by other groups, with the suggestion that an overly-strict VAS threshold results in sub-optimal specificity of the ACR/EULAR 2011 remission criteria when applied to clinical practice – see Introduction 1.5.1 and discussed further by Kuriya *et al.* (2012); Masri *et al.* (2012); Studenic *et al.* (2012); Vermeer *et al.* (2012); Svensson *et al.* (2013); Thiele *et al.* (2013); Baker *et al.* (2017). A pragmatic decision was therefore made to change the clinical remission definition to the composite scoring system of DAS28-CRP, which is extensively used in current clinical practice (Box 3.1). The use of CRP was preferred owing to its greater specificity for inflammation than compared with ESR, which can be affected by non-inflammatory factors such as age, gender, red blood cell morphology and hyperglobulinaemia (Ward, 2004). A threshold of DAS28-CRP < 2.4 was used as the definition of clinical remission – this is lower than the remission threshold for DAS28-ESR (<2.6) and is in line with recently published data that recommends this lower remission threshold (Fleischmann *et al.*, 2015). Values of CRP below the detectable threshold of the local clinical laboratory (<5mg/L) were recorded as zero for the purposes of DAS28-CRP calculation.

Within the design of the study, it was acknowledged that circumstances may arise where DAS28-CRP ≥ 2.4 in the absence of active arthritis – for example, during systemic infection causing a rise in CRP, or after trauma causing local joint pain/swelling. In these circumstances, clinician discretion to overlook a single DAS28-CRP ≥ 2.4 measurement was permitted only if there was a clear alternative explanation, with the requirement that a further

Box 3.1 – The final clinical remission definition used in the BioRRA study. CRP, C-reactive protein in mg/L; DAS28, disease activity score in 28 joints; SJC28, swollen joint count in 28 joints; TJC28, tender joint count in 28 joints; $VAS_{patient}$, patient visual analogue score in millimetres (on 0-100mm scale).

DAS28-CRP remission **(Fleischmann *et al.*, 2015)**	**$[0.56\sqrt{(TJC28)} + 0.28\sqrt{(SJC28)} + 0.36\ln(CRP+1) + 0.014(VAS_{patient}) + 0.96] < 2.4$**

review as performed within the following four weeks. If DAS28-CRP ≥ 2.4 at this subsequent visit, then the patient exited the study regardless of whether an alternative explanation was present, and was referred back to their rheumatology clinical team for recommencement of DMARD therapy.

3.3 Study design and sample size estimation

The design of the study together with sample size estimations are summarised in Figure 3.1 Patients who satisfied both DAS28-CRP < 2.4 and an absence of PD signal on ultrasound assessment (see Methods 3.6) at the baseline visit were eligible for DMARD cessation. Further administrations of methotrexate, sulfasalazine and/or hydroxychloroquine were immediately stopped without tapering. Non-DMARDs, including folic acid, remain unaltered and non-steroidal anti-inflammatory drug (NSAID) use was permitted. Research blood tests including serum, plasma, peripheral blood mononuclear cells (PBMCs), $CD4^+$ T cell isolation and whole blood RNA TEMPUS™ tubes (Applied Biosystems, Foster City, California, USA) were collected at baseline, month 3 and month 6 following DMARD cessation. An US scan was performed again at the month 6 visit. If a patient experienced a flare of their arthritis (defined as DAS28 ≥ 2.4) or if they developed PD synovitis on the month 6 scan, then they could receive rescue corticosteroids and were discharged from the study with their DMARD therapy promptly recommenced by their referring rheumatologist. Those patients who remained in remission at 6 months with no PD synovitis were discharged back to their referring rheumatologist without DMARD therapy. Patients could request additional appointments at any time should they believe their arthritis may be flaring. A schedule of events for the study is detailed in Table 3.2.

The BioRRA study was conducted with the ethos of an exploratory study. Indeed, it was impossible to conduct a formal power calculation owing to the complete absence of information regarding the nature of a putative gene expression and/or cytokine signature of DMARD-free remission. In consultation with local bioinformaticians, and based upon the previous demonstration of an ability to detect significant differences in $CD4^+$ gene expression at the 1.1 to 1.4-fold level (Pratt *et al.*, 2012), it was estimated that a total of 60 patients would be required for transcriptomic analysis. This is based on the assumption of a 50% flare rate within 6 months of DMARD cessation (Step C, Figure 1), as was observed in previous studies (Klarenbeek *et al.*, 2011b; Haschka *et al.*, 2016).

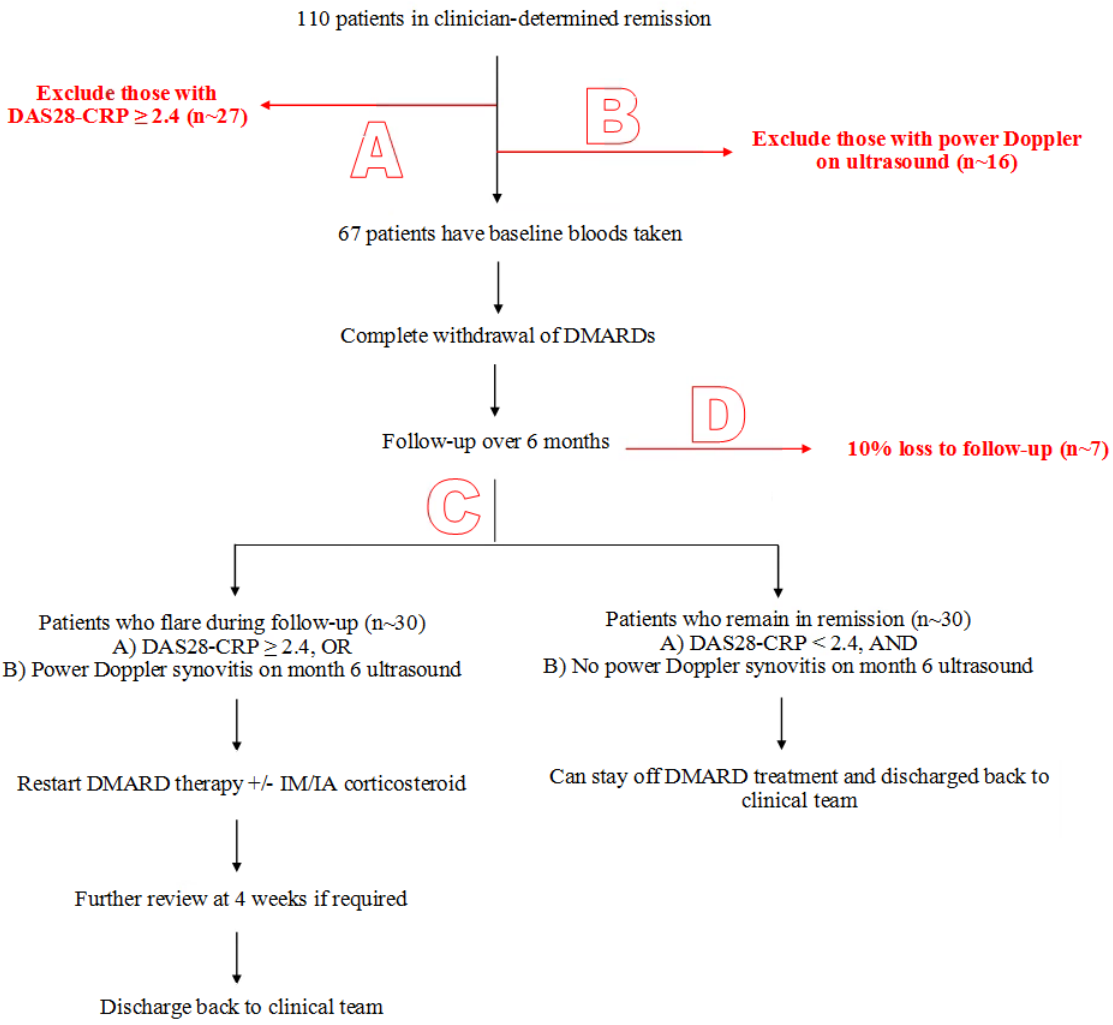


Figure 3.1 – Design of the BioRRA study together with sample size estimations. Figure reproduced from the BioRRA study protocol (version 6). CRP, C-reactive protein; DAS, disease activity score; DMARD, disease-modifying anti-rheumatic drug; IA, intra-articular; IM, intra-muscular.

It was estimated that 110 patients were needed to be recruited to the study in order to achieve 60 patients stopping DMARD therapy. Of these 110 patients, an estimated 75% were expected to have a DAS28-CRP < 2.4 (Step A, Figure 3.1). This was based on data from my Newcastle-based MRes project, where 13/17 (76%) of patients referred to the study satisfied this criteria (Baker, 2014). Of those patients in clinical remission, an estimated 20% were anticipated to display PD signal and hence not be eligible for DMARD withdrawal (Step B, Figure 3.1). Finally, a 10% drop-out rate was estimated during the six-month follow-up period (Step D, Figure 3.1).

During the course of conducting the study, it became apparent that no patients were being lost to follow-up and that substantially greater proportion of patients satisfied DAS28-CRP remission than previously anticipated. The overall recruitment target was therefore reduced

Table 3.2 – Schedule of events for the BioRRA study. Patients were able to request an additional review at any time if they thought they might be experiencing an arthritis flare between routine scheduled visits. ACPA: anti-citrullinated peptide antibody; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; HAQ-DI: Health assessment questionnaire disability index; RhF: rheumatoid factor.

Activity	Baseline	Month 1	Month 3	Month 6	Patient-requested
Informed consent	X				
Focussed medical history	X				
Blinded DAS28-CRP	X				
Unblinded DAS28-CRP		X	X	X	X
US7 ultrasound scan	X			X	
HAQ-DI	X			X	
CRP	X	X	X	X	X
ESR	X				
RhF	X				
ACPA	X				
Research bloods	X[1]	X[2]	X	X	X[2]
DMARD cessation	X[1]				
Response to arthritis flare		If DAS28-CRP ≥ 2.4 at any time following DMARD cessation, then: [3] 1. IM steroid injection given at discretion of study clinician 2. Patient exits study and is referred back to rheumatology clinical team for recommencement of DMARDs			

Footnotes

1 Only if DMARD cessation criteria fulfilled at baseline.

2 Were originally only collected if joint count and $VAS_{patient}$ not compatible with DAS28-CRP remission, later amended to be collected at all visits (substantial amendment 2)

3 A single measure of DAS28-CRP ≥ 2.4 was permitted if alternative explanation (e.g. concurrent infection, joint trauma) – see Methods 3.2.

from 110 to 90 patients, whilst maintaining the same target of 60 patients to complete the study following DMARD cessation.

3.4 Patient recruitment

Patients were referred to the BioRRA study from routine outpatient rheumatology clinic appointments by their clinician or nurse specialist. Patients were referred to the study from five separate rheumatology centres across the North East of England, namely: Newcastle upon Tyne Hospitals NHS Foundation Trust; Northumbria Healthcare NHS Foundation Trust; Gateshead Health NHS Foundation Trust; County Durham and Darlington NHS Foundation Trust; and Sunderland City Hospitals NHS Foundation Trust. All study procedures were performed at a single research site (Newcastle). Recruitment to the study commenced on 7/8/14 and ended on 31/10/16. Study visits were performed between 9am and 1pm where possible to minimise circadian variation in laboratory samples.

3.5 Clinical variable assessment

3.5.1 Prospective clinical variable assessment

Pre-specified clinical variables were recorded prospectively at the baseline study visit, as listed in Table 3.3. Included in these variables is the Health Assessment Questionnaire Disability Index (HAQ-DI), a self-completed questionnaire (Appendix A) that quantifies physical disability that has been extensively validated in the setting of RA and other chronic diseases (Bruce and Fries, 2003).

3.5.2 Retrospective clinical variable assessment

Data were obtained for a range of pre-specified variables, as listed in Table 3.4. This data was obtained both by patient interview at baseline visit, and from clinical notes review. Historical clinical records were available for all patients and were assessed by a single reviewer (KB). It was acknowledged that the most reliable source of information might differ depending on the variable of interest. A systematic methodology of recording clinical variables was therefore implemented (Table 3.4).

Table 3.3 – Baseline clinical variables recorded prospectively in the BioRRA study. CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; RhF: rheumatoid factor; ACPA: anti-citrullinated peptide antibody; HAQ-DI:

Baseline variable	Data type
Age	Continuous
Sex	Binary
28 tender joint count	Discrete
28 swollen joint count	Discrete
Patient arthritis visual analogue score (range 0-100)	Continuous
ESR	Continuous
CRP	Continuous
RhF positive	Binary
ACPA positive	Binary
DAS28-CRP	Continuous
DAS28-ESR	Continuous
Fulfilment of ACR/EULAR Boolean remission	Binary
HAQ-DI (range 0 – 3)	Continuous
Patient global health score (range 0-100)	Continuous
Patient pain score (range 0-100)	Continuous

Data regarding smoking and alcohol history were frequently missing or out-dated in the medical notes, and hence patient-reported values for these variables at the baseline visit were recorded. Although glucocorticoid prescription for RA was recorded in the medical notes, systemic glucocorticoid use for other indications (e.g. asthma, chronic obstructive pulmonary disease etc.) was frequently not recorded in hospital records. It was therefore decided to record the shortest of either the value recorded in the medical notes or that reported by the patient at baseline. Values of all other retrospective clinical variables were recorded as per the value stated in the medical notes.

3.5.3 Assessment of RA classification criteria

As previously discussed, it was not practically possible to formally assess fulfilment of classification criteria at the point of patient recruitment to the study owing to a lack of availability of clinical notes access for patients referent from external hospital sites. Patients

Table 3.4 – Baseline clinical variables recorded retrospectively in the BioRRA study.

Variable	Data type	Data source
Year of RA diagnosis	Continuous	Medical notes
Months from symptom onset to first rheumatology clinic review	Continuous	Medical notes
Months from first rheumatology clinic review to commencement of first DMARD	Continuous	Medical notes
Months since last change in DMARD therapy (dose and/or drug)	Continuous	Medical notes
Months since last glucocorticoid	Continuous	Most recent of either medical notes or patient interview
Smoking status	Categorical (current/previous/never)	Patient interview
Weekly alcohol unit intake	Continuous	Patient interview
Methotrexate use	Categorical (current/previous/never)	Medical notes
Sulfasalazine use	Categorical (current/previous/never)	Medical notes
Hydroxychloroquine use	Categorical (current/previous/never)	Medical notes
Other previous DMARDs	Free text	Medical notes

were therefore recruited on the grounds of a clinical diagnosis of RA made by a consultant rheumatologist, with satisfaction of formal classification criteria assessed retrospectively after patient recruitment.

Fulfilment of both 1987 ARC (Arnett *et al.*, 1988) and 2010 ACR/EULAR classification criteria for RA (see Introduction 1.1.5, Tables 1.3 – 1.4) were assessed for all patients by medical notes review, to account for those diagnosed before and after publication of the 2010 criteria. Both the clinic letters and original hand-written clinical notes were reviewed by the

same researcher (KB) for all patients. Where there was ambiguity as to the extent of joint involvement for the 2010 classification criteria assessment, the most conservative value was recorded - for example, if a clinic entry states that 'the MCP joints were swollen', then a value of 2 was recorded for the joint count. In cases where there was no documentation of a classification parameter (e.g. duration of morning stiffness), this parameter was treated as absent for the purposes of classification assessment.

3.6 Musculoskeletal ultrasonography

The emerging role of musculoskeletal US in predicting long-term outcomes in RA holds promise for its use in predicting DMARD-free remission. It was felt important not to withdraw DMARDs from patients with PD synovitis on US, given the emerging evidence of its potential value in predicting future flare and radiographic progression in the context of active disease (see Introduction 1.4.4.c) and RA remission (see Introduction 1.5.2). The US7 protocol of Backhaus (Backhaus *et al.*, 2009) was used in this study as it is externally validated, realistic to perform within a 20 minute timeframe and has been shown to yield comparable results to more laborious 78-joint scans (Hammer and Kvien, 2011). Furthermore, in my previous MRes thesis I demonstrated good levels of both intra and inter-observer agreement in PD and GS synovitis scores in scans performed by myself according to the US7 protocol, and rescored in a blinded fashion by a rheumatology consultant experienced in musculoskeletal ultrasound (Baker, 2014).

All US scans were performed using the same machine (Xario XG Diagnostic Ultrasound System model SSA-680A, Toshiba Medical Systems Corporation, Otawara, Tochigi Prefecture, Japan) by the same operator (KB) who is trained in the use of musculoskeletal US. All scans were performed in the same darkened room using the same linear mixed array transducer (part number PLT-1204BT). B-mode frequency was fixed at 12MHz for all scans, and B-mode gain was individually set to a level providing optimal contrast between soft tissue, tendons and bony surfaces. Power Doppler images were acquired at a Doppler frequency of 5.3MHz for all scans, with Doppler gain individually set to the maximum level possible without cortical bone artefact. It was not possible to alter the pulse-repetition frequency (PRF) on this ultrasound machine, which automatically adjusts PRF according to the default musculoskeletal settings as calibrated by a Toshiba ultrasound technical representative.

A minimum of 30 still images were recorded per scan, corresponding to the individual views of the seven joints of the US7 protocol: the dominant wrist; 2nd and 3rd metacarpophalangeal joints; 2nd and 3rd proximal interphalangeal joints; and 2nd and 5th metatarsophalangeal joints (Figure 3.2 and Appendix B). The level of GS at each joint, and the levels of PD at each joint and tendon complex, were scored using the semi-quantitative scales as per the approach of Scheel *et al.* (2005) and Szkudlarek *et al.* (2003) respectively (Table 3.5 and Figure 3.3). Tendon-associated GS and joint erosions were scored as either present (1) or absent (0). Low-level PD signal is often difficult to interpret at the wrist, owing to small blood vessels that traverse the joint. For pragmatic purposes, minor vessel-related Doppler signal at the wrist was not scored as power Doppler signal so long as all of the following criteria were satisfied:

a) Only a single vessel was present, and

b) The origin of the vessel could be easily visualised as arising from a vessel superficial to the tendons of extensor digitorum, and

c) No further branching of the vessel occurred below deep to the tendons of extensor digitorum

d) The vessel did not traverse any areas of any level of greyscale change.

Such an approach is in keeping with representative images from a published atlas of musculoskeletal ultrasonographic scoring for use in clinical research (Hammer *et al.*, 2011).

Table 3.5 – Scoring systems used to grade GS and PD change in the BioRRA study.

Greyscale (GS) Grading **Scheel *et al.* (2005)**	**Power Doppler (PD) Grading** **Szkudlarek *et al.* (2003)**
0 = absent	0 = absent
1 = mild (small hypoechoic line)	1 = single vessel signal
2 = moderate (hypoechoic area within joint capsule to level of joint)	2 = confluent vessel signals in <50% of joint/tendon area
3 = severe (joint capsule markedly distended)	3 = confluent vessel signals in >50% of joint/tendon area

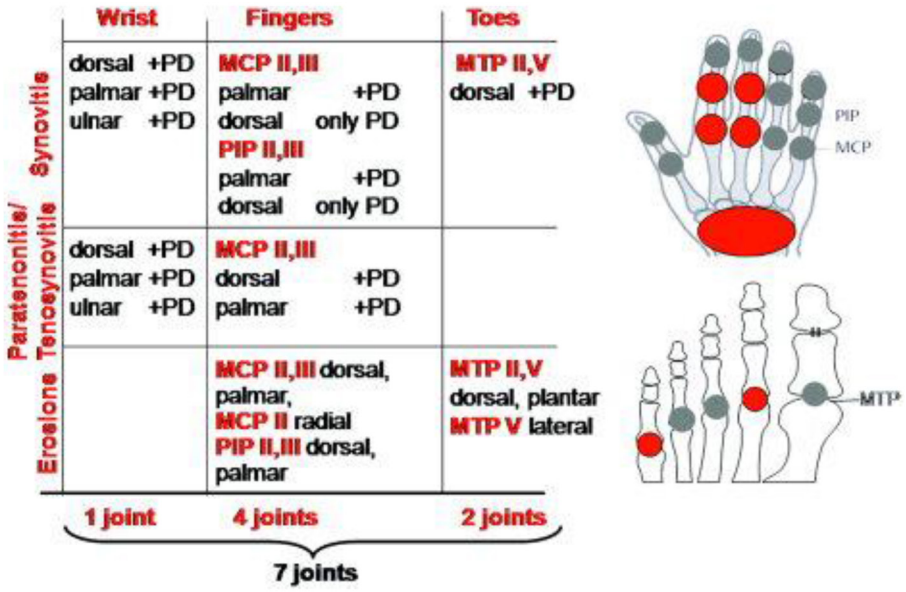


Figure 3.2 – The images obtained in the US7 scan protocol. Reproduced with permission from Backhaus *et al.* (2009). Copyright © 2009 by the American College of Rheumatology.

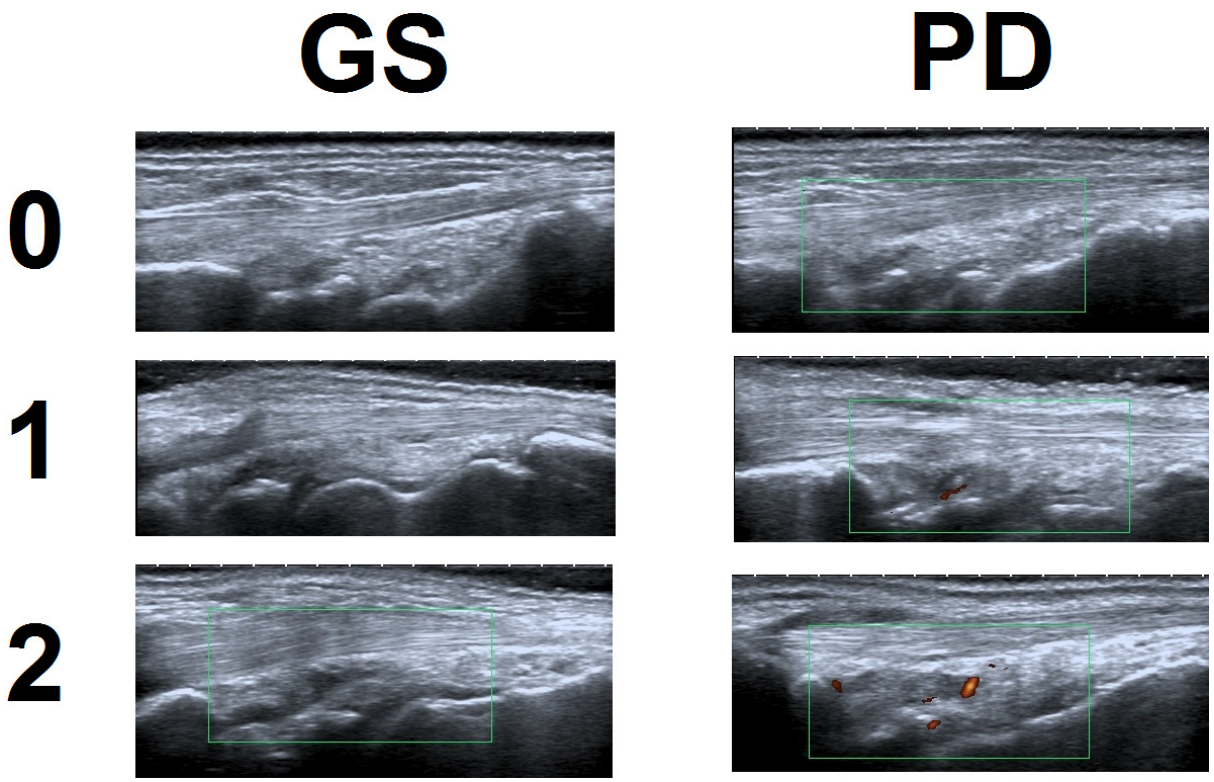


Figure 3.3 – Representative ultrasound images from the BioRRA study demonstrating semi-quantitative scoring of greyscale (GS) and power Doppler (PD) at the dorsal view of the wrist, as agreed by both ultrasound assessors. No instances of grade 3 GS or PD at the dorsal view of the wrist were recorded.

Individual summed scores across all 7 joints were calculated for each patient to obtain total scores for joint GS (possible range 0 – 27), joint PD (possible range 0 – 39), tendon GS (possible range 0 – 7), tendon PD (possible range 0 – 21), and joint erosions (possible range 0 – 14).

Baseline DAS28-CRP assessments were performed by a research nurse, thus blinding the ultrasound operator (KB) to the DAS28-CRP score at baseline to maintain the objectivity of the baseline ultrasound assessments. All scan images were scored at the time of image acquisition by KB. The ultrasound images were later reviewed by KB and a second observer (BT), blinded to the original ultrasound images and disease activity scores, in order to calculate the intra- and inter-rater agreement respectively (see Methods 3.10.1).

3.7 Study amendments

A number of amendments were made to the study protocol and documentation to respond to unanticipated issues as they arose. All amendments received research ethics committee approval (see Methods 3.11) before they were implemented (Table 3.6).

There were three substantial amendments made to the protocol during the recruitment period. The first substantial amendment was to change the remission criteria from ACR/EULAR Boolean remission to DAS28-CRP<2.4, as is further discussed in Methods 3.2. Consequently, a greater proportion of patients were eligible for DMARD cessation and the recruitment target was reduced (from 170 to 110 patients).

During study design it had been anticipated that the majority of patients would experience an arthritis flare between 3 and 6 months of follow-up, and collection of research bloods had not been planned at the month one visit to reduce financial expenditure. However, during the conduct of the study it became apparent that most flare events occurred before 12 weeks of follow-up, and thus before the subsequently scheduled routine research blood sampling at month three. The second substantial amendment therefore allowed the collection of research blood samples at month one for the purposes of longitudinal analysis.

The original study protocol specified that microarray technology would be used for the analysis of differential gene expression. However, in the three years that elapsed between the start of study design and the end of recruitment, microarray technology became obsolete and was discontinued by the manufacturer (Illumina). It was therefore decided to use next-generation RNAseq technology, and the third substantial amendment was necessary to effect

Table 3.6– Amendments to study protocol and documentation during the course of patient recruitment.

Amendment	Date of ethical committee approval	Purpose
Minor amendment 1	2/10/14	• Correction to error in order of events in baseline visit • Update of contact telephone numbers on clinician information forms
Minor amendment 2	10/10/14	• Correction to error in version number on front cover of protocol
Substantial amendment 1	22/12/14	• Change remission criteria from ACR/EULAR Boolean to DAS28-CRP<2.4 (see Methods 3.2) • Reduce recruitment target from 170 to 110 • Explicitly seek consent to reproduce anonymised ultrasound images
Minor amendment 3	23/12/14	• To allow patients who were previously ineligible for DMARD withdrawal owing to failure to meet ACR/EULAR Boolean remission (i.e. prior to substantial amendment 1) to be re-recruited to the study should they so wish.
Substantial amendment 2	23/7/15	• Collection of research blood samples from patients in remission at month one visit • Update to clinician information sheets to reflect change to DAS28-CRP remission criterion
Substantial amendment 3	23/8/16	• Change from microarray to RNAseq technology for analysis of differential gene expression • Reduction of recruitment target from 110 to 90 patients • Minor changes to protocol wording to explicitly state that serum samples may be sent to external parties for processing where possible (already included in patient consent form)

this change. Furthermore, higher-than-anticipated rates of eligibility for DMARD-cessation allowed a further reduction in recruitment target from 110 to 90 patients (see Methods 3.4).

3.8 Healthy control participants

In order to provide a control group for subsequent transcriptomic analyses, four healthy participants were recruited. Each participant donated blood at four time points to mirror those of the patient participants – i.e. baseline, month 1, month 3 and month 6. Healthy participants donated blood between 9am and 1pm where possible to minimise circadian variation in

laboratory samples. Blood was left to stand at room temperature to mimic the equivalent period of transit for patient samples, before being processed for CD4 T cell extraction and subsequent downstream RNA/DNA applications using identical protocols to those of the patient participants.

3.9 Laboratory procedures

The reagents and equipment used in the laboratory procedures together with manufacturer and catalogue number details are listed in Appendix C. All procedures were performed at room temperature using aseptic technique in a positive-pressure laminar flow tissue culture hood unless otherwise stated.

3.9.1 $CD4^+$ T cell Isolation

$CD4^+$ T cells were isolated from 27ml of whole blood drawn into three 9ml ethylenediaminetetraacetic acid (EDTA) tubes following the protocol of Pratt (2011). Briefly, monocytes were first depleted by the use of an anti-CD34 antibody (Rosettesep® human monocyte depletion cocktail) that crosslinks monocytes to erythrocytes forming immunorosettes, which were separated by centrifugation after the addition of the erythrocyte aggregation agent HetaSep®. The supernatant was then collected and the remaining $CD4^+$ T cells were positively selected by automated anti-CD4 antibody-based magnetic isolation (Easisep® whole blood $CD4^+$ selection kit and Robosep® automated cell separator). By the prior removal of $CD4^{lo}$ expressing monocytes this extraction method is able to obtain $CD4^+$ T cell purities of over 98%, compared to approximately 90% for single step column-based immunoprecipitation methods (Pratt *et al.*, 2012). After cell counting (Section 3.9.6**)**, 2 x 10^5 cells were transferred to a single well of a 96-well V-bottom plate for flow cytometry processing (Methods 3.9.7).

3.9.2 $CD4^+$ T cell lysis

Purified CD4+ T cell isolates were transferred to 30ml universal tube and washed by addition of cold (4°C) calcium- and magnesium-free Hanks balanced salt solution (HBSS) + 1% foetal calf serum (FCS) to a total volume of 25ml. The tube was then centrifuged at 400g for 7 minutes at 4°C, after which the supernatant was removed by aspiration using a vacuum-driven

glass pipette. In an RNase-free open workbench area, the pellet was then resuspended in cold (4°C) Qiagen Buffer RLT Plus + 1% β-mercaptoethanol dependent on the number of T cells to be lysed; if <5 million cells then 350µL of Buffer/ β-mercaptoethanol was added, whereas if ≥5 million cells then 600µL of Buffer/β-mercaptoethanol was added (in line with the manufacturer's protocol). The suspension was thoroughly mixed by pipetting and vortexing before transfer to a QIA-shredder column. Lysis of the T cells was completed by centrifugation of the column at 13,000g for 2 minutes, and the lysate was then stored at -80°C.

3.9.3 Peripheral blood mononuclear cell isolation

PBMCs were isolated from 18ml of whole blood drawn into two 9ml EDTA tubes. The blood was diluted in HBSS + 2mM EDTA to a total volume of 40ml. The diluted blood was then split to two 20ml volumes that were layered by slow pipetting above 15ml of Lymphoprep® within a 50ml centrifuge tube. The layered tubes were then centrifuged at 895g for 30 minutes with minimal acceleration and deceleration speeds to maintain layering of the sample. After centrifugation, PBMCs were removed from the interface layer using a Pasteur pipette and transferred to a fresh 50ml centrifuge tube. The PBMCs were then immediately washed in cold (4°C) HBSS + 1% FCS to a total volume of 50ml, and centrifuged at 600g for 7 minutes at 4°C to remove any residual Lymphoprep®. The supernatant was discarded, and the pellet resuspended in 50ml of cold (4°C) HBSS + 1% FCS before centrifuging at 250g for 7 minutes at 4°C to remove any platelets. The supernatant was discarded, and the pellet resuspended in 7ml of cold (4°C) HBSS + 1% FCS and strained through a 70µm nylon filter to a new 50ml centrifuge tube(to exclude clumped cells), which was kept on ice for immediate cell counting. After cell counting, 2 x 10^5 cells were transferred to a well of a 96-well V-bottom plate and stored at 4°C for flow cytometry analysis (Methods 3.9.7). The remaining volume was centrifuged at 400g for 7 minutes at 4°C, and the supernatant discarded. The pellet was then resuspended in freezing medium (90% FCS + 10% dimethyl sulphoxide [DMSO]) and transferred to cryovials such that each aliquot contained no less than 5 million cells. The cryovials were then frozen at -80°C in a polystyrene foam box before transfer to long-term Biobank storage at -150°C.

3.9.4 Plasma separation

Blood drawn in to a single 9ml EDTA tube was centrifuged at 1,800g for 12 minutes. The supernatant (i.e. plasma) was then carefully removed using a Pasteur pipette and transferred to a 30ml universal tube. Plasma proteases were then inhibited by the addition of one cOmplete mini protease cocktail inhibitor tablet, which was dissolved within the sample. The resultant volume was then divided to 1ml aliquots, which were frozen at -80°C for future analysis.

3.9.5 Serum separation

Blood drawn in to a single 8ml serum separator clot activator tube was centrifuged at 1,800g for 12 minutes. The supernatant (i.e. serum) was then carefully removed by pipetting and divided to 1ml aliquots, which were frozen at -80°C for future analysis.

3.9.6 Cell counting

PBMCs and $CD4^+$ T cells were counted within 10µL of diluted sample placed underneath a cover slip mounted upon a Bürker haemocytometer chamber. The number of cells within 25 squares was then manually counted using a light microscope. The total number of cells present in the sample was calculated as per Formula 3.1.

Formula 3.1 – calculation of cell number by Bürker haemocytometer chamber counting.

$$Total\ cells = [volume\ (ml)] \times [dilution\ factor] \times [number\ of\ cells\ in\ 25\ squares] \times 10^4$$

3.9.7 $CD4^+$ purity check by flow cytometry

Unfixed samples of PBMCs and $CD4^+$ T cells stored in 96-well V-bottom plates at 4°C were stained and analysed by flow cytometry within 18 hours of sample isolation. Firstly, the plate was centrifuged at 400g for 3 minutes and the supernatant removed by flicking. Cells were then resuspended in 50µL of flow cytometry antibody mixture by thorough pipetting and incubated in the dark for 30 minutes at 4°C. After this, 100µL of flow cytometry buffer was added to each well and the plate then centrifuged at 400g for 3 minutes. After removal of the supernatant by flicking, the stained cells were then resuspended in 150µL of flow cytometry buffer and the plate centrifuged at 400g for 3 minutes. After removal of the supernatant by flicking, the cells were resuspended in 200µL of flow cytometry buffer and transferred to

individual cytometry tubes. Flow cytometry data was recorded using a FACSCanto-II cytometer and FACSDiva software (Becton Dickinson Biosciences, San Jose, California, USA). Analysis of flow cytometry data was performed using FlowJo software (FlowJo LLC Data Analysis Software, Ashland, Oregon, USA). PBMC samples were first gated on side-scatter area (SSC-A) and width (SSC-W) to identify singlets, which were then gated on SSC-A and forward-scatter area (FSC-A) to exclude debris. The resulting population was then gated on compensated CD3 and compensated CD4 to identify $CD3^{+}CD4^{+}$ T cells, SSC-A and compensated CD14 to identify $CD14^{+}$ monocytes, and SSC-A and compensated CD19 to identify $CD19^{+}$ B-cells. These gates were then applied to the $CD4^{+}$ T cell isolate matched to the individual patient where available to assess purity (Figure 3.4).

3.9.8 TEMPUS™ tube storage

Blood was drawn into TEMPUS™ tubes (3ml blood per tube) and stored at -80°C for future analysis.

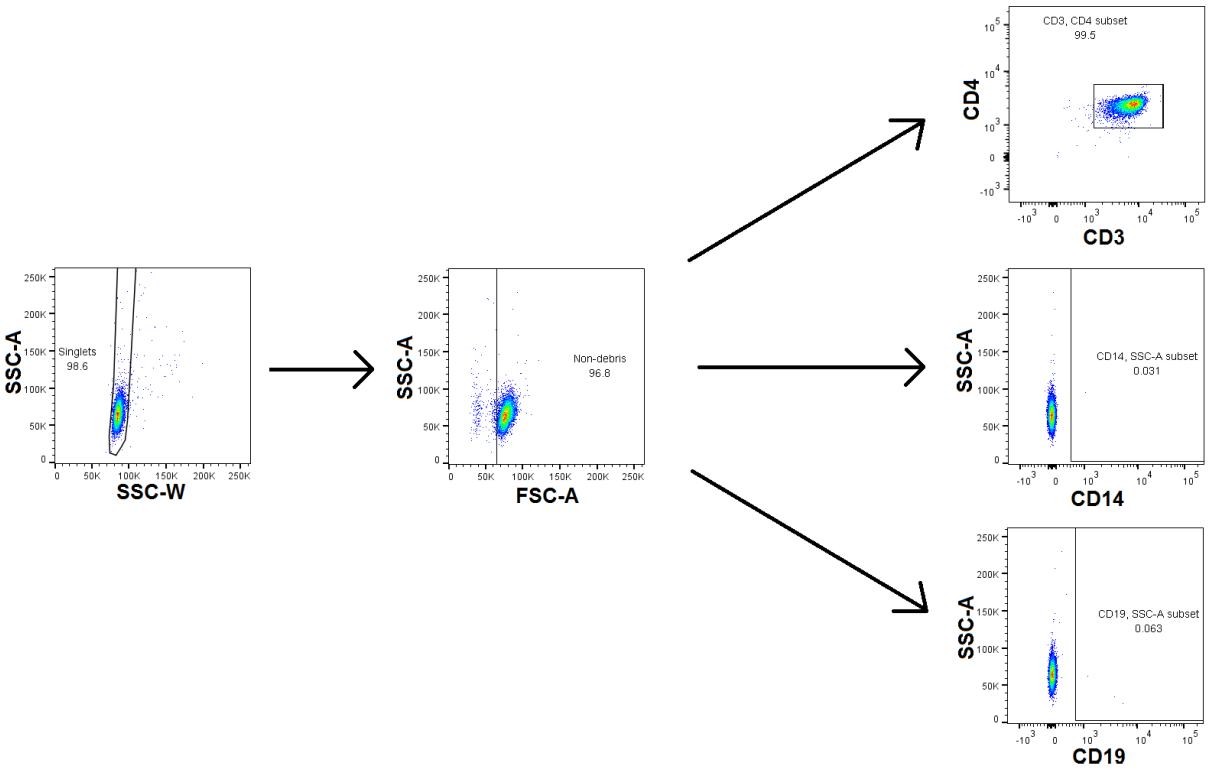


Figure 3.4 – Gating strategy for analysis of CD4+ T cell purity by flow cytometry. FSC-A: forward-scatter area; SSC-A: side-scatter area; SSC-W: side-scatter width.

3.9.9 CD4+ T cell RNA extraction

Frozen CD4+ T cell lysates (see Methods 3.9.2) were thawed at room temperature in an RNase-free open workbench area. Thawed lysates were mixed by pipetting and transferred to RNase-free conical-bottom 2ml microcentrifuge tubes. In order to remove residual magnetic nanoparticles remaining from the CD4+ T cell isolation procedure, the lysates were centrifuged at maximum speed (13000 rpm) for 2 minutes. The supernatant was then carefully removed using a P1000 pipette (taking care not to disturb the pellet of magnetic nanoparticles at the bottom of the tube) and transferred to a Qiagen AllPrep DNA Mini spin column placed within a 2ml collection tube. The sample was then centrifuged at maximum speed (13000 rpm) for 30 seconds, following which the column was placed in a new 2ml collection tube and stored at 4°C for processing (see Methods 3.9.10) after completion of the RNA extraction protocol below.

The collection tube containing the flow-through from the DNA column was then processed according to the manufacturer's instructions, including on-column DNase digestion and all steps designated as 'optional' according to the manufacturer's instructions. Final purified RNA was eluted from the RNeasy Mini spin column using a volume of RNase-free water determined by total number of CD4+ T cells present in the initial sample (see Methods 3.8.6) as follows: 30µL for $<5 \times 10^6$ cells, or 40µL for $\geq 5 \times 10^6$ cells. The amount of RNA present in each sample was then quantified using a NanoDrop™ ND1000 ultraviolet (UV) spectrophotometer (Thermo Fisher Scientific), with 2µg of each sample (or the total sample if <2µg) stored at -80°C for RNA sequencing processing (see Methods 3.9.11). Any remaining RNA solution was divided in to 1µg aliquots and stored at -80°C for potential future analysis.

3.9.10 CD4+ T cell DNA extraction

Immediately following completion of the RNA extraction protocol, the DNA Mini spin column (see Methods 3.9.5) was processed according to the manufacturer's instructions. Final purified DNA was eluted from the DNA Mini spin column using 200µL of Buffer EB. The amount of DNA present in each sample was then quantified using a NanoDrop™ ND1000 UV spectrophotometer (Thermo Fisher Scientific). The samples were then divided to three equal aliquots (66.3µL volume) and stored at -80°C for potential future analysis.

3.9.11 Next-generation RNA sequencing (RNAseq)

The laboratory processing detailed in this section 3.9.11 was performed by Raf Hussain and Jonathan Coxhead at the Newcastle University Core Genomics Facility.

The quantitiy and estimated RNA integrity (RINe) of RNA samples was measured by gel electrophoresis using a Tapestation™ 4200 machine (Agilent). Following quantification, 1.5µg of total RNA per sample was used for RNAseq processing; where total RNA < 1.5µg, the entire sample was used. Total RNA was processed using the TruSeq™ Stranded mRNA Library Prep Kit (Illumina), according to the 'High Sample Protocol' section of the manufacturer's instructions. First, messenger RNA (mRNA) was enriched from the purified total RNA by poly-A selection using poly-T oligo attached magnetic beads in two rounds of purification. Enriched mRNA was then fragmented by heating with magnesium cations, and then incubated with reverse transcriptase to synthesise first strand cDNA for each sample. This step was performed in the presence of Actinomycin D to prevent DNA-dependent synthesis of a second strand. The mRNA was then degraded with RNase and second strand cDNA was then synthesised by incubation with DNA Polymerase I. Double-stranded cDNA was then isolated using solid phase reversible immobilisation (SPRI) paramagnetic beads (Agencourt™ AMPure™ XP beads, Beckman Coulter Genomics), after which the 3' ends were adenylated to facilitate sequencing adaptor binding. The Illumina sequencing adaptors contained three key functional elements: an amplification element required for amplification of the cDNA by polymerase chain reaction, a sequencing element required for the sequencing reaction, and a unique index element to allow identification of each individual patient sample. cDNA that had successfully ligated with adaptors was selectively amplified by polymerase chain reaction. Amplified cDNA was then diluted to equimolar concentrations and pooled before sequencing.

RNA sequencing was performed using an Illumina NextSeq™ 500 in high-output mode. This configuration delivered 400 million reads over 75 cycles for 40 samples loaded across 4 lanes per flow cell. Sequencing was performed in batches across 4 separate flow cell sequencing runs. Samples were allocated to sequencing batches such that computational correction for any batch-to-batch variation at the level of either the RNA extraction (6 batches) or RNA sequencing (4 batches) could be achieved, according to a predetermined experimental design using the duplicate correlation command of the 'limma' Bioconductor/R package (v3.32.5) (Ritchie *et al.*, 2015). This sequencing batch allocation was performed by Andrew Skelton, Experimental Scientific Officer, Bioinformatics Support Unit, Newcastle University. Using

this approach, it was possible to sequence all 136 CD4+ T cell samples to a depth of 10 million reads per sample, with 75bp single-end reads.

3.9.12 Multiplex cytokine/chemokine electrochemiluminescence assays

Previously separated serum samples stored at -80°C (see Methods 3.8.5) were thawed at 37°C, mixed by vortexing and then centrifuged for 2 minutes at maximum speed (13000 rpm) to separate any contaminating debris. Serum was then transferred to 96-well V-PLEX™ plates (MesoScale Discovery) and processed according to the manufacturer's instructions. The volumes of sample loaded per well, together with the fold dilution and dilution method for each plate is detailed in Table 3.7, as specified by the manufacturer. The assays included on each plate are detailed in Table 3.8.

Owing to their large number, samples were processed across pairs of each plate type. Each pair of plates was processed on the same day to minimise variation between plates for each set of analytes. Although V-PLEX™ plates are specifically designed and certified to have minimal variation in assay performance between separate plates of the same lot number, additional steps were taken to minimise any potential for plate-to-plate variation to affect data analysis. First, for each plate pair, all baseline samples were included together on the same individual plate. This allowed for comparison of baseline samples between patients independent of any effect of plate-to-plate variation. Secondly, it was ensured that all samples from each individual patient were included together on the same individual plate. This

Table 3.7 – Volume, fold dilution and dilution method for samples according to V-PLEX™ plate.

V-PLEX™ plate	Sample volume (µL)	Fold dilution	Dilution method
Cytokine panel 1 (human)	25	2	In-plate
Chemokine panel 1 (human)	12.5	4	In-plate
Proinflammatory panel 1 (human)	25	2	In-plate
Th17 panel 1 (human)	12.5	4	In-plate
Vascular injury panel 2 (human)	10	1000	Three serial 10-fold dilutions prior to addition to plate

allowed for comparison between study time points at the level of each individual patient independent of any effect of plate-to-plate variation in absolute quantification.

Following processing, plates were immediately analysed by electrochemiluminescence (ECL) using a MESO™ QuickPlex SQ120 (Meso Scale Diagnostics, LLC.) according to

Table 3.8 – Cytokine and chemokine assays by V-PLEX™ plate type. CCL: C-C motif chemokine ligand; CXCL: C-X-C motif chemokine ligand; GM-CSF: granulocyte-macrophage colony-stimulating factor; hsCRP: high-sensitivity C-reactive protein; ICAM: intercellular adhesion molecule; IFN: interferon; IL: interleukin; MCP: monocyte chemoattractant protein; MDC: macrophage-derived chemokine; MIP: macrophage inhibitory protein; IP-10: interferon-γ induced protein 10kDa; SAA: serum amyloid A; TARC: thymus and activation-regulated chemokine; TNF: tumour necrosis factor; VCAM: vascular cell adhesion molecule; VEGF: vascular endothelial growth factor.

V-PLEX™ plate	**Assays**	
Cytokine panel 1 (human)	GM-CSF IL-1α IL-5 IL-7 IL-12/23 p40 subunit	IL-15 IL-16 IL-17A TNF-β VEGF-A
Chemokine panel 1 (human)	Eotaxin (CCL11) MIP-1β (CCL4) Eotaxin-3 (CCL26) TARC (CCL17) IP-10 (CXCL10)	MIP-1α (CCL3) IL-8(HA) MCP-1 (CCL2) MDC (CCL22) MCP-4 (CCL13)
Proinflammatory panel 1 (human)	IFN-γ IL-1β IL-2 IL-4 IL-6	IL-8 IL-10 IL-12p70 subunit IL-13 TNF-α
Th17 panel 1 (human)	IL-17A IL-21 IL-22 IL-23	IL-27 IL-31 MIP-3α (CCL20)
Vascular injury panel 2 (human)	SAA hsCRP	VCAM-1 ICAM-1

themanufacturer's instructions. Signal – concentration curves based on serial dilutions of a known supplied standard were generated for each assay to calculate the concentration of analytes in each sample using the Discovery Workbench™ software version 4.0 (Meso Scale Diagnostics, LLC.). For each assay, the upper limit of detection (ULOD) for was defined as the concentration of the highest calibrator, and the lower limit of detection (LLOD) was defined as 2.5 x standard deviation above the lowest calibrator concentration. Analytes with an ECL signal corresponding to a calculated concentration above the ULOD were assigned a calculated concentration equal to the ULOD. Analytes with an ECL signal corresponding to calculated concentration below the LLOD were assigned a calculated concentration equal to the LLOD. Where a sample was duplicated on the same plate (for internal quality control purposes), the mean calculated concentration value was used for analysis.

Owing to time constraints imposed by the PhD programme, MSD plates were processed before the final clinical study visit, though after recruitment had closed. Therefore, whilst all baseline samples were processed, there were some longitudinal follow-up samples for patients who remained under follow-up in the study at the time of plate processing which were not available for analysis.

3.10 Data analysis

Two general approaches to analysing data in this study were possible, dependent on the outcome measure of interest. One approach was to categorise patients by flare status – i.e. a binary outcome of 'flare' vs. 'remission' for each patient upon their completion of the study. A major drawback of this intuitive approach was that it makes no distinction between those patients who flared early versus those who flared at a later time point following DMARD cessation, and thus did not make full use of the richness of the available study dataset. Nevertheless, it allowed standard statistical tests to be performed (e.g. comparison of means using parametric tests), which form the basis of many of the well-optimised analysis pipelines that have been developed for application in computationally-intensive large datasets such as those generated by next-generation RNAseq. For this reason, the core analyses in the RNAseq results chapter were based on analyses that dichotomised patients by flare as a binary outcome measure.

An alternative approach to data analysis was to use time-to-flare as the outcome measure. In this approach, it was possible to use more powerful survival analysis approaches such as Cox

regression, which are able to account for differences in the time between DMARD cessation and onset of flare. In this approach, patients who remain in remission at the end of the study were treated as being censored at the time of their final study visit. Owing to its superior statistical power in this setting, the majority of analyses of non-transcriptomic data were performed using this survival-based approach.

3.10.1 Analysis of clinical, ultrasound and cytokine data

Analysis of clinical, ultrasound and cytokine data was performed in the R environment, version 3.3.2 (R Core Team, 2016), with additional packages installed as listed in Table 3.9.

Table 3.9 – Additional R packages used for data analysis.

Package name	Package version	Citation
biomaRt	2.32.1	Durinck *et al.* (2009)
checkmate	1.8.2	Lang (2016)
dplyr	0.7.2	Wickham *et al.* (2017)
forestplot	1.7	Gordon and Lumley (2017)
futile.logger	1.4.3	Rowe (2016)
ggpubr	0.1.2	Kassambara (2017)
ggplot2	2.2.1	Wickham (2009)
irr	0.84	Gamer *et al.* (2012)
limma	3.32.5	Ritchie *et al.* (2015)
lpSolve	5.6.13	Berkelaar (2015)
magrittr	1.5	Bache and Wickham (2014)
MASS	7.3-47	Venables and Ripley (2002)
ordinal	6-28	Christensen (2015)
pls	2.6-0	Bjørn-Helge *et al.* (2016)
pROC	1.10.0	Robin *et al.* (2011)
rmcorr	0.1.0	Bakdash and Marusich (2017)
survival	2.41-3	Therneau (2015)
survminer	0.3.1	Kassambara and Kosinski (2017)
tximport	1.4.0	Soneson *et al.* (2015)
VennDiagram	1.6.17	Chen (2016)

Analyses of all three types of data (clinical, ultrasound and cytokine) was performed according to the structure detailed below. All Cox regression models used Breslow approximation for handling of tied survival times (Therneau, 2015).

1. The quality of data was assessed, and where necessary, low quality data was excluded.
2. The distributions of data were summarised by descriptive statistics and, for continuous data, were visualised by standard methods including boxplots, Q-Q plots and histograms.
3. Where the distributions of data were skewed, appropriate transformations of data groups was performed (e.g. natural logarithmic transformation).
4. The association of each individual variable at baseline with time-to-flare was assessed by univariate Cox regression using the 'survival' package.
5. Baseline variables were selected based on their univariate p-value to be taken forward to a multivariate Cox regression model. For clinical, ultrasound and cytokine data, an elevated significance threshold ($p<0.2$) was used in order to reduce the risk of type II error at this preliminary stage, in keeping with established precedent (Dales and Ury, 1978; Mickey and Greenland, 1989). In comparison, a more stringent significance threshold ($p<0.001$) was utilised for RNAseq univariate analysis (see Methods 3.10.2) in reflection of the several log-fold greater number of variables analysed.
6. Backward stepwise variable selection based on the Akaike information criterion (AIC) using the 'stepAIC' function of the 'MASS' package was used to create a stepwise multivariate Cox regression model. The regression coefficient (equivalent to the natural logarithm of the hazard ratio) and its corresponding 95% confidence interval for each variable was visualised in a forest plot format using the package 'forestplot' (and its dependent packages 'checkmate' and 'magrittr').
7. Patients were dichotomised by the levels of variables measured at baseline. In the case of binary variables, patients were dichotomised simply by presence or absence of the variable at baseline. For continuous variables, thresholds were determined by receiver operating characteristic (ROC) analysis (using the 'pROC' package). For each continuous variable, two optimum thresholds were set to maximise negative predictive value (NPV) and positive predictive value (PPV) for flare, corresponding to biomarker thresholds for remission and flare respectively. Confidence intervals for these metrics

and the area under the ROC curve (ROC_{AUC}) were calculated by bootstrapping (2000 replicates) and the DeLong procedure (DeLong *et al.*, 1988) respectively.

8. Variables that were significantly associated with time-to-flare in the stepwise multivariate Cox regression model at the $p<0.05$ threshold were then combined in composite scores, weighted by their respective coefficients in multivariate Cox regression analysis.

9. Survival curves were compared between the dichotomised groups (using the 'survminer', and its dependent 'ggpubr' and 'ggplot2', packages) by the log-rank test as a measure of their utility in predicting time-to-flare after DMARD cessation.

10. Finally, where data allowed, the relationship between longitudinal changes in variables at the individual patient level and time-to-flare were explored.

A key assumption of Cox regression is that that the hazard function for each stratum of the dataset is proportional over time – in other words, the proportional change in hazard attributable to a given variable must be constant across all time points. In a seminal paper, Schoenfeld defined residuals of the proportional hazards model that, if proportionality of hazards is true, show no significant correlation with time (Schoenfeld, 1982). Following this approach, proportionality of hazards was tested for each individual variable in univariate and stepwise multivariate Cox regression models by plotting scaled Schoenfeld residuals against time, and fitting a smoothed spline curve with four degrees of freedom using the survival package (Therneau, 2015). Proportionality of hazards was assumed if no significant association at the 0.05 threshold was observed between scaled Schoenfeld residuals versus time.

The Akaike information criterion (AIC) was used to drive stepwise backwards selection of variables in the multivariate Cox regression models. The AIC is a well-established method of nested model selection, which penalises goodness of model fit by the number of variables contained within the model (Bozdogan, 1987). The best model, displaying the optimum balance between predictive utility and number of variables, is distinguished the lowest AIC score. In backwards stepwise selection, a set of reduced models is created by dropping each of variables in turn from the starting model. The model with the lowest AIC is then taken forward to the next round of variable selection, and the process repeated until no further reduction in AIC is possible – the model with the lowest AIC in the final step is accepted as the final stepwise model. Stepwise backwards variable selection of Cox regression models based on AIC has been previously described in analyses of differential gene expression in

cancer studies, using both microarray technology (Wozniak *et al.*, 2013) and next-generation sequencing data (Falco *et al.*, 2016; Fan and Liu, 2016).

Additional analyses were performed for the ultrasound data. Cohen's kappa statistic (Jacob, 1960) was used to quantify intra- and inter-rater agreement in ultrasound image scoring. This technique allows for adjustment of agreement in scoring that would be expected by chance alone, and thus has substantial advantage over simple measures of agreement such as percentage agreement. It is also better suited to analysis of categorical data, as opposed to intra-class correlation which is arguably better suited to analysis of continuous data (Mandrekar, 2011). Cohen's kappa was calculated for scores of each individual ultrasound image both with and without linear weighting using the 'irr' package (and its dependent 'lpSolve' package) – linear weighting was used to account for the ordinal nature of the ultrasound scores. The strength of agreement measured by the kappa statistic was interpreted as per the approach of Landis and Kock (1977), summarised in Table 3.10. For assessment of inter-rater agreement, scores recorded by the observers at the time of image review were compared rather than with those recorded at the time of image acquisition, to allow for minimisation of any differences in image visualisation on the ultrasound machine screen versus computer display.

Although the total ultrasound scores were made more continuous by virtue of summation, they were nevertheless derived from the individual ordinal ultrasound scores. Therefore, in order to appropriately acknowledge the ordinal derivation of these data, ordinal logistic regression was performed to assess the correlation between clinical and ultrasound parameters at baseline using the 'ordinal' package.

Table 3.10 – Interpretation of Cohen's kappa according to Landis and Kock (1977).

Cohen's kappa value	**Strength of agreement**
<0	Poor
0.00 – 0.20	Slight
0.21 – 0.40	Fair
0.41 – 0.60	Moderate
0.61 – 0.80	Substantial
0.81 – 1.00	Almost perfect

3.10.2 Analysis of next-generation RNAseq data

Computer programming scripts, bioinformatics analysis and figure generation for all RNAseq data (with the exception of multivariate Cox regression and ROC analysis) were performed by Andrew Skelton, Experimental Scientific Officer, Bioinformatics Support Unit, Newcastle University. I am grateful to Andrew for his help in the writing of the methods for this section 3.10.2.

The quality of raw RNA sequencing data within each individual FASTQ file was assessed using the FastQC tool (v0.11.5) (Andrews, 2016). Quality metrics measured for each sample included number of sequences, sequencing length distribution, Phred score distribution by sequence position, percentage GC content, and percentage adaptor sequence contamination. Graphical visualisations of quality metrics were produced using the MultiQC tool (v0.7) (Ewels *et al.*, 2016). Correlation between laboratory sample processing time and RIN^e measurements was analysed using the 'rmcorr' package, accounting for clustering effect of repeated measurements at the individual patient level.

Transcript abundance was estimated from the raw FASTQ files using Kallisto software (v0.43.0) (Bray *et al.*, 2016) ran in single-end mode, and using an index based on Gencode v24 transcript sequences (Harrow *et al.*, 2012). Read counts were imported to R (v3.4.1) using the 'tximport' package, removing genes with mean read count of <60. The 'dplyr' package was used to aid data manipulation. Gene annotation using the Ensembl GRCh38 assembly (Yates *et al.*, 2016) was performed using the 'biomaRt' package. Read counts were normalised using trimmed mean of M-values normalisation (TMM), and were then logarithmically transformed to log counts per million (logCPM) using the variance modelling at the observational level (voom) approach described by Law *et al.* (2014). This transformation effectively renders the data to be similar to the output of microarray platforms, thus allowing for well-established and validated microarray packages to be utilised (Law *et al.*, 2014).

Differential gene expression (DGE) between binary contrast groups was analysed within a linear model using the 'limma' package, applying correction for RNA sequencing batch and $CD4^+$ T cell purity. For longitudinal analyses, the 'duplicateCorrelation()' function of the 'limma' package was used to account for pairing of patient samples. Statistical significance of DGE was assessed using the moderated t-test with empirical Bayes moderation, with false-discovery rate correction according to approach of Benjamini and Hochberg (1995). DGE which exceeded a 1.5 fold-change threshold at a corrected p value of <0.05 was considered as

significant. Exploratory analyses were performed using a relaxed significance threshold (unadjusted $p<0.001$).

The association between baseline gene expression and time-to-flare was analysed by univariate Cox regression across all genes using the 'survival' package. Genes which were significantly (unadjusted $p<0.001$) associated with time-to-flare were then advanced to multivariate Cox regression and ROC analysis following the procedure detailed in Methods 3.10.1 (steps 6 – 8). Volcano plots were generated using the 'ggplot2' package. Venn diagrams were produced using the 'VennDiagram' package, and its dependent package 'futile.logger'.

3.11 Ethical approval and study governance

The study was approved by an NHS Health Research Authority (HRA) Research Ethics Committee (REC) and received NHS Research & Development approval before recruitment of the first participant (REC reference 14/NE/1042). The study was registered online at clinicaltrials.gov (NCT02219347). Professor John Isaacs was the Chief Investigator for the study, and the Newcastle upon Tyne Hospitals NHS Foundation Trust acted as study Sponsor. A Trial Steering Committee comprising of BioRRA investigators and researchers from outside the study team met at least once every six months during the active recruitment period to review study progress, with reports made available to the study Sponsor, NHS REC and the Wellcome Trust (as study funder). Healthy participants were recruited as part of an approved project within the Newcastle Academic Health Partners Bioresource (NAHBP), under the ethical approval of Newcastle University. All patients and healthy participants provided written informed consent before participation in the study, which was conducted in accordance with the Declaration of Helsinki (World Medical Association, 2013).

Chapter 4. Results 1 – Clinical data

4.1 Introduction

Rheumatoid arthritis was historically a disease with only limited treatment options, with irreversible joint destruction and disability an inevitable consequence for many patients. In contrast, the past two decades have witnessed remarkable therapeutic advances in terms of both novel drugs and improved treatment paradigms, including early combination DMARDs and the treat-to-target approach (see Introduction 1.3). Such has been the success of these strategies that remission is now a realistic treatment target for the majority of patients. This changing face of RA in modern clinical practice poses a novel and increasingly encountered dilemma – when is it appropriate to withdraw DMARDs in RA remission?

Such a question is clearly important, not least because of the significant side effects of DMARD therapy, and the expense and inconvenience of regular safety monitoring. The concept of DMARD withdrawal and cessation has been explored by several recent studies, with encouraging results. Of the handful of studies that have addressed complete drug-free remission in RA as a primary outcome, it appears that approximately half of patients can achieve this status following DMARD cessation (see Introduction 1.6). However, the majority of studies have explored only partial DMARD withdrawal, usually in the form of bDMARD tapering and cessation, and often as secondary or exploratory endpoints. Evidence surrounding the potential for biomarkers that can predict DFR in patients with established RA controlled with csDMARD therapy is thus lacking (see Introduction 1.7), and is the primary focus of this study.

In this results chapter I focus on the clinical aspects of the BioRRA study. The primary aim of this work was to identify baseline clinical variables that are predictive of sustained DFR versus flare following DMARD cessation. First, general clinical aspects of the study, including patient recruitment, cohort demographics, and clinical outcomes are presented. Clinical variables identified *a priori* at the study design stage are then compared between flare and remission groups, and their association with time-to-flare analysed by Cox regression. Finally, I present a composite clinical score and assess its utility in predicting flare versus DFR within the study population.

The structure of the remainder of this results chapter is as follows:

4.2 Study procedures

4.3 Quality control

4.4 Descriptive analysis

4.5 Comparison of clinical variables between flare versus remission groups

4.6 Survival analysis

4.7 Composite clinical biomarker score

4.8 Long-term clinical outcomes

4.9 Discussion

4.10 Summary

4.2 Study procedures

4.2.1 Patient recruitment

A total of 78 patients attended a baseline study visit. Of these, 3 patients did not meet the eligibility criteria and were not recruited: one was enrolled in another long-term clinical trial, and 2 patients were taking leflunomide. A further patient did not receive the patient information sheet prior to their baseline visit - as the ethical approval of the study mandated that all patients receive the information sheet at least a day in advance of their baseline visit, this patient was provided with an information sheet and their baseline visit rescheduled. Unfortunately, the patient did not attend this rescheduled appointment, and despite multiple attempts could not be contacted before the closure of the study recruitment period and hence was not enrolled in the study. A further patient had received systemic glucocorticoids within 3 months of their baseline visit – in this case, their baseline visit was rescheduled to a later date and they were subsequently successfully enrolled.

Of the 74 patients who were enrolled in the study, 30 (41%) patients did not meet the criteria for DMARD cessation. 19/74 (26%) patients failed DMARD cessation criteria owing only to the presence of PD signal on baseline ultrasound scan, 4/74 (9%) failed solely due to DAS28-CRP≥2.4, and 3 (4%) failed owing to both DAS28-CRP≥2.4 and PD signal on ultrasound. A further 5 patients, who were recruited prior to the protocol amendment to change the

remission criteria to DAS28-CRP<2.4 (see Methods 3.2), satisfied DAS28-CRP remission but did not satisfy ACR/EULAR Boolean remission and hence did not stop DMARD therapy in accordance with the protocol version in force at the time. Following the protocol amendment, ethical approval was granted to offer these patients a further study appointment using the amended remission criteria – only one patient accepted this offer, and they were subsequently eligible for DMARD cessation.

4.2.2 Patient outcomes

Of the 44 patients who stopped DMARDs, 21 patients maintained DAS28-CRP remission for the 6 months of study follow-up. One of these patients had synovitis of both ankles and a 5th MTP joint at review 176 days after DMARD cessation, demonstrated both by clinical and ultrasonographic examination. Despite clearly exhibiting objective evidence of active disease, their DAS28-CRP score (which does not include assessment of the ankles or feet) was within the remission range (1.58). Nevertheless, the patient was deemed to have experienced an arthritis flare, received an intramuscular steroid injection and was referred back to their rheumatology team for recommencement of DMARD therapy. This did not constitute a breach of the study protocol, which permits recommencement of DMARDs in those patients with PD signal at the month 6 ultrasound scan. A further 3 patients had grade 1/3 PD signal at the wrist on their month 6 ultrasound scan, but no clinical synovitis, and maintained DAS28-CRP remission. As ultrasound findings do not form part of the clinical remission criteria, these patients were classified as maintaining clinical remission for the purposes of data analysis.

A DAS28-CRP score ≥ 2.4 was recorded for 22 patients during the follow-up period, who were classified as having experienced an arthritis flare. An additional patient was reviewed 69 days after DMARD withdrawal before the first substantial protocol amendment – although this patient had a DAS28-CRP score within the remission range (1.46), they did not satisfy ACR/EULAR Boolean remission. Thus, according to the study protocol in force at the time they were treated as having experienced an arthritis flare, and were referred back to their rheumatology team for recommencement of DMARD therapy. Therefore, had this visit occurred after implementation of the first substantial amendment, this patient would in effect have been lost to further follow-up. Nevertheless, the remainder of all patients successfully completed the follow-up period.

In summary, of the 44 patients who discontinued DMARD therapy, 23 (52%) were classified as experiencing an arthritis flare, 20 (45%) maintained DFR and 1 (2%) was effectively lost to follow-up (Figure 4.1). In order to maintain consistency in data analysis, two separate approaches have been implemented to account for this latter patient dependent on the outcome measure of interest. For analyses where the outcome is binary (i.e. flare vs. remission), the patient has been excluded as it is unclear whether they would have maintained DAS28-CRP remission to the end of the 6 month study period. For analyses where the outcome is time-to-flare, the patient has been analysed as being censored in remission after 69 days follow-up.

4.2.3 Adverse events

Arthritis flare was recorded as an adverse event in 24 patients (i.e. including the patient who was classified as flare prior to the first substantial amendment). Routine influenza and pneumococcal vaccination during study follow-up were also recorded as adverse events (11 events, 10 patients) to allow for subsequent identification during longitudinal data analysis. A further 66 adverse events were recorded, none of which were judged to be a consequence of DMARD cessation (Table 4.1). There were no serious adverse events.

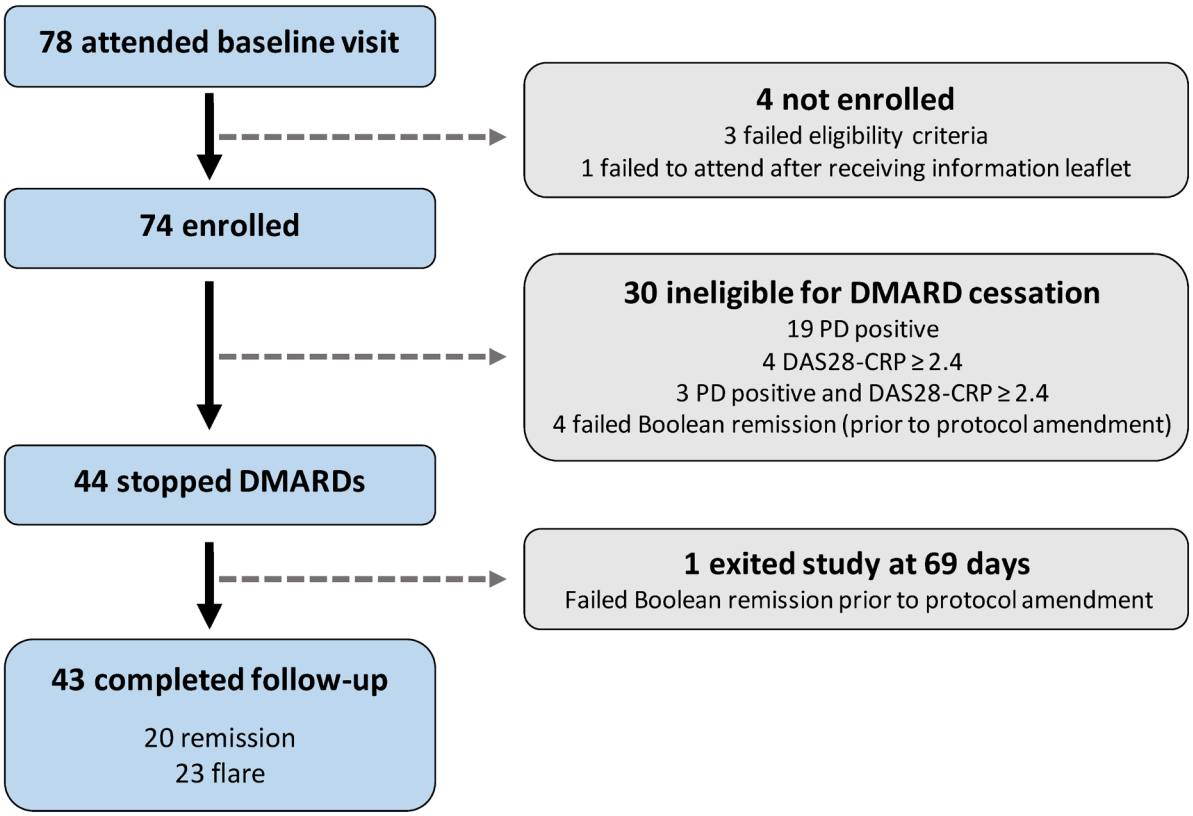


Figure 4.1 – Flow diagram showing patient recruitment and outcomes.

Table 4.1 – Adverse events occurring in the study.

Category	Adverse Event	Number of Events
Infection	Nasopharyngitis	12
	Pneumonia	2
	Skin infection	2
	Influenza-like illness	1
	Herpes zoster	1
	Oral herpes	1
	Urinary tract infection	1
Respiratory	Breathlessness	2
	Incidental finding of asbestos-related pleural plaque	1
	Nasal polyposis	1
Metabolic	Hypercholesterolaemia	3
	Increase in diabetes mellitus medications	1
Circulatory	Outpatient coronary imaging	2
	Increase in ischaemic heart disease medications	1
Musculoskeletal	Arthritis flare	24
	Elbow epicondylitis	2
	Muscle cramp	1
	Lower back pain	1
	Myalgia & lethargy following intravenous bisphosphonate	1
	Pain around knee replacement	1
	Sialadenitis	1
Skin	Actinic keratosis	3
	Basal cell carcinoma	2
	Dry skin	1
	Itch	1
Gastrointestinal	Diarrhoea	2
	Abdominal pain	2
	Irritable bowel syndrome	1
	Inguinal hernia	1
	Routine elective screening colonoscopy	1
	Fatty liver change on ultrasound	1
Ophthalmological	Red/dry eyes	2
	Elective phacoemulsification	2
	Elective ocular punctoplasty	1
Other	Influenza vaccination	10
	Fall	5
	Pneumococcal vaccination	1
	Dental extraction	1
	Fatigue	1
	Hay fever	1

Of note, one patient was treated with a 7-day course of oral prednisolone for nasal polyposis at 5 months after DMARD cessation. This patient subsequently maintained DFR at their month six follow-up visit. This was annotated on the study database to allow for subsequent identification during longitudinal data analysis.

4.3 Quality control

4.3.1 Study visits

A total of 184 study visits were conducted, the timings of which are detailed in Table 4.2

All visits were performed according to the study protocol with no protocol deviations. Two patients were unable to attend their month 3 visit owing to personal/family commitments – both of these patients subsequently attended their month 6 visit as scheduled.

4.3.2 Missing data

4.3.2.a Prospectively recorded clinical data

ESR measurements were not available for 3 patients at baseline owing to insufficient blood sample (2 patients) and failure by clinical laboratory to perform test (1 patient). These values were left missing in the final dataset, with exclusion of these records as necessary in analyses based on ESR values. Aside from this, prospectively recorded clinical data were otherwise complete for all other variables.

4.3.2.b Retrospectively recorded clinical data

Medical notes were available for all patients and the quality of documentation was generally excellent. Symptom duration before first rheumatology review was not documented in three medical records. For two cases the patient-recollected value was recorded as a substitute – however, one patient could not recollect their symptom duration and this value was left

Table 4.2– Number of study visits.

Timing of visit	Number
Baseline	74
Month 1	42
Month 3	26
Month 6	23
Unscheduled (patient-requested)	19
Total	184

missing in the final dataset. Similarly, time from first rheumatology review to commencement of first DMARD was not recorded in two medical records. Both of these patients could not recall this duration, and hence these two values were left as missing in the final dataset.

Aside from the above, retrospectively recorded clinical data were otherwise complete for all other variables.

4.4 Descriptive analysis

4.4.1 Patient demographics

Demographic details of the patients who stopped DMARD therapy are listed in Table 4.3. Demographics of all 74 patients who were recruited to the study are listed in Appendix D.

Overall, the 44 patients who stopped DMARDs are largely representative of an established RA outpatient population, with a median of 5.5 years since diagnosis. Half of patients were women, slightly less than the expected 2.5:1 female:male ratio for RA. An extended period of stable disease control prior to study enrolment was suggested by the prolonged time since both last DMARD change and last steroid use (median 22.5 and 30 months respectively). Three-quarters of patients were seropositive for either RhF or ACPA in keeping with the expected proportion in RA. A similar proportion of patients had ultrasonographic evidence of erosions on the baseline ultrasound scan, in keeping with what would be expected clinically for an established RA cohort. All patients who stopped DMARDs satisfied the 2010 ACR/EULAR RA diagnostic criteria, and all patients who stopped DMARDs were Caucasian.

Systemic markers of inflammation were generally low at baseline– of note, one patient did have a significantly raised ESR (77mm/hr) though this was secondary to comorbid polyclonal hypergammaglobulinaemia as part of secondary Sjögren's syndrome, and both their CRP and DAS28-CRP were low (<5mg/L and 1.07 respectively). A further patient had an elevated ESR of 44 leading to an elevated DAS28-ESR (3.23) at baseline, though this patient satisfied both DAS28-CRP and ACR/EULAR Boolean remission. Two thirds of patients fulfilled Boolean remission – reasons for failure to achieve Boolean remission were: $VAS_{patient}$ >10/100 (11 patients), two swollen joints (3 patients) and CRP>10mg/L (1 patient). Of the 11 patients who had a $VAS_{patient}$>10/100, the median (range) score was 21 (13-35).

Table 4.3 – Demographics of the patients who stopped DMARD therapy. HCQ: hydroxychloroquine; MTX: methotrexate; SJC: swollen joint count; SFZ: sulfasalazine; TJC: tender joint count.

Demographic	Value
Number of patients stopped DMARDs	44
Satisfied 2010 ACR/EULAR RA diagnostic criteria: n(%)	44 (100%)
Age: median (IQR) [range]	66.5 (54.5 – 71.3) [35 – 82]
Female: n(%)	23 (52%)
Years since RA diagnosis: median (IQR) [range]	5.5 (3 – 11) [1 – 40]
Symptom duration in months prior to first rheumatology review: median (IQR) [range]	5 (2 – 8.5) [1 – 60]
Months from first rheumatology review to starting first DMARD: median (IQR) [range]	1 (0 – 3) [0 – 210]
Months since last steroid: median (IQR) [range]	30 (12 – 46.5) [0 – 95]
Months since last change in DMARDs: median (IQR) [range]	22.5 (12 – 48.5) [2 – 132]
Current smoker: n (%)	5 (11%)
Previous smoker: n (%)	21 (48%)
Never smoker: n(%)	18 (41%)
Weekly alcohol unit intake: median (IQR) [range]	5 (0 – 10.3) [0 – 50]
Total DMARDs since diagnosis: median [range]	2 [1 – 4]
Current MTX monotherapy: n(%)	23 (52%)
Current SFZ monotherapy: n(%)	4 (9%)
Current HCQ monotherapy: n(%)	1 (2%)
Current MTX+SFZ: n(%)	5 (11%)
Current MTX+HCQ: n(%)	8 (18%)
Current SFZ+HCQ: n(%)	1 (2%)
Current MTX+SFZ+HCQ: n(%)	2 (5%)
RhF positive: n(%)	25 (57%)
ACPA positive: n(%)	24 (55%)
RhF or ACPA positive: n(%)	32 (73%)
RhF and ACPA positive: n(%)	17 (39%)
Baseline 28 SJC: median (IQR) [range]	0 (0 – 0) [0 – 2]
Baseline 28 TJC: median (IQR) [range]	0 (0 – 0) [0 – 2]
Baseline patient VAS (mm): median (IQR) [range]	3 (1 – 10) [0 – 35]
Baseline CRP in mg/L: median (IQR) [range]	0 (0 – 0) [0 – 13]
Baseline ESR in mm/hr: median (IQR) [range]	9 (2 – 15) [1 – 77]
Baseline DAS28-CRP: median (IQR) [range]	1.07 (0.99 – 1.63) [0.96 – 2.34]
Baseline DAS28-ESR: median (IQR) [range]	1.66 (0.71 – 2.38) [0.48 – 3.23]
ACR/EULAR Boolean remission: n(%)	29 (66%)
Presence of joint erosion on baseline 7-joint ultrasound scan: n(%)	29 (70%)

Current methotrexate use was common in those patients who went on to discontinue DMARDs, reflecting the popularity of this medication in current rheumatology practice. Half of patients were taking methotrexate monotherapy, with methotrexate forming part of combination DMARD therapy in a further 15 (34%) patients. Only 6 (14%) of patients were not taking methotrexate at the time of DMARD cessation – of these patients, 3 had been prescribed methotrexate previously.

4.4.2 Distribution of arthritis flare events

Of the 44 patients who stopped DMARDs, 23 (52%) experienced an arthritis flare (defined as DAS28-CRP ≥ 2.4) in the 6 months following DMARD cessation. The median (IQR) time to flare was 48 (31.5 – 86.5) days, and ranged from 16 – 187 days (Figure 4.2). The median flare-free survival was 176 days (95% confidence interval: lower limit 83 days, upper limit undefined) (Figure 4.3).

The median (IQR) DAS28-CRP value at the time of flare was 3.12 (2.62 – 3.94), and ranged from 1.58 – 4.51 (Figure 4.4). Note that one patient was classified as experiencing a flare with a DAS28-CRP value of 1.58 owing to the presence of synovitis in the ankles and feet (see Results 4.2.2) – removing this patient gave a DAS28-CRP range of 2.45 – 4.51 at the time of flare.

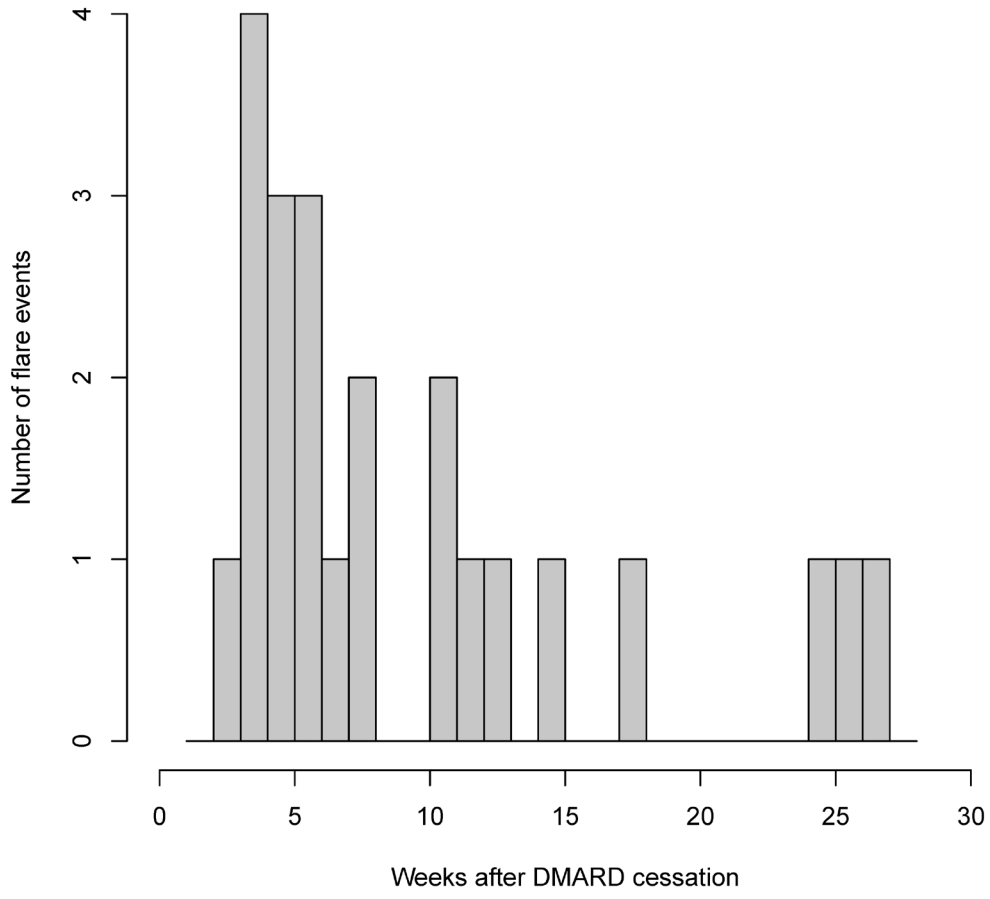


Figure 4.2 – Distribution of flare events by weeks after DMARD cessation.

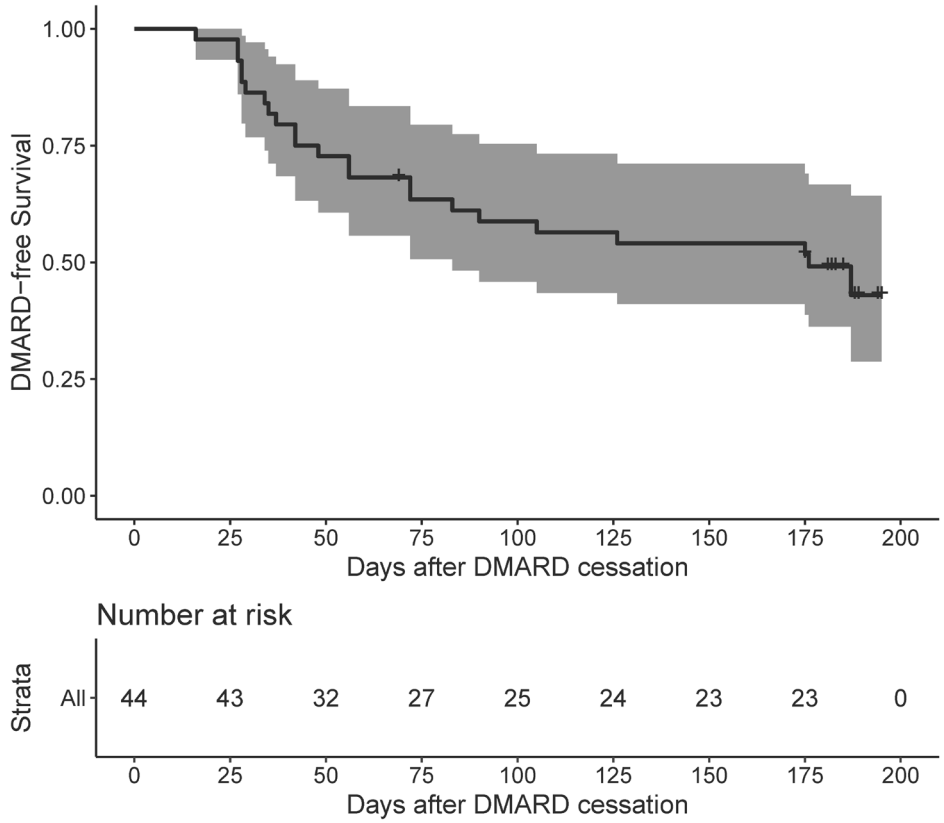


Figure 4.3 – Kaplan-Meier plot of flare-free survival for all patients who stopped DMARDs. Shaded region depicts 95% confidence interval of survival estimate.

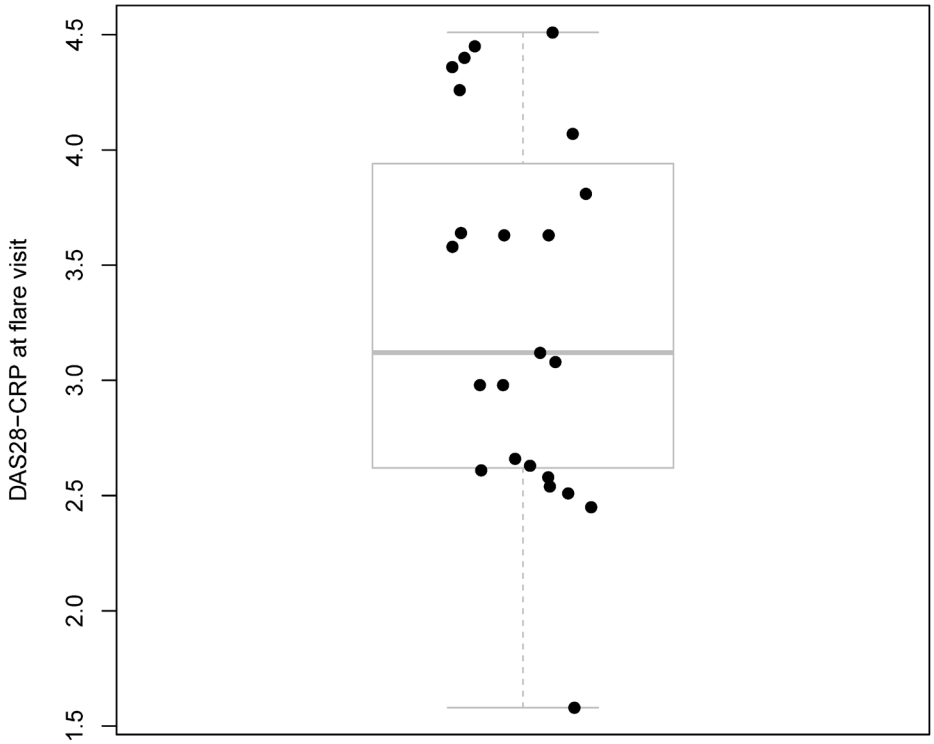


Figure 4.4 – Distribution of DAS28-CRP score at the time of flare.

4.5 Comparison of clinical variables between flare versus remission groups

Patients who stopped DMARDs were stratified by subsequent flare status (n=43), and the statistical significance of differences in values of baseline clinical parameters between flare vs. remission groups was assessed by univariate binary logistic regression (Table 4.4).

The 13 variables with a univariate p-value<0.2 were then entered in a multivariate binary logistic regression model with backwards stepwise variable selection based on the Akaike information criterion (AIC). After 7 rounds of selection, 6 variables remained in the final stepwise multivariate binary logistic regression model (Table 4.5). Three variables were significantly ($p<0.05$) associated with increased occurrence of arthritis flare following DMARD cessation, namely: failure to satisfy ACR/EULAR Boolean remission at baseline, current methotrexate therapy and shorter time since last change in DMARD therapy, though the effect size of the latter was negligible (Figure 4.5).

4.6 Survival analysis

The association between baseline clinical variables and time to flare following DMARD cessation was analysed by univariate Cox regression for all 44 patients who stopped DMARDs (Table 4.6). Proportionality of hazards was assessed for each univariate variable by correlation of scaled Schoenfeld residuals with transformed flare-free survival time (see Methods 3.10.1). No significant departure from proportional hazards was observed for any of the variables.

The 15 variables with a univariate p value < 0.2 were then entered simultaneously in a multivariate Cox regression model. Stepwise backward selection based on AIC was then performed using the same variables to fit a stepwise Cox regression model. After 6 rounds of selection, 9 variables remained in this stepwise model (Table 4.7).

Table 4.4 – Association of clinical variables with arthritis flare following DMARD cessation by univariate binary logistic regression. For continuous variables, logistic regression coefficients (B) and odds ratios (OR) are calculated for a 1 unit change in that variable. HCQ: hydroxychloroquine; MTX: methotrexate; SFZ: sulfasalazine.

Variable	Value in flare group (n = 23)	Value in remission group (n = 20)	B	OR_{flare}	95% CI OR_{flare}	Univariate p-value
Median current number of DMARDs	1	1	1.272	3.57	1.00 – 12.64	0.049
Median months since last change in DMARDs	20	41.5	-0.022	0.98	0.96 – 1.00	0.052
Median cumulative number of DMARDs since diagnosis	2	1.5	0.716	2.05	0.98 – 4.28	0.057
Proportion ACR/EULAR Boolean remission	0.52	0.8	-1.299	0.27	0.07 – 1.07	0.063
Proportion both RhF and ACPA positive	0.52	0.25	1.186	3.27	0.89 – 12.03	0.074
Proportion current MTX	0.96	0.75	1.992	7.33	0.78 – 69.24	0.082
Median baseline patient VAS (mm)	5	3	0.066	1.07	0.99 – 1.15	0.086
Proportion male	0.61	0.35	1.061	2.89	0.83 – 10.01	0.094
Proportion current HCQ	0.35	0.15	1.106	3.02	0.68 – 13.51	0.148
Median baseline 28 TJC	0	0	-0.982	0.37	0.10 – 1.45	0.156
Median months since last steroid	24	40.5	-0.019	0.98	0.96 – 1.01	0.173
Proportion RhF positive	0.65	0.45	0.829	2.29	0.67 – 7.84	0.186
Proportion ACPA positive	0.65	0.45	0.829	2.29	0.67 – 7.84	0.186
Proportion ever smoker	0.52	0.7	-0.760	0.47	0.13 – 1.65	0.236
Median symptom duration in months prior to first rheumatology review	6.5	4.5	0.045	1.05	0.97 – 1.13	0.264
Proportion baseline DAS28-ESR remission	0.90	0.80	0.930	2.53	0.41 – 15.8	0.319
Proportion either RhF or ACPA positive	0.78	0.65	0.662	1.94	0.50 – 7-49	0.337
Median weekly alcohol unit intake	6	5	0.031	1.03	0.96 – 1.11	0.398
Median months from first rheumatology review to starting first DMARD	1	1	0.074	1.08	0.90 – 1.29	0.409
Median years since diagnosis	6	5.5	0.028	1.03	0.95 – 1.11	0.467
Median age (years)	69	64	0.017	1.02	0.96 – 1.07	0.524
Proportion current smoker	0.09	0.15	-0.617	0.54	0.08 – 3.61	0.525
Median baseline HAQ-DI score	0	0.125	-0.356	0.70	0.23 – 2.12	0.528
Median baseline ESR (mm/hr)	9	6	0.009	1.01	0.97 – 1.05	0.673
Median baseline 28 SJC	0	0	0.169	1.18	0.43 – 3.25	0.742
Proportion current SFZ	0.26	0.3	-0.194	0.82	0.22 – 3.13	0.776
Median baseline CRP (mg/L)	<5	<5	0.024	1.02	0.83 – 1.26	0.822
Median baseline DAS28-ESR	1.66	1.54	-0.024	0.98	0.48 – 1.98	0.946
Median baseline DAS28-CRP	1.35	1	-0.006	0.99	0.25 – 3.97	0.993

Table 4.5 - Association of clinical variables with occurrence of arthritis flare following DMARD cessation, using a backwards stepwise multivariate binary logistic regression model. For continuous variables, logistic regression coefficients (B) and odds ratios (OR) are calculated for a 1 unit change in that variable.

Variable	B	OR_{flare}	95% CI OR_{flare}	Multivariate p
Baseline ACR/EULAR Boolean remission	-5.011	0.01	0.007 – 0.32	0.011
Months since last change in DMARDs	-0.055	0.95	0.90 – 0.99	0.019
Current methotrexate	2.841	17.13	1.27 – 231.10	0.032
ACPA positive	1.964	7.13	0.91 – 56.07	0.062
Baseline 28 TJC	-1.722	0.18	0.02 – 1.46	0.108
RhF positive	1.572	4.82	0.70 – 33.27	0.111

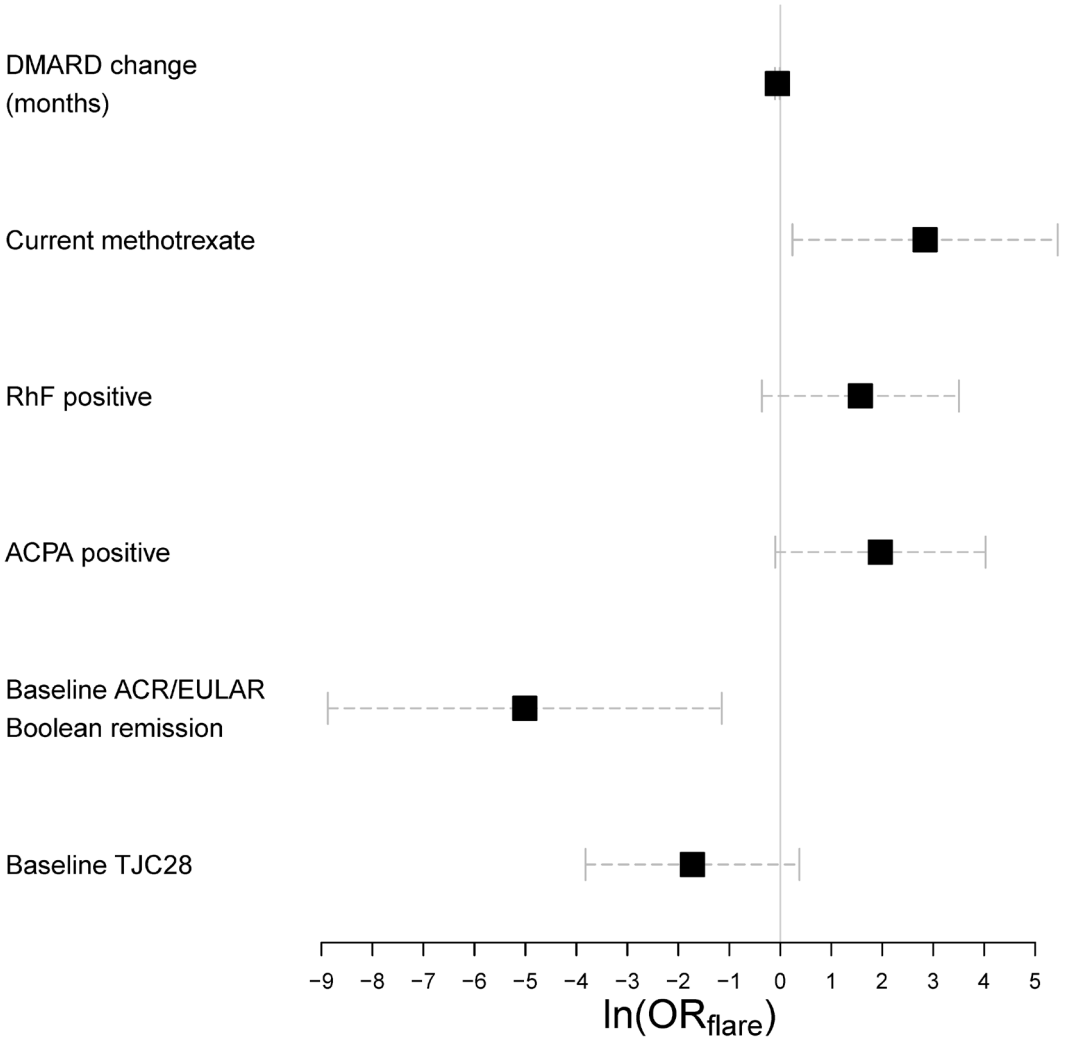


Figure 4.5 – Association of baseline clinical variables with occurrence of flare following DMARD cessation within a stepwise multivariate binary logistic regression model. OR: odds ratio. DMARD change: months since last change in DMARD therapy.

Table 4.6 – Association of clinical variables with occurrence of arthritis flare following DMARD cessation by univariate Cox regression. For continuous variables, hazard ratios (HR) and the Cox regression coefficients (B) are presented for a 1 unit change in that variable. P values calculated by the Wald test. HCQ: hydroxychloroquine; MTX: methotrexate; SFZ: sulfasalazine.

Variable	B	HR_{flare}	95% CI HR_{flare}	Univariate p-value
Double seropositive	0.982	2.67	1.17 – 6.09	0.019
Symptom duration prior to first rheumatology review (months)	0.036	1.04	1.00 – 1.07	0.032
Current number of DMARDs	0.704	2.02	1.06 – 3.86	0.033
Cumulative number of DMARDs since diagnosis	0.401	1.49	0.98 – 2.27	0.060
Months since last change in DMARD therapy	-0.015	0.98	0.97 – 1.00	0.067
Male sex	0.763	2.14	0.93 – 4.96	0.075
ACPA positive	0.752	2.12	0.90 – 5.01	0.087
Current hydroxychloroquine	0.752	2.12	0.89 – 5.04	0.089
Baseline ACR/EULAR Boolean remission	-0.692	0.50	0.22 – 1.14	0.098
Baseline $VAS_{patient}$	0.032	1.03	0.99 – 1.07	0.100
RhF positive	0.698	2.01	0.85 – 4.77	0.113
Months from first rheumatology review to starting first DMARD	0.007	1.01	1.00 – 1.02	0.141
Current methotrexate	1.422	4.14	0.56 – 30.84	0.165
Weekly alcohol unit intake	0.033	1.03	0.99 – 1.08	0.167
Disease duration (years)	0.034	1.03	0.99 – 1.09	0.172
Baseline 28 TJC	-0.700	0.50	0.16 – 1.53	0.222
Either RhF or ACPA positive	0.612	1.84	0.68 – 4.99	0.229
Months since last steroid	-0.011	0.99	0.97 – 1.01	0.252
Ever smoker	-0.405	0.67	0.29 – 1.52	0.334
Baseline DAS28-ESR remission	0.579	1.79	0.42 – 7.67	0.436
Baseline CRP (mg/L)	0.055	1.06	0.92 – 1.21	0.440
Baseline HAQ-DI	-0.295	0.74	0.32 – 1.74	0.495
Baseline ESR (mm/hr)	0.009	1.01	0.98 – 1.04	0.526
Age (years)	0.011	1.01	0.97 – 1.05	0.569
Current smoker	-0.314	0.73	0.17 – 3.12	0.671
Current sulfasalazine	-0.109	0.90	0.35 – 2.28	0.819
Baseline DAS28-CRP	0.080	1.08	0.45 – 2.60	0.858
Baseline 28 SJC	0.049	1.05	0.54 – 2.02	0.885
Baseline DAS28-ESR	0.019	1.02	0.63 – 1.64	0.938

Table 4.7 - Association of clinical variables with occurrence of arthritis flare following DMARD cessation, using a backwards stepwise multivariate Cox regression model. For continuous variables, hazard ratios and the Cox regression coefficients (B) are presented for a 1 unit change in that variable. P values calculated by the Wald test.

Variable	B	HR_{flare}	95% CI	p value
Months from first rheumatology review to starting first DMARD	0.034	1.03	1.01 – 1.06	0.008
RhF positive	1.629	5.10	1.48 – 17.6	0.010
ACPA positive	1.589	4.90	1.36 – 17.7	0.015
Baseline ACR/EULAR Boolean remission	-1.126	0.32	0.12 – 0.90	0.031
Current methotrexate	2.435	11.41	1.25 – 104	0.031
Months since last change in DMARD therapy	-0.025	0.98	0.95 – 1.00	0.034
Disease duration (years)	-0.127	0.88	0.76 – 1.02	0.092
Male sex	0.975	2.65	0.80 – 8.73	0.109
Symptom duration prior to first rheumatology review (months)	0.042	1.04	0.98 – 1.11	0.158

Proportionality of hazards was again assessed for each variable in the final stepwise multivariate Cox regression model. A significant departure from proportional hazards was observed only for current methotrexate use ($p = 0.04$), though this was only notable for a single outlier with no discernible trend in the remainder of the data (Figure 4.6). The global Schoenfeld test was non-significant ($p = 0.49$), indicating proportionality of hazards for the model as a whole.

Four variables were associated with shorter time-to-flare at the 5% significance level, namely: ACPA positivity, RhF positivity, current methotrexate and time from diagnosis to commencement of first DMARD. In contrast, fulfilment of ACR/EULAR Boolean remission criteria and time since last change in DMARD therapy were associated with a longer time-to-flare (Figure 4.7).

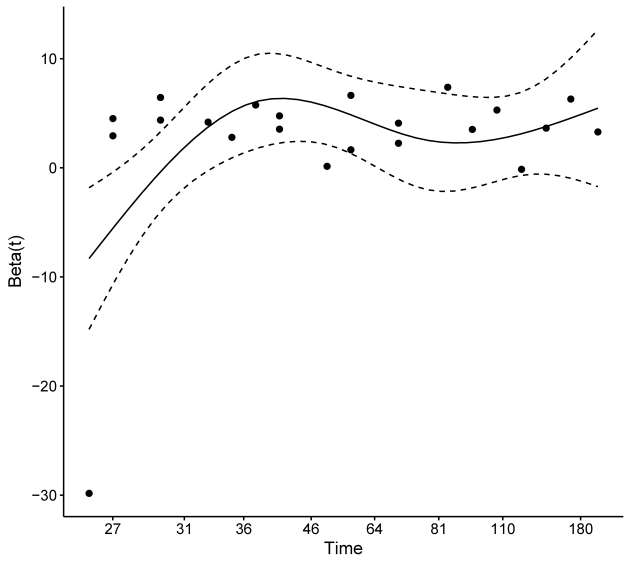


Figure 4.6 – Correlation of scaled Schoenfeld residuals (Beta(t)) against Kaplan-Meier-transformed flare-free survival time for current methotrexate in the stepwise multivariate Cox regression model. Dashed lines indicate ± 2 standard errors of the smoothed spline fit with 4 degrees of freedom (solid line). Discounting the single outlier, there does not appear to be any observable correlation between the scaled residuals and survival time.

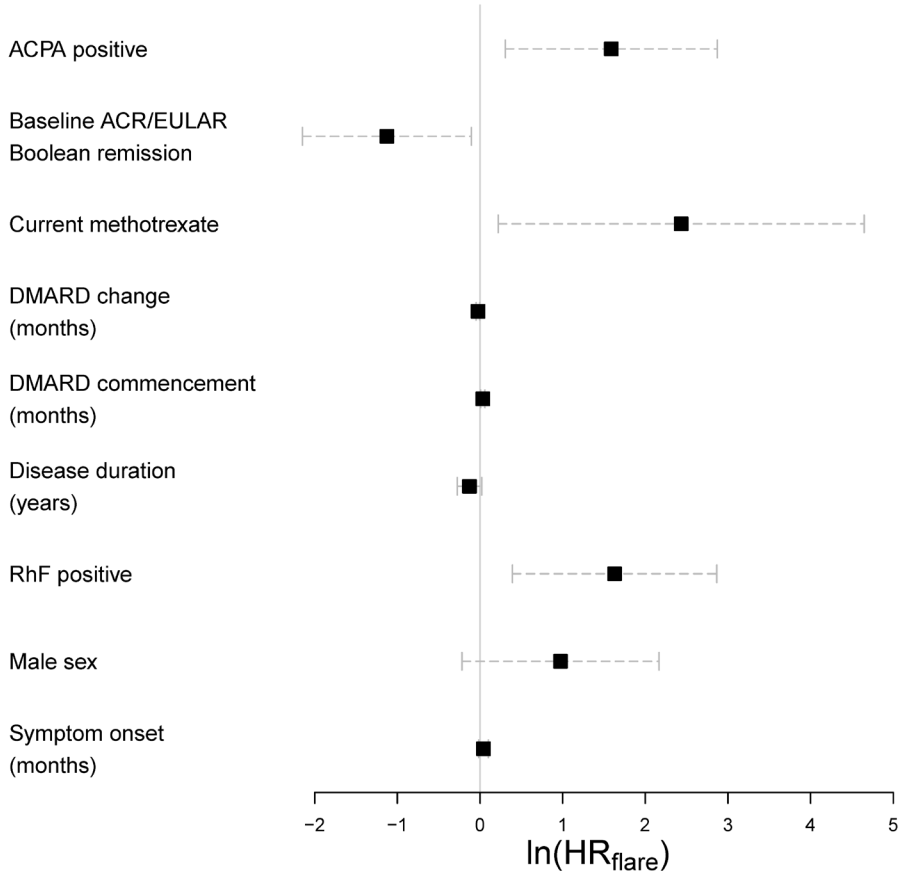


Figure 4.7 - Association of baseline clinical variables with occurrence of flare following DMARD cessation within a stepwise multivariate Cox regression model. HR: hazard ratio.

The study cohort was dichotomised by presence of these four variables, and the statistical significance of differences in survival distributions between groups compared by the log-rank test. Presence of RhF or ACPA was associated with a shorter time-to-flare (Figure 4.8 and Figure 4.9) – the difference in survival distribution was marginally greater for double seropositivity (p = 0.048, Figure 4.10). The survival distributions for presence or absence of baseline ACR/EULAR Boolean remission were comparable for the first 3 months – beyond this point, the rate of flare in those patients who satisfied Boolean remission plateaued, whereas those who failed to satisfy Boolean remission at baseline continued to experience a constant ongoing rate of flare. However, the difference in overall survival distributions failed to meet statistical significance (Figure 4.11). All but one of the patients not taking methotrexate at baseline maintained remission to the end of the follow-up period, though the statistical significance of the difference in survival distribution was low owing to the small sample size (n=6) (Figure 4.12).

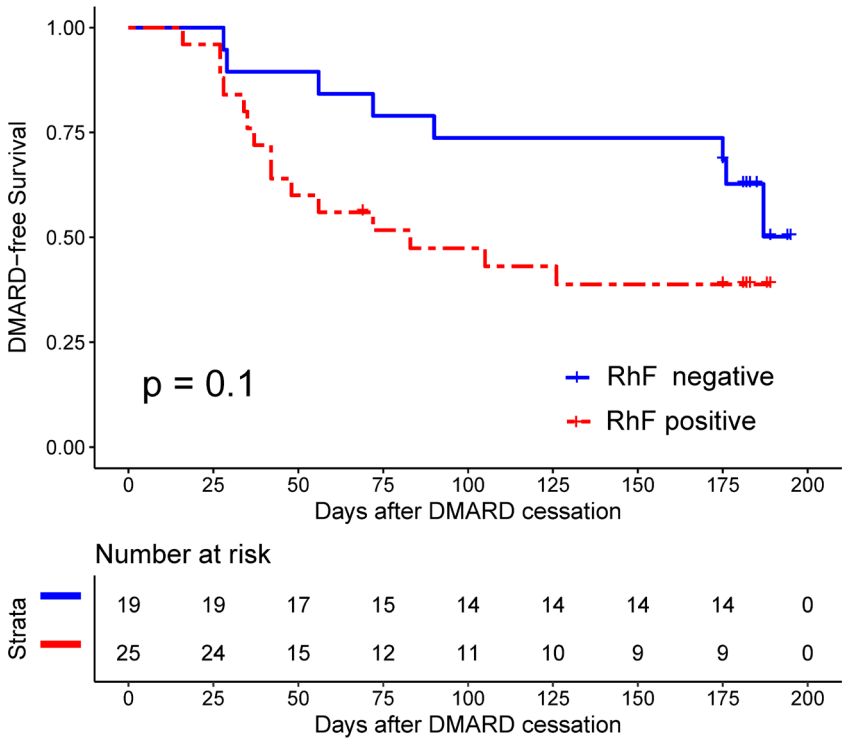


Figure 4.8 – Kaplan-Meier plot of DMARD-free survival time stratified by presence (red) or absence (blue) of rheumatoid factor. Significance of difference between survival curves was assessed by the log-rank test.

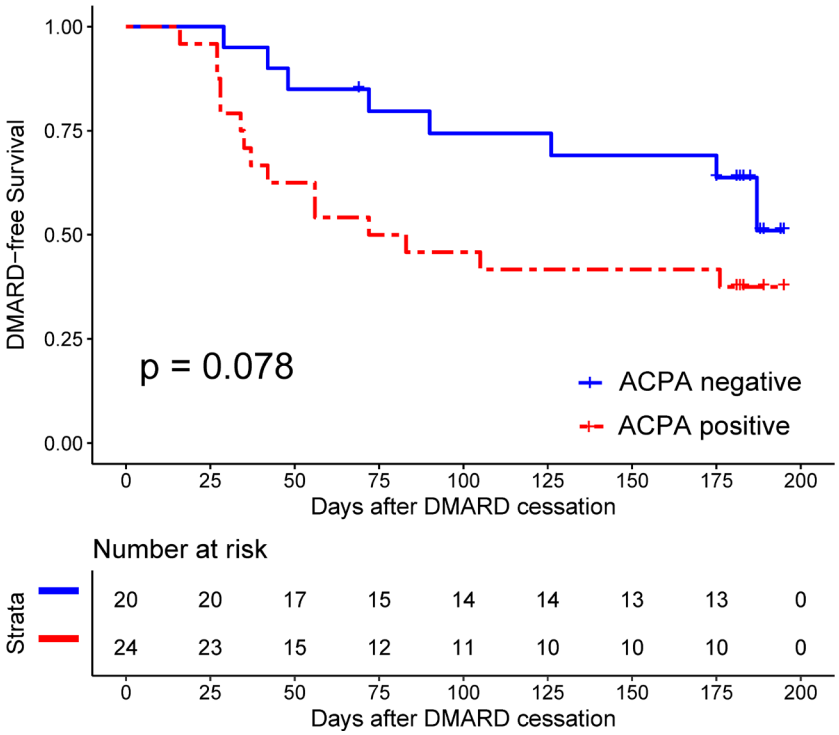


Figure 4.9 – Kaplan-Meier plot of DMARD-free survival time stratified by presence (red) or absence (blue) of ACPA. Significance of difference between survival curves was assessed by the log-rank test.

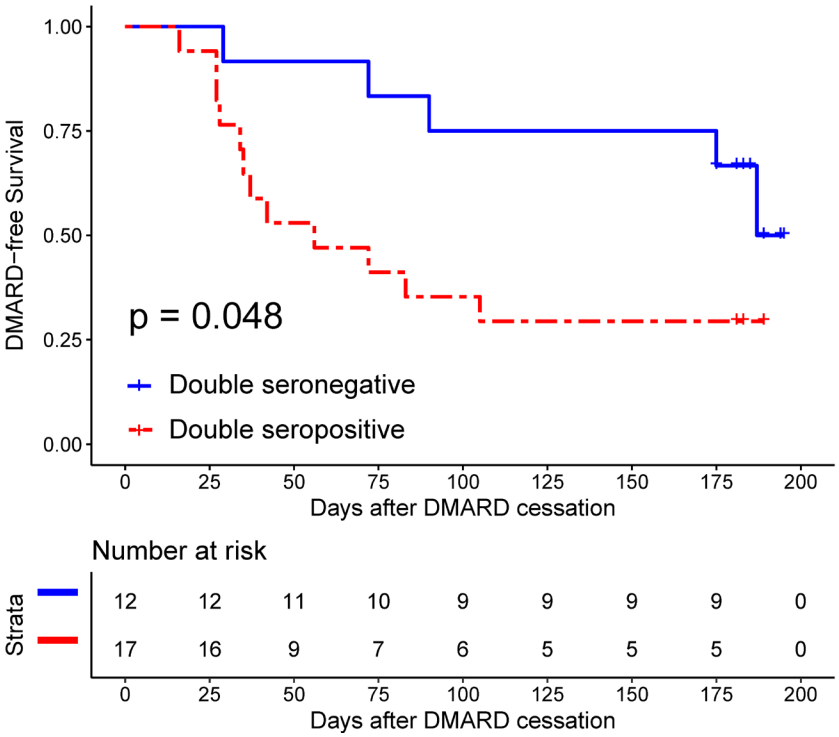


Figure 4.10 – Kaplan-Meier plot of DMARD-free survival time stratified by double seropositivity (red) or double seronegativity (blue) of RhF and ACPA. Significance of difference between survival curves was assessed by the log-rank test.

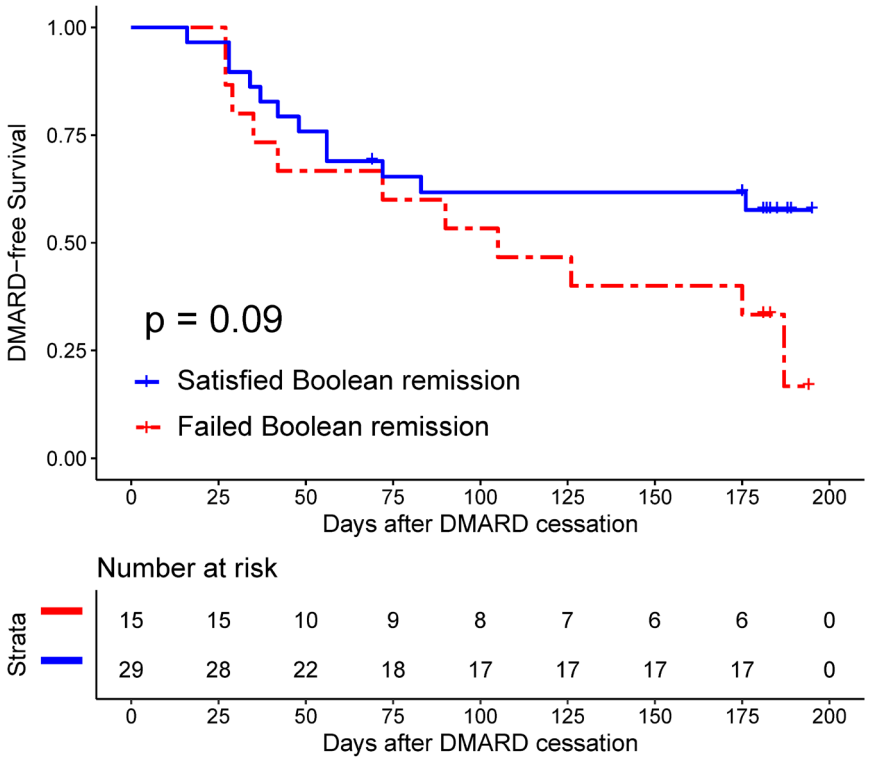


Figure 4.11 – Kaplan-Meier plot of DMARD-free survival time stratified by failure (red) or satisfaction (blue) of ACR/EULAR Boolean remission at baseline. Significance of difference between survival curves was assessed by the log-rank test.

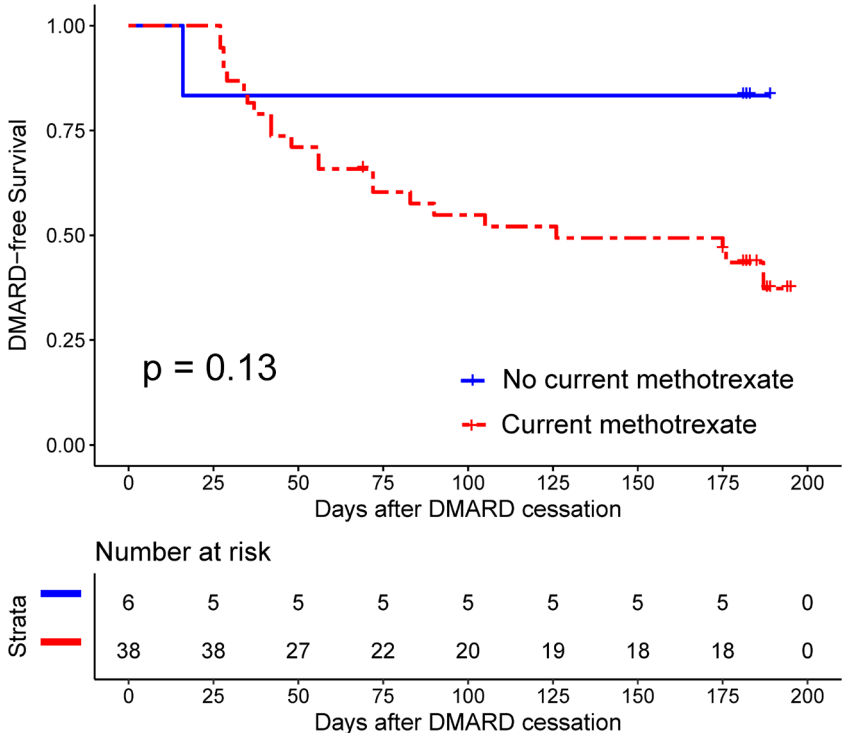


Figure 4.12 – Kaplan-Meier plot of DMARD-free survival time stratified by current use (red) or non-use (blue) of methotrexate at baseline. Significance of difference between survival curves was assessed by the log-rank test.

For the continuous variables, thresholds were determined by receiver operating characteristic (ROC) analysis. For time since last change in DMARD therapy, two optimum thresholds were set to maximise negative and positive predictive values for flare, corresponding to biomarker thresholds for the prediction of remission and flare respectively (Figure 4.13A). Patients with a time since last change in DMARD therapy greater than the 'remission threshold' (i.e. 43 months) had a significantly longer time-to-flare than those below this level (log-rank p = 0.014) (Figure 4.13B). In contrast, the distribution of flares in patients with a time since last DMARD change shorter than the 'flare threshold' (i.e. 13 months) only differed from the remainder of the study population towards the end of the follow-up period, with no significant difference between overall survival distributions (p = 0.14) (Figure 4.13C). Time from first rheumatology review to commencement of first DMARD was a relatively poor predictor of outcome during the six months of study follow-up (Figure 4.14).

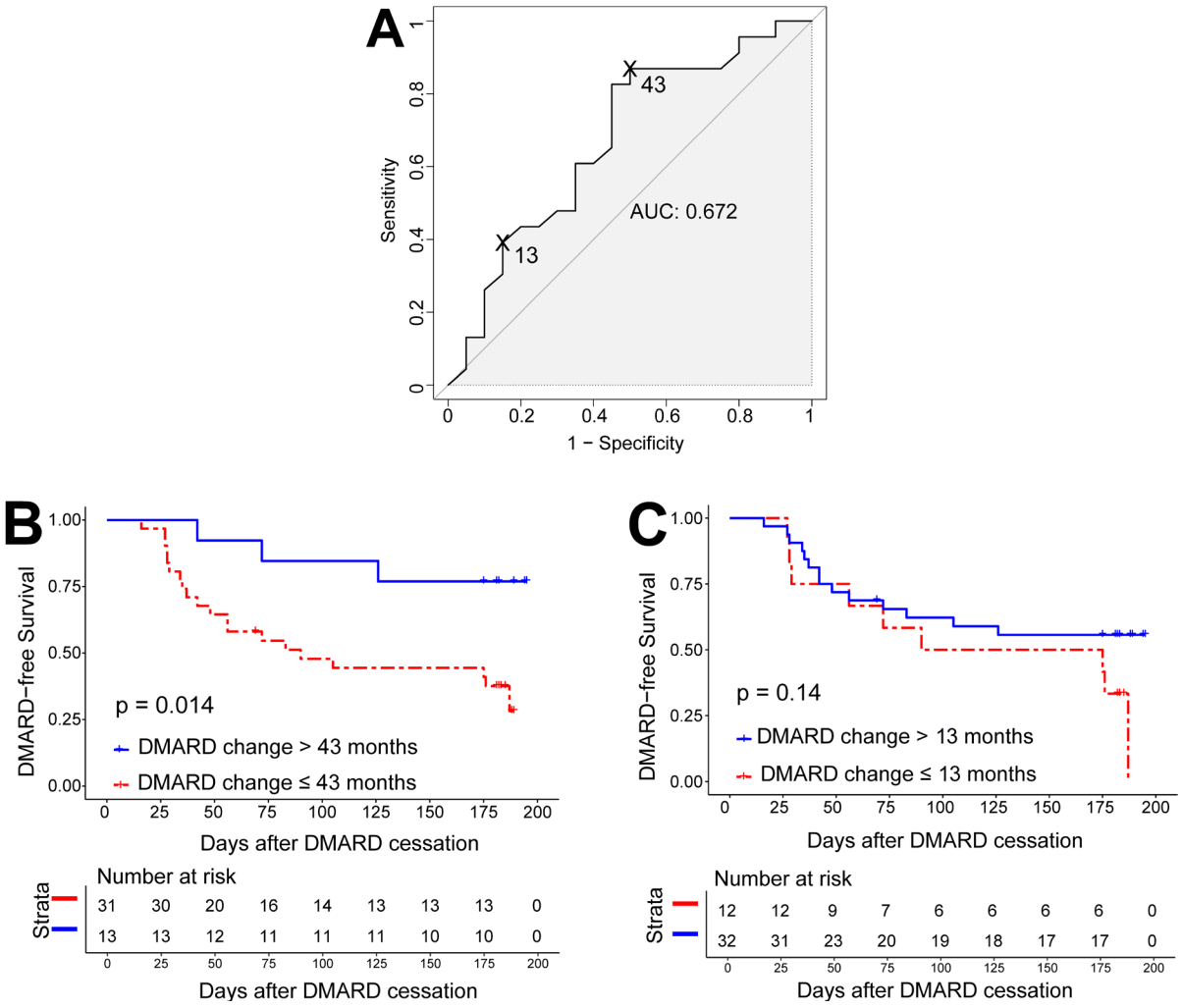


Figure 4.13 – A: Receiver operating characteristic (ROC) curve for sensitivity and specificity for flare, as predicted by time since last change in DMARD therapy. Crosses indicate the remission (43 months) and flare (13 months) thresholds. B: Kaplan-Meier plot of DMARD-free survival stratified by time since last change in DMARD therapy of >43 months (blue) or ≤43 months (red). C: Kaplan-Meier plot of DMARD-free survival stratified by time since last change in DMARD therapy of >13 months (blue) or ≤13 months (red). P-values calculated by log-rank test.

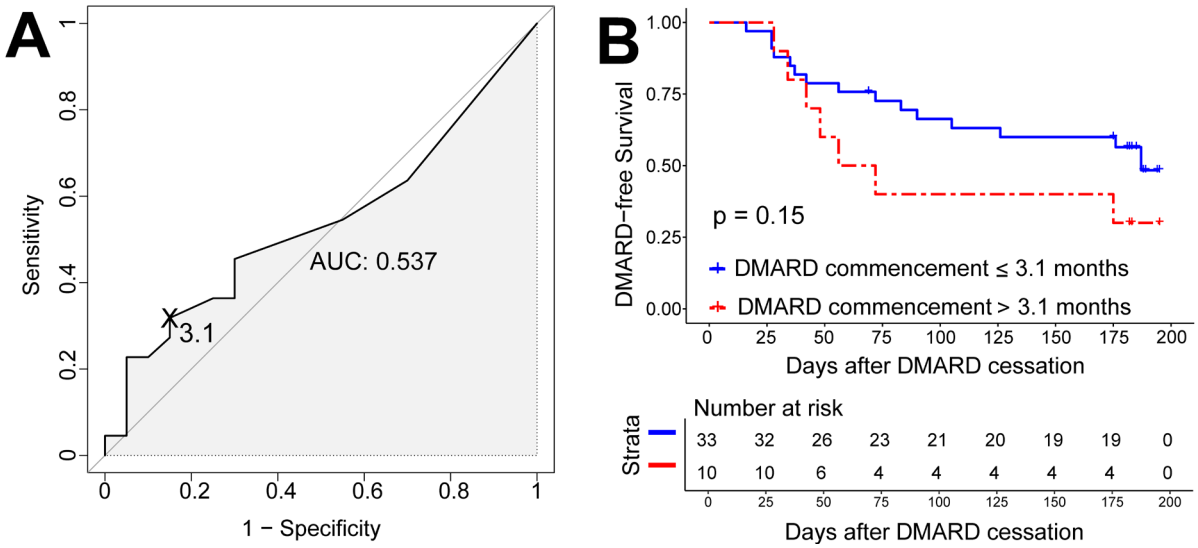


Figure 4.14 – A: Receiver operating characteristic (ROC) curve for sensitivity and specificity for flare, as predicted by time from first rheumatology review to commencement of first DMARD. The cross indicates the 3.1 month threshold. B: Kaplan-Meier plot of DMARD-free survival stratified by DMARD commencement time of >3.1 months (red) or ≤3.1 months (blue). P-value calculated by log-rank test.

4.7 Composite clinical biomarker score

Values of the six variables that were significantly ($p<0.05$) associated with time-to-flare at in the multivariate stepwise Cox regression model were multiplied by their respective coefficients in the model and then summed to create composite scores. The predictive value of all 63 possible combinations of these variables to predict flare and remission following DMARD cessation was then compared by area under the receiver-operating characteristic curve (ROC_{AUC}). The ten composite scores with the highest ROC_{AUC} are listed in Table 4.8 (for a full listing, see Appendix E).

Table 4.8 - The top ten clinical composite scores ranked by ROC_{AUC}. Variables included within each score are indicated in green, and those excluded are indicated in red.

RhF positive	ACPA positive	ACR/EULAR Boolean remission	DMARD change (months)	Current methotrexate	DMARD commencement (months)	ROC_{AUC}
✗	✓	✓	✓	✓	✗	0.850
✓	✓	✓	✓	✓	✗	0.848
✗	✗	✓	✓	✓	✗	0.837
✓	✗	✓	✓	✓	✗	0.833
✓	✓	✓	✓	✓	✓	0.805
✗	✓	✓	✓	✓	✓	0.798
✗	✓	✗	✓	✓	✗	0.787
✓	✗	✗	✓	✓	✗	0.786
✓	✗	✓	✓	✓	✓	0.782
✓	✓	✓	✓	✗	✓	0.777

Optimal performance was observed for a four-variable composite score incorporating ACPA positivity, ACR/EULAR Boolean remission, time since last change in DMARD therapy, and current use of methotrexate (Formula 4.1). The composite clinical score performed well its ability to discriminate flare versus remission following DMARD cessation, with a total area under the ROC curve (ROC_{AUC}) of 0.85 (95% CI 0.73 – 0.97) (Figure 4.15). Optimal thresholds were determined to maximise detection of flare (1.82) and remission (0.51) (Figure 4.15). Both thresholds performed well in the study population, with a positive predictive value (PPV) of 0.90 (95% CI 0.78 – 1.00) for the flare threshold, and a negative predictive value (NPV) of 0.87 (95% CI 0.69 – 1.00) for the remission threshold (Table 4.9).

Formula 4.1 – Composite clinical biomarker score. Values for binary variables, as indicated by the square brackets, are only added to the equation if the variable is present.

$$\textit{Clinical score} = 0.654[\textit{ACPA positive}] + 1.130[\textit{current methotrexate}] - 0.521[\textit{ACR/EULAR Boolean remission}] - 0.012(\textit{months since last DMARD change})$$

Table 4.9 – Predictive utility of the clinical composite clinical score in predicting flare following DMARD cessation, with a positive test defined by either flare or remission thresholds. NPV: negative predictive value; PPV: positive predictive value.

Positive test threshold	Sensitivity	Specificity	PPV	NPV
Flare (>1.82)	**0.78** (0.61 – 0.91)	**0.90** (0.75 – 1.00)	**0.90** (0.78 – 1.00)	**0.78** (0.67 – 0.91)
Remission (>0.51)	**0.91** (0.78 – 1.00)	**0.60** (0.40 – 0.80)	**0.72** (0.62 – 0.85)	**0.87** (0.69 – 1.00)

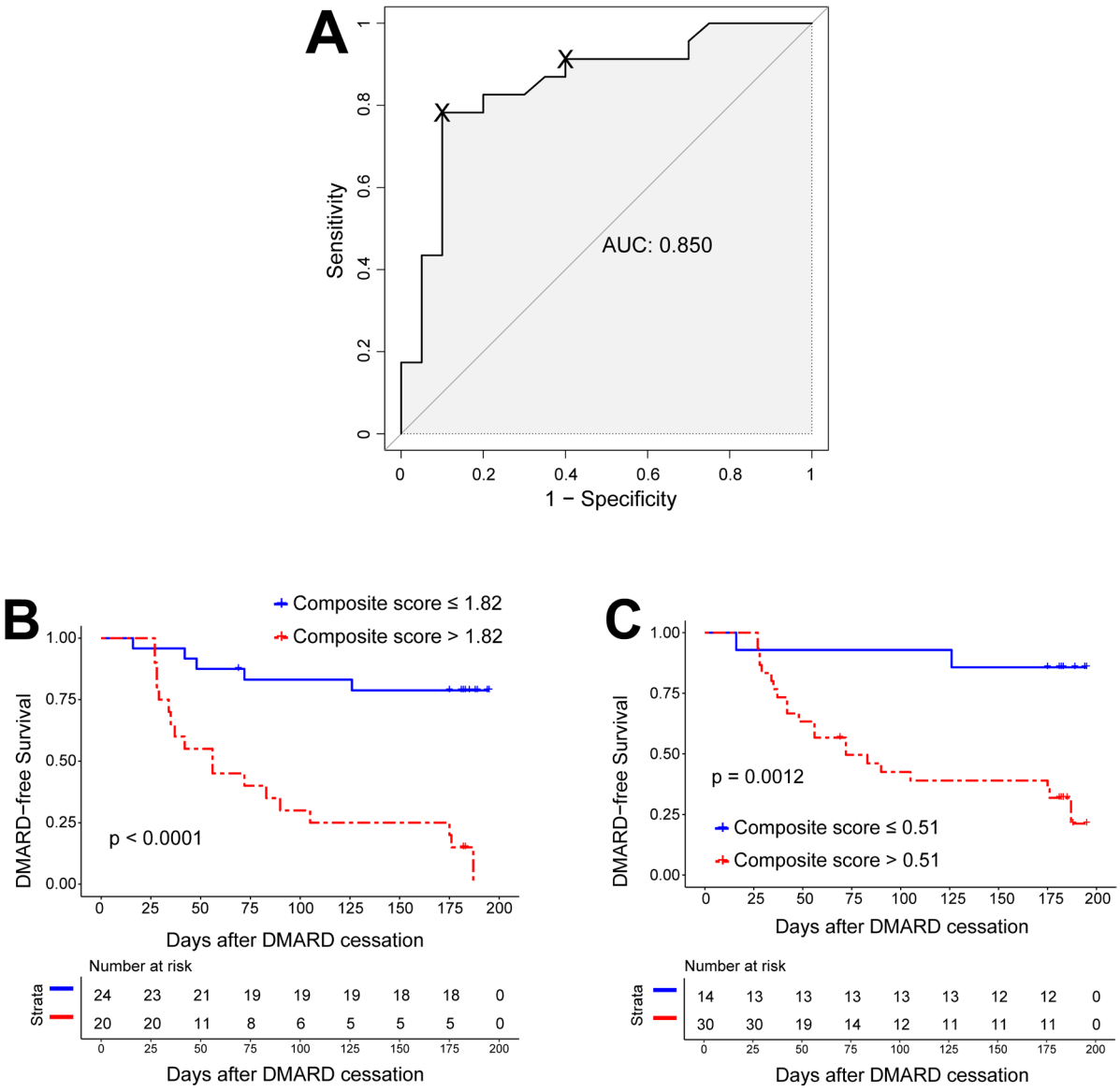


Figure 4.15 - A: Receiver operating characteristic (ROC) curve for sensitivity and specificity for flare, as predicted the composite clinical score. B: Kaplan-Meier plot of DMARD-free survival stratified by composite clinical score >1.82 (red) versus ≤ 1.82 (blue). C: Kaplan-Meier plot of DMARD-free survival composite clinical score >0.51 (red) or ≤0.51 (blue). P-values calculated by log-rank test.

4.8 Long-term clinical outcomes

The medical records of patients who participated in the BioRRA study were reviewed 5 to 6 months after the end of study recruitment. Formal measures of disease activity were frequently not recorded, and hence sustained remission was pragmatically defined as satisfaction of all three of: clinical impression of remission by the assessing clinician, no escalation of DMARD therapy, and no use of systemic glucocorticoids. Drug-free remission was defined as satisfying the above sustained remission definition in the absence of DMARDs.

4.8.1 Outcomes following arthritis flare

Follow-up data was available for 20 of the 23 patients who experienced an arthritis flare and restarted DMARD therapy. Of these patients, 15 (75%) were documented to have regained remission following study discharge. The infrequent nature of outpatient clinic attendances prohibited the analysis of time to regain remission in this group. Of these 15 patients, 10 had follow-up data extending beyond 6 months after study discharge, all of whom maintained remission, suggesting that remission status was stable once re-achieved.

Five patients failed to re-achieve remission following study discharge. Of note, 4 of these patients elected to restart DMARDs at a lower dose than was prescribed at baseline. Follow-up data was limited to 1 month following study discharge in the remaining patient.

4.8.2 Outcomes following sustained DFR

Follow-up data was available for 12 of the 20 patients who maintained DFR during the six-month study period. Of these, 9/12 (75%) maintained DFR after a median (range) of 5 (2 – 22) months of follow-up.

Of the three patients who failed to maintain DFR following discharge from the study:

1. One patient elected to restart DMARDs at the end of the study period (clinical remission, grade 1/3 PD at wrist on ultrasound) and subsequently remained in clinical remission after 13 months of follow-up.
2. One patient received an IM steroid for a possible arthritis flare one month after study discharge, though was not clinically reviewed at the time. This patient subsequently

remained in clinical remission without DMARDs or further steroids after 9 months of follow-up.

3. One patient restarted DMARDs one month after study discharge following clinician-observed arthritis flare, though at a lower dose than that prescribed at entry to the study. After 19 months of follow-up, this patient was yet to re-achieve remission though remained on a lower dose of DMARDs than at study enrolment.

4.9 Discussion

4.9.1 Occurrence and timing of arthritis flare

In this study, 23/44 (52%) patients experienced an arthritis flare during the six-month follow-up period. Although few studies have explored complete DMARD cessation in the setting of established RA (reviewed in Introduction 1.6), the occurrence of flare in these studies was broadly similar. In the RETRO study, randomisation to complete cessation of a variety of biological (bDMARD) and conventional synthetic DMARDs (csDMARDs) was associated with arthritis flare (DAS28-ESR > 2.6) in 14/27 (52%) patients by six months after complete DMARD withdrawal (Haschka *et al.*, 2016). In the placebo-controlled RCT conducted by ten Wolde *et al.* (1996), cessation of csDMARDs (many of which are now considered historical) was associated with a clinical definition of arthritis flare in 53/143 (37%) patients after 12 months follow-up. In the BeST study, arthritis flare (DAS44 > 1.6) was observed in 56 (49%) of 115 patients who tapered DMARDs to complete cessation (Klarenbeek *et al.*, 2011b).

The apparently consistent proportion of patients who maintain drug-free remission following DMARD cessation in this and other studies is even more remarkable given the range of different definitions of flare and heterogeneity in DMARDs studied. Indeed, this has been cited by some as circumstantial evidence of a possible inherent biological characteristic of RA, whereby drug-free remission represents a final end-point in the natural history of the disease for a small subset of patients (Scott *et al.*, 2013a).

Alternative explanations are conceivable; for example, the consistent rate of flare observed in the published studies above may represent a common failure of clinical definitions of remission to identify patients with low-level subclinical synovitis. Nevertheless, a similar rate of flare is observed in this study, despite the mandatory absence of power Doppler signal on a 7-joint ultrasound scan as a prerequisite for DMARD cessation. Although synovitis may have been present in joints outside the scan protocol, one would expect that the exclusion of 19

patients on the grounds of PD-positivity alone should have resulted in a lower rate of flare in BioRRA in comparison to the above published studies if, indeed, the sole explanation for flare was persistent low-grade synovitis at baseline. This raises the possibility that the low-grade PD signal observed may not have been indicative of active synovitis (discussed further in Chapter 5). Nevertheless, direct comparison between this and other studies is difficult owing to heterogeneity in patient demographics, baseline DMARD therapy and methodology of DMARD withdrawal.

In this study, the median (IQR) time-to-flare was 48 (31.5 – 86.5) days after DMARD cessation, which was shorter than that observed in other studies. For example, ten Wolde *et al.* (1996) observed flare at a mean (sd) of 24 (16) weeks after DMARD cessation, whereas arthritis flare occurred at a median (IQR) of 5 (2 – 16) months after achievement of complete DMARD cessation in the BeST study (Klarenbeek *et al.*, 2011b). In the RETRO study, patients who completely stopped mediation first tapered DMARDs to 50% dose for 6 months, with the majority of flares occurring towards the end of this six-month tapering phase (Haschka *et al.*, 2016). Indeed, during the design of the BioRRA study it was anticipated that most flare events would occur in the final 3 months of follow-up; the fact that the opposite occurred was unexpected.

The reason that arthritis flares occurred sooner in my study in comparison to others is not immediately apparent. Assessment of disease activity in my study was performed only slightly more frequently during the six month follow-up period than in the RETRO study (one more visit), and less frequently than by ten Wolde *et al.* (1996) (one less visit). It is thus unlikely that frequency of assessment would have substantially affected the detection of flare events. Another explanation is that my study included a greater proportion of patients with subclinical active synovitis – however, as discussed above, this would seem counterintuitive given the use of musculoskeletal ultrasound. Alternatively, patients recruited to my study may have had a more severe or unstable RA phenotype, which more readily transitioned to flare following DMARD cessation. However, a difference in clinical disease phenotype is not suggested by the broadly equivalent prevalence of RhF positivity in my study (57%) as compared to ten Wolde *et al.* (1996) (66%) and in the DMARD-discontinuation arm of the RETRO study (67%). In addition, the prevalence of ACPA positivity was similar in both my study (55%) and the DMARD discontinuation arm of the RETRO study (63%). Furthermore, the long time since last steroid use or change in DMARD therapy (median 30 and 22.5 months respectively) in my study would suggest an extended period of excellent, as opposed to unstable, disease control.

One possible explanation for the earlier occurrence of arthritis flare is an excessively stringent flare definition. In my study, a single DAS28-CRP ≥ 2.4 measurement at any point during the follow-up period was sufficient for categorisation of arthritis flare and exit from the study. This decision was driven by concern at the study design phase that patients may experience severe arthritis flares, and hence a desire for early intervention to prevent this. However, 8/23 (35%) of patients who experienced an arthritis flare exited the study with a DAS28-CRP score < 2.9, which arguably may have settled back to remission levels with time in a subset of patients. Indeed, an alternative flare definition may have been more appropriate to account for this borderline group – for example, either i) DAS28-CRP ≥ 2.9, or ii) 2.4≥DAS28-CRP > 2.9 on two occasions 7 days apart. Similar leniency for such borderline patients has been permitted in the flare definitions of other DMARD withdrawal studies; for example, the U-Act-Early study permitted ≤2 visits with 2.6≤DAS28-ESR<3.2 within its sustained remission definition (Bijlsma *et al.*, 2016). Nevertheless, such definitions would risk losing specificity for drug-free remission, which was the primary focus of this study. Indeed, the robust inflammatory signatures seen in longitudinal cytokine and gene expression analyses – as discussed in Chapters 6 and 7 – corroborate an active inflammatory state at the time of flare within the flare patient group.

4.9.2 Recovery from arthritis flare

One concern when designing this study was that arthritis flare may prove difficult to control, even after recommencement of DMARDs. It was therefore reassuring to observe that clinical remission was regained for the majority (15/20) of patients for whom outcome data was available following study discharge. Furthermore, 10 of these 15 patients had follow-up data extending beyond 6 months after study discharge, suggesting that remission was stable once re-achieved. Importantly, 4/5 of the patients who failed to regain remission elected to restart DMARDs at a lower dose than that prescribed at baseline, and the remaining patient had no follow-up data beyond just one month after study discharge. It would thus appear that robust disease remission is relatively easily regained, provided prompt administration of DMARDs at the time of flare and at the original dose, with additional intramuscular glucocorticoid as required. Longer-term outcome data is required, but these provisional findings are in keeping with the generally high rates of restoration of remission following arthritis flare that have been observed in other DMARD tapering and withdrawal studies (see Introduction 1.6).

4.9.3 Stability of drug-free remission

A weakness of this study is the short six-month duration of follow-up, which was an unavoidable consequence of the limited study resources and time available within this PhD Fellowship. It is thus entirely conceivable that patients who exited the study in drug-free remission (DFR) may have experienced an arthritis flare in the immediate few months following study discharge. Long-term outcome data for this group was limited, partly owing to the infrequent outpatient reviews of patients in clinical remission, which are often conducted at yearly intervals. Nevertheless, follow-up data was available for 12/20 patients who maintained DFR for the duration of the study, of which 9/12 maintained DFR after a median (range) of 5 (2 – 22) months of follow-up after study discharge. Again, longer-term outcome data is required, though this data is clearly encouraging and is in keeping with the long-duration of DFR (median follow-up of 23 months) that was observed in the BeST study (Klarenbeek *et al.*, 2011b).

4.9.4 Baseline predictors of drug-free remission

Of the six variables significantly associated with time-to-flare in the multivariate stepwise Cox regression model, four had discriminatory value in a composite score for predicting flare and remission in the six months after DMARD cessation. ACPA and RhF positivity were predictive of increased likelihood of flare; and ACR/EULAR Boolean remission and months since last DMARD change were predictive of increased likelihood of remission.

4.9.4.a Autoantibody status

There is in general a dearth of evidence surrounding biomarkers of drug-free remission in RA. Nevertheless, data is emerging from a small number of recent studies that suggests autoantibody status is predictive of flare following DMARD cessation. RhF positivity has been consistently associated with an increased risk of arthritis flare in three independent prospective DMARD withdrawal studies (ten Wolde *et al.*, 1996; Tanaka *et al.*, 2010; Fautrel *et al.*, 2016). Indeed, RhF positivity was associated with an approximately 2 – 4 times increase in risk of arthritis flare in multivariate analysis in these studies (see Introduction 1.7), similar to the multivariate HR_{flare} of 5.10 (95% CI 1.48 – 17.6) presented herein. Similarly, ACPA positivity has been associated with an approximately 5 – 10 times increased risk of arthritis flare in three independent prospective studies (Klarenbeek *et al.*, 2011b; El Miedany

et al., 2016; Haschka *et al.*, 2016), which again is in keeping with the stepwise multivariate HR_{flare} of 4.90 (95% CI 1.36 – 17.67) observed in my study. Both ACPA and RhF positivity are well-established prognosticators of poor outcome in RA, identifying a subset of patients who are at greater risk of joint erosions and extra-articular manifestations of the disease. It is entirely conceivable that patients with more aggressive disease, as defined by autoantibody positivity, are at greater risk of relapse following DMARD cessation. Compelling evidence now links seropositive RA with a breakdown of tolerance to citrullinated peptide autoantigens, likely generated by environmental insults in the lung and other mucosal sites (see Introduction 1.2.2). If DMARDs provide a pharmacological brake to such processes, it is thus conceivable that latent autoimmunity could be unleashed following DMARD cessation in the presence of continued exposure to citrullinated autoantigens. Indeed, the very presence of circulating autoantibodies confirms the presence of autoreactive B/plasma cells (and likely $CD4^+$ T cells), which could be the effectors in such a process. Longitudinal analysis of the circulating autoantibody repertoire in the approach to arthritis flare may shed further light on this topic and would be a possible extension to this current work.

4.9.4.b Time since last DMARD change

The inverse association between time since last DMARD change and risk of arthritis flare observed in this study does have face validity, given that this is a surrogate measure of recent stability of arthritis control. Due to the design of my 'real-world' study of patients with established RA, it was not possible to formally assess disease activity in the period before study enrolment. Such an assessment was however possible in the BeST study, in which DMARD-naïve patients were recruited, received treatment in a prospective clinical trial setting, and then discontinued DMARDs once remission was achieved. In the BeST study, higher mean disease activity (DAS-44) in the period before DMARD cessation was indeed predictive of an increased risk of flare (OR_{flare} 4.7, 95% CI 1.5 – 15.2). Owing to different measures of disease stability and different methods of statistical analysis, it is not possible to directly compare this finding to the results of my study. Nevertheless, these data do suggest that period of stable remission prior to DMARD cessation is important in increasing the chance of successful DMARD withdrawal. Whether this reflects a necessary period of immune homeostasis – perhaps permissive for a pro-tolerogenic modulation of immunity mediated by mechanisms such as epigenetic modification – or is simply indicative of a less severe clinical phenotype, remains to be determined.

4.9.4.c Current methotrexate use

Current use of methotrexate was significantly associated with increased flare in the six months following DMARD cessation in my study, albeit with a wide confidence interval (HR_{flare} 11.41, 95% CI 1.25 – 104.47, p = 0.031). This finding does not appear to have been replicated in the published literature, and indeed is contradicted by the BeST study that found a lower rate of flare in those patients who discontinued methotrexate versus sulfasalazine (Klarenbeek *et al.*, 2011b). When interpreting this result, it is prudent to note that only 6/44 (14%) patients in my study were not receiving methotrexate at baseline, only one of whom experienced an arthritis flare. Despite its statistical significance in the multivariate Cox analysis of clinical variables, it is thus possible that non-use of methotrexate was confounded by another unmeasured variable. Furthermore, given the popularity of methotrexate as a first-line DMARD in current clinical practice, it is likely that patients who were not receiving methotrexate at baseline were fundamentally different from their counterparts, for example in terms of comorbidities or disease activity. Taken together, there is reasonable grounds to suspect that the observed effect of current methotrexate use in this study could be artefactual – further study with a larger independent cohort is required to confirm this.

4.9.4.d ACR/EULAR Boolean remission

A notable finding of this study is that fulfilment of ACR/EULAR Boolean remission at baseline is associated with a 3-fold lower risk of arthritis flare in the six months following DMARD cessation. Given the relatively new introduction of this remission definition, data surrounding its utility in the prediction of DFR following DMARD cessation remains scarce. The only study to have previously addressed this issue is the RETRO study, which demonstrated only a non-significant trend towards lower flare rate in those satisfying ACR/EULAR Boolean remission (OR_{flare} 0.673, 95% CI 0.211 – 2.144, p = 0.503) (Haschka *et al.*, 2016).

The precise properties of ACR/EULAR Boolean remission that confer its predictive value in this study remain open to question. Any of the individual components that constitute ACR/EULAR Boolean remission failed to demonstrate a significant association with time-to-flare in the multivariate analysis, suggesting that it is the combination of variables within the definition that is of importance. Although not designed specifically for clinical use, a major limitation of the use of ACR/EULAR Boolean remission in clinical practice lies in its stringent $VAS_{patient} \leq 10/100$ threshold (see Introduction 1.5.1). Indeed, 10/74 (15%) of

patients recruited to my study satisfied DAS28-CRP remission at baseline yet failed to achieve ACR/EULAR remission solely on grounds of $VAS_{patient}$ alone (median 22/100, range 12 – 34). A modified Boolean remission criteria incorporating a higher $VAS_{patient}$ threshold (for example ≤20/100) may have greater specificity for flare without losing sensitivity. Such analysis is however not possible using my small dataset, and would be of interest in future larger cohorts.

4.10 Summary

A similar proportion of patients experienced an arthritis flare in my study (44/23, 52%) compared to other published studies. However, arthritis flares occurred after an unexpectedly short duration following DMARD cessation in my study compared to others, for reasons that are not readily apparent. Rapid resumption of DMARD therapy at the same dose as baseline, with supplementary intramuscular steroid depot injection, was sufficient to quickly restore clinical remission in the vast majority of patients. Many patients who maintained DFR for the duration of study follow-up remained in remission beyond the end of the study, though further long-term follow-up data is required.

Autoantibody status (ACPA and RhF positivity) predicted flare following DMARD cessation, corroborating the observations of other studies. Current methotrexate use was also positively associated with flare, though there is uncertainty surrounding the validity of this finding. In contrast, longer time since last DMARD change and ACR/EULAR Boolean remission were both negatively associated with flare, suggesting a robust and stable clinical remission phenotype at baseline in those patients who subsequently achieved sustained drug-free remission.

Chapter 5. Results 2 – Ultrasound data

5.1 Introduction

Musculoskeletal ultrasound provides a semi-quantitative measure of disease activity that is complementary to clinical and laboratory assessments (see Introduction 1.4). By direct visualisation of synovial thickening and increased vascularity, US imaging can be used both to corroborate clinical findings and to detect subclinical synovitis. However, there remains considerable uncertainty surrounding the optimum number of joints to scan, and the acceptable threshold of ultrasonographic parameters that constitute clinically relevant synovial pathology in the absence of symptoms of disease (see Introduction 1.5). Furthermore, the potential role of ultrasound as a treatment target in RA has been challenged by negative results from recent large treat-to-ultrasound-target clinical trials (see Introduction 1.5). Despite these limitations, US imaging is a unique non-invasive modality by which subclinical disease activity can be assessed, and thus is ideally placed to serve as a potential biomarker of DFR in RA. However, virtually no studies have explored the predictive utility of musculoskeletal ultrasound in the setting of DMARD-cessation (see Introduction 1.7).

In this chapter, I outline the ultrasound data of the BioRRA study. I first describe the quality of ultrasound image acquisition and scoring, followed by a descriptive analysis of differences between ultrasound parameters at baseline between flare and remission groups, and longitudinal change from baseline to month six. I then explore the association of ultrasound parameters with both baseline clinical variables and time-to-flare following DMARD cessation.

The structure of the remainder of this chapter is as follows:

5.2 Quality control

A total of 97 seven-joint US7 ultrasound scans (see Methods 3.6) comprising 3089 images were recorded. Ultrasound scans were performed at baseline for all 74 patients who were enrolled in the study, and for all 23 patients who attended a month 6 appointment. The median (IQR) time taken to perform the scans was 21 (19 – 23) minutes, ranging from 14 – 35 minutes.

Ultrasound scores recorded at the time of image acquisition were compared with scores recorded at subsequent image re-grading by the same ultrasound operator (KB) to calculate the intra-rater agreement. Intra-rater agreement in ultrasound scores was high, as measured by Cohen's kappa statistic (see Methods 3.10.1). The strength of agreement was greater when linear weighting was employed, indicating that the majority of disagreements were small in magnitude (Table 5.1). Weaker agreement was observed for tendon greyscale (GS) change, owing in part to fewer observed events as each individual disagreement contributed proportionately more to the Kappa statistic in comparison to, for example, joint GS scores. The overall kappa value for intra-rater agreement was 0.73, which compares favourably to the value of 0.64 observed in the original US7 publication (Backhaus *et al.*, 2009).

Inter-rater agreement was assessed by comparison of ultrasound scores recorded at the time of image review between the original ultrasound operator (KB) and a second blinded observer (BT). Inter-rater agreement was, as expected, lower than intra-rater agreement in ultrasound scores, though generally followed a comparable profile. Both intra- and inter-rater agreement compares favourably with those observed by the authors of the original US7 scan publication (Table 5.2) (Backhaus *et al.*, 2009).

5.3 Descriptive analysis

5.3.1 Prevalence of ultrasound-detected abnormalities at baseline

The prevalence of ultrasonographic findings at baseline is detailed in Table 5.3. As per the design of the study, no patient with any degree of PD signal was permitted to stop DMARDs. All patients had GS change recorded in at least one joint, reflecting a high prevalence of GS change in this established RA cohort.

Table 5.1 – Intra- and inter-rater agreement in ultrasound scores, as assessed by Cohen's kappa statistic. Kappa scores are listed with and without linear weighting. Number of events was calculated as the number of images containing any degree of the specified ultrasound finding as recorded in the original ultrasound score. For binary parameters (i.e. tendon GS and erosions), weighted kappa scores are identical to their unweighted counterparts and are omitted here for clarity.

			Intra-rater agreement		Inter-rater agreement	
Parameter	**Number of scores**	**Number of events**	**Unweighted κ**	**Weighted κ**	**Unweighted κ**	**Weighted κ**
Joint GS	864	356	0.62	0.69	0.46	0.56
Joint PD	1248	40	0.92	0.95	0.74	0.79
Tendon GS	672	45	0.45		0.37	
Tendon PD	672	4	1.00	1.00	0.57	0.77
Erosions	1344	181	0.79		0.71	
Total	4800	626	0.73	0.77	0.62	0.68

Table 5.2 – Comparison of inter-rater agreement in ultrasound scans between this study and the original US7 publication by (Backhaus et al., 2009). κ: unweighted Cohen's kappa statistic.

	Inter-rater unweighted κ	
Parameter	**BioRRA**	**Backhaus**
Joint GS	0.46	0.55
Joint PD	0.74	0.67
Erosions	0.71	0.56

Table 5.3 – Prevalence and distribution of scoring of ultrasonographic findings at baseline.

Parameter	**All patients (n = 74)**	**Patients who stopped DMARDs (n = 44)**
Proportion with joint GS: n(%)	74 (100)	44 (100)
Proportion with joint PD n(%)	21 (28)	0 (0)
Proportion with tendon GS: n(%)	31 (42)	21 (48)
Proportion with tendon PD: n(%)	2 (3%)	0 (0)
Proportion with erosions: n(%)	51 (69)	29 (66)
Total joint GS score: median (IQR) [range]	5 (3 – 6) [1 – 10]	5 (3 – 6) [1 – 8]
Total joint PD score: median (IQR) [range]	0 (0 – 1) [0 – 7]	n/a
Total tendon GS score: median (IQR) [range]	0 (0 – 1) [0 – 3]	0 (0 – 1) [0 – 2]
Total tendon PD score: median (IQR) [range]	0 (0 – 0) [0 – 5]	n/a
Total erosion score: median (IQR) [range]	0 (0 – 3) [0 – 10]	2 (0 – 3) [0 – 8]

The baseline distribution of total joint GS, tendon GS and erosion scores by flare status in those patients who stopped DMARDs is depicted in Figures 5.1 – 5.3. No significant differences were observed in the distributions of total baseline ultrasound scores between the two groups ($p = 0.27$, $p = 0.67$, and $p = 0.28$ for joint GS, tendon GS and erosion total scores respectively by Mann-Whitney U test). Analysis at the individual joint level also failed to demonstrate any significant differences between flare and remission groups ($p = 0.76$, $p = 0.26$, and $p = 0.45$ for number of joints with GS≥1, GS≥2, and erosive change respectively).

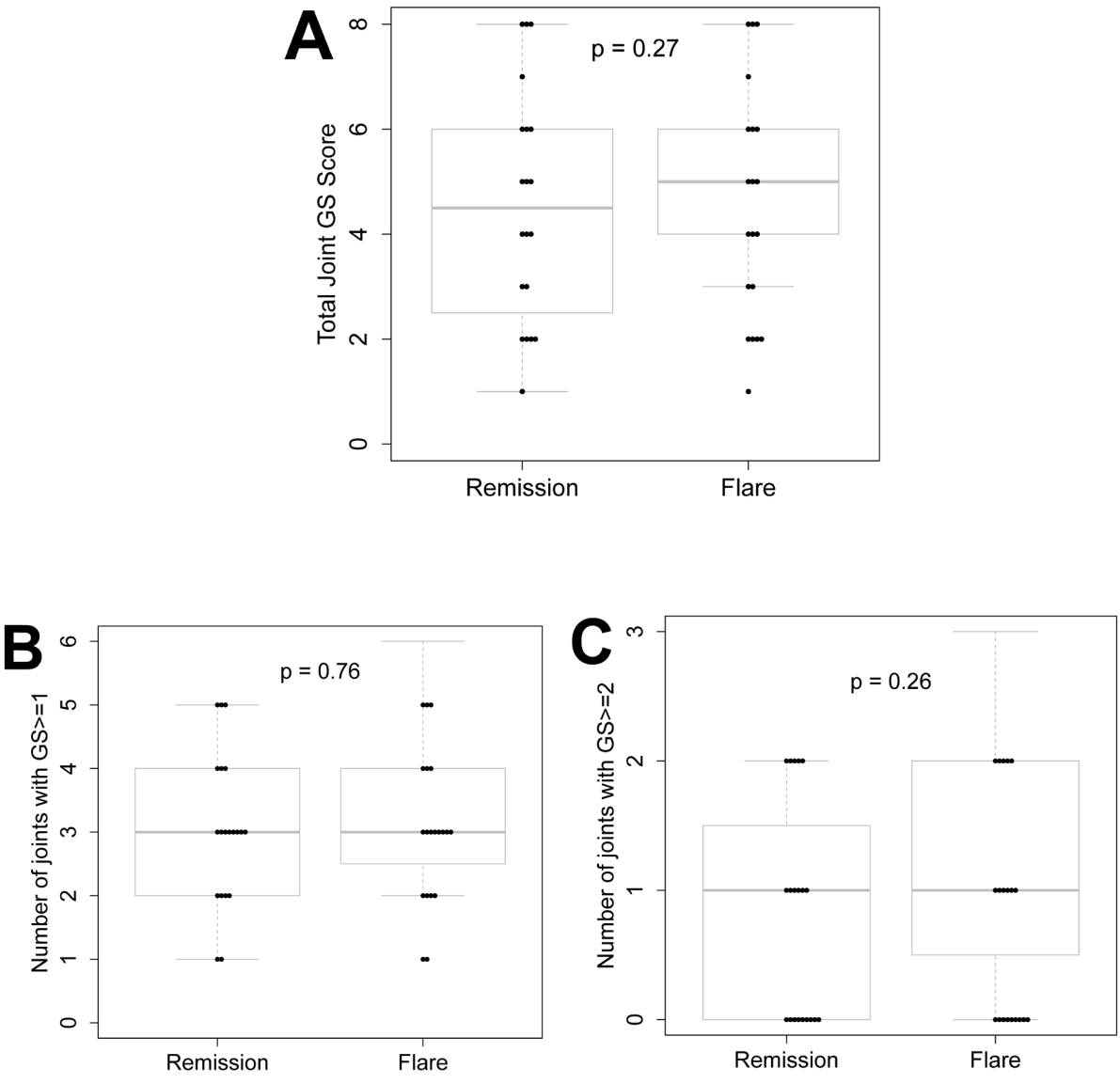


Figure 5.1 – Distribution by flare status of total joint GS score (A), number of joints with GS≥1 (B), and number of joints with GS≥2 (C) at baseline in patients who stopped DMARDs (p value calculated by Mann-Whitney U test).

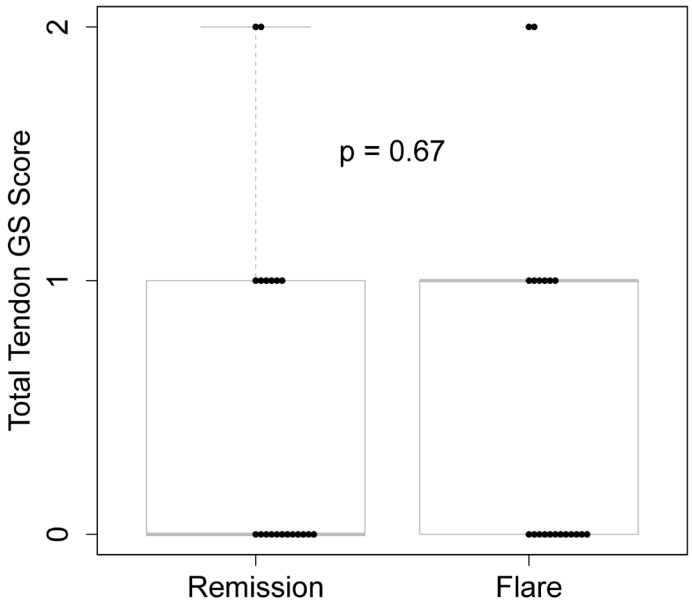


Figure 5.2 – Distribution by flare status of total tendon GS score at baseline in patients who stopped DMARDs (p value calculated by Mann-Whitney U test).

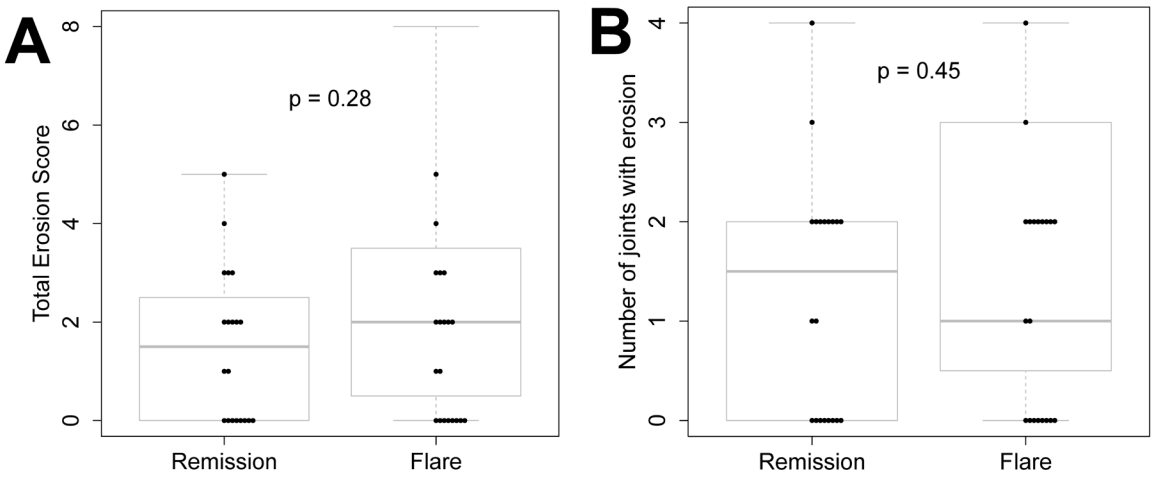


Figure 5.3 – Distribution by flare status of total erosion score (A), and number of joints with erosive change (B) at baseline in patients who stopped DMARDs (p value calculated by Mann-Whitney U test).

5.3.2 Longitudinal change in ultrasound scores

The distributions of total ultrasound scores at baseline and month six visits in the 20 patients who maintained DFR for the duration of study follow-up are summarised in Table 5.4. Tendon PD was not observed in any of these patients on the month 6 scans. No significant differences were observed in the distributions of total ultrasound scores between baseline and

month 6 scans ($p > 0.999$, $p = 0.174$, $p = 0.402$, and $p = 0.098$ for joint GS, joint PD, tendon GS and erosion total scores respectively by paired Wilcoxon signed rank sum test) (Figure 5.4).

Of the 23 patients who experienced a flare during study follow-up, only 3 attended a month six appointment and thus had a repeat ultrasound scan. All three patients had developed grade 1/3 PD in a single joint at month 6, and 2/3 demonstrated increased joint GS compared to baseline (Figure 5.5A). There was no change in tendon GS score and no patient had tendon PD signal. There was no increase in total erosion score compared to baseline (Figure 5.5B). The small sample size prohibited statistical analysis in this group.

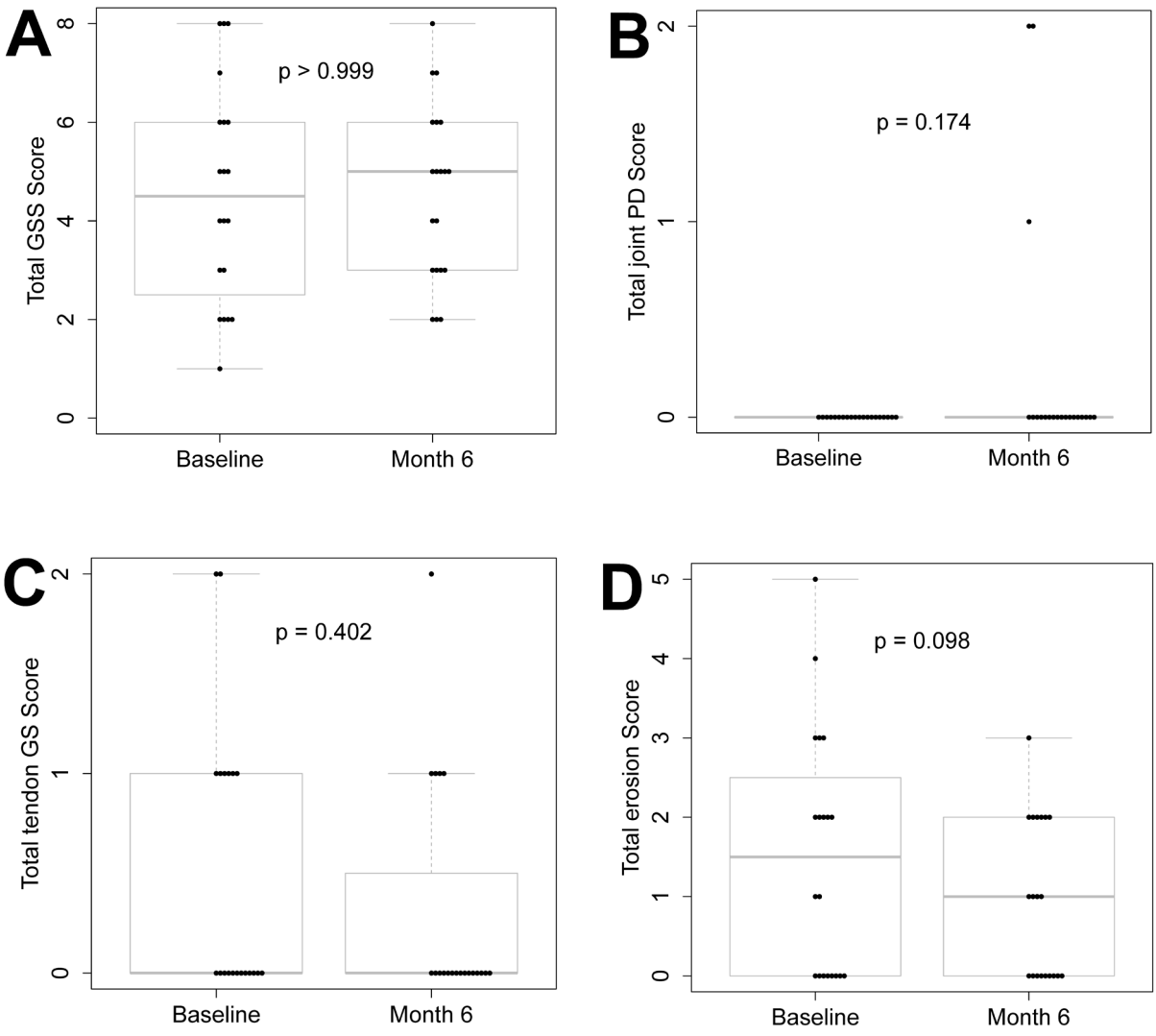


Figure 5.4 – Distribution of total joint GS scores (A), total joint PD scores (B), total tendon GS scores (C), and total joint erosion scores (D) at baseline and month 6 for patients who maintained DFR (p value calculated by paired Wilcoxon signed rank sum test).

Table 5.4 – Distribution of ultrasound scores at baseline and month six visits for the 20 patients who maintained DMARD-free remission for the duration of the study. PD (joint or tendon) was not observed at baseline by virtue of the study design, and none of the patients had tendon PD at month 6.

Parameter	Baseline visit	Month 6 visit
Total joint GS score: median (IQR) [range]	4.5 (2.7 – 6) [1 – 8]	5 (3 – 6) [2 – 8]
Total joint PD score: median (IQR) [range]	n/a	0 (0 – 0) [0 – 2]
Total tendon GS score: median (IQR) [range]	0 (0 – 1) [0 – 2]	0 (0 – 0.3) [0 – 2]
Total tendon PD score: median (IQR) [range]	n/a	n/a
Total erosion score: median (IQR) [range]	1.5 (0 – 2.3) [0 – 5]	1 (0 – 2) [0 – 3]

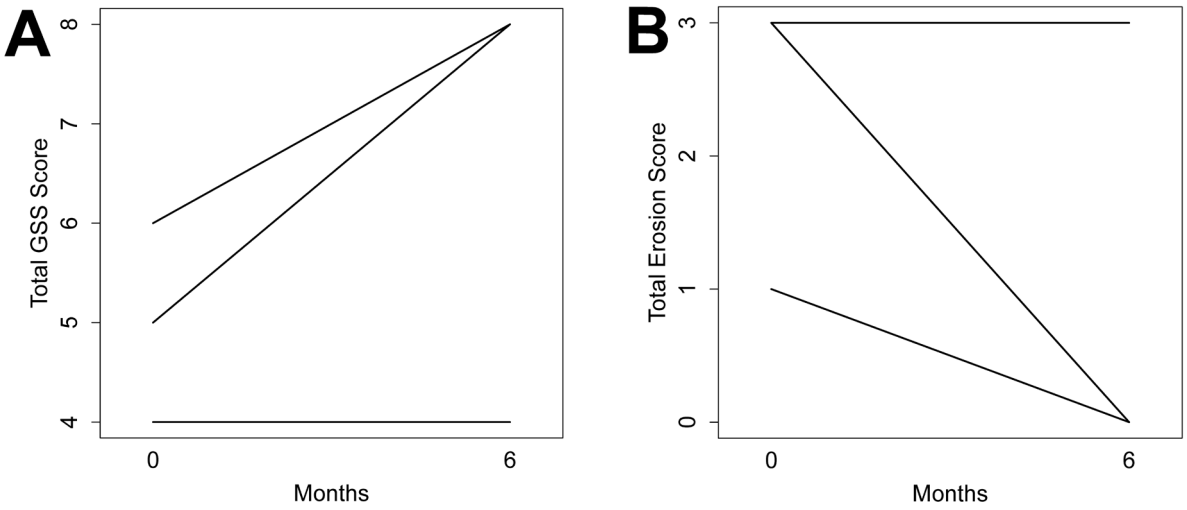


Figure 5.5 – Change in total joint GS score (A), and total erosion score (B) for the three patients who experienced an arthritis flare and had an ultrasound scan at six months. Each line represents the trajectory of an individual patient.

5.4 Association between clinical and ultrasound parameters in RA remission

The association between baseline clinical and ultrasound parameters was explored by multivariate ordinal logistic regression. Clinical parameters that were felt likely to correlate with ultrasound measures were selected, namely: sex, age, disease duration, smoking history, alcohol intake, RhF and ACPA positivity, ACR/EULAR Boolean remission, baseline HAQ-DI score, ESR and the individual components of the DAS28-CRP score. Patients who either had a baseline DAS28-CRP ≥ 2.4 (n = 7) or who had failed to meet either 1987 or 2010 RA diagnostic criteria (n=1) were excluded, leaving 66 patients with RA in clinical remission for

analysis. Regression was performed separately with each individual ultrasound parameter as the dependent variable (Appendix F); however, there were insufficient occurrences of tendon PD signal to allow for its statistical analysis.

Seven variable-score associations were statistically significant at the unadjusted $p<0.05$ level: male sex and ESR versus joint GS score; disease duration, tender joint count and ESR versus erosion score; and swollen joint count and alcohol intake versus tendon GS. (Figure 5.6). The largest effect was observed for male sex, which was associated with increased total joint GS score (OR_{GS} 5.04, 95% CI 1.47 – 17.26, $p = 0.010$). Higher ESR was also associated with higher joint GS score, though the effect size was small and of borderline significance (OR_{GS} 1.05 per year of disease, 95% CI 1.00 – 1.09, $p = 0.038$). Joint erosion was positively associated with disease duration ($OR_{erosion}$ 1.16, 95% CI 1.06 – 1.27, $p = 0.002$) though was negatively associated with tender joint count ($OR_{erosion}$ 0.17, 95% CI 0.05 – 0.56, $p = 0.004$) and ESR ($OR_{erosion}$ 0.92, 95% CI 0.85 – 0.99, $p = 0.022$). For tendon GS, swollen joint count showed a positive association ($OR_{tendon.GS}$ 5.37, 95% CI 1.46 – 19.72, $p = 0.011$) and alcohol intake showed a borderline negative association ($OR_{tendon.GS}$ 0.88, 95% CI 0.77 – 1.00, $p = 0.044$). All other variables failed to show a significant association with any of the ultrasound scores at the unadjusted 5% significance level.

To take account of multiple testing, p values were corrected according to the Benjamini-Hochberg procedure. After multiple test correction, only two associations remained significant, namely disease duration versus erosions (corrected $p = 0.024$) and tender joint count versus erosions ($p = 0.025$).

5.5 Survival analysis

The association between baseline ultrasound parameters (joint GS, tendon GS and erosions) and time-to-flare was analysed by Cox regression for all 44 patients who stopped DMARD therapy. Patients with higher total joint GS and erosion scores at baseline tended towards a shorter time-to-flare, though this was not statistically significant in either univariate or multivariate analysis (Table 5.5, Table 5.6 and Figure 5.7).

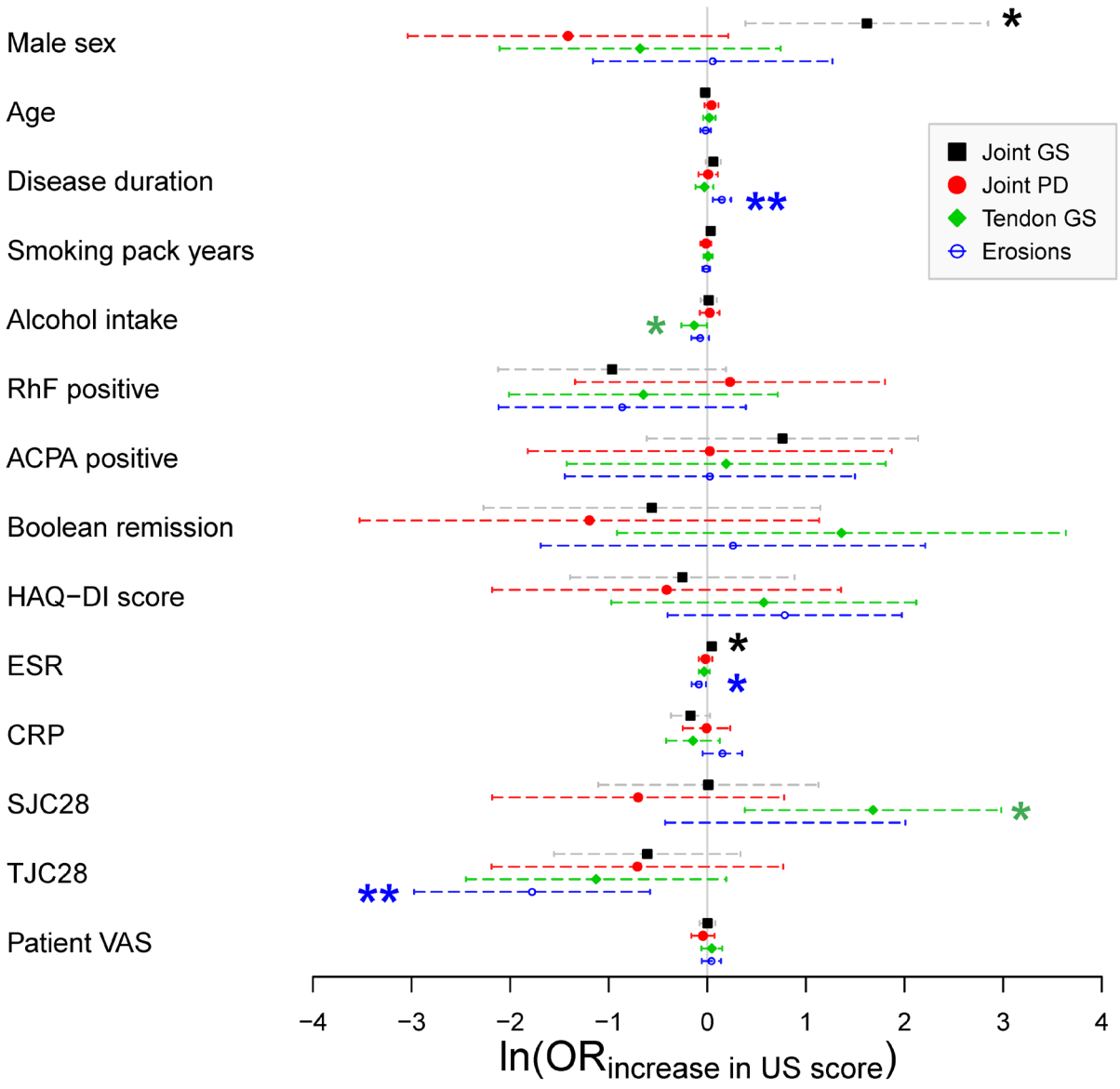


Figure 5.6 – Association between clinical and ultrasound parameters at baseline in a multivariate ordinal logistic regression model. OR: odds ratio. * = unadjusted $p < 0.05$, ** = Benjamini-Hochberg corrected $p < 0.05$)

Table 5.5 – Association of baseline ultrasound parameters with time-to-flare following DMARD cessation by univariate Cox regression.

Variable	HR_{flare}	95% CI	Univariate p-value
Total joint GS score	1.14	0.93 – 1.40	0.216
Total tendon GS score	1.01	0.52 – 1.97	0.980
Total erosion score	1.17	0.96 – 1.43	0.114

Table 5.6 – Association of baseline ultrasound parameters with time-to-flare following DMARD cessation by multivariate Cox regression.

Variable	HR_{flare}	95% CI	Multivariate p-value
Total joint GS score	1.15	0.93 – 1.43	0.208
Total tendon GS score	1.00	0.49 – 2.07	0.995
Total erosion score	1.18	0.96 – 1.44	0.109

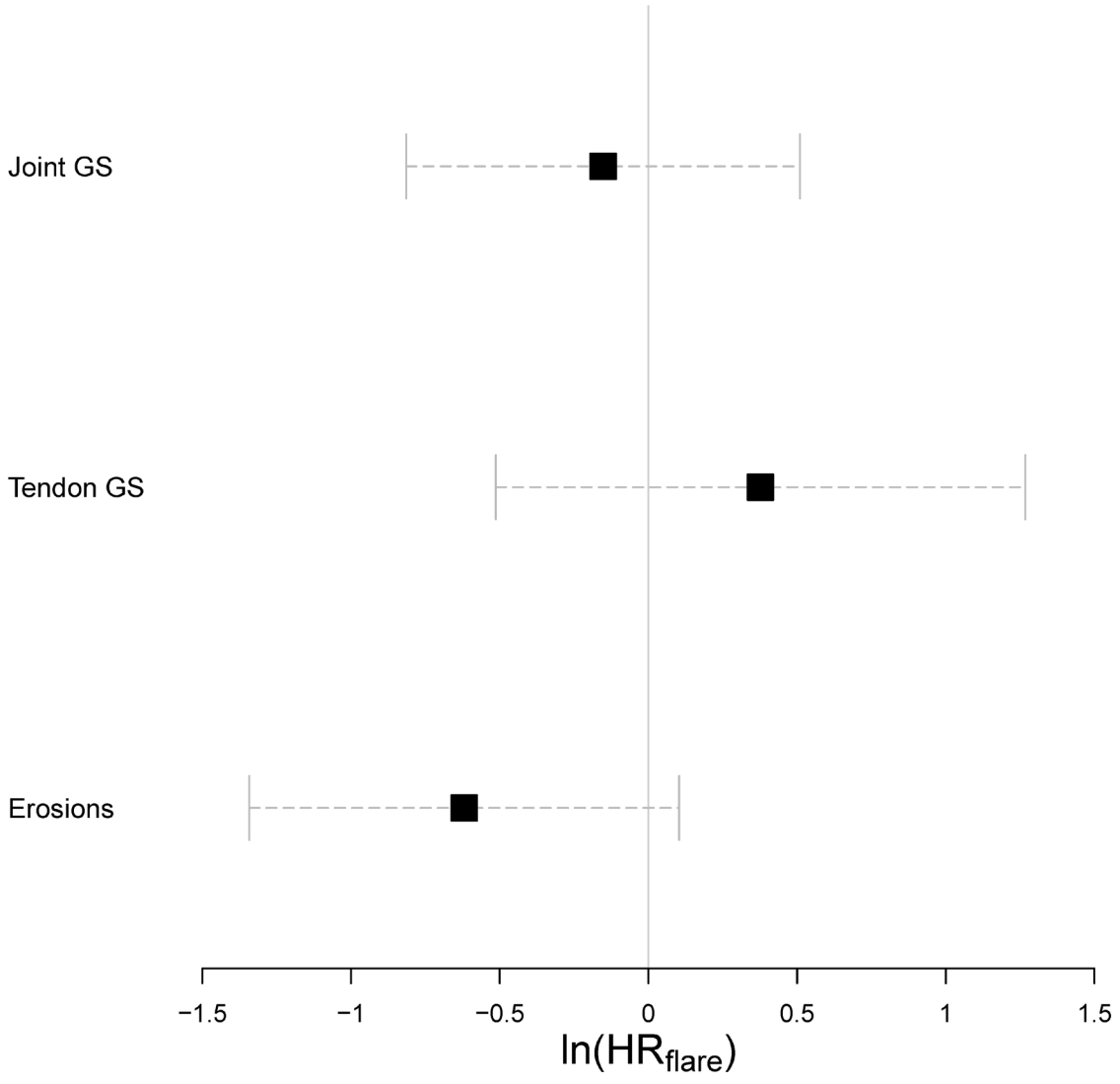


Figure 5.7 – Association between baseline ultrasound parameters and time-to-flare following DMARD cessation in a multivariate Cox regression model.

5.6 Discussion

5.6.1 Power Doppler

The primary focus of this study was to identify biomarkers of drug-free remission in RA. As such, it was considered advantageous to ensure as far as possible that all patients who were recruited to the study were genuinely in remission with no demonstrable evidence of synovitis. With this in mind, a decision was made during the design of the study not to withdraw DMARDs from patients with any grade of PD signal on a 7-joint musculoskeletal ultrasound scan. In doing so, it was acknowledged that low-level non-specific PD signal unrelated to RA activity might preclude DMARD cessation in otherwise eligible patients. Indeed, PD positivity has been observed in approximately 5% of healthy individuals (Millot *et al.*, 2011; Padovano *et al.*, 2016). Nevertheless, data emerging at the time of study design that supported a prognostic role of PD signal in predicting future flare (Saleem *et al.*, 2012) and joint erosion (Foltz *et al.*, 2012), combined with evidence of a correlation between PD-positivity and increased circulating angiogenic biomarkers (Ramirez *et al.*, 2014), was deemed sufficiently strong evidence to support the need for exclusion of PD-positive patients in this study.

Several criteria were specified for the definition of PD-positivity at the wrist in order to minimise the unnecessary exclusion of patients from DMARD withdrawal. These pragmatic criteria allowed for the inclusion of patients with single vessel PD signal, provided its origin could be easily traced, there was no branching within the joint space, and there was no surrounding greyscale change (see Methods 3.6). Despite these modifications, 19/30 (63%) of the patients who were excluded from DMARD withdrawal did so on the grounds of PD positivity alone, of which 7 had a single measurement of grade 1/3 PD signal at the wrist only.

Following the design of this study, several studies of biologic withdrawal in RA have identified PD signal as a predictor of arthritis relapse (Iwamoto *et al.*, 2014; Naredo *et al.*, 2015; Lamers-Karnebeek *et al.*, 2016). However, other studies have raised questions as to the value of ultrasound in the management of RA, with two recent large prospective trials (ARCTIC and TaSER) both failing to demonstrate a benefit of treat-to-ultrasound-target strategies over clinical targets alone (Dale *et al.*, 2016; Haavardsholm *et al.*, 2016). These findings have led to a recent shift away from a focus on ultrasound-defined treatment targets (Caporali and Smolen, 2018), though further research is needed. Given the current lack of clarity surrounding the role of ultrasound in the management of RA, hindsight suggests that

excluding patients with positive PD signal may have been a weakness in study design, both limiting recruitment and precluding assessment of its predictive utility in this setting. Nevertheless, analysis of the utility of other ultrasound parameters was possible, including greyscale hypertrophy at both joints and tendons, and joint bony erosions.

5.6.2 Baseline predictors of drug-free remission

Joint greyscale hypertrophy, joint erosions, and tendon greyscale change were explored for their utility in predicting DFR following DMARD cessation. Total scores for all three measures, as well as counts at the individual joint level, all failed to show a significant association with time-to-flare in univariate Cox regression analysis.

Few studies that have explored the predictive value of ultrasound in the setting of DMARD withdrawal, the results of which are often contradictory with wide confidence intervals, particularly for GS and erosion measures. In a study of bDMARD tapering in 77 patients in remission (Naredo *et al.*, 2015), total GS score in 12 joints was a significant predictor of flare at 12 months in univariate analysis (p=0.028), though not at 6 months (p=0.370) and not in the full 42-joint set (p = 0.187 at 12 months). In another study of 42 RA patients in remission discontinuing biologic therapy, both total GS and PD scores were significant univariate predictors of flare ($p < 0.01$), though a multivariate analysis was not performed (Iwamoto *et al.*, 2014). In a retrospective study of 40 patients discontinuing bDMARD therapy (Kawashiri *et al.*, 2017), bone erosion on ultrasound was the only significant predictor of flare in a multivariate analysis (OR_{flare} 8.35, 95% CI 1.78 – 53.2, p = 0.006). Finally, in a prospective study of csDMARD and bDMARD withdrawal, baseline ultrasound parameters demonstrated no utility for prediction or flare or drug-free remission (El Miedany *et al.*, 2016).

When comparing these results to those of my study, it is important to remember that the setting of the majority of these trials is very different, with a focus largely upon bDMARD withdrawal (with continued csDMARDs) and not complete DMARD cessation. It is thus conceivable that the patients within these studies have a more aggressive disease phenotype that may respond differently to the challenge of DMARD withdrawal. The results of my study therefore add useful data to the field and suggest, within the confines of limited sample size, that ultrasound-defined GS hypertrophy and erosions have limited role in the prediction of DFR following complete DMARD cessation.

5.6.3 Association of baseline ultrasound and clinical parameters

The association between baseline clinical and ultrasound parameters was explored for all 66 patients who both satisfied DAS28-CRP remission at baseline and who satisfied either 1987 or 2010 RA diagnostic criteria at baseline. After multiple test correction, two clinical variables demonstrated a significant association with joint erosions at baseline: disease duration ($OR_{erosion}$ 1.16, 95% CI 1.06 – 1.27, adjusted p = 0.024), and baseline tender joint count ($OR_{erosion}$ 0.17, 95% CI 0.05 – 0.56, p = 0.025).

The positive association between disease duration and erosions is expected, given that patients who have had RA for a longer period of time are likely to have been exposed to a longer cumulative duration of active synovitis. Furthermore, those with longer disease duration are likely to have ben diagnosed at a time when intensive early DMARD therapy was not standard-of-care for RA, and thus may have been more at risk of developing erosions in the early phases of the disease. The observation that patients with higher tender joint counts had less erosions is however unexpected, and perhaps suggests joint pain unrelated to RA activity in these patients – such as comorbid osteoarthritis for example. Intriguingly, Cheung *et al.* (2016) also observed a negative correlation between joint tenderness and future radiographic progression in RA patients starting anti-TNF therapy, though only in the absence of ultrasound-defined synovitis. Whether these observations reflect a distinct subset of 'high pain, low erosion' patients remains to be determined.

Few studies have examined the correlation between clinical and ultrasound measures of remission. In a cross-sectional study of 94 patients with RA, Peluso *et al.* (2011) found that early disease was associated with greater odds of combined GS and PD negativity (OR 7.6, 95% CI 2.3 – 25.8), in keeping with the non-significant trend towards higher GS with longer disease duration in my study. In contrast to my study, Peluso *et al.* (2011) demonstrated a positive association between ACR/EULAR Boolean remission and combined GS/PD negativity. However, in a cross-sectional analysis of 128 patients with RA in remission (DAS28-ESR < 2.6), Saleem *et al.* (2011) found no significant association of ACR/EULAR Boolean remission, CRP or tender joint count with either total GS nor total PD scores, in keeping with the negative findings of my study.

In summary, there was little correlation between clinical variables and ultrasound measures at baseline in those patients in clinical remission after application of multiple-test correction, notably including the key measure of PD positivity. If low-grade PD does indeed reflect clinically significant active synovitis despite clinical remission – as suggested by

histopathological studies (Alivernini *et al.*, 2017) – this would suggest that ultrasound assessment is necessary in addition to clinical assessment in order to detect subclinical inflammation prior to contemplating DMARD cessation. Nevertheless, the negative results of recent treat-to-ultrasound target initiatives, such as the ARCTIC and TaSER studies, suggest that such low-level PD signal may perhaps not be of clinical significance in the setting of clinical remission. This issue cannot be addressed further with the data from this study – further research in an independent cohort is required to validate these observations as well as address the significance of low-level PD in the setting of DMARD cessation.

5.6.4 Longitudinal ultrasound data

Ultrasound scans were repeated at month six, thus allowing for assessment of longitudinal change in those patients who maintained DFR. No significant change was seen in any of the ultrasound parameters between baseline and month six, demonstrating that maintenance of clinical remission was mirrored by a lack of accumulation of further ultrasound abnormalities. Three patients who experienced a flare at the month six appointment also had a second scan – this revealed an increase in joint PD and joint GS scores in 3/3 and 2/3 patients respectively, in keeping with the clinical flare phenotype. However, no increase in joint erosion score was apparent, suggesting no accumulation of joint damage during the study period in these patients, at least within the 7-joint set of the US7 scan protocol.

Although intra- and inter-rater reliability were good, a potential issue arose with the sensitivity to change of erosion score. For the 20 patients who maintained DFR, there was a non-significant trend towards a reduced joint erosion score at month six compared to baseline. Although it has been reported that erosions can resolve with time in some RA patients if their disease is controlled (Sharp *et al.*, 2003), this is unlikely to have occurred in the short duration of this study. In reality, the apparent reduction in erosion score more likely relates to issues surrounding the reproducibility of repeated ultrasound measures of joint erosion. The same effect was observed in those patients who experienced a flare at month six – for example, the total erosion score reduced from 3 to 0 for one patient. There are several possible explanations for these observations. Owing to the relatively advanced age of the cohort, it was sometimes difficult to discriminate osteoarthritic change from joint erosions, which may have led to misclassification in some patients. Furthermore, it is recognised that additional topographic features such as the anatomical joint neck and vascular bone channels can also be erroneously classified as erosions by ultrasound imaging (Kawashiri *et al.*, 2017). It would thus seem that

structures previously defined as erosions at baseline were either not seen or not classified as erosions on the repeat scan, perhaps due to differences in probe orientation. Alternatively, given the month six scans were performed unblinded to previous disease activity scores, unconscious bias by the ultrasound operator may have influenced the month six scan results. A more robust approach would have been to enlist an ultrasound operator who was blinded to both previous disease activity and study visit number; however, this was not possible given the limited study resources. Alternatively, joint erosions could have been assessed by plain radiographs at baseline and month six and interpreted by a blinded radiologist – however, it is unlikely that erosions large enough to be visible on plain radiographs would have developed over the short duration of the study. Nevertheless, it is important to emphasise that analysis of longitudinal change in imaging measures was not an aim of this study. To address this issue adequately, further research is required using more sensitive imaging modalities such as MRI, thus allowing visualisation of bone marrow oedema which would likely be the most sensitive to change over the six-month follow-up period.

5.7 Summary

In summary, this study provides no evidence to support a role for ultrasound-defined erosions and GS hypertrophy in the prediction of outcome following DMARD cessation in RA remission. Longitudinal observations serve to both corroborate a robust remission phenotype in those patients who maintained DFR, and confirm an increase in ultrasonographic measures of joint inflammation in the three patients who experienced an arthritis flare at month six. Furthermore, ultrasound measures were largely independent of clinical parameters, in keeping with the opinions of others that ultrasound assesses complementary though distinct aspects of disease activity compared to clinical assessment alone. Whether or not the detection of subclinical PD signal is important in guiding DMARD cessation cannot be addressed by the data from this study, and represents an important area of future research.

Chapter 6. Results 3 – Cytokine and Chemokine Data

6.1 Introduction

The measurement of circulating chemokines and cytokines provides an attractive approach to the development of biomarkers of DFR in RA. Suitable biological samples (serum or plasma) can be obtained with simple venepuncture, and require only a single centrifugation step before suitable for long-term freezer storage. Furthermore, many laboratory assays for the sensitive and specific detection of a wide range of human cytokines are already in widespread research and clinical use. A robust serum-based circulating cytokine/chemokine biomarker of DFR would thus be ideally placed for translation to a high-throughput assay suitable for use in clinical practice.

Surprisingly little research has been conducted to date that explores circulating serum predictors of DFR in RA beyond acute-phase markers. One notable exception is the multibiomarker disease activity (MBDA) score, a composite biomarker of 12 cytokines/chemokines that has shown potential in predicting DFR in a single study of DMARD cessation (see Introduction 1.7.3). Nevertheless, available tools such as the MBDA assay have been developed for the measurement of disease activity, and not specifically for the prediction of DFR. It is thus possible that such measures could overlook more nuanced mediators that are not directly involved in the acute phase response, but nonetheless play crucial roles maintaining the balance between sustained DFR and arthritis flare.

In this chapter, I present analysis of the cytokine and chemokine data of the BioRRA study. The specific aim of this work was to develop a cytokine/chemokine biomarker that, when measured immediately prior to DMARD cessation, could differentiate those patients who subsequently developed arthritis flare versus those who remained in sustained DFR. A secondary aim of these analyses was to explore the longitudinal change in circulating cytokine/chemokine profile in both flare and remission groups, thus providing insights to the underlying immunobiology of flare and DFR.

The structure of the remainder of this results chapter is as follows:

6.2 Quality control

6.3 Baseline predictors of drug-free remission and flare

6.4 Longitudinal analysis

6.5 Discussion

6.6 Summary

6.2 Quality control

In this study, commercially available multiplex electrochemiluminescence assays (MesoScale Discovery) were used for the detection of 39 chemokines and cytokines (see Methods 3.9.12). These kits benefit from both a highly specific antibody-based capture mechanism, together with robust quality control mechanisms to ensure reliable assay performance.

6.2.1 Sample collection

Serum was not collected for one patient at baseline due to difficult venous access – thus of the 44 patients who stopped DMARDs, baseline samples from 43 patients were available for analysis. Prior to the second substantial protocol amendment, serum was not collected at the month one visit for patients who satisfied remission criteria (see Methods 3.7). Consequently, serum was available for 29/42 month one visits and for 15/19 patient-requested unscheduled visits. Serum was collected for all month 3 and month 6 visits. Owing to time constraints, some of the MSD plates were processed before the final clinical study visit. Therefore, samples from later time points for patients who were still under follow-up within the study at the time of plate processing were not available for inclusion within the analysis (Table 6.1). Serum was available at the time of flare for 22/23 patients who experienced an arthritis flare – serum was not collected for the remaining patient as failure to achieve remission criteria was only apparent after the CRP result was available.

Time from blood draw to centrifugation was recorded for 133/136 serum samples collected, with a median value of 50 (IQR: 43 – 87, range: 30 – 210) minutes (Figure 6.1).

Table 6.1 – Collection of serum samples by study visit for patients who stopped DMARDs.

Visit type	Number of visits	Number of serum samples: n(% visits)	Number of samples processed on chemokine, cytokine and proinflammatory plates: n (% visits)	Number of samples processed on Th17 and vascular plates: n (% visits)
Baseline	44	43 (98)	43 (98)	43 (98)
Month one	42	29 (69)	29 (69)	29 (69)
Month three	26	26 (100)	22 (85)	26 (100)
Month six	23	23 (100)	16 (70)	23 (100)
Unscheduled	19	15 (79)	15 (79)	15 (79)
Total	**154**	**136 (88)**	**125 (81)**	**136 (88)**

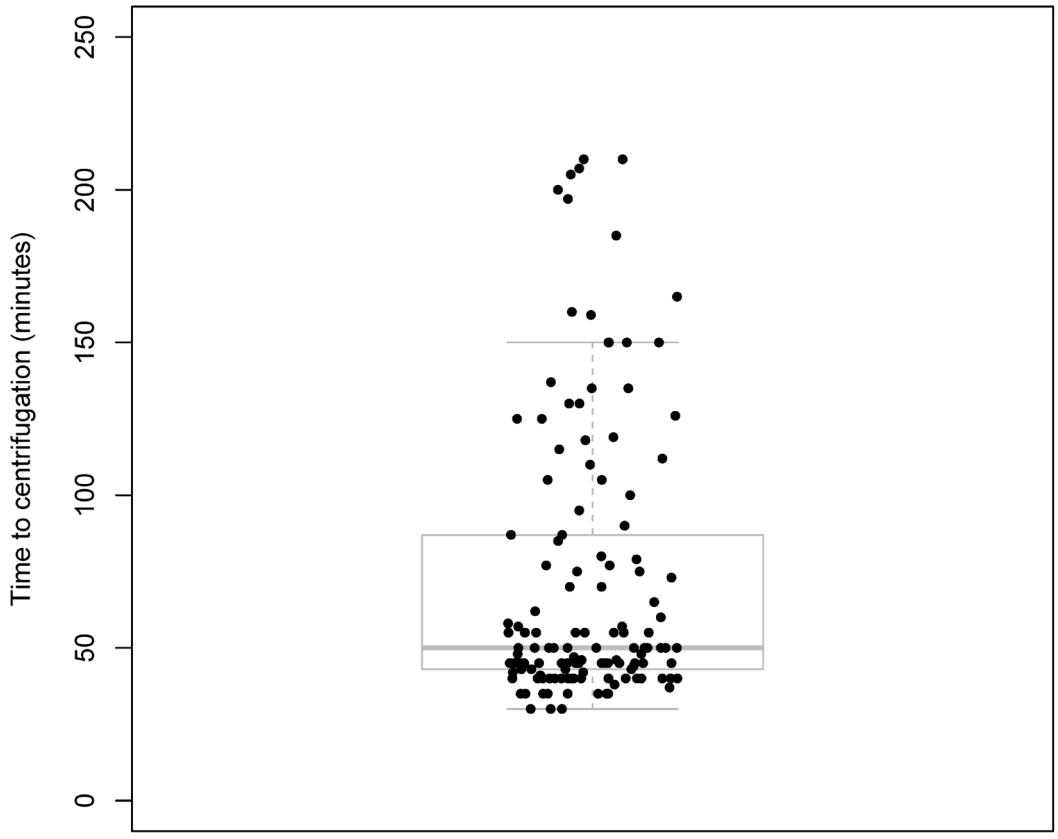


Figure 6.1 – Distribution of timed lengths between blood draw and serum centrifugation.

6.2.2 Calibrator coefficient of variation

Seven manufacturer-supplied calibrator solutions containing known concentrations of analytes were prepared by serial dilution for each plate. This allowed calculation of the percentage coefficient of variation (%CV) of calculated concentration for each calibrator on each plate (Appendix G). The manufacturer states that the %CV is typically less than 20% for repeat measurements. Over the 10 individual plates there were 656 pairs of calibrator measurements, of which %CV>20 in 60 (9%) of measurement pairs. Of these measurement pairs, 37/60 (62%) were within the three lowest concentration calibrators.

6.2.3 Limits of detection

A total of 6324 assay measurements (excluding calibrators and blanks) were recorded over the 10 plates. Upper and lower limits of detection (ULOD and LLOD respectively) were calculated for each assay as previously described (see Methods 3.9.12). No samples exceeded the ULOD, whereas 3391 (54%) had a calculated concentration below the LLOD. Samples where the calculated concentration fell below the LLOD were assigned a calculated concentration equal to the LLOD for each respective assay.

Two IL-8 assays were included with differing ranges of detection: a standard IL-8 assay and an alternative IL-8 assay (IL-8 (HA)) with a higher detection range. Concentration measurements frequently fell below the limits of detection for the IL-8(HA) assay, and thus results from the standard IL-8 assay were used for analysis. Similarly, two IL-17A assays were included based on different isotypes of detection antibody. A greater proportion of assays fell below the LLOD for the IL-17A assay on the Th17 panel plates in comparison to the IL-17A assay on the cytokine panel plates, and thus the former was excluded from analysis.

For analyses based solely on baseline samples, 13 assays where <20% of measurements were below the LLOD were excluded (Figure 6.2). For longitudinal analysis of flare patients, six assays where all measurements were below the LLOD at both baseline and time of flare were excluded. For longitudinal analysis of remission patients, eight assays where all measurements were below the LLOD at both baseline and month six visit were excluded. The assays included within each analysis following the above quality control steps are summarised in Table 6.2.

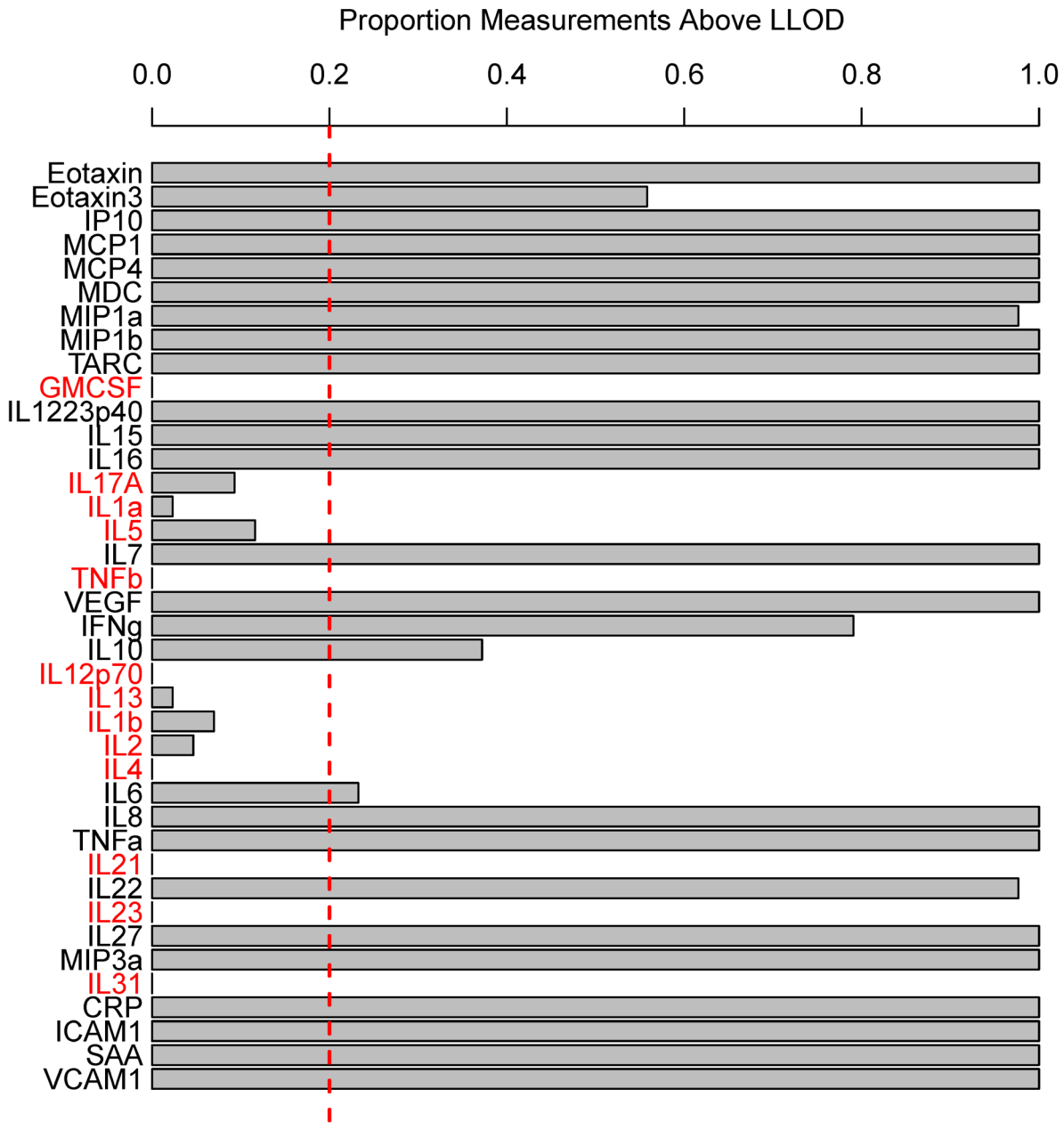


Figure 6.2 – Proportion baseline samples where calculated concentration was above the assay-specific LLOD. Assays which fell below the 20% threshold (red line) were excluded from analysis.

Table 6.2 – Inclusion of assays by type of analysis.

		Inclusion of assay by analysis type		
MSD plate	**Assay**	**Baseline (all patients)**	**Baseline to flare visit (flare patients)**	**Baseline to month six visit (remission patients)**
Cytokine panel 1 (human)	GM-CSF	✗	✗	✗
	IL-12/IL-23p40 subunit	✓	✓	✓
	IL-15	✓	✓	✓
	IL-16	✓	✓	✓
	IL-17A	✗	✓	✓
	IL-1α	✗	✓	✓
	IL-5	✗	✓	✓
	IL-7	✓	✓	✓
	TNF-β	✗	✗	✓
	VEGF	✓	✓	✓
Chemokine panel 1 (human)	Eotaxin	✓	✓	✓
	Eotaxin-3	✓	✓	✓
	IL-8(HA)	✗	✗	✗
	IP-10	✓	✓	✓
	MCP-1	✓	✓	✓
	MCP-4	✓	✓	✓
	MDC	✓	✓	✓
	MIP-1α	✓	✓	✓
	MIP-1β	✓	✓	✓
	TARC	✓	✓	✓
Proinflammatory panel 1 (human)	IFN-γ	✓	✓	✓
	IL-10	✓	✓	✓
	IL-12p70 subunit	✗	✗	✗
	IL-13	✗	✓	✗
	IL-1β	✗	✓	✗
	IL-2	✗	✓	✓
	IL-4	✗	✓	✗
	IL-6	✓	✓	✓
	IL-8	✓	✓	✓
	TNF-α	✓	✓	✓
Th17 panel 1 (human)	IL-17A	✗	✗	✗
	IL-21	✗	✗	✗
	IL-22	✓	✓	✓
	IL-23	✗	✗	✗
	IL-27	✓	✓	✓
	MIP-3α	✓	✓	✓
	IL-31	✗	✗	✗
Vascular injury panel 2 (human)	CRP	✓	✓	✓
	ICAM-1	✓	✓	✓
	SAA	✓	✓	✓
	VCAM-1	✓	✓	✓

6.2.4 Logarithmic data transformation

The distribution of calculated concentrations for each assay was typically positively skewed. To create a more even distribution of data, the natural logarithm of each analyte concentration measurement was calculated before further data analysis. A constant value (+ 1) was added to each analyte concentration before logarithmic transformation to allow for zero values. An illustrative effect of this logarithmic transformation is depicted in Figure 6.3.

6.2.5 Plate equilibration

It was not possible to analyse all serum samples together on the same plate, as the total number of samples (136) exceeded the number of available wells (80) on a single plate. It was therefore necessary to process samples across pairs of each type of MSD plate. Each pair of plates was processed on the same day to minimise variation. Furthermore, all baseline samples were run together on the same plate within each plate pair, thus permitting analysis of baseline samples without any effect of plate-to-plate variation.

By replicating baseline samples where space allowed, it was attempted to keep all samples from the same participant together on the same single plate for the purposes of longitudinal analysis – however, this was not possible in all cases. Despite the manufacturer's claims of excellent reproducibility of assay performance between plates of identical lots, it was deemed necessary to apply a data equilibration procedure to minimise any potential effect upon

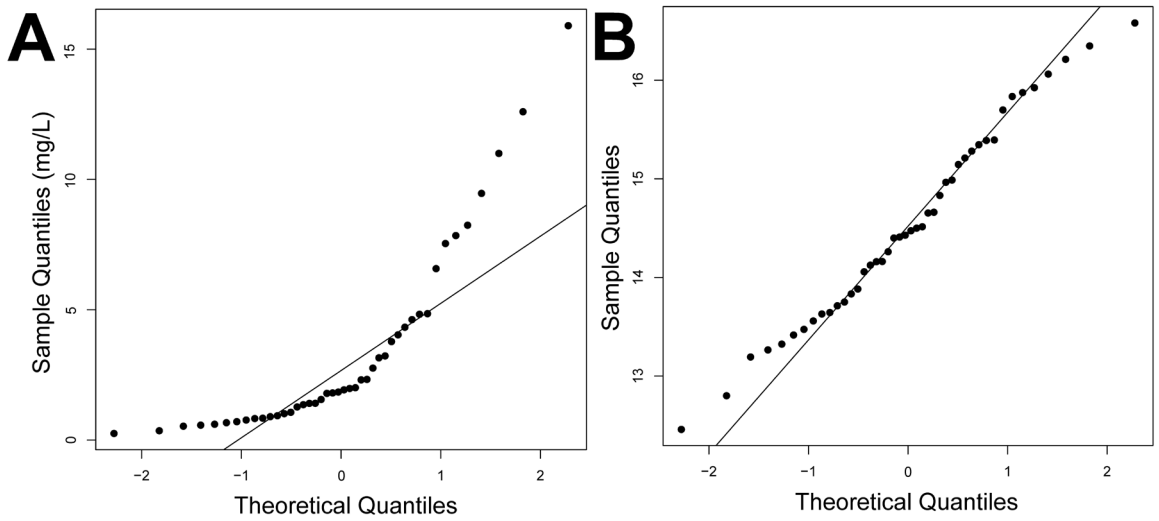


Figure 6.3 – Normal quantile-quantile (Q-Q) plot for baseline CRP concentration before (A) and after (B) natural logarithmic transformation.

longitudinal analyses. First, the relationship between the concentrations of each individual analyte for duplicated samples (i.e. present on both plates within a pair) was modelled by linear regression as detailed in Formula 6.1.

Formula 6.1 – linear regression equation for plate equilibration procedure

$$Plate_2\ concentration = m * (plate_1\ concentration) + c$$

The regression formula was then applied to each individual concentration measurement for each assay within each $plate_2$, thus equilibrating $plate_2$ with $plate_1$ measurements in each plate pair. Regression coefficients and constants for each assay are listed in Appendix H. Finally, to avoid any bias introduced by differing LLODs between plate pairs, the highest LLOD of either plate within each pair was applied for the purposes of longitudinal data analyses. Where a sample had been analysed on both plates, the sample measurement on the same plate as the majority of other samples for that patient was used for analysis to reduce further any bias introduced by plate-to-plate variation.

There were 11 assays where only two or fewer samples measured above the LLOD on both plates – these assays were thus excluded from plate equilibration, namely: GM-CSF, IL-1α, TNFβ, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-21, IL-23 and IL-31. In addition, MIP1α concentration demonstrated a poor correlation between plates and was also excluded. Plate equilibration was not performed for these samples, but rather only concentrations from a single plate (i.e. excluding values from the other plate) at the level of each patient were used for analysis.

6.3 Baseline predictors of drug-free remission and flare

6.3.1 Baseline survival analysis

The association between baseline cytokine/chemokine levels and time-to-flare following DMARD cessation were analysed by univariate Cox regression (Table 6.3) for all 26 assays that passed quality control checks (see Results 6.1.3). Proportionality of hazards was observed for all variables with the exception of ln(ICAM1+1). There was no statistical association between ln(ICAM1+1) and flare by univariate Cox regression ($p = 0.95$). To confirm that this lack of association was not a spurious effect of poor survival modelling, the relationship

Table 6.3 – Association between the circulating concentration of cytokines/chemokines at baseline and time-to-flare following DMARD cessation, analysed by univariate Cox regression. Hazard ratios are calculated for a 1-unit change in log-transformed cytokine/chemokine concentration. B: Cox regression coefficient. P values calculated by the Wald test.

Variable	B	HR_{flare}	HR_{flare} 95% CI	Univariate p value
ln(MCP1+1)	2.212	9.13	1.97 – 42.32	0.005
ln(CRP+1)	0.426	1.53	1.02 – 2.31	0.042
ln(Eotaxin+1)	1.386	4.00	0.97 – 16.5	0.055
ln(IL6+1)	0.730	2.08	0.97 – 4.45	0.060
ln(TNFa+1)	1.273	3.57	0.93 – 13.7	0.063
ln(IP10+1)	0.592	1.81	0.97 – 3.38	0.064
ln(IL10+1)	1.737	5.68	0.65 – 49.8	0.117
ln(IL27+1)	0.948	2.58	0.78 – 8.53	0.120
ln(MCP4+1)	0.522	1.69	0.84 – 3.38	0.140
ln(IL15+1)	1.572	4.82	0.56 – 41.6	0.153
ln(Eotaxin3+1)	0.310	1.36	0.81 – 2.28	0.238
ln(VCAM1+1)	0.654	1.92	0.55 – 6.72	0.306
ln(IL16+1)	0.694	2.00	0.52 – 7.65	0.311
ln(IL7+1)	0.628	1.87	0.51 – 6.84	0.342
ln(IL22+1)	-0.502	0.61	0.20 – 1.87	0.384
ln(MIP1a+1)	-0.485	0.62	0.19 – 2.04	0.427
ln(TARC+1)	0.283	1.33	0.64 – 2.76	0.448
ln(MIP1b+1)	0.342	1.41	0.47 – 4.17	0.538
ln(MDC+1)	-0.376	0.69	0.15 – 3.07	0.623
ln(IL8+1)	0.171	1.19	0.55 – 2.57	0.664
ln(IFNg+1)	0.074	1.08	0.73 – 1.59	0.712
ln(VEGF+1)	-0.043	0.96	0.46 – 1.99	0.909
ln(MIP3a+1)	0.033	1.03	0.58 – 1.85	0.912
ln(SAA+1)	0.017	1.02	0.66 – 1.56	0.940
ln(ICAM1+1)	0.033	1.03	0.34 – 3.15	0.954
ln(IL1223p40+1)	0.019	1.02	0.47 – 2.21	0.961

between ln(ICAM1+1) and occurrence of flare was assessed by univariate binomial logistic regression, which also demonstrated a similar lack of association (OR_{flare} 0.66, 95% CI 0.12 – 3.72, p = 0.64).

Following univariate modelling, the 10 variables with a univariate p-value < 0.2 were then entered to a multivariate Cox regression model, and stepwise backward variable selection based on AIC was performed. After seven rounds of selection, three variables remained in this stepwise model (Table 6.4 and Figure 6.4). Proportionality of hazards was confirmed for all three variables by the Schoenfeld residual test.

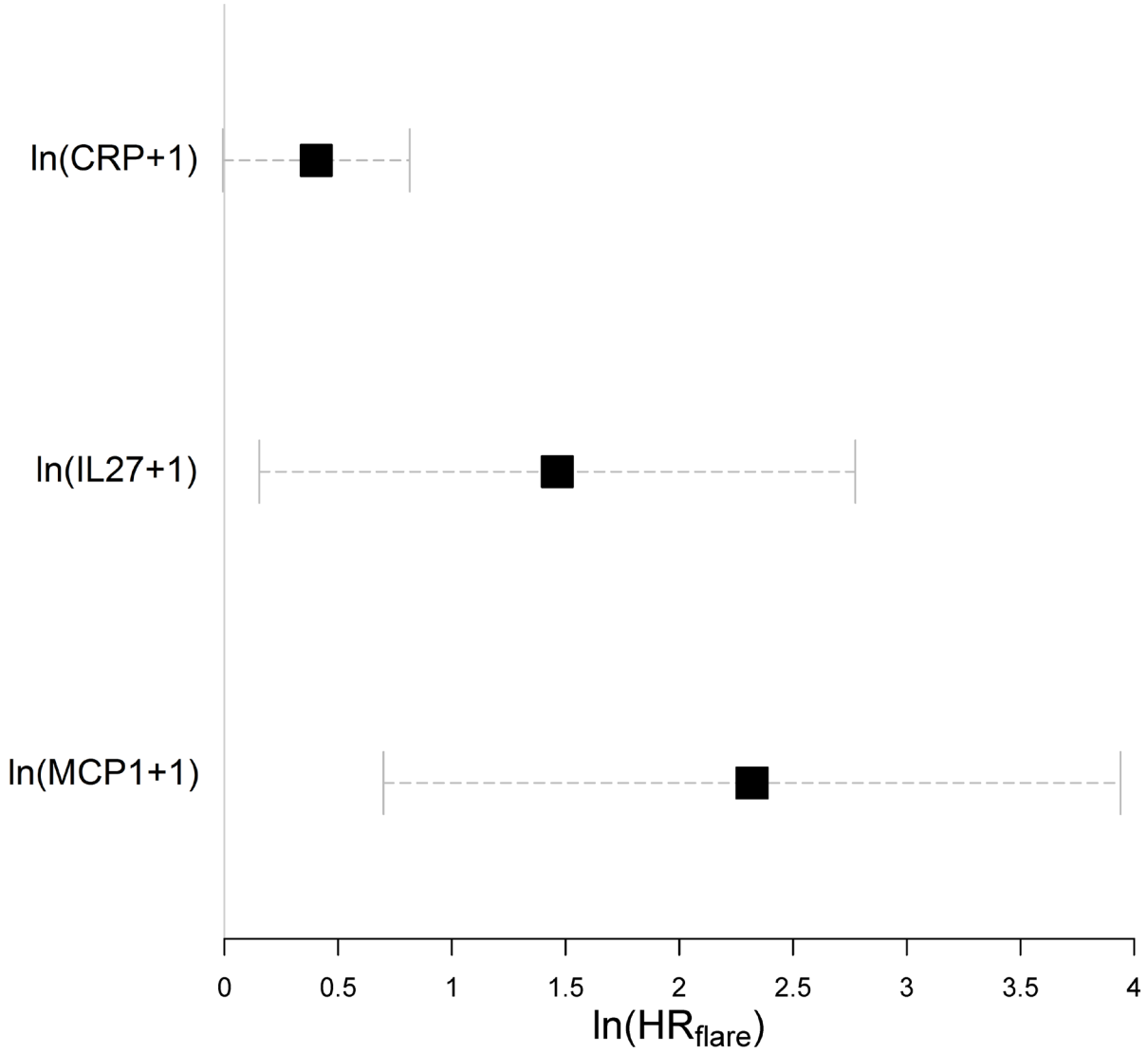


Figure 6.4 – Summary of the three variables included in the stepwise multivariate Cox regression model.

The distributions of baseline ln(MCP1+1), ln(IL27+1) and ln(CRP+1) by flare status are summarised in Figure 6.5. Baseline MCP1 levels were significantly higher in those patients who experienced an arthritis flare (p=0.012, unpaired Student's T-test). There was a trend towards higher baseline CRP and IL-27 in the flare group, though this was not statistically significant (p = 0.121 and p = 0.224 respectively).

6.3.2 ROC analysis and biomarker thresholds

Patients were dichotomised based on their baseline levels of MCP1, IL-27 and CRP using two thresholds determined by ROC analysis optimised for the prediction of flare and remission, as

Table 6.4 - Association between the circulating concentration of cytokines/chemokines at baseline and time-to-flare following DMARD cessation, analysed in a backward stepwise multivariate Cox regression model. Hazard ratios are calculated for a 1-unit change in log-transformed cytokine/chemokine concentration. B: Cox regression coefficient. P values calculated by the Wald test.

Variable	B	HR_{flare}	95% CI HR_{flare}	Multivariate p
ln(MCP1+1)	2.320	10.2	2.01 – 51.4	0.005
ln(IL27+1)	1.464	4.32	1.17 – 16.0	0.029
ln(CRP+1)	0.404	1.50	0.99 – 2.26	0.054

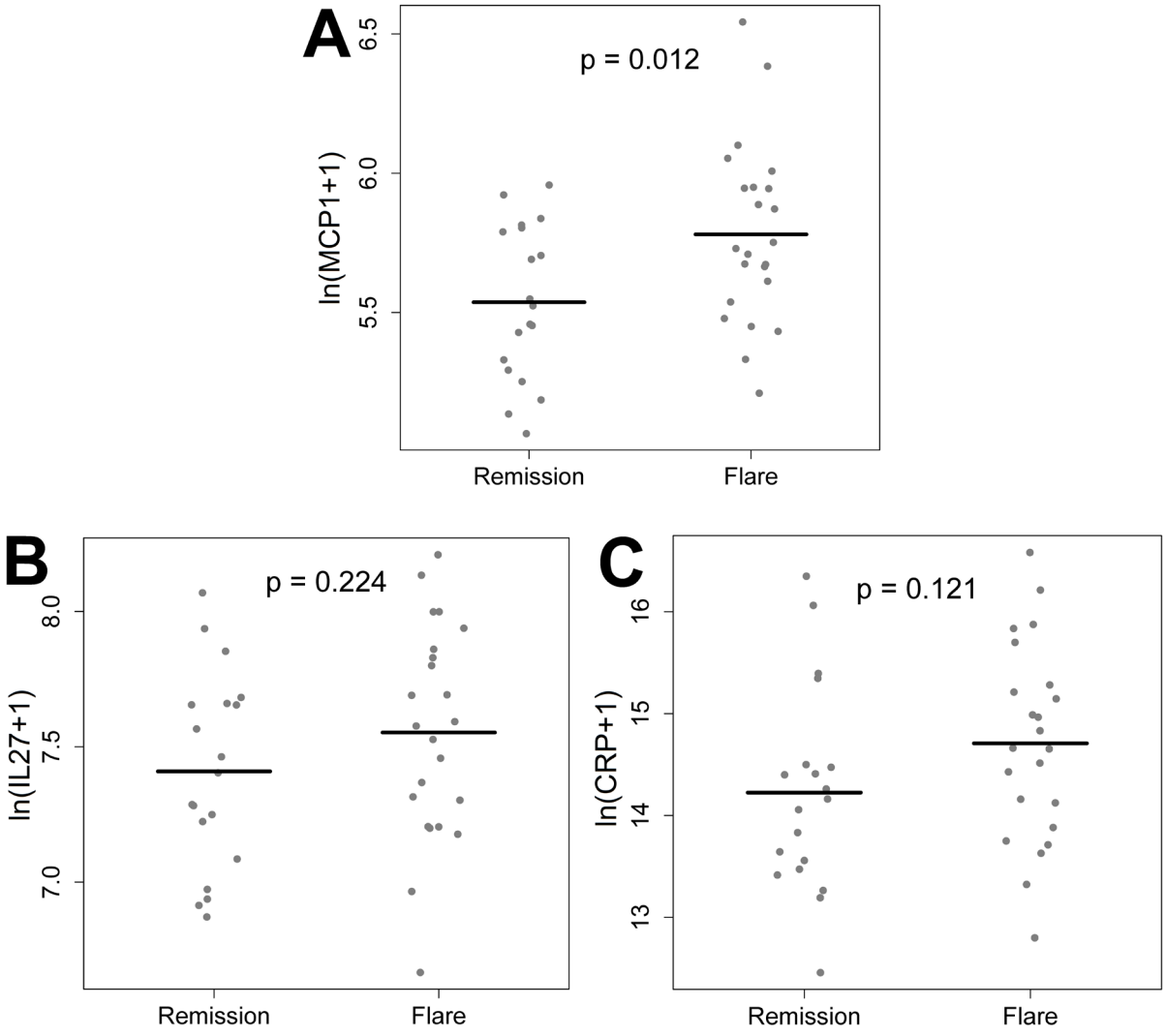


Figure 6.5 – Distribution of log2-transformed concentrations of MCP1 (A), IL-27 (B), and CRP (C) at baseline in flare and remission groups. Solid line represents mean value, statistical significance of difference in means calculated by unpaired Student's T-test.

previously discussed (Methods 3.10.1). Variables were also combined to form composite scores, weighted by their respective coefficients from the stepwise multivariate Cox regression model (Table 6.5). The ROC curves for each score are presented in Figure 6.6.

Whereas MCP1 performed reasonably well in isolation, the discriminative value of IL-27 and CRP when used on their own was relatively poor. The best results were observed for composite measures, namely MCP1+CRP and MCP1+IL27, which performed best for the identification of future remission (Figure 6.7) and flare (Figure 6.8) respectively. A composite score encompassing all three variables did not yield any additional predictive value (Table 6.6).

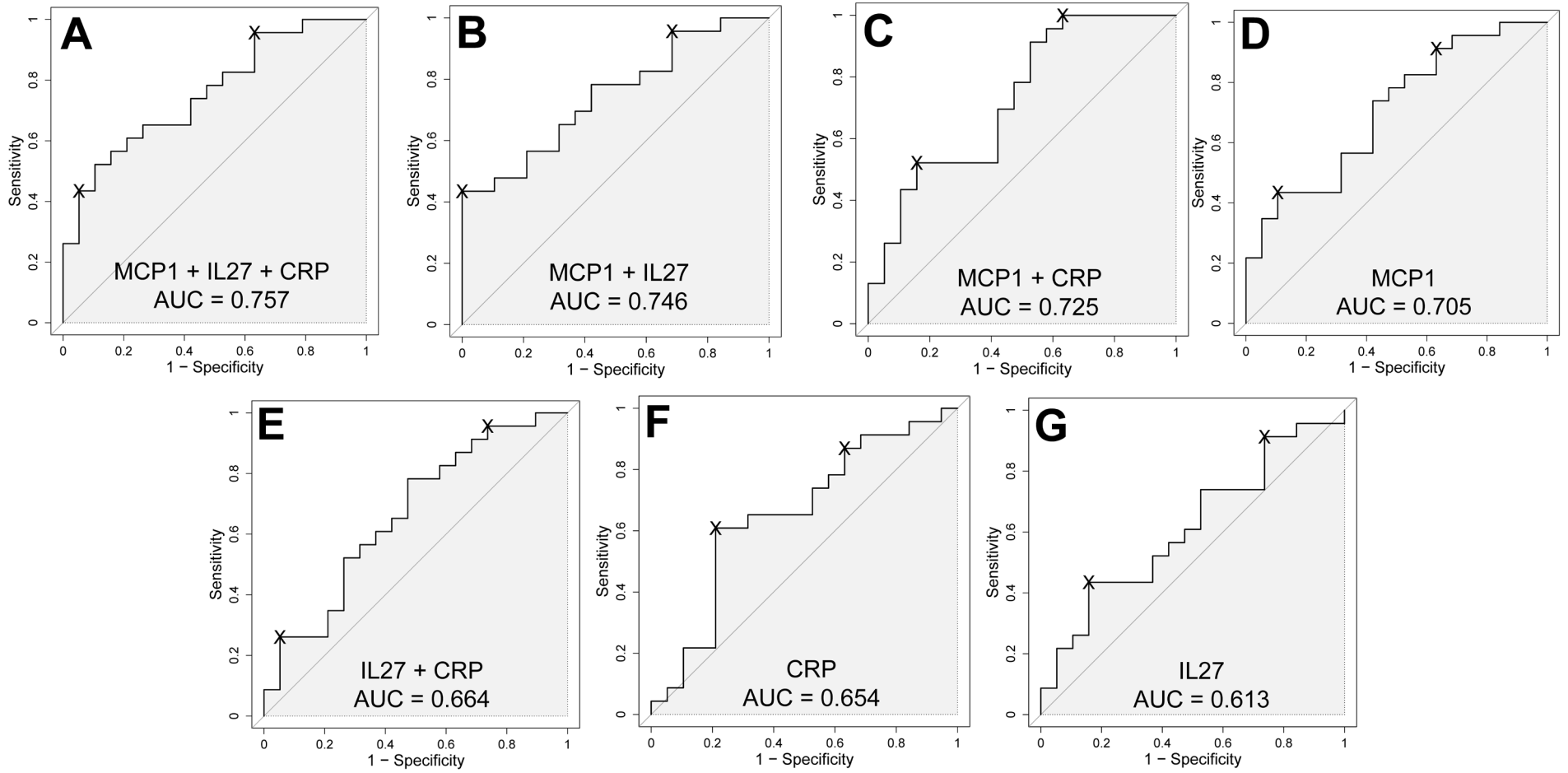
Table 6.5 – Cytokine/chemokine composite scores ranked by ROC_{AUC}. Variables included within each score are indicated in green, and those excluded are indicated in red.

ln(MCP1+1)	ln(IL27+1)	ln(CRP+1)	Remission threshold	Flare threshold	ROC_{AUC}
✓	✓	✓	29.31	30.54	0.757
✓	✓	✗	23.01	24.695	0.746
✓	✗	✓	18.17	19.29	0.725
✓	✗	✗	5.43	5.85	0.705
✗	✓	✓	16.15	17.45	0.664
✗	✗	✓	13.68	14.51	0.654
✗	✓	✗	7.13	7.69	0.613

Table 6.6 - Predictive utility of cytokine/chemokine variables in predicting flare following DMARD cessation, with a positive test defined by either flare or remission thresholds. Optimum pairs of predictive metrics are highlighted in **bold**. NPV: negative predictive value; PPV: positive predictive value.

Variable	ROC_{AUC} (95% CI)	Threshold	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
MCP1+CRP	0.73 (0.57 – 0.88)	Flare (19.29)	0.52 (0.30 – 0.70)	0.84 (0.68 – 1.00)	0.80 (0.63 – 1.00)	0.59 (0.48 – 0.72)
		Remission (18.17)	1.00 (1.00 – 1.00)	**0.37** (0.16 – 0.58)	0.66 (0.59 – 0.74)	**1.00** (1.00 – 1.00)
MCP1+IL27	0.75 (0.60 – 0.89)	Flare (24.695)	**0.43** (0.26 – 0.65)	1.00 (1.00 – 1.00)	**1.00** (1.00 – 1.00)	0.59 (0.53 – 0.70)
		Remission (23.01)	0.96 (0.87 – 1.00)	0.32 (0.11 – 0.53)	0.63 (0.56 – 0.72)	0.88 (0.57 – 1.00)
MCP1+IL27+CRP	0.76 (0.61 – 0.90)	Flare (30.54)	0.43 (0.22 – 0.65)	0.95 (0.84 – 1.00)	0.92 (0.71 – 1.00)	0.58 (0.50 – 0.69)
		Remission (29.31)	0.96 (0.87 – 1.00)	0.37 (0.16 – 0.58)	0.65 (0.58 – 0.74)	0.89 (0.60 – 1.00)

Figure 6.6 – Receiver-operating characteristic curves for cytokine/chemokine composite biomarker scores for the prediction of flare following DMARD cessation. A: MCP1+IL27+CRP, B: MCP1+IL27, C: MCP1+CRP, D: MCP1, E: IL27+CRP, E: CRP, F: IL27.



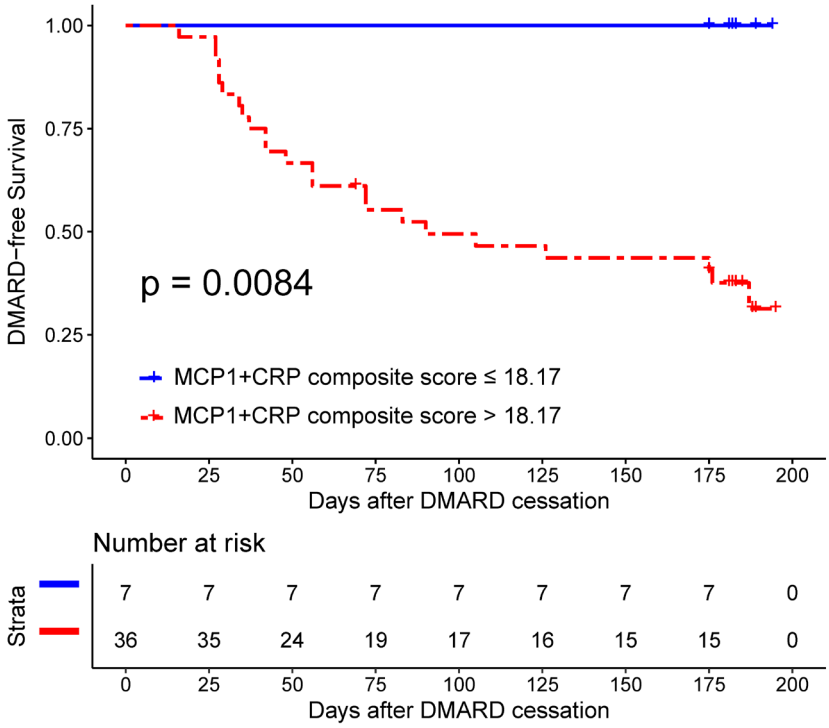


Figure 6.7 – Kaplan-Meier plot of DMARD-free survival for the study population dichotomised by baseline composite MCP1/CRP score using the remission threshold. All patients with a negative composite score maintained DFR.

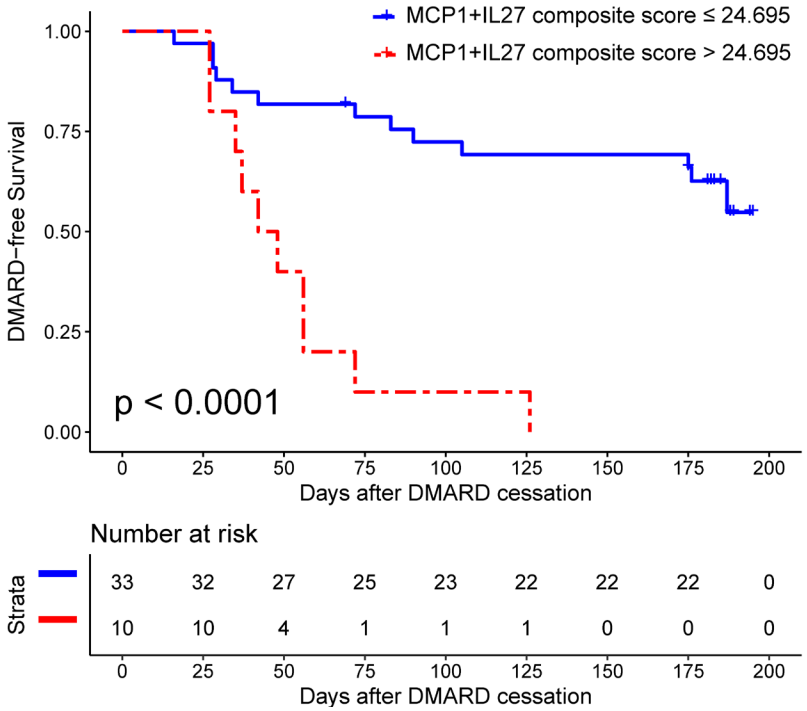


Figure 6.8 - Kaplan-Meier plot of DMARD-free survival for the study population dichotomised by baseline composite MCP1/IL-27 score using the flare threshold. All patients with a positive composite score experienced an arthritis flare.

6.3.3 Sensitivity analysis for time to centrifugation

Cytokines and chemokines are known to degrade in whole blood that has been left to stand for a prolonged period before centrifugation (Jackman *et al.*, 2011). To assess whether this may have influenced the biomarker analysis, the three variables within the composite scores (i.e. ln(MCP1+1), ln(IL27+1) and ln(CRP+1)), and time from blood draw to centrifugation, were entered to a multivariate Cox regression model. In the three visits where centrifugation delay was not recorded, the median centrifugation delay across all visits was imputed. No significant association was observed between centrifugation delay (minutes) and arthritis flare (HR_{flare} 1.00, 95% CI 0.99 – 1.00, p = 0.465). Similar coefficients and statistical significance were observed for the three cytokine/chemokine variables as per the main analysis, demonstrating that their utility for predicting arthritis flare was not affected by inclusion of centrifugation delay within the model (Table 6.7). Proportionality of hazards was observed for all individual variables and the model as a whole.

6.4 Longitudinal analysis

6.4.1 Baseline to flare visit

Cytokine and chemokine levels were compared at baseline and flare visits in the 22 patients who both experienced a flare and had serum available at the time of flare. The statistical significance of differences between log-transformed cytokine/chemokine concentrations at baseline and flare visits was assessed by Student's paired T-test (Table 6.8). Four analytes demonstrated a >1.5 fold change in concentration at the 5% significance level after multiple-test correction using the Benjamini-Hochberg procedure: CRP, SAA, IL-6 and IP-10 (Figure 6.9).

Table 6.7 – Sensitivity analysis incorporating time from blood draw to centrifugation within a multivariate Cox regression model. Hazard ratios are calculated for a 1-unit change in log-transformed cytokine/chemokine concentration, or for a 1 minute change in centrifugation delay.

Variable	B	HR_{flare}	95% CI	p
ln(MCP1+1)	2.24	9.41	1.83 – 48.42	0.007
ln(IL27+1)	1.47	4.34	1.18 – 15.94	0.027
ln(CRP+1)	0.37	1.45	0.97 – 2.18	0.070
Centrifuge delay (minutes)	0.00	1.00	0.99 – 1.01	0.465

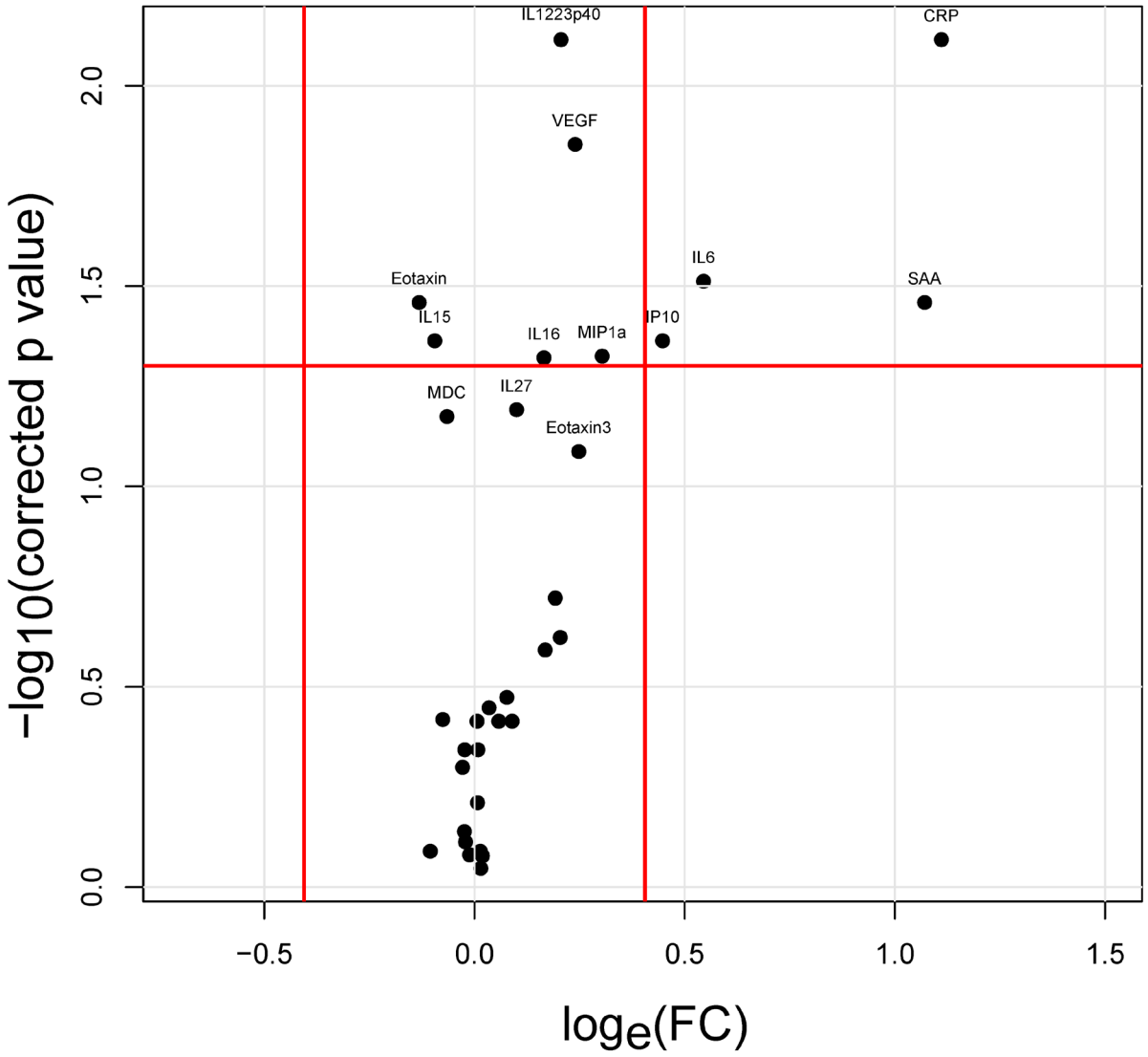


Figure 6.9 – Volcano plot of $\log_e$(fold change) in chemokine/cytokine concentration between baseline versus flare visit, and associated adjusted p values (Student's paired T-test, Benjamini-Hochberg) for those patients who experienced an arthritis flare. Thresholds for significance are shown at fold-change > 1.5 and adjusted $p < 0.05$. FC: fold-change.

Table 6.8 – Change in cytokine/chemokine concentration from baseline to flare visits in those patients who experienced an arthritis flare following DMARD cessation. Statistical significance was assessed by Student's paired T-test, with multiple test-correction using the Benjamini-Hochberg procedure. FC: fold-change.

Variable	Number of sample pairs	Mean $\log_e$(FC) from baseline to flare	Unadjusted p value	Adjusted p value
ln(IL1223p40+1)	20	0.206	<0.001	0.008
ln(CRP+1)	22	1.111	<0.001	0.008
ln(VEGF+1)	20	0.239	0.001	0.014
ln(IL6+1)	20	0.545	0.004	0.031
ln(Eotaxin+1)	20	-0.132	0.006	0.035
ln(SAA+1)	22	1.071	0.006	0.035
ln(IP10+1)	20	0.447	0.010	0.043
ln(IL15+1)	20	-0.095	0.010	0.043
ln(MIP1α+1)	20	0.304	0.013	0.047
ln(IL16+1)	20	0.165	0.014	0.048
ln(IL27+1)	22	0.100	0.021	0.064
ln(MDC+1)	20	-0.066	0.024	0.067
ln(Eotaxin3+1)	20	0.248	0.032	0.082
ln(ICAM1+1)	22	0.192	0.081	0.190
ln(MIP3α+1)	22	0.204	0.108	0.238
ln(IL1α+1)	20	0.168	0.124	0.256
ln(VCAM1+1)	22	0.077	0.173	0.336
ln(IL5+1)	20	0.034	0.194	0.356
ln(TARC+1)	20	-0.076	0.219	0.381
ln(IL17A+1)	20	0.006	0.257	0.385
ln(IL10+1)	20	0.058	0.248	0.385
ln(TNFα+1)	20	0.089	0.240	0.385
ln(IL2+1)	20	-0.023	0.330	0.454
ln(IL4+1)	20	0.008	0.330	0.454
ln(IL13+1)	20	-0.029	0.380	0.502
ln(IL1β+1)	20	0.007	0.485	0.615
ln(MCP1+1)	20	-0.024	0.594	0.726
ln(IL7+1)	20	-0.022	0.654	0.771
ln(MCP4+1)	20	0.014	0.739	0.813
ln(IFNγ+1)	20	-0.105	0.732	0.813
ln(MIP1β+1)	20	-0.012	0.779	0.829
ln(IL22+1)	22	0.019	0.810	0.835
ln(IL8+1)	20	0.015	0.897	0.897

6.4.2 Baseline to month six remission visit

Cytokine and chemokine levels were compared at baseline and month six visits in the 19 patients who maintained DFR and had serum available at baseline and month six. The statistical significance of differences between log-transformed cytokine/chemokine concentrations at baseline and month six visits was assessed by Student's paired T-test (Table 6.9). No analytes exceeded a >1.5 fold change in concentration at the 5% significance level (Figure 6.10).

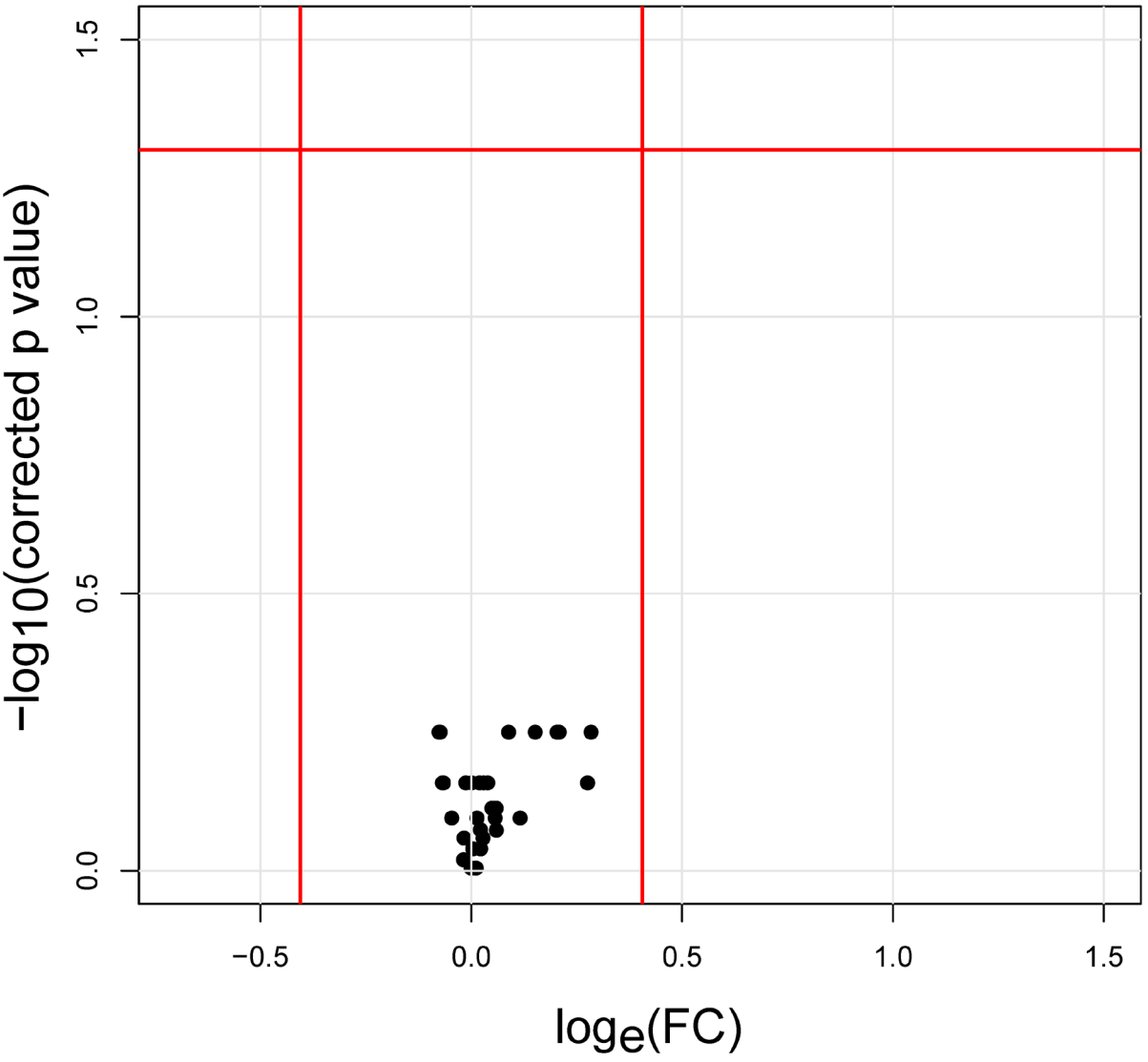


Figure 6.10 - Volcano plot of $\log_e$(fold change) in chemokine/cytokine concentration between baseline and month six visits and associated adjusted p values (Student's paired T-test, Benjamini-Hochberg) for those patients who remained in remission. Thresholds for significance are shown at fold-change > 1.5 and adjusted $p < 0.05$. FC: fold change.

Table 6.9 – Change in cytokine/chemokine concentration from baseline to month six visits in those patients who remained in remission following DMARD cessation. Statistical significance was assessed by Student's paired T-test, with multiple test-correction using the Benjamini-Hochberg procedure.

Assay	Number of sample pairs	Mean $\log_e$(fold change) from baseline to month 6	Unadjusted p value	Adjusted p value
ln(TNFα+1)	14	0.203	0.051	0.562
ln(MCP1+1)	14	0.088	0.064	0.562
ln(IL15+1)	14	-0.074	0.077	0.562
ln(IL1223p40+1)	14	0.151	0.099	0.562
ln(MIP3α+1)	19	0.208	0.100	0.562
ln(IP10+1)	14	0.284	0.121	0.562
ln(TARC+1)	14	-0.077	0.127	0.562
ln(IL1α+1)	14	-0.069	0.188	0.693
ln(IFNγ+1)	14	0.276	0.216	0.693
ln(IL5+1)	14	0.029	0.259	0.693
ln(IL10+1)	14	0.019	0.260	0.693
ln(IL7+1)	14	-0.066	0.294	0.693
ln(IL27+1)	19	0.039	0.309	0.693
ln(TNFβ+1)	14	0.001	0.336	0.693
ln(IL2+1)	14	-0.013	0.336	0.693
ln(VEGF+1)	14	0.059	0.419	0.771
ln(VCAM1+1)	19	0.049	0.423	0.771
ln(ICAM1+1)	19	0.057	0.534	0.803
ln(IL16+1)	14	-0.046	0.541	0.803
ln(SAA+1)	19	0.116	0.542	0.803
ln(IL17A+1)	14	0.013	0.544	0.803
ln(MIP1β+1)	14	0.022	0.598	0.843
ln(Eotaxin3+1)	14	0.059	0.626	0.844
ln(IL22+1)	19	0.028	0.688	0.872
ln(MDC+1)	14	-0.017	0.704	0.872
ln(IL6+1)	14	0.003	0.768	0.912
ln(MCP4+1)	14	0.022	0.794	0.912
ln(MIP1α+1)	14	-0.018	0.862	0.955
ln(IL8+1)	14	0.010	0.929	0.988
ln(CRP+1)	19	0.011	0.968	0.988
ln(Eotaxin+1)	14	-0.001	0.988	0.988

6.4.3 Longitudinal change in selected cytokines and chemokines

The longitudinal change in circulating concentrations of selected cytokines and chemokines was explored across all study visits where serum samples were available for analysis. Owing to infrequent sampling, particularly within patients who flare, it was not possible to use formal hypothesis-testing methods such as spline analysis or multi-level modelling beyond comparison of baseline to final study visit (Figure 6.11). Therefore, descriptive analysis with the aid of longitudinal 'spaghetti plots' is presented herein (Figure 6.12).

Figure 6.11 – Longitudinal change in selected cytokines and chemokines from baseline to flare visit in patients who experienced an arthritis flare, and baseline to month six visit for patients who remained in remission following DMARD cessation. Solid line shows mean value, unadjusted statistical significance of difference between means calculated by paired Student's t-test.

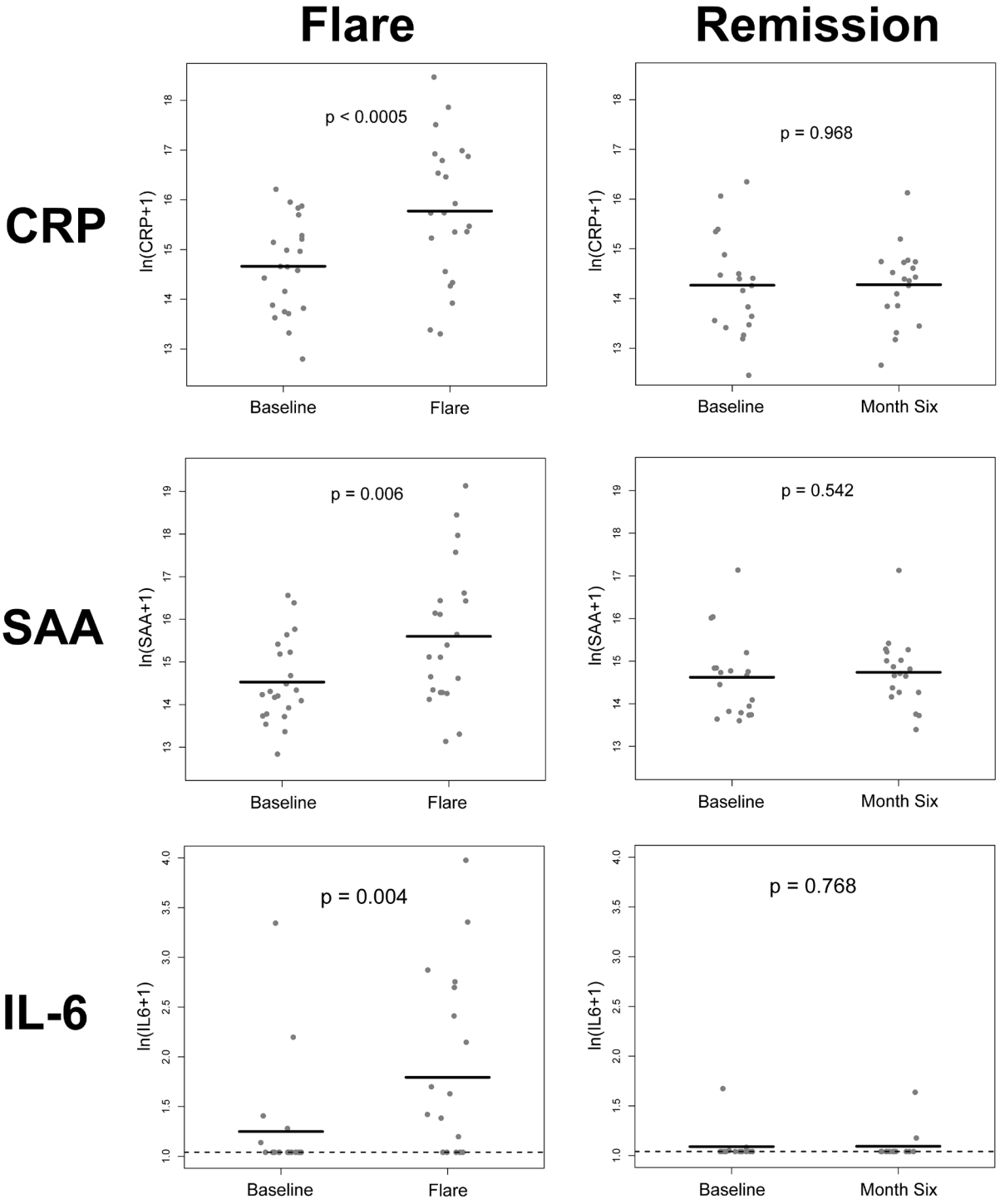


Figure 6.11 (continued)

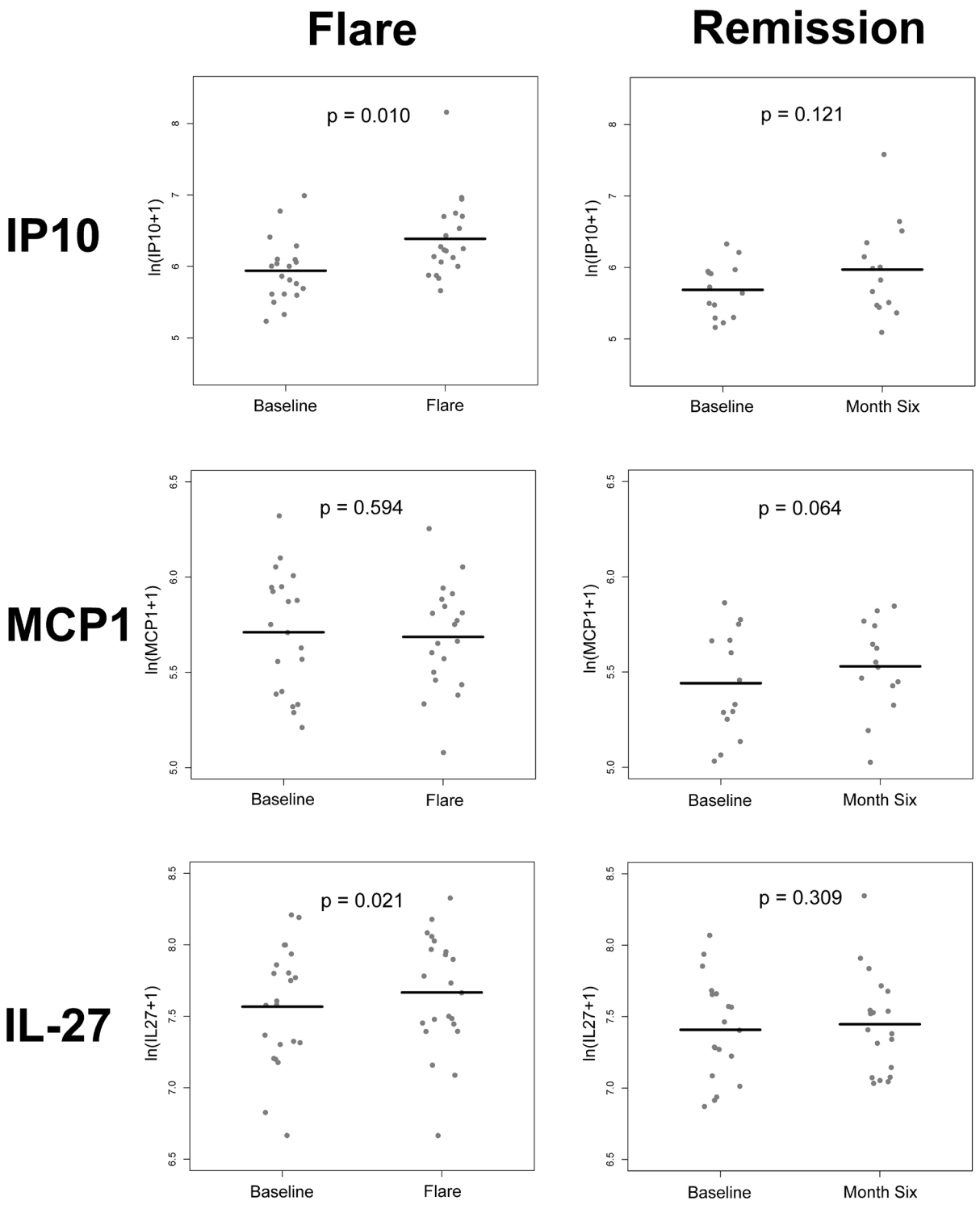
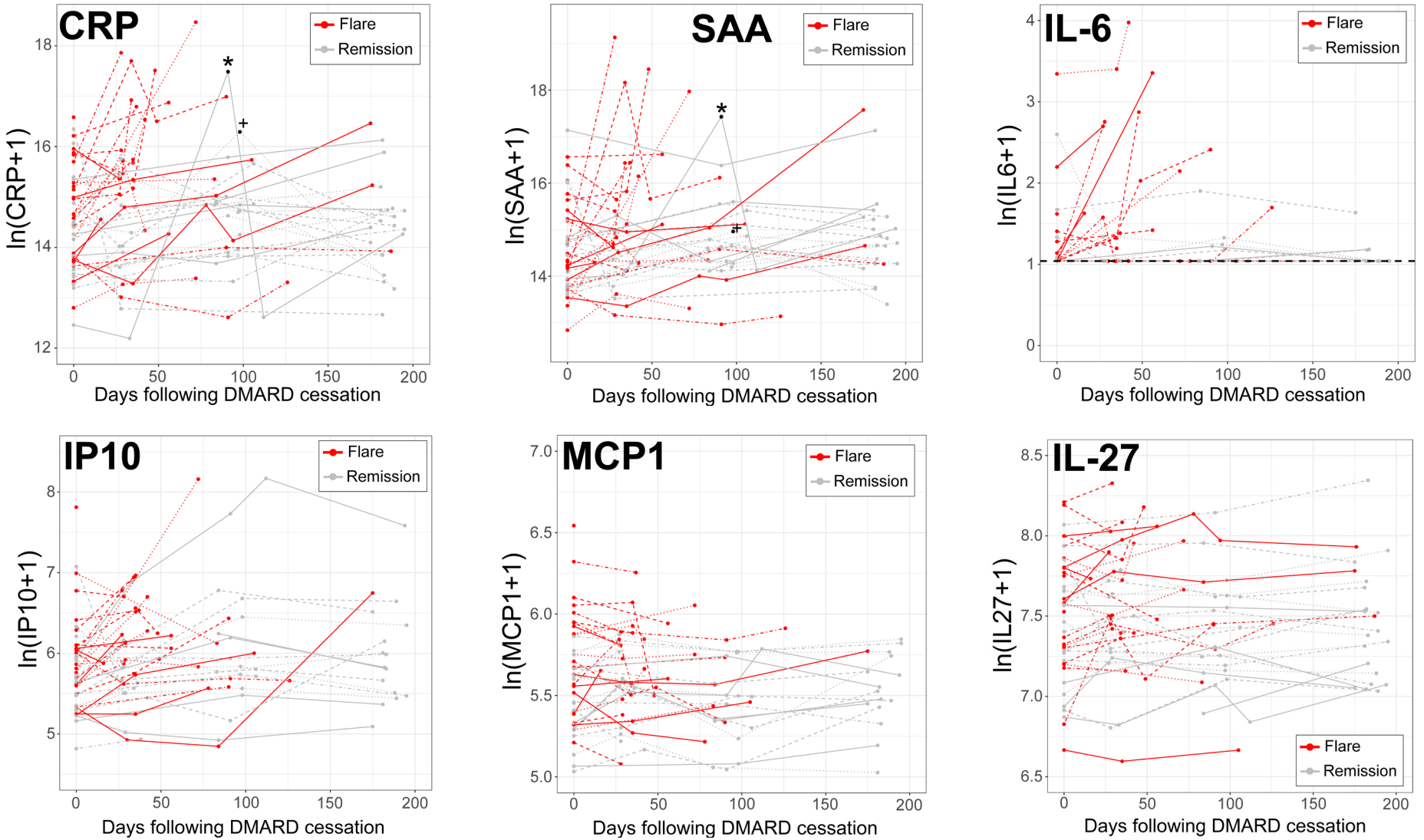


Figure 6.12 – Longitudinal change in selected cytokines and chemokines. Each line represents an individual patient; those who experienced an arthritis flare are shown in red, whereas those who remained in remission are shown in grey. Spikes in CRP and SAA secondary to urinary tract infection (*) and chest infection (+) are highlighted.



Both CRP and SAA concentrations rose in the approach towards arthritis flare, and remained essentially static for patients in remission, with the exception of two remission patients who had a concomitant urinary tract and lower respiratory tract infections respectively at the time of their month 3 study visit. IL6 concentrations abruptly rose at the time of flare, and remained static for patients in remission, although many samples fell below the plate-merged LLOD. Overall, IP10 concentrations rose higher in the flare versus remission group, though there was greater heterogeneity in the individual patient trends.

There was a modest overall increase in IL-27 from baseline to flare which was not observed in patients who remained in remission, although the individual patient trends were somewhat mixed. There were no clear longitudinal trends in MCP1, in contrast to its utility for predicting flare when measured at baseline.

6.5 Discussion

6.5.1 Baseline predictors of drug-free remission

Studies of DMARD withdrawal in RA remission to-date have focussed largely on clinical parameters, and surprisingly few data have been published regarding the use of cytokines in the prediction of DFR. The only studies of note to have addressed this issue utilise a 12-cytokine score known as the multi-biomarker disease activity (MBDA) score, commercially marketed as Vectra® DA (Crescendo Bioscience Inc., San Francisco, USA) (Centola *et al.*, 2013) (see Introduction 1.4.5). Of the 12 cytokines included in the MBDA score, five were also measured in this study: serum amyloid A (SAA), C-reactive protein (CRP), IL-6, vascular endothelial growth factor (VEGF) and vascular cell adhesion molecule 1 (VCAM-1).

In a sub-analysis of 94 patients of the RETRO study, MBDA score was significantly higher ($p = 0.0001$) at baseline in patients who subsequently experienced an arthritis flare following DMARD tapering/cessation compared to those remaining in remission (Rech *et al.*, 2016). Furthermore, the predictive value of MBDA score appeared to be multiplicative with that of ACPA status, such that 76% of $ACPA^+$ patients with a high MBDA score experienced an arthritis flare compared with 13% of $ACPA^-$ patients with a low MBDA score ($p = 0.0001$). Of note, the statistically significant higher MBDA score in those patients who flared was a result of a combination of relatively small and individually non-significant elevations of 8 of the 12 cytokine score components, namely SAA, CRP, IL-6, matrix metalloproteinase 1 and 3 (MMP-1 and -3), leptin, epidermal growth factor (EGF), and VEGF (Rech *et al.*, 2016). The

results of the RETRO study therefore corroborate the elevated baseline CRP and IL-6 measurements in patients who flared in my study, and corroborate the lack of predictive value of VCAM-1. In contrast, no predictive value was seen at baseline for SAA or VEGF in my study, though the levels of SAA did rise at the time of flare.

The predictive value of the MBDA score has also been studied in the setting of bDMARD tapering and withdrawal, though with mixed results. In an exploratory analysis of the 439 patients who stopped anti-TNF therapy as part of the POET study, high MBDA score was associated with a modest increased risk of physician-reported flare (OR_{flare} 2.00, 95% CI 1.06 – 3.77) (Lamers-Karnebeek *et al.*, 2015). A description of the components of the MBDA score that contributed to this observation was not presented. In contrast to the above two studies, a sub-analysis of 171 patients from the DRESS study – an RCT of anti-TNF tapering and cessation in RA – found no significant association between MBDA score and future arthritis flare in those patients tapering anti-TNF therapy (Bouman *et al.*, 2017). Again, a breakdown of individual cytokine trends was not presented by the authors of this study. However, neither of these studies addressed DFR as csDMARDs were continued in all patients.

6.5.2 Monocyte chemoattractant protein 1 (MCP-1/CCL2)

Monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) is a 13kDa protein composed of 76 amino acids, and encoded on the long arm of chromosome 17 (Deshmane *et al.*, 2009). MCP-1 is secreted by a wide range of different cell types, and has potent chemoattractant properties for monocytes, and also memory T cells and NK cells (Deshmane *et al.*, 2009). MCP1 acts via the G-protein coupled receptor CCR2, which exists in 2 isoforms: CCR2A and CCR2B. Upregulation of CCR2 expression has been observed in in several autoimmune diseases, including by myocytes and mononuclear inflammatory infiltrates in inflammatory myopathy (Bartoli *et al.*, 2001), and by synovial fibroblasts in RA (Cho *et al.*, 2007). Studies of single nucleotide polymorphisms (SNPs) affecting MCP-1 have also shed light on the potential importance of the chemokine in human autoimmunity. In a recent meta-analysis of 26 studies exploring the association between autoimmunity and the 2518A/G SNP in the MCP-1 promotor, Chen *et al.* (2016) found a positive association of A vs G allele in Asian patients with RA (OR 1.616, 95% CI 1.027 – 2.542, p = 0.038) and European patients with Crohn's disease (OR 1.383, 95% CI 1.142 – 1.676, p = 0.022). However, a converse association was observed for AA vs. AG/GG genotype in European

patients with lupus nephritis (OR 0.713, 95% CI 0.545 – 0.933, p = 0.014) (Chen *et al.*, 2016). In contrast however, an independent though smaller meta-analysis of 14 studies found no significant association of the MCP-1-2518A/G SNP with susceptibility to RA, vasculitis or multiple sclerosis (Lee and Bae, 2016).

MCP-1 has been implicated in the pathogenesis of RA by several studies. Higher concentration of MCP-1 has been observed in the synovial fluid of patients with RA versus non-RA controls (Chen *et al.*, 2017), and higher CCR2 expression has been demonstrated in synovial fibroblasts (Cho *et al.*, 2007) and circulating neutrophils (Talbot *et al.*, 2015) in RA versus OA and healthy controls respectively. Furthermore, in a nested case-control study of 220 women who later developed RA compared to 675 controls, Arkema *et al.* (2015) demonstrated a positive association between circulating MCP-1 concentration in the five years before disease onset and future risk of developing RA (OR 2.42, 95% CI 1.22 – 4.89) adjusted for confounding factors including smoking, alcohol intake and obesity. Finally, circulating MCP-1 concentration has been shown to correlate closely with DAS28-CRP score in an observational cohort study of 111 RA patients (Liou *et al.*, 2013).

In my study, MCP-1 is predictive of outcome following DMARD cessation, with low levels at baseline demonstrating particular discrimination for those patients who maintained DFR. This observation is biologically plausible - low circulating levels of MCP-1 could conceivably reflect lower production by stromal and immune cell mediators within the synovium, thus reducing recruitment of monocytes, T cells and NK cells to the joints. Interestingly, no significant increase in MCP-1 concentration was observed in longitudinal analysis of patients who experienced an arthritis flare. This observation suggests a possible modulatory effect of MCP-1 at baseline, as opposed to representing a surrogate measure of disease activity. However, whether the observed low levels of circulating MCP-1 reflect a qualitative difference in immune homeostasis in those patients who maintain DFR, rather than simply a quantitative difference in subclinical synovitis, remains open to speculation and would require the study of matched synovial biopsy tissue in these patients.

6.5.3 Interleukin-27

IL-27 is a relatively recently discovered member of the IL-12 family, which also includes IL-12, IL-23 and IL-35. IL-27 is a heterodimer composed of the 34kDa glycopeptide Epstein-Barr virus-induced gene 3 (EBI3) – which is also a component of IL-35 – together with the 24.5 kDa polypeptide IL-27p28 (Yoshida and Hunter, 2015). IL-27 is produced primarily by

antigen presenting cells including dendritic cells, monocytes and macrophages (Meka *et al.*, 2015). The receptor for IL-27 (IL-27R) is a heterodimer of IL-27Rα and gp130, the latter of which also forms part of several other cytokine receptors including the receptors for IL-6 and IL-35 (Yoshida and Hunter, 2015). IL-27R is expressed on T and B lymphocytes, NK cells, mast cells and antigen-presenting cells, and signals via the Janus kinase (JAK) / signal transducer and activation of transcription (STAT) and p38 mitogen-activated protein kinase (MAPK) pathways (Meka *et al.*, 2015).

The role of IL-27 in the modulation of immune processes is complex, with sometimes contradictory effects dependent on the cell type, local cytokine milieu, and experimental setting or human disease in question. Initial animal studies demonstrated a pro-Th_1 effect of IL-27 attributed to an increase in STAT1 signalling synergistic to the effects of IL-12 (Pflanz *et al.*, 2002), and in human cells to lead to enhanced $CD8^+$ T cell cytolytic activity (Schneider *et al.*, 2011). However, further studies have shown the ability of IL-27 to induce type 1 regulatory cells (Tr1) – which produce IL-10 – and suppress the differentiation of Th17 cells (Awasthi *et al.*, 2007; Murugaiyan *et al.*, 2009). As discussed by Yoshida and Hunter (2015), Il-27 also appears to be important in the development of a subset of regulatory T cells in mice that express the transcriptional factor T-bet and CXCR3, which can function to control Th1 responses via production of IL-10 at the sites of inflammation in *Toxoplasma gondii* infection (Hall *et al.*, 2012). Such a process is hypothesised to be important in the resolution of inflammation after clearance of intracellular infection, and similar IL-27-induced IL-10 production by NK cells has also been demonstrated in murine models (Chong *et al.*, 2015). Thus in different contexts, IL-27 can serve to either promote a Th1 response or promote a regulatory T cell phenotype.

Several studies have highlighted a role for IL-27 in the pathogenesis of RA. An SNP in *IL27* (-924A/G) has been associated with susceptibility to RA in a Polish population (Paradowska-Gorycka *et al.*, 2014). Circulating (Shen *et al.*, 2011; Lai *et al.*, 2016) and synovial fluid (Tanida *et al.*, 2011) concentrations of IL-27 are higher in RA patients versus healthy and OA controls respectively. When stimulated by IL-27 *ex vivo*, RA synovial fibroblasts can induce a dose-dependent increase in the production of pro-inflammatory mediators such as matrix metalloproteinase 1 (MMP1) (Wong *et al.*, 2010). Furthermore, IL-27 knock-out mice show a reduced severity and delayed onset of disease in the proteoglycan-induced arthritis model (Cao *et al.*, 2008). However, evidence also suggests a beneficial role of IL-27 in inflammatory arthritis dependent on the setting. In the murine collagen-induced arthritis model, exogenous IL-27 can reduce synovitis when administered at the time of onset of disease (Niedbala *et al.*,

2008). Furthermore, high expression of *IL27* (which encodes IL-27p28) in human RA synovial tissue has also been shown to associate with reduced ectopic lymphoid structure formation, mirroring an increased number of synovial Th17 cell aggregates seen in an IL-27R knock-out murine model (Jones *et al.*, 2015).

In my study, circulating levels of IL-27 at baseline were predictive of outcome following DMARD cessation, with high levels at baseline demonstrating particular discrimination for those patients who experienced an arthritis flare. Furthermore, there was a significant though small increase in circulating levels of IL-27 at the time of flare (mean fold change 1.07, unadjusted $p = 0.021$), though this latter observation was not robust to multiple test correction. Taken together, these results would imply a positive association between circulating IL-27 and arthritis flare in this study. This would be in keeping with the pro-Th1 actions of IL-27 described above, though it is impossible to infer causality based on these data. Indeed, higher levels of IL-27 at baseline may serve to modulate the response to upstream perturbations of the immune response following DMARD cessation rather than play a causative role *per se*, for example by altering the signalling of other STAT-pathway cytokines such as IL-6. The robust increase in IL-6 observed at the time of flare in this study (discussed further below) lends some support to this hypothesis. Alternatively, it is even conceivable that high IL-27 may reflect a regulatory response to pro-inflammatory mechanisms, but that this regulatory response is somehow deficient in those patients who subsequently develop an arthritis flare. Clearly, there are many limitations of circulating cytokine data, leading to substantial uncertainty in how this reflects immune processes at the synovium. Again, synovial joint biopsies would be a vital source of corroborative data, though this was not feasible in this small exploratory study.

6.5.4 Longitudinal cytokine data

Although not the primary focus of this study, longitudinal observation of trends in circulating cytokine levels provides a tantalising insight in to the mechanisms underlying the emergence of RA flare. The most striking observation was that significant changes in cytokine levels were only observed in the flare group, whereas those patients who maintained DFR demonstrated very little, if any, longitudinal change in cytokine levels. This presumably reflects the substantial dysregulation of immunity that occurs at the time of arthritis flare, which is readily detected in the peripheral circulation. In contrast, the continued immune

homeostasis underlying those who maintain DFR is likely to generate more subtle signals, which may furthermore be detectable only at the individual joint level.

The acute-phase proteins CRP and serum amyloid A (SAA) demonstrated the greatest increase from baseline to time of flare (mean fold change 2.16 [adjusted p = 0.008] and 2.10 [adjusted p = 0.035] respectively). CRP is the most widely used measure of the acute phase response in clinical practice, and is an integral component of many disease activity scores including DAS28-CRP, the Simple Disease Activity Index (SDAI), and ACR/EULAR Boolean remission (see Introduction 1.4.1). Increased levels of SAA in inflammatory states are well established, and can lead to amyloidosis in cases of chronic uncontrolled inflammation – typically historically observed in diseases such as RA and ankylosing spondylitis, though now relatively rare in the era of effective modern DMARD therapy (Nakamura, 2011). Circulating SAA has been shown to correlate with clinical disease activity (Hwang *et al.*, 2016), and can itself mediate pro-inflammatory effects via Toll-like receptor 2 (TLR2) ligation in RA synovial fibroblasts (Connolly *et al.*, 2016). Of note, both CRP and SAA are components of the aforementioned 12-cytokine MBDA score (Centola *et al.*, 2013). Taken together, the observed increased concentrations of CRP and SAA at the time of flare in my study provide biochemical evidence of a robust systemic inflammatory response in patients who experienced an arthritis flare.

Further analysis of longitudinal cytokine trends in this study yields additional insights beyond only an increase in acute-phase response. IL-6 concentration was higher at the time of flare than baseline (mean fold change 1.50, corrected p = 0.031) despite many results falling below, and thus being assigned the value of, the lower limit of detection for the assay. Given this technological limitation in assay performance, it is likely that the true difference in IL-6 concentration between baseline and flare is actually greater than that observed. IL-6 is a 26kDa glycoprotein known to be of central importance in the pathogenesis of a range of autoimmune conditions including RA, vasculitis and juvenile idiopathic arthritis (Kishimoto, 2010). IL-6 can bind to either membrane-bound or soluble IL-6 receptor, which after signal transduction via gp130 signals by the JAK/STAT pathway to increase the levels of intracellular STAT3 (Kim *et al.*, 2015). The effects of IL-6 are vigorously pro-inflammatory, including immunoglobulin production by plasma cells and the activation of osteoclasts (Kim *et al.*, 2015). The observation that circulating IL-6 levels closely correlate with intracellular phosphorylated STAT3 in peripheral $CD4^+$ T cells in early ACPA-negative RA further underscores the importance in the early pathophysiological events of the disease (Anderson *et al.*, 2016). IL-6 also plays a key role in Th17 differentiation in murine T cells, though other

cytokines including TGF-β, IL-21 and IL-23 are also known to modulate Th17 differentiation in human T cells (Yang *et al.*, 2008). Toclizumab, a monoclonal antibody that blocks the IL-6 receptor, is now in widespread clinical use in the treatment of RA and several more IL-6 targeting biologic therapies are in the advanced stages of clinical development (June and Olsen, 2016).

The observation that IL-6 levels rise in the approach to arthritis flare in my study is in keeping with the well-established role of IL-6 in the pathogenesis of the disease. Additionally, co-stimulation of Toll-like receptor 3 (TLR3) and TLR7 pathways has been observed to lead to transcriptional synergy of IL-6 and IL-12 in mouse macrophages, an effect mediated by interaction of the transcription factors Jun B, CCAAT/enhancer-binding protein beta (C/EBPβ) and IRF1 at the *IL6* and *IL12b* promoters (Liu *et al.*, 2015). This effect may be of relevance given the statistically significant though small increase in circulating IL-12 at the time of flare in my study (fold change 1.15, adjusted $p < 0.008$).

Circulating concentration of the chemokine interferon-γ inducible protein 10 (IP-10, also known as CXCL10) was significantly higher at the time of flare (fold change 1.36, adjusted $p = 0.043$). IP-10 is a 10 kDa protein produced by a wide variety of cell types in response to IFN-γ (Liu *et al.*, 2011). IP-10 signals via the CXCR3 G-protein coupled receptor, and has a potent chemoattractive effect for numerous immune cells including activated T lymphocytes, NK cells, dendritic cells and macrophages (Liu *et al.*, 2011). IP-10 is thus thought to be a key player in a positive-feedback loop of recruitment of Th1 cells, which themselves secrete IFN-γ, to the sites of inflammation (Antonelli *et al.*, 2014). Circulating concentrations of IP-10 are higher in RA patients versus healthy controls and correlate with disease activity (Kuan *et al.*, 2010; Pandya *et al.*, 2017). Furthermore, blockade of IP-10 can reduce both synovial inflammatory cell infiltrate and bone erosions in the murine collagen-induced arthritis model (Kwak *et al.*, 2008), and IP-10 knock-out mice are resistant to collagen antibody-induced arthritis (Lee *et al.*, 2017).

The observation of higher circulating levels of IP-10 at the time of flare in my study would therefore be in keeping with an active flare phenotype where activated lymphocytes are recruited to the inflamed synovium. Furthermore, the postulated role of IP-10 in amplifying the recruitment of Th1 cells may be of significance given the increased levels of the pro-Th1 cytokine IL-12 at the time of flare.

6.6 Summary

In summary, circulating concentrations of IL-27, MCP1 and CRP at baseline were predictive of flare and remission following DMARD cessation. Longitudinal analysis demonstrated increased levels of acute phase reactants (CRP and SAA) at the time of flare, together with the pro-Th17 cytokine IL-6, and the pro-Th1 mediators IL-12 and IP-10. Taken together, one can hypothesise a potent pro-inflammatory cytokine and chemokine milieu at the time of arthritis flare, characterised potentially by dysregulated Th17 and Th1 responses.

There are several limitations to these cytokine data, most notably the lack of corroborative synovial tissue in which to validate the peripheral cytokine findings. Without such tissue, it is impossible to confirm how these findings relate to the pathophysiological processes occurring in the joint. These difficulties are most pronounced where mediators are known to have pleiotropic effects – for example, it is unclear whether the increased IL-27 concentration observed at baseline in the flare population represents a pro-Th1 state, or reflects an activated yet defective Tr1-like regulatory response in these patients. A further limitation was encountered with the assay technology – 13/39 analytes had >80% of measurements below the lower limit of detection at baseline and were thus excluded from the analysis. This included several key mediators of interest, including GM-CSF, IL-17A, IL-2 and IL-12. In longitudinal analysis, measurements falling below the LLOD were assigned the value of the LLOD for that assay. This conservative approach is likely to have artificially reduced the magnitude of observed increases in cytokine concentrations, such as that observed for IL-6 for example. Nevertheless, the electrochemiluminescence assays used in this study represent the most sensitive and specific method of multiplex cytokine measurement possible given the financial and equipment resources available. Finally, the exploratory nature of this study necessitated analysis of a broad range of cytokines and chemokines. Multiple significance test correction was employed to limit the rate of type I error, though could have resulted in significant associations being overlooked. Validation of these cytokine findings with a reduced variable set focussed on key mediators (e.g. CRP, SAA, MCP1, IL-27, IP-10, and IL-6) in a larger cohort of patients with more frequent sampling is necessary to address these concerns.

Chapter 7. Results 4 – $CD4^+$ T cell RNAseq data

7.1 Introduction

The results discussed in the first two results chapters of this Thesis relate to data gained by clinical and ultrasound measurements. Although distinct in their nature, these parameters all share the common characteristic of being global measures of disease phenotype and/or activity, rather than a measure of disease-specific processes *per se*. In Chapter 6, I described circulating cytokine data may reflect trends in underlying immune processes, albeit still at a systemic level. Conceivably, measuring parameters within a cellular compartment of the immune system of particular relevance to disease pathogenesis may hold additional promise in the identification of disease-specific biomarkers.

RA is undeniably a heterogeneous disease that involves many different cellular and humoral pathogenic pathways. Nevertheless, as outlined in Introduction 1.2, compelling evidence supports a key role for $CD4^+$ T cells in the pathogenesis of the disease. Circulating $CD4^+$ T cells therefore provide a potential compartment that is both important in RA pathobiology as well as easily obtainable without the need for invasive sample collection procedures. In previous work by the Newcastle University Musculoskeletal Research Group, a protocol was developed to isolate $CD4^+$ T cells from whole blood with a high cell yield and purity (Pratt, 2011). Furthermore, microarray analysis of genome-wide gene expression in $CD4^+$ T cells isolated by this method identified a 12-gene signature, which had prognostic utility in predicting which patients with undifferentiated inflammatory arthritis subsequently progressed to a diagnosis of RA (Pratt *et al.*, 2012). Therefore, robust data support both the feasibility of gene expression analysis in highly purified $CD4^+$ T cells and the utility of this data in biomarker identification. Transcriptional profiling of circulating $CD4^+$ T cells therefore represents a logical target upon which to focus efforts to identify a cell-specific biomarker of drug-free remission. Furthermore, the increased transcriptome coverage, specificity and sensitivity of modern next-generation sequencing techniques offer unparalleled opportunities to study this in greater detail than previously possible using microarray platforms.

In this results chapter I present analysis of $CD4^+$ T cell gene expression data as generated by next-generation RNA sequencing. The aims of this chapter are:

1. To demonstrate the feasibility of $CD4^+$ T cell isolation from whole blood, RNA extraction from these cells, and RNA sequencing to a high quality.
2. To use gene expression data to create a biomarker signature which, when applied to baseline samples, has predictive utility in discriminating patients who flare versus those who remain in remission following DMARD cessation.
3. To explore differential gene expression both at baseline between contrast groups, and longitudinally at an individual patient level, in order to gain insights in to the immunological mechanisms underlying DFR and arthritis flare.

The structure of the remainder of this results chapter is as follows:

7.2 Quality control

7.2.1 Sample collection

7.2.1.a Patient samples

$CD4^+$ T cell samples were available at all baseline, month three and month six study visits. Prior to the second substantial protocol amendment, blood was not collected at the month one visit for patients who satisfied remission criteria. Consequently, $CD4^+$ T cell samples were available for 26/42 month one visits and for 14/19 patient-requested unscheduled visits. Owing to time constraints, RNA extraction and sequencing was performed before the final

clinical study visit. Therefore, samples from later time points for patients who were still under follow-up within the study at the time of sample processing were not available for inclusion within the analysis (Table 7.1). In total, $CD4^+$ T cell RNA was available for sequencing for 120/154 (78%) of study visits. $CD4^+$ T cell RNA was available for sequencing at the time of flare for 18/23 (78%) patients who experienced an arthritis flare, and was available at month six for 15/20 (75%) patients who maintained drug-free remission.

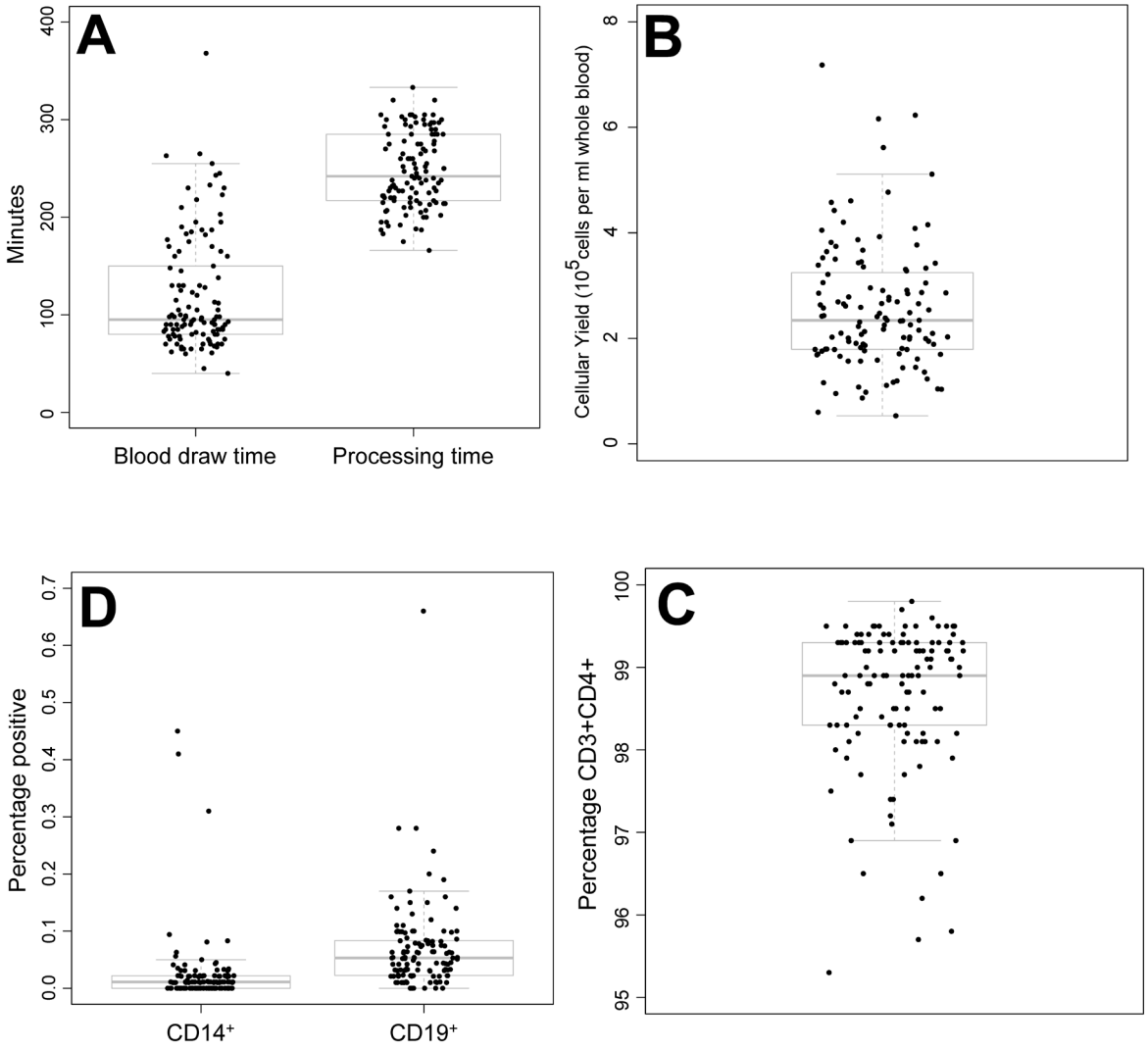
Time from blood draw to start of $CD4^+$ T cell processing, and time from start of T cell processing to freezing of T cell lysate, were recorded for 117/120 of the sequenced patient samples (Figure 7.1A). The median (IQR, range) time from blood draw to start of processing was 95 (80-150, 40-368) minutes. The median (IQR, range) time from start of processing to freezing of lysate was 242 (217-285, 166-333) minutes.

The number of cells in every T cell isolate was recorded to calculate the cell yield, with a median (IQR, range) value of 2.3×10^5 cells per ml whole blood (1.8 – 3.2, 0.5 – 7.2) (Figure 7.1B). The purity of every $CD4^+$ T cell isolate was confirmed by flow-cytometric analysis as described in Methods 3.9.7 (Figure 7.1C). The median (IQR, range) percentage of $CD3^+CD4^+$ cells in patient T cell isolates was 98.9 (98.3 – 99.3, 95.3-99.8). The percentage of contaminating monocytes and B-cells, defined as $CD14^+$ or $CD19^+$ respectively, were generally low and are summarised in Figure 7.1D. To account for small variations in $CD4^+$ T cell purity, the percentage of $CD3^+CD4^+$ cells was included as a covariate in gene expression models together with sequencing batch.

Table 7.1 – Collection and sequencing of $CD4^+$ T cell samples by study visit for patients who stopped DMARDs.

Visit type	Number of visits	Number of $CD4^+$ samples: n(% visits)	Number of $CD4^+$ T cell samples sequenced: n(% visits)
Baseline	44	44 (100)	44 (100)
Month one	42	26 (62)	26 (62)
Month three	26	26 (100)	21 (81)
Month six	23	23 (100)	16 (70)
Unscheduled	19	14 (74)	13 (68)
Total	**154**	**133 (86)**	**120 (78)**

Figure 7.1 – Quality control data for sequenced patient CD4+ T cell isolates: time delays (A), cellular yield (B), percentage of $CD3^+CD4^+$ cells (C), and percentage of contaminating cells positive for CD14 (monocytes) and CD19 (B cells) (D). 'Blood draw time': time from venepuncture to start of laboratory processing; 'Processing time': time from start of laboratory processing to freezing of T cell lysate.



7.2.1.b Healthy controls

Four healthy volunteers were recruited to donate blood as a control arm for comparison with the study population. The volunteers were aged 50 years (male), 40 years (male), 31 years (female) and 31 years (female) at the time of first donation. Sampling was performed at four time points (baseline, month 1, month 3 and month 6) from all participants giving 16 healthy $CD4^+$ T cell samples in total. Blood was drawn where possible between 9am-1pm and left to stand before processing to mimic the collection and transport of the patient samples. Time from blood draw to start of $CD4^+$ T cell processing, and time from start of T cell processing to freezing of T cell lysate, were recorded for all samples. The median (IQR, range) time from

blood draw to start of processing was 67 (56-90, 28-198) minutes. The median (IQR, range) time from start of processing to freezing of lysate was 254 (231 – 305, 170 – 333) minutes. There was no significant difference in mean blood draw or processing times between healthy controls and patients (p = 0.141 and p = 0.538 respectively, Mann-Whitney U test) (Figure 7.2A).

The median (IQR, range) cell yield for healthy control T cell isolates was 3.1 (2.3 – 3.7, 1.2 – 5.0) x10^5 cells per ml whole blood. The mean cellular yield was not significantly different between healthy controls and patients (p = 0.267, Mann-Whitney U test) (Figure 7.2B). The median (IQR, range) percentage of $CD3^+CD4^+$ cells in healthy control T cell isolates was 98.1 (97.6 – 98.9, 96.8-99.2). The mean percentage $CD3^+CD4^+$ cells was not significantly

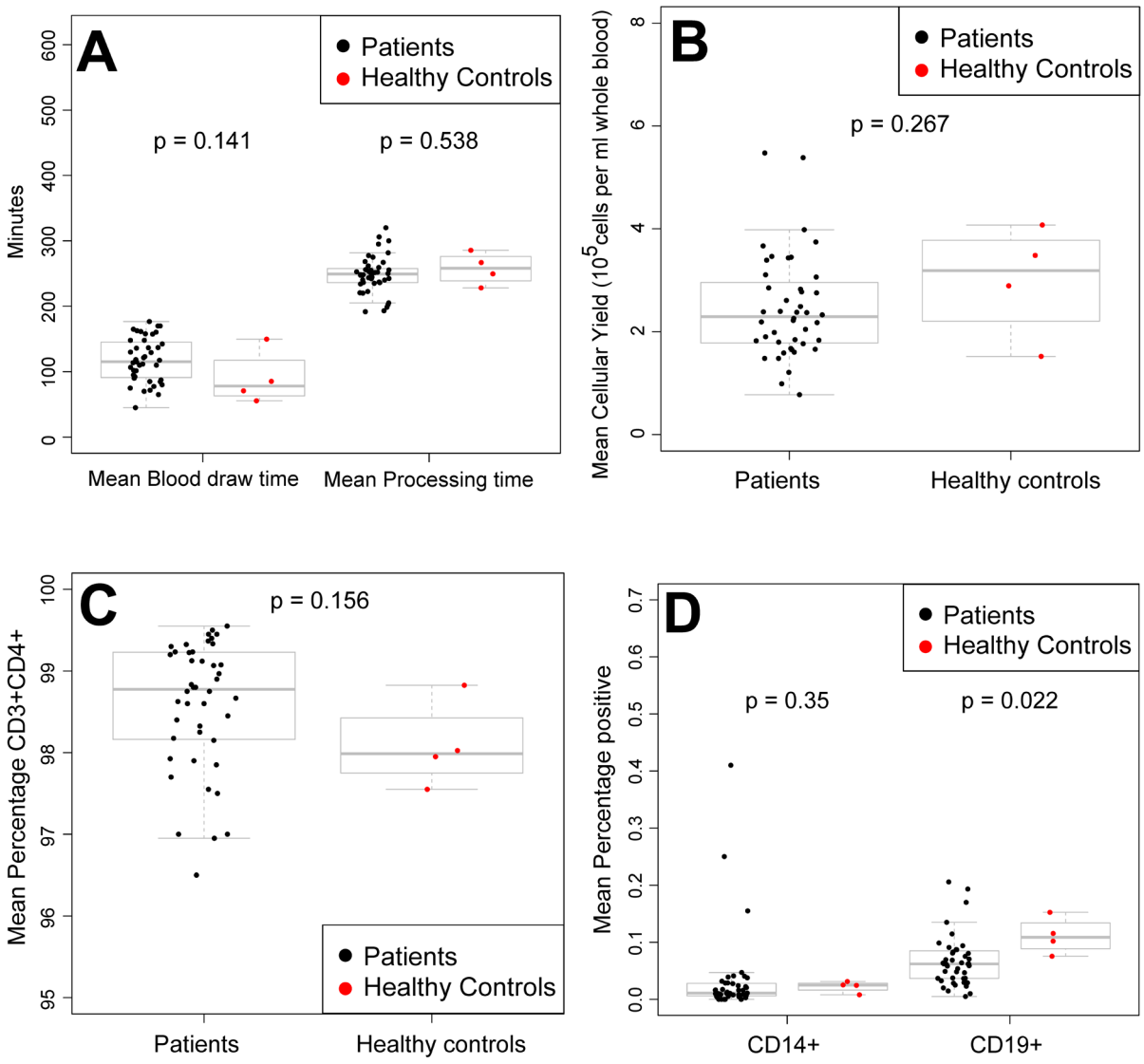


Figure 7.2 – Comparison of quality control data between sequenced patient samples and hea;thy controls: mean blood draw and mean processing times (A), mean cell yield (B), mean percentage $CD3^+CD4^+$ (C) and mean percentage contaminating cells positive for CD14 (monocytes) and CD19 (B cells) (D). P values calculated by Mann-Whitney U test.

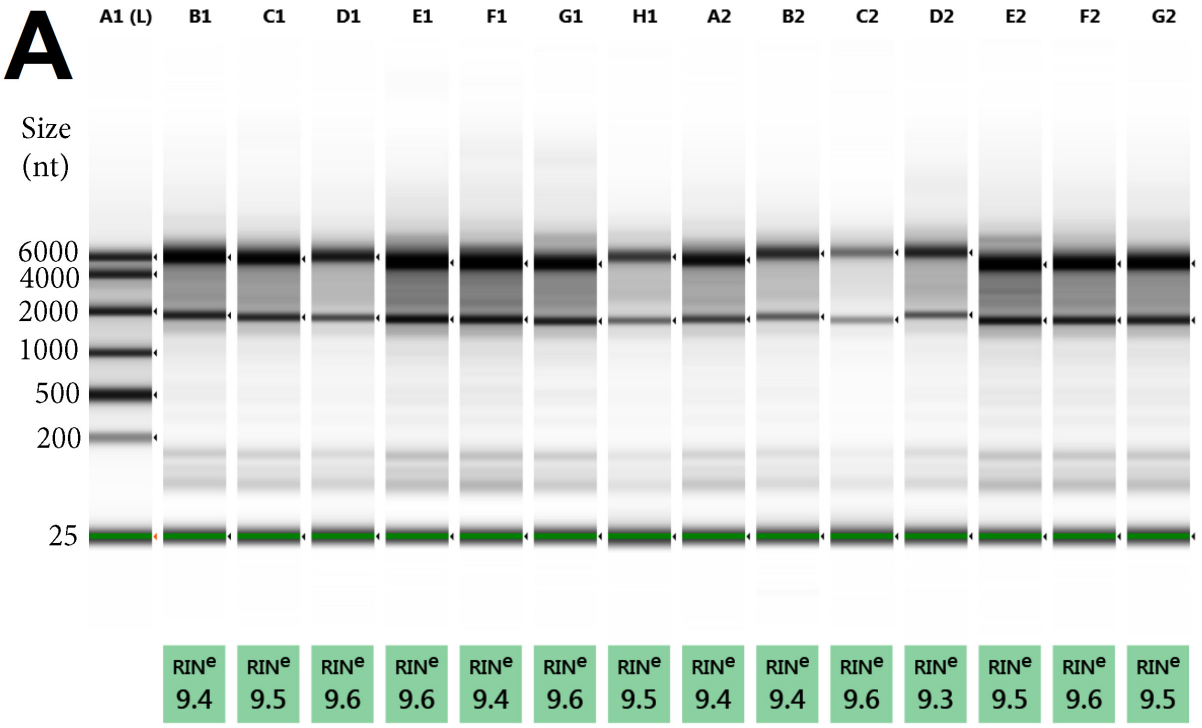
different between healthy controls and patients (p = 0.156, Mann-Whitney U test) (Figure 7.2C). The percentage of contaminating monocytes and B-cells, defined as $CD14^+$ or $CD19^+$ respectively, are summarised in Figure 7.2D. Significantly greater mean $CD19^+$ contamination was seen for healthy controls versus patients (p = 0.022). The explanation for this is not immediately apparent. Where data was available, the mean yield of $CD19^+$ cells in parallel PBMC isolations was not significantly different between patients and healthy controls (p = 0.843, Mann-Whitney U test). This suggests that the higher $CD19^+$ contamination in healthy T cell isolates was not due to a greater number of $CD19^+$ cells healthy control versus patient blood – however, an absolute $CD19^+$ cell count in whole blood, as opposed to the $CD19^+$ cell yield following density centrifugation, would be required to confirm this.

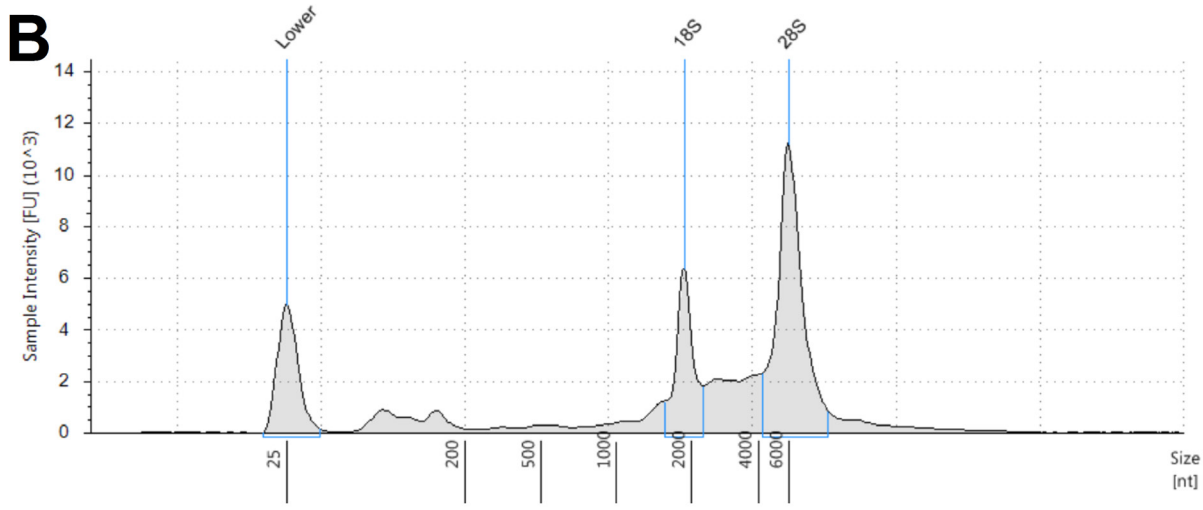
One participant had a mild upper respiratory tract infection at the time of the month one sample, and another had mild hay fever (not requiring any treatment) at the month three time point. Data from these two individuals, at the relevant time-point in each case, were excluded after read count normalisation and before differential gene expression analysis.

7.2.3 RNA yield and integrity

The quantity and quality of RNA in each T cell lysate was measured by gel electrophoresis using a Tapestation™ 4200 machine (Agilent) (Figure 7.3). The median (IQR, range) RNA yield was 870 (660 – 884, 277 – 2275) ng per million cells lysed. (Figure 7.4A). Prior to sequencing, the 28S/18S ribosomal RNA (rRNA) ratio and estimated RNA integrity number (RIN^e) were calculated for each sample. Lower RIN^e and 28S/18S ratios suggest RNA degradation; the RNAseq platform manufacturer (Illumina) recommends a $RIN^e > 8$ and a 28S/18S rRNA ratio > 2.0 for high quality RNAseq analysis. The median (IQR, range) RIN^e was 9.4 (9.2 – 9.6, 8.1 – 10.0) (Figure 7.4B). The median (IQR, range) 28S/18S rRNA ratio was 2.6 (2.5 – 2.7, 2.1 – 2.9) (Figure 7.4C). There was no correlation between RIN^e and total time from venepuncture to freezing of T cell lysate (repeated measures $R^2 = -0.023$, p = 0.833), suggesting that the RNA integrity was not affected by laboratory processing time during the T cell isolation procedure (Figure 7.4D). There was no significant difference in mean RIN^e between patients and healthy controls (p = 0.104, Mann-Whitney U test).

Figure 7.3 – Assessment of RNA integrity. A. Gel electrophoresis of a representative set of RNA samples as visualised using a Tapestation™ 4200 machine. The ladder was loaded in lane A1, and patient samples loaded in the remaining lanes. Arrows in the sample lanes highlight the lower marker, 18S and 28S bands. B. Electropherogram for sample B1 showing the lower marker, 18S and 28S peaks. $RIN^{e:}$ estimated RNA integrity number; nt: nucleotide. Images copyright Agilent Technologies Inc. 2015, reproduced with permission.





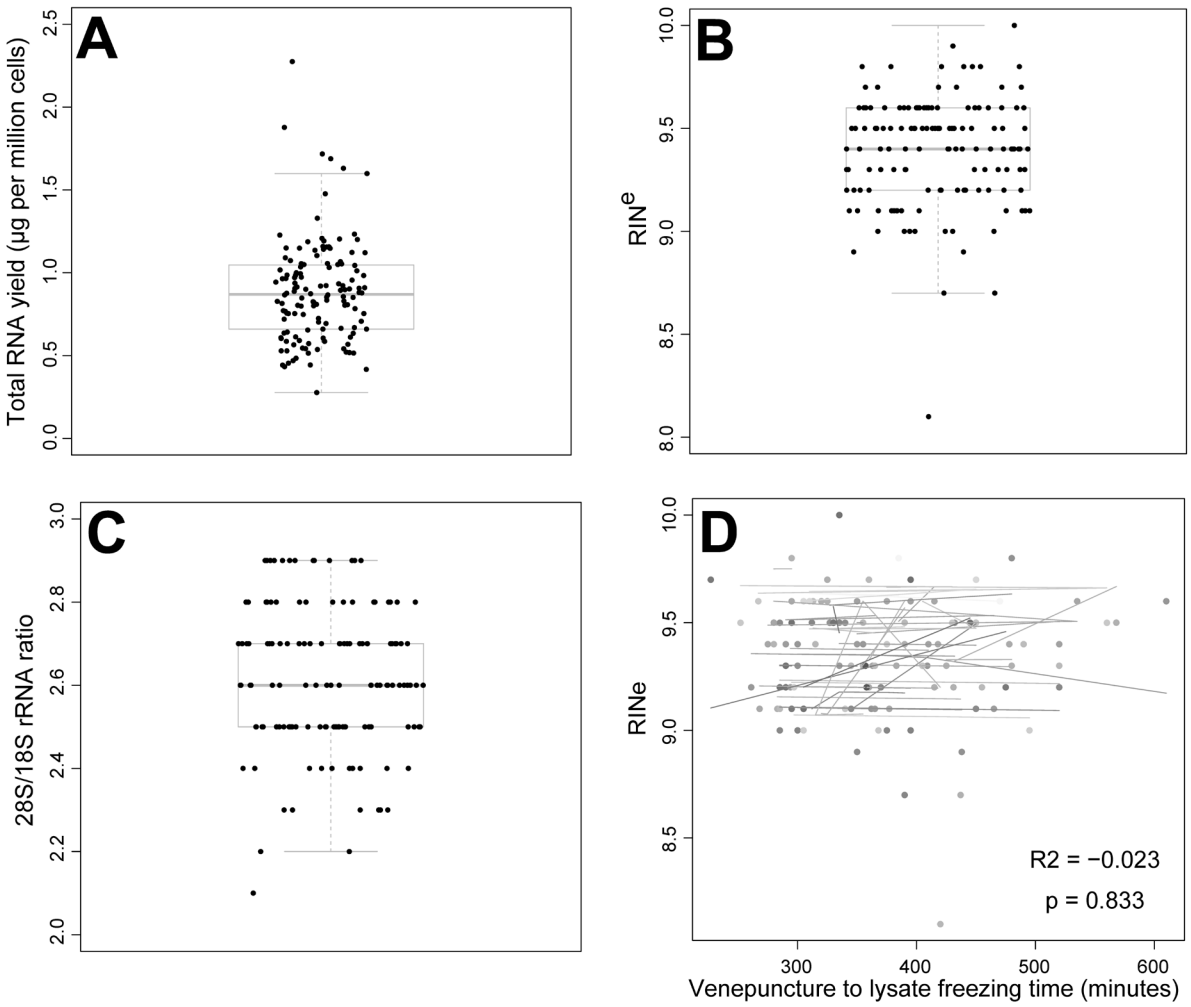


Figure 7.4 – Quality control of sequenced RNA samples. $CD4^+$ T cell lysates RNA yield (A), estimated RNA integrity number (RIN^e) (B), 28S/18S ribosomal RNA (rRNA) ratio (C), and correlation between RINe and time from venepuncture to freezing of T cell lysate (D). Lines represent individual patient linear regression lines, and R2/p-values refer to overall correlation accounting for repeated measures.

7.2.4 Sequencing quality

The quality of RNA sequencing data was assessed as described in Methods 3.10.2. Samples were sequenced to a mean (range) depth of 12.3 (9.2 – 18.4) reads per sample (Figure 7.5A). Sequencing read length was tightly clustered around the expected 75bp (Figure 7.5B), and no samples were found with adaptor contamination >0.1%. Quality of the sequencing was excellent with a mean Phred score >30 across all read positions (Figure 7.5C) and no read trimming was necessary.

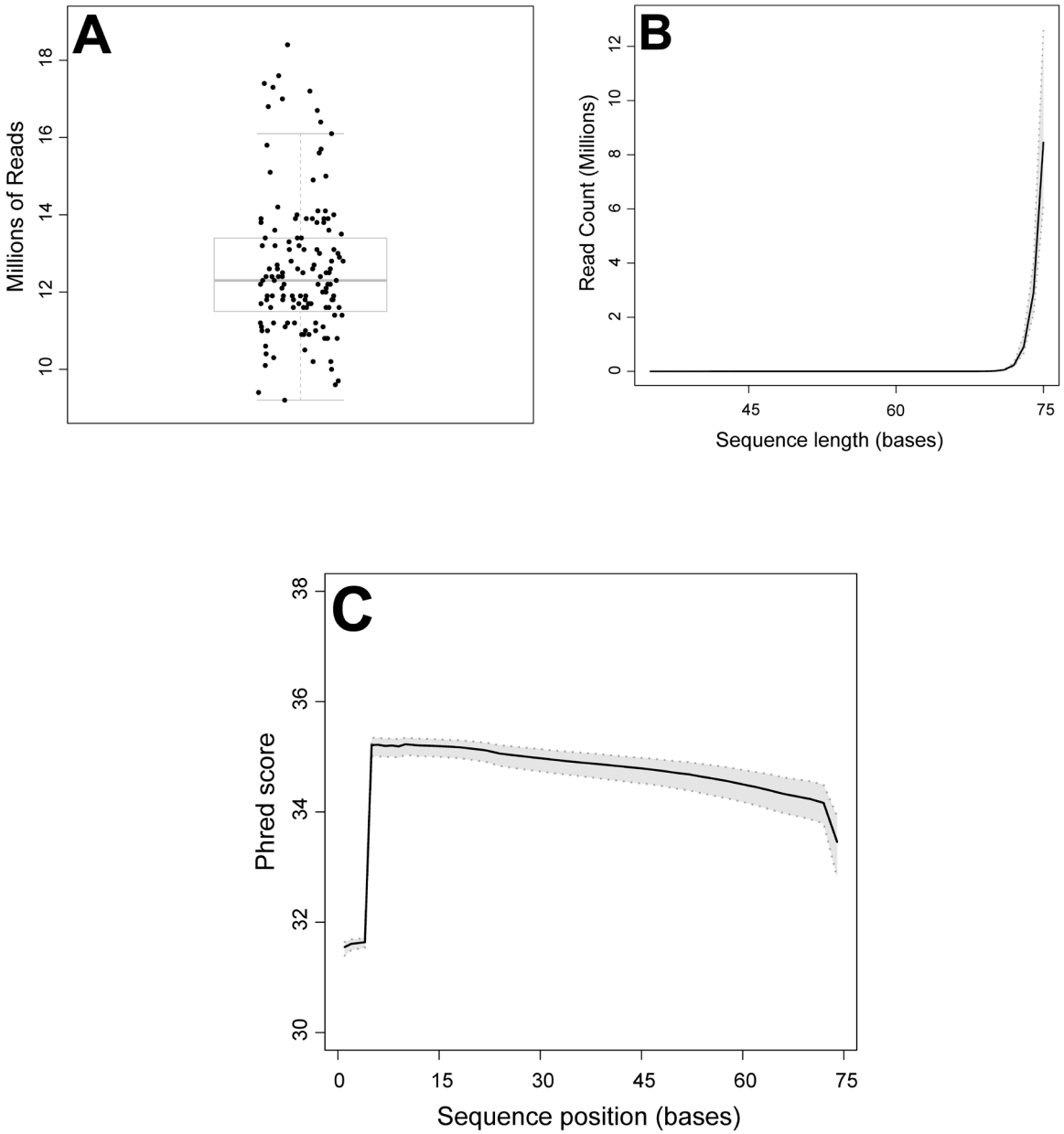


Figure 7.5 – Quality control of RNA sequencing. A: Number of sequences of each patient sample RNA library. B: Distribution of mean number of reads by RNA sequence length. Read count range for each sequence length is shown in grey. C: Distribution of mean Phred score by sequence position across all samples. Phred score range at each sequence position is shown in grey.

7.3 Baseline analyses

All analyses were performed with adjustment for RNA sequencing batch and sample $CD4^+$ T cell purity (see Methods 3.10.2). Change in gene expression was considered significant if there was >1.5 fold-difference in expression between groups at the 0.05 significance level

according to the moderated t-test, after false-discovery rate (FDR) correction using the Benjamini-Hochberg procedure. Where changes in gene expression failed to meet statistical significance, unadjusted p-values were used as in a secondary exploratory analysis with a post hoc significance threshold of $p < 0.001$. Computer programming scripts, bioinformatics analysis and figure generation for all RNAseq data (with the exception of multivariate Cox regression and ROC analysis) were performed by Andrew Skelton, Experimental Scientific Officer, Bioinformatics Support Unit, Newcastle University.

7.3.1 Flare versus remission

The comparison of $CD4^+$ T cell gene expression at baseline between patients who experienced a flare versus those who remained in DFR following DMARD cessation was the primary analysis for this study. Substantial variation in gene expression between flare and remission groups by log-fold change values was evident, but none of these differences reached statistical significance after FDR adjustment (Figure 7.6A).

An exploratory analysis was performed without FDR correction, which identified 11 genes that were differentially expressed with an unadjusted p-value < 0.001 (Figure 7.6B and Table 7.2). Pathway analysis was performed with functional gene annotation using Ingenuity™ Pathway Analysis (IPA™) software (Qiagen, Redwood City, USA). Log fold-change of all 118 genes that demonstrated differential expression with an unadjusted p-value threshold of <0.01 were analysed. The top identified network contained the functions “cell cycle, cell death and survival, and inflammatory response”, for which 11/35 were up-regulated and 1/35 down-regulated in flare versus remission patients. Nevertheless, visualisation of this network did not identify any clear edges between the differentially expressed nodes (Figure 7.8).

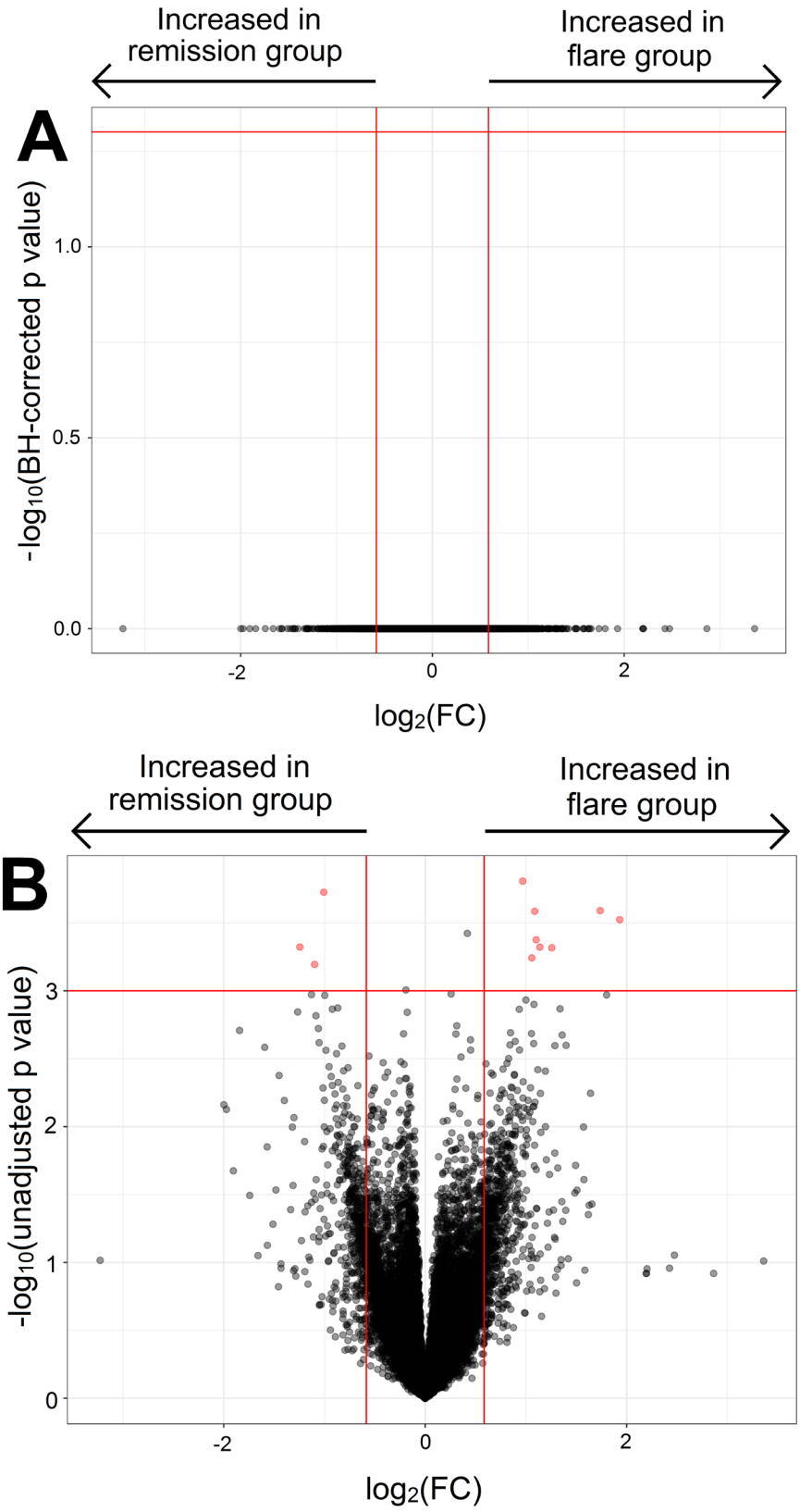
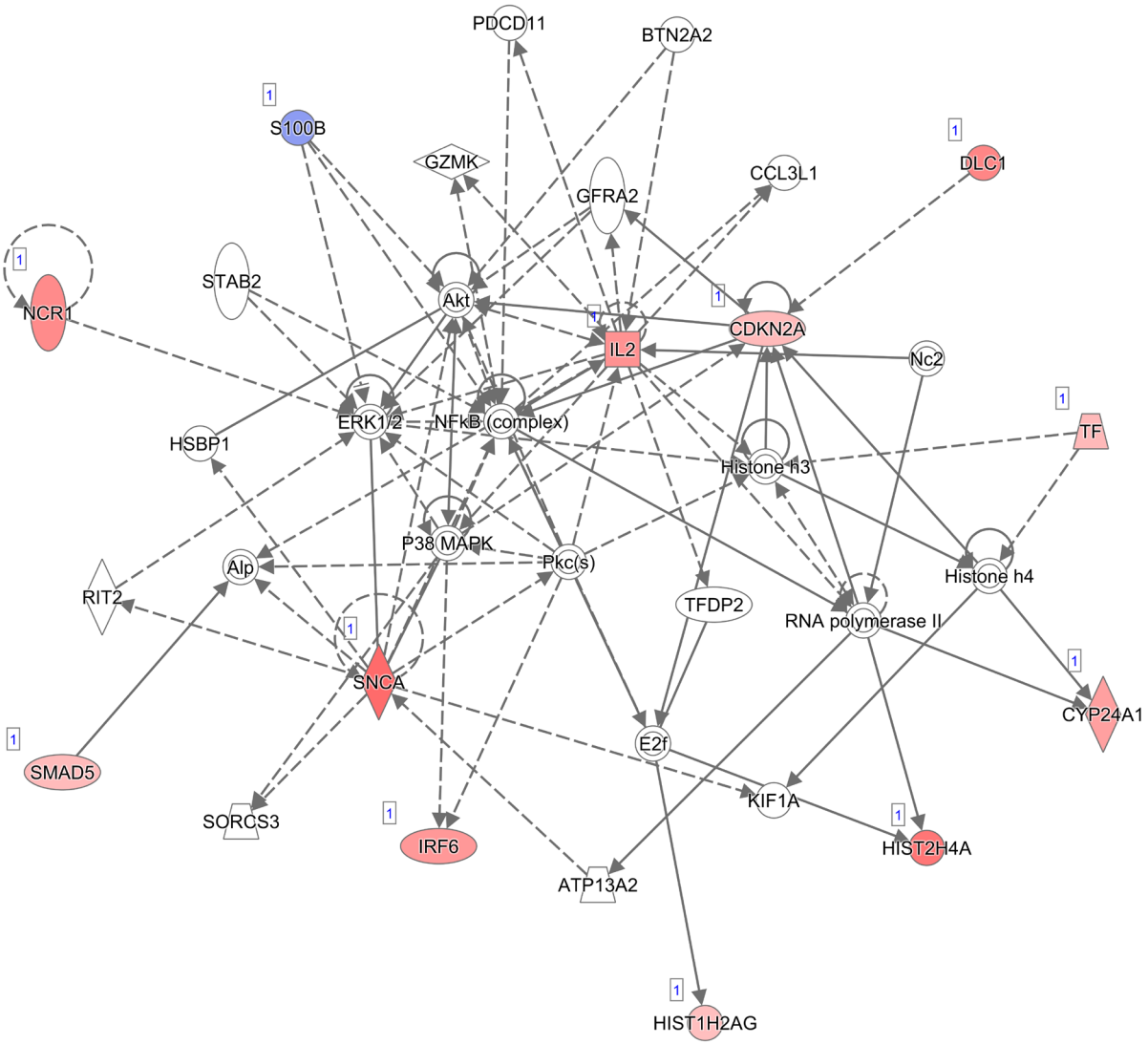


Figure 7.6 – Volcano plots showing baseline differential gene expression in circulating $CD4^+$ T cells between patients who subsequently experienced an arthritis flare versus those who remained in drug-free remission following DMARD cessation. Plots are shown with (A: FDR-corrected p-value < 0.05) and without (B: unadjusted p < 0.001) multiple test correction. Horizontal lines represent log-fold change (log_2FC) > 1.5.

Table 7.2 – Differential expression of genes at baseline between flare and remission groups, using an unadjusted significance threshold of $p<0.001$. Positive log-fold change indicates higher expression in the flare group, whereas negative log-fold change indicates higher expression in the remission group. FC: fold-change; HGNC: HUGO gene nomenclature committee; lincRNA: long intergenic non-coding RNA.

Ensembl gene ID	Log_2 FC	Average expression	t	Unadjusted p value	HGNC symbol	Description
ENSG00000102362	0.97	-1.229	3.907	0.000156	*SYTL4*	synaptotagmin like 4
ENSG00000213296	-1.01	-3.627	-3.855	0.000188		(known processed pseudogene)
ENSG00000247033	1.74	-2.527	3.769	0.000257		(novel antisense)
ENSG00000255330	1.09	2.602	3.766	0.00026	*SOGA3*	suppressor of glucose, autophagy associated (SOGA) family member 3
ENSG00000221957	1.93	-3.008	3.726	0.0003	*KIR2DS4*	killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 4
ENSG00000260876	1.10	-3.327	3.629	0.000421	*LINC01229*	long intergenic non-protein coding RNA 1229
ENSG00000182489	-1.24	-0.281	-3.593	0.000476	*XKRX*	XK related, X-linked
ENSG00000164741	1.14	-2.246	3.593	0.000477	*DLC1*	Deleted In Liver Cancer 1 (DLC1) Rho GTPase activating protein
ENSG00000144366	1.26	-2.506	3.590	0.000482	*GULP1*	engulfment adaptor PTB domain containing 1
ENSG00000234199	1.06	-2.748	3.540	0.000573	*LINC01191*	long intergenic non-protein coding RNA 1191
ENSG00000214803	-1.10	-2.297	-3.507	0.00064		(known lincRNA)

Figure 7.7 – Visualisation of differential expression of genes within a predicted "cell cycle, cell death and survival, and inflammatory response" network between patients who experienced an arthritis flare versus those who remained in remission following DMARD cessation. Genes highlighted in red were up-regulated in the flare group, whereas those highlighted in blue were down-regulated in the flare group relative to the remission group. Copyright Qiagen 2017, reproduced with permission.



7.3.2 Flare versus healthy control

$CD4^+$ T cell gene expression was compared between baseline samples of patients who flared following DMARD cessation versus healthy controls. Three genes were differentially expressed after FDR adjustment (Figure 7.8A), and a further 55 were differentially expressed using an unadjusted significance threshold of $p<0.001$ (Figure 7.8B). The entire 58-gene list is detailed in Appendix I, and the top 20 genes (by unadjusted p value) are presented in Table 7.3.

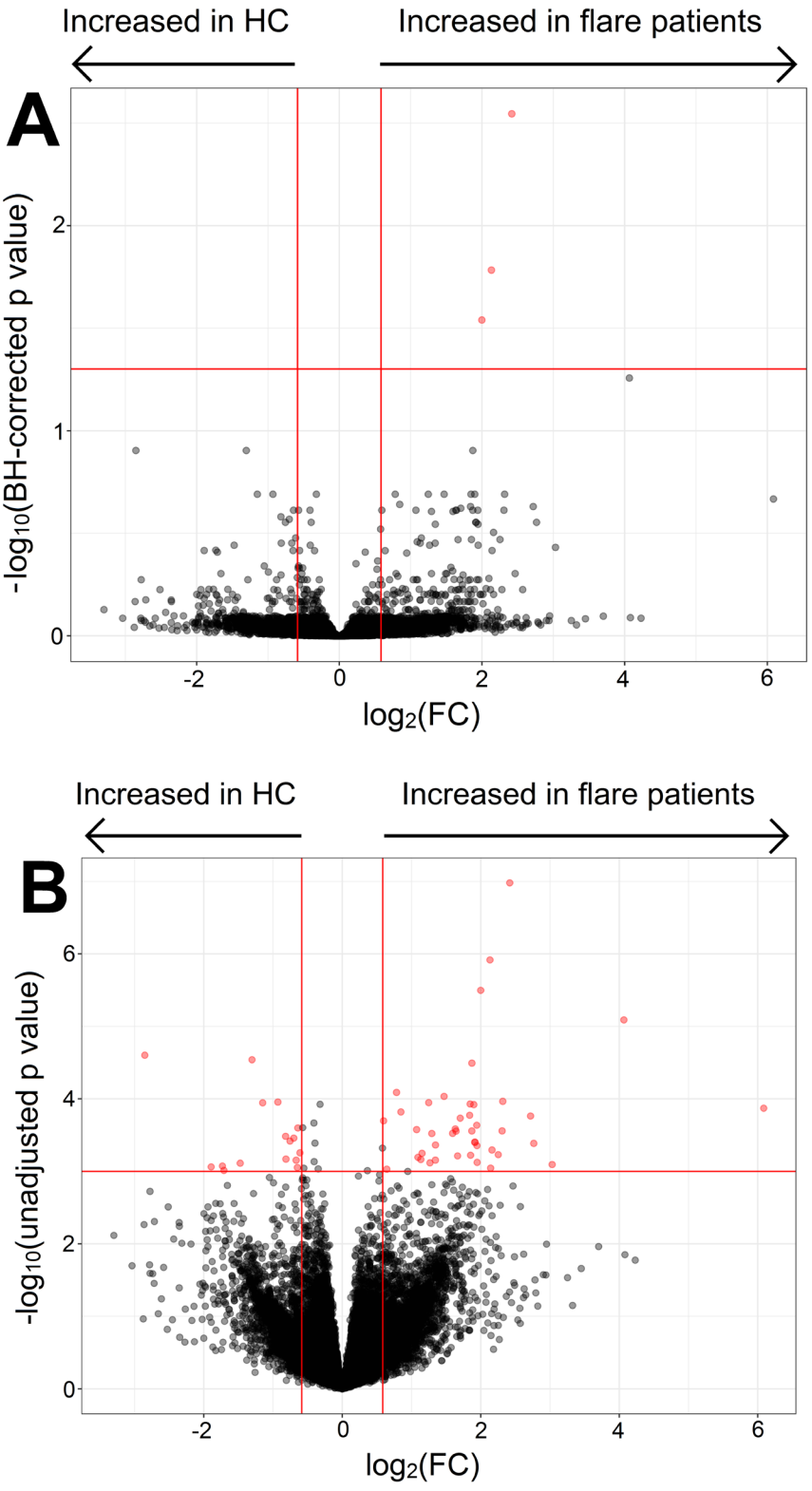


Figure 7.8 - Volcano plots showing baseline differential gene expression in circulating $CD4^+$ T cells between patients who subsequently experienced an arthritis flare following DMARD cessation versus healthy controls (HC). Plots are shown with (A: FDR-corrected p-value < 0.05) and without (B: unadjusted $p < 0.001$) multiple test correction. Horizontal lines represent log-fold change (log_2FC) > 1.5. Genes that exceeded both thresholds are highlighted in red.

Table 7.3 - Top 20 (by unadjusted p value) differentially expressed genes at baseline between flare patients and healthy controls. Positive log-fold change indicates higher expression in the patient group, whereas negative log-fold change indicates higher expression in the control group. FC: fold-change; HGNC: HUGO gene nomenclature committee. * = significant after FDR adjustment (corrected p<0.05).

Ensembl gene ID	Log_2 FC	Average expression	t	Unadjusted p-value	HGNC symbol	Description
ENSG00000171560	**2.42**	-3.929	5.664	**1.05E-07***	***FGA***	**fibrinogen alpha chain**
ENSG00000106927	**2.13**	-3.961	5.116	**1.21E-06***	***AMBP***	**alpha-1-microglobulin/bikunin precursor**
ENSG00000171564	**2.00**	-4.006	4.890	**3.19E-06***	***FGB***	**fibrinogen beta chain**
ENSG00000163631	4.07	-2.059	4.665	8.15E-06	*ALB*	albumin
ENSG00000182489	-2.85	-0.281	-4.387	2.50E-05	*XKRX*	Kell Blood Group Complex Subunit-Related, X-Linked
ENSG00000198538	-1.30	2.898	-4.350	2.89E-05	*ZNF28*	zinc finger protein 28
ENSG00000223551	1.87	-0.957	4.323	3.22E-05	*TMSB4XP4*	thymosin beta 4, X-linked pseudogene 4
ENSG00000226029	0.78	2.868	4.081	8.16E-05	*LINC01772*	long intergenic non-protein coding RNA 1772
ENSG00000141622	1.47	0.279	4.048	9.25E-05	*RNF165*	ring finger protein 165
ENSG00000251411	2.32	-2.195	4.006	1.08E-04		(known processed pseudogene)
ENSG00000197841	-0.93	3.455	-3.999	1.11E-04	*ZNF181*	zinc finger protein 181
ENSG00000164136	1.25	2.113	3.994	1.13E-04	*IL15*	interleukin 15
ENSG00000172985	-1.15	2.204	-3.993	1.13E-04	*SH3RF3*	SH3 domain containing ring finger 3
ENSG00000247311	1.85	-0.006	3.983	1.18E-04		(novel antisense)
ENSG00000112139	6.09	2.575	3.946	1.35E-04	*MDGA1*	meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu (MAM) domain containing glycosylphosphatidylinositol anchor 1
ENSG00000088538	0.85	2.753	3.914	1.52E-04	*DOCK3*	dedicator of cytokinesis 3
ENSG00000131080	1.84	-0.562	3.886	1.68E-04	*EDA2R*	ectodysplasin A2 receptor
ENSG00000229314	2.72	-3.203	3.879	1.73E-04	*ORM1*	orosomucoid 1
ENSG00000125726	1.70	0.422	3.860	1.85E-04	*CD70*	CD70 molecule
ENSG00000152242	0.60	6.523	3.837	2.01E-04	*C18orf25*	chromosome 18 open reading frame 25

7.3.3 Remission versus healthy control

$CD4^+$ T cell gene expression was compared between baseline samples of patients who remained in FDR following DMARD cessation versus healthy controls. No genes were differentially expressed after FDR adjustment (Figure 7.9A); 39 were differentially expressed using an unadjusted significance threshold of $p<0.001$ (Figure 7.9B). The entire gene list is detailed in Appendix I, and the top 20 genes (by unadjusted p-value) are presented in Table 7.4.

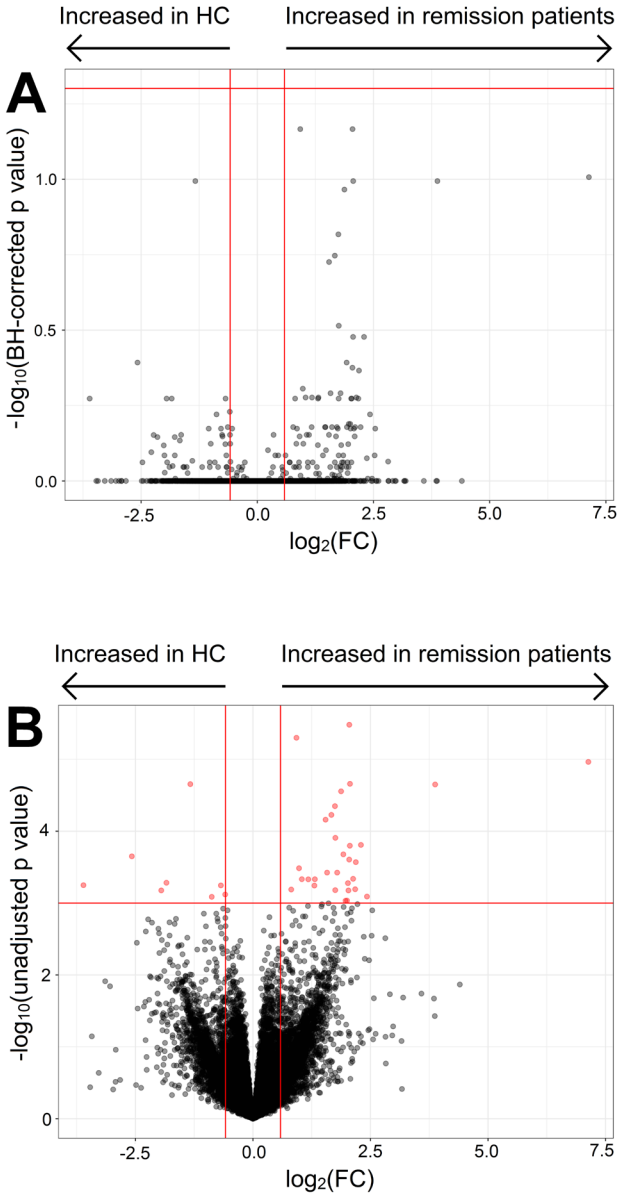


Figure 7.9 - Volcano plots showing baseline differential gene expression in circulating $CD4^+$ T cells between patients who subsequently remained in drug-free remission following DMARD cessation versus healthy controls (HC). Plots are shown with (A: FDR-corrected p-value < 0.05) and without (B: unadjusted $p < 0.001$) multiple test correction. Horizontal lines represent log-fold change (log_2FC) > 1.5. Genes that exceeded both thresholds are highlighted in red.

Table 7.4 - Top 20 (by unadjusted p value) differentially expressed genes at baseline between remission patients and healthy controls. Positive log-fold change indicates higher expression in the patient group, whereas negative log-fold change indicates higher expression in the control group. FC: fold-change; HGNC: HUGO gene nomenclature committee. TEC = to be experimentally confirmed.

Ensembl gene ID	Log_2 FC	Average expression	t	Unadjusted p value	HGNC symbol	Description
ENSG00000106927	2.05	-3.961	4.882	3.31E-06	*AMBP*	alpha-1-microglobulin/bikunin precursor
ENSG00000226029	0.92	2.868	4.782	5.02E-06	*LINC01772*	long intergenic non-protein coding RNA 1772
ENSG00000112139	7.13	2.575	4.595	1.09E-05	*MDGA1*	meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu (MAM) domain containing glycosylphosphatidylinositol anchor 1
ENSG00000247311	2.06	-0.006	4.420	2.19E-05		(novel antisense)
ENSG00000198538	-1.33	2.898	-4.418	2.21E-05	*ZNF28*	zinc finger protein 28
ENSG00000163631	3.88	-2.059	4.415	2.24E-05	*ALB*	albumin
ENSG00000171560	1.87	-3.929	4.359	2.79E-05	*FGA*	fibrinogen alpha chain
ENSG00000171564	1.74	-4.006	4.238	4.48E-05	*FGB*	fibrinogen beta chain
ENSG00000256913	1.67	-0.059	4.165	5.94E-05		(novel processed pseudogene)
ENSG00000259657	1.54	0.037	4.124	6.92E-05	*PIGHP1*	phosphatidylinositol glycan anchor biosynthesis class H pseudogene 1
ENSG00000265293	1.75	-0.842	3.969	1.24E-04	*ARGFXP2*	arginine-fifty homeobox pseudogene 2
ENSG00000204380	2.30	-1.803	3.908	1.55E-04	*PKP4-AS1*	Plakophilin 4 - antisense RNA 1
ENSG00000228382	2.06	-1.596	3.901	1.59E-04	*ITPKB-IT1*	Inositol-Trisphosphate 3-Kinase B - intronic transcript 1
ENSG00000279148	1.92	-1.618	3.825	2.10E-04		(known TEC)
ENSG00000214081	-2.58	-3.722	-3.807	2.24E-04	*CYP4F30P*	cytochrome P450 family 4 subfamily F member 30, pseudogene
ENSG00000165259	2.05	-0.644	3.778	2.48E-04	*HDX*	highly divergent homeobox
ENSG00000251411	2.19	-2.195	3.755	2.70E-04		(known processed pseudogene)
ENSG00000154099	0.98	0.726	3.700	3.28E-04	*DNAAF1*	dynein axonemal assembly factor 1
ENSG00000261487	1.58	0.395	3.660	3.77E-04		(novel processed transcript)
ENSG00000253676	1.79	-0.299	3.660	3.77E-04	*TAGLN2P1*	transgelin 2 pseudogene 1

7.4 Longitudinal analysis

7.4.1 Flare visit versus baseline: flare patients

$CD4^+$ T cell gene expression was compared between time of flare and baseline for the 17 patients who experienced an arthritis flare following DMARD cessation and where both baseline and flare samples were included in the RNAseq experiment. A total of 81 genes were differentially expressed between the two groups at an unadjusted significance level of $p<0.001$, of which 2 were robust to FDR correction (Figure 7.10). The entire list of 81 genes is presented in Appendix I, and the top 20 genes by p value are listed in Table 7.5.

Log fold-change of the 284 genes that demonstrated differential expression with an unadjusted p-value threshold of <0.01 were analysed by Ingenuity Pathway Analysis. Canonical pathways were dominated by genes involved in cellular proliferation. The top network identified was related to "cell cycle; cellular assembly and organisation; DNA replication, recombination and repair" processes, with robust upregulation of 31/35 genes across the virtually the entire network (Figure 7.11).

7.4.2 Month six versus baseline visits: remission patients

$CD4^+$ T cell gene expression was compared between baseline and the final month six study visit for the 15 patients who remained in DFR following DMARD cessation and where both baseline and month six samples were included in the RNAseq experiment. There was no significant differential expression after FDR adjustment (Figure 7.12A). Using unadjusted p values, 19 genes were differentially expressed at the $p<0.001$ significance level (Figure 7.12B, Table 7.6).

Ingenuity pathway analysis was performed using the 183 genes that were differentially expressed at an unadjusted p threshold of $p<0.01$between month 6 and baseline visits in the remission group. The top network that was identified involved genes with roles in "cancer; cell-to-cell signalling and interaction; and organismal injury and abnormalities". Differential expression was observed in 16/35 nodes in this predicted network, the majority of which were down-regulated at month six verses baseline (Figure 7.13). There were however very few edges between differentially expressed nodes in the network.

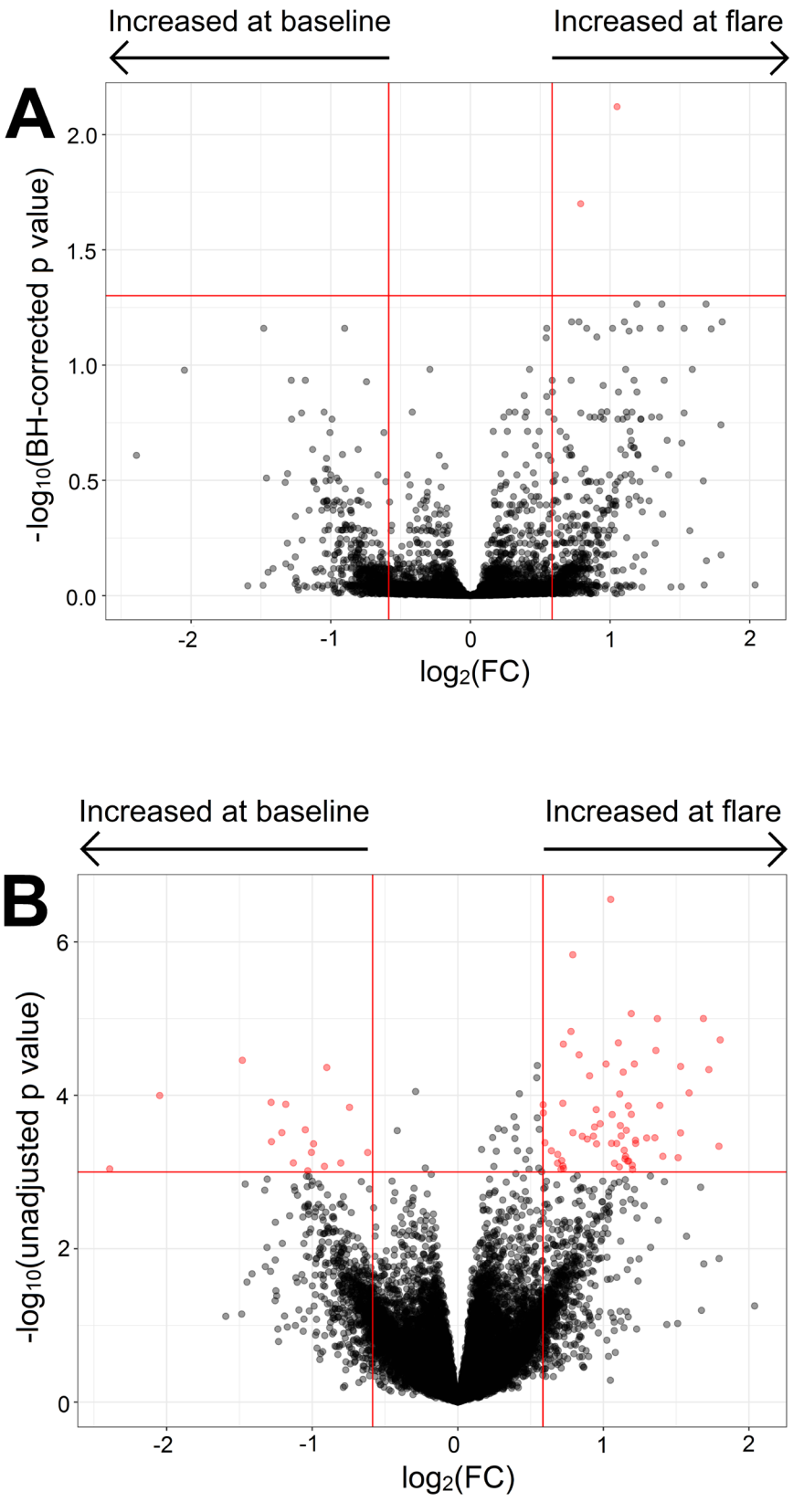


Figure 7.10 - Volcano plots showing longitudinal change in gene expression between time of arthritis flare versus baseline for patients who experienced an arthritis flare following DMARD cessation. Plots are shown with (A: FDR-corrected p-value < 0.05) and without (B: unadjusted p < 0.001) multiple test correction. Horizontal lines represent log-fold change (log_2FC) > 1.5. Genes that exceeded both thresholds are highlighted in red.

Table 7.5 – Top 20 (by unadjusted p value) differentially expressed genes between flare versus baseline visits in 17 patients who experienced an arthritis flare. Positive log-fold change indicates higher expression at the flare visit, whereas negative log-fold change indicates higher expression at baseline. HGNC: HUGO gene nomenclature committee. * = significant after multiple test correction ($p<0.05$).

Ensembl gene ID	Log_2 FC	Average expression	t	Unadjusted p value	HGNC symbol	Description
ENSG00000144354	**1.05**	**2.811**	**5.489**	**2.79E-07**	***CDCA7***	**cell division cycle associated 7 ***
ENSG00000130164	**0.79**	**4.180**	**5.105**	**1.47E-06**	***LDLR***	**low density lipoprotein receptor ***
ENSG00000165409	1.19	-0.350	4.678	8.59E-06	*TSHR*	thyroid stimulating hormone receptor
ENSG00000171533	1.69	-2.505	4.641	9.98E-06	*MAP6*	microtubule associated protein 6
ENSG00000137474	1.37	0.283	4.640	1.00E-05	*MYO7A*	myosin VIIA
ENSG00000156127	0.78	3.068	4.544	1.47E-05	*BATF*	basic leucine zipper ATF-like transcription factor
ENSG00000251537	1.80	-2.089	4.480	1.89E-05		(known protein coding)
ENSG00000088325	1.10	2.241	4.458	2.07E-05	*TPX2*	TPX2, microtubule nucleation factor
ENSG00000156535	0.72	1.385	4.447	2.15E-05	*CD109*	CD109 molecule
ENSG00000138180	1.36	0.809	4.399	2.60E-05	*CEP55*	centrosomal protein 55
ENSG00000137812	0.83	1.516	4.365	2.97E-05	*KNL1*	kinetochore scaffold 1
ENSG00000216819	-1.48	-3.326	-4.323	3.50E-05	*TUBB2BP1*	tubulin beta 2B class IIb pseudogene 1
ENSG00000131747	1.21	3.351	4.294	3.91E-05	*TOP2A*	topoisomerase (DNA) II alpha
ENSG00000170312	1.02	1.016	4.293	3.91E-05	*CDK1*	cyclin dependent kinase 1
ENSG00000148773	1.53	4.054	4.274	4.20E-05	*MKI67*	marker of proliferation Ki-67
ENSG00000267496	-0.90	-0.920	-4.266	4.34E-05	*FAM215A*	family with sequence similarity 215 member A (non-protein coding)
ENSG00000263218	1.72	-3.428	4.250	4.62E-05		(known antisense RNA)
ENSG00000237649	1.14	1.173	4.230	4.98E-05	*KIFC1*	kinesin family member C1
ENSG00000137804	0.91	2.575	4.201	5.56E-05	*NUSAP1*	nucleolar and spindle associated protein 1
ENSG00000175063	1.59	-0.144	4.063	9.31E-05	*UBE2C*	ubiquitin conjugating enzyme E2 C

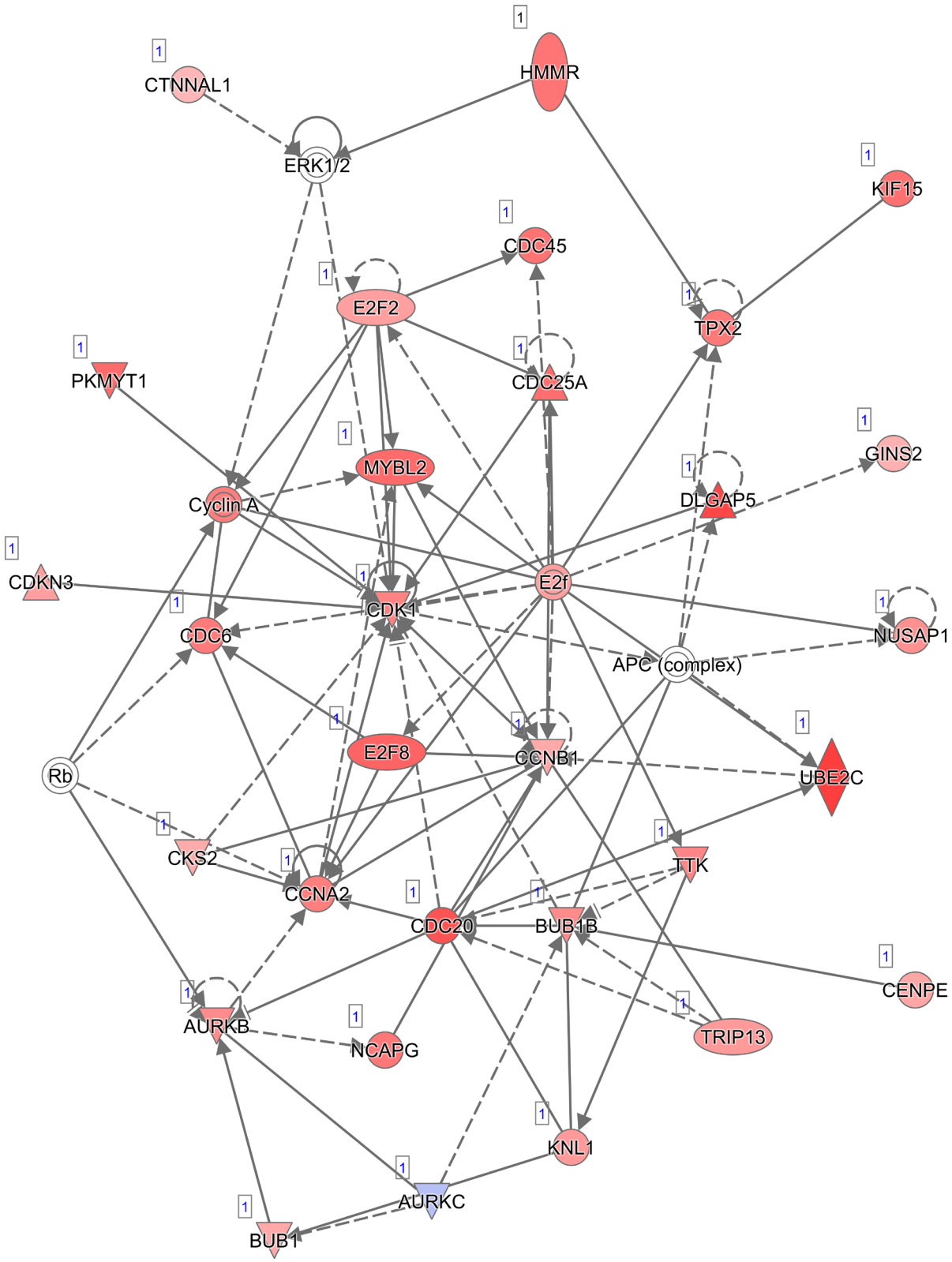


Figure 7.11 – Visualisation of differential expression of genes within a predicted "cell cycle; cellular assembly and organisation; DNA replication, recombination and repair" network between baseline and flare visit samples in those patients who experienced an arthritis flare following DMARD cessation. Genes highlighted in red were up-regulated at the flare visit, whereas those highlighted in blue were down-regulated at the flare visit relative to baseline. Copyright Qiagen 2017, reproduced with permission.

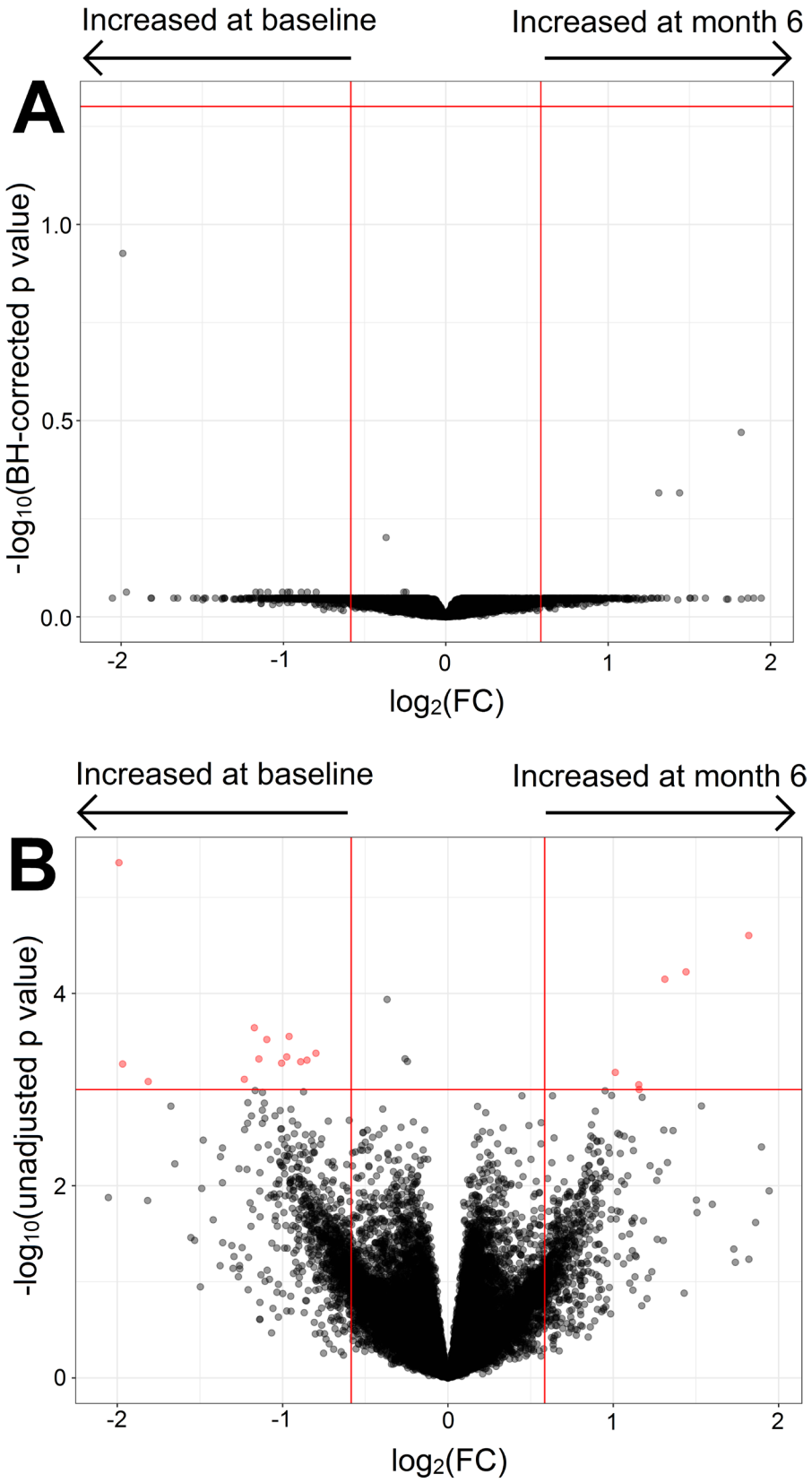


Figure 7.12 - Volcano plots showing longitudinal change in gene expression between month six versus baseline for patients who remained in drug-free remission following DMARD cessation. Plots are shown with (A: FDR-corrected p-value < 0.05) and without (B: unadjusted p < 0.001) multiple test correction. Horizontal lines represent log-fold change (log_2FC) > 1.5. Genes that exceeded both thresholds are highlighted in red.

Table 7.6– Differential expression of genes between month six versus baseline visits in 15 patients who remained in DFR, using an unadjusted significance threshold of p<0.001. Positive log-fold change indicates higher expression at the month six visit, whereas negative log-fold change indicates higher expression at baseline. HGNC: HUGO gene nomenclature committee.

Ensembl gene ID	Log_2 FC	Average expression	t	Unadjusted p value	HGNC symbol	Description
ENSG00000211677	-1.99	-0.565	-4.844	4.36E-06	*IGLC2*	immunoglobulin lambda constant 2
ENSG00000240036	1.82	-2.709	4.410	2.49E-05		(processed pseudogene)
ENSG00000244357	1.44	-1.978	4.182	5.97E-05	*RN7SL145P*	RNA, 7SL, cytoplasmic 145, pseudogene
ENSG00000238260	1.31	-3.377	4.135	7.12E-05		(known antisense RNA)
ENSG00000254230	-1.17	-3.649	-3.817	0.000227		(known antisense RNA)
ENSG00000271153	-0.96	-4.036	-3.759	0.00028	*RPL23AP88*	ribosomal protein L23a pseudogene 88
ENSG00000260896	-1.10	-2.058	-3.738	0.000301	*LINC02170*	long intergenic non-protein coding RNA 2170
ENSG00000255757	-0.80	0.052	-3.643	0.000419		(processed pseudogene)
ENSG00000104067	-0.98	-1.610	-3.618	0.000457	*TJP1*	tight junction protein 1
ENSG00000255045	-1.14	-2.449	-3.603	0.00048		(known antisense RNA)
ENSG00000237593	-0.85	-0.704	-3.595	0.000495		(processed pseudogene)
ENSG00000136634	-0.89	-0.556	-3.584	0.000514	*IL10*	interleukin 10
ENSG00000254095	-1.01	-3.776	-3.574	0.000531		(processed transcript)
ENSG00000211895	-1.97	0.058	-3.568	0.000542	*IGHA1*	immunoglobulin heavy constant alpha 1
ENSG00000101384	1.01	-0.338	3.509	0.000663	*JAG1*	jagged 1
ENSG00000256651	-1.23	-2.981	-3.459	0.000782		(processed pseudogene)
ENSG00000211890	-1.81	-2.260	-3.443	0.000825	*IGHA2*	immunoglobulin heavy constant alpha 2 (A2m marker)
ENSG00000224842	1.15	-3.139	3.419	0.000892		(known antisense RNA)
ENSG00000272945	1.16	-2.857	3.385	0.000999		(sense intronic)

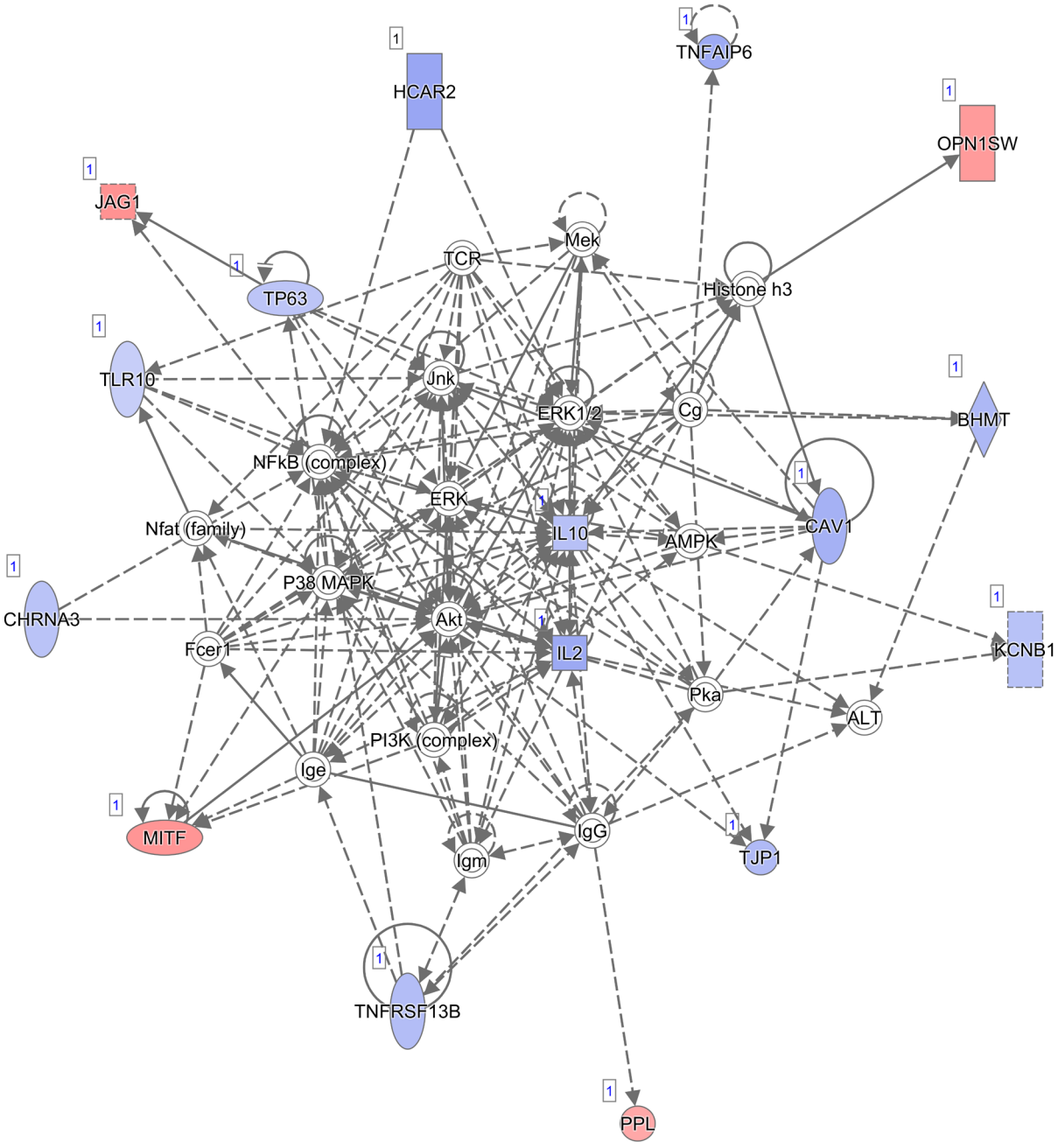


Figure 7.13 – Visualisation of differential expression of genes within a predicted network of "cell cycle; cellular assembly and organisation; DNA replication, recombination and repair" genes between month six and baseline visit samples in those patients who maintained drug-free remission following DMARD cessation. Genes highlighted in red were up-regulated at month six, whereas those highlighted in blue were down-regulated at month six relative to baseline.

7.5 Comparison between contrast groups

In a further exploratory analysis, genes that were differentially expressed at the unadjusted p<0.001 threshold in the various contrasts described above were compared (Figure 7.14). Very little overlap was observed in differentially expressed genes (DEGs) between contrast pairs, with the exception of flare vs. controls and remission vs. controls analyses, which shared 23 common DEGs (Table 7.7). The substantially higher number of common DEGs in these comparisons is to be expected, given the common comparator group of healthy controls. These DEGs could conceivably represent gene expression specific to RA disease processes, or a gene expression profile common to the effects of DMARD treatment.

A further single gene (XKRX - XK related, X-linked, which encodes for a Kell blood group antigen) was down-regulated in both $flare_{baseline}$ vs. $remission_{baseline}$ (log_2FC -1.24) and $flare_{baseline}$ vs. $HC_{baseline}$ (log_2FC -2.85) comparisons.

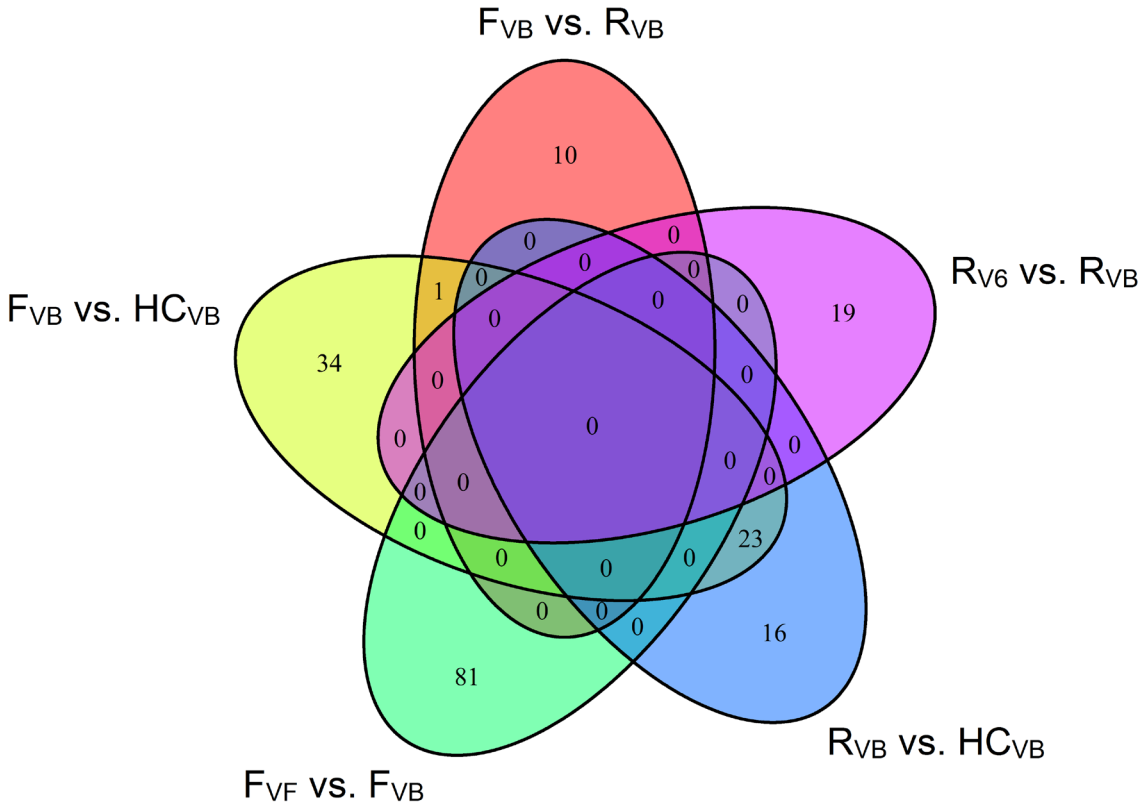


Figure 7.14 – Overlap of differentially expressed genes identified in different contrast pairs at the unadjusted p<0.001 significance threshold. F: flare patient; FV: flare visit; HC: healthy control; V6: month 6 visit; VB: baseline visit; VF: flare visit; R: remission patient.

Table 7.7 – Differentially expressed genes common to both $flare_{baseline}$ versus healthy control ($HC_{baseline}$) and $remission_{baseline}$ versus $HC_{baseline}$ analyses at the unadjusted $p<0.001$ significance threshold. Positive log_2(fold change) (log_2FC) indicates higher expression in patients, whereas a negative log_2FC indicates lower expression in patients relative to healthy controls. HGNC: HUGO gene nomenclature committee. lincRNA: long intergenic non-coding RNA; TEC = to be experimentally confirmed.

Ensembl gene ID	log_2FC $flare_{baseline}$ vs $HC_{baseline}$	log_2FC $remission_{baseline}$ vs. $HC_{baseline}$	HGNC symbol	Description
ENSG00000106927	2.13	2.05	*AMBP*	alpha-1-microglobulin/bikunin precursor
ENSG00000226029	0.78	0.92	*LINC01772*	long intergenic non-protein coding RNA 1772
ENSG00000112139	6.09	7.13	*MDGA1*	MAM domain containing glycosylphosphatidylinositol anchor 1
ENSG00000247311	1.85	2.06		(antisense RNA)
ENSG00000198538	-1.30	-1.33	*ZNF28*	zinc finger protein 28
ENSG00000163631	4.07	3.88	*ALB*	albumin
ENSG00000171560	2.42	1.87	*FGA*	fibrinogen alpha chain
ENSG00000171564	2.00	1.74	*FGB*	fibrinogen beta chain
ENSG00000259657	1.35	1.54	*PIGHP1*	phosphatidylinositol glycan anchor biosynthesis class H pseudogene 1
ENSG00000265293	1.64	1.75	*ARGFXP2*	arginine-fifty homeobox pseudogene 2
ENSG00000228382	1.91	2.06	*ITPKB-IT1*	ITPKB intronic transcript 1
ENSG00000279148	1.87	1.92		(TEC)
ENSG00000165259	1.94	2.05	*HDX*	highly divergent homeobox
ENSG00000251411	2.32	2.19		(processed pseudogene)
ENSG00000261487	1.59	1.58		(processed transcript)
ENSG00000141622	1.47	1.32	*RNF165*	ring finger protein 165
ENSG00000115129	1.15	1.18	*TP53I3*	tumour protein p53 inducible protein 3
ENSG00000246016	1.95	2.02	*LINC01513*	long intergenic non-protein coding RNA 1513
ENSG00000159882	-0.67	-0.68	*ZNF230*	zinc finger protein 230
ENSG00000272086	1.27	1.31		(known LincRNA)
ENSG00000267939	2.31	2.17		(known LincRNA)
ENSG00000273598	-1.89	-1.95		(unprocessed pseudogene)
ENSG00000229314	2.72	2.43	*ORM1*	orosomucoid 1

7.6 Predictive biomarker analyses

7.6.1 Univariate Cox regression

Standard bioinformatics pipelines analyse differential gene expression dependent on the presence or absence of a binary outcome measure. Whilst this approach benefits from many years of accumulated knowledge and refined computer packages, it is inherently underpowered in comparison to survival analysis when analysing time-to-event data. Therefore, in a further analysis, the association between gene expression at baseline and time-to-flare following DMARD cessation was analysed across all sequenced genes using univariate Cox regression. Using this approach, 19 genes were identified that were significantly associated with time-to-flare by a post hoc unadjusted p-value threshold of <0.001 (Figure 7.15). None of the genes were robust to multiple test correction, although the Benjamini-Hochberg procedure appeared particularly conservative in its correction relative to the two-way analyses (Table 7.8). No significant departure from proportional hazards was observed for any of these 19 genes.

7.6.2 Multivariate Cox regression

The 19 genes that were significantly associated with time-to-flare at unadjusted significance level of <0.001 by univariate Cox regression were entered in to a multivariate Cox regression model. Stepwise backward selection based on Akaike Information Criterion (AIC) was then performed in order to reduce the number of variables to a practical size for the purposes of a biomarker signature, where a lower AIC score indicates a better model (see Methods 3.10.1). After five selection steps, 14 variables remained in the preliminary backwards stepwise multivariate Cox model (Table 7.9), with a reduction in AIC from 86.97 to 79.42. The 13 variables in this model with a p value < 0.2 were then taken forward to a second round of stepwise backward selection. Although this 13 variable model had an AIC slightly greater AIC (79.81) than the 14 variable model, this could be reduced further by an additional two selection steps. This generated a final stepwise multivariate Cox regression model with 11 variables and an AIC (77.69) that was lower than the 14 variable model (Table 7.10). Proportionality of hazards was demonstrated for all variables in both the 14-variable and 11-variable stepwise models, and for both models as a whole.

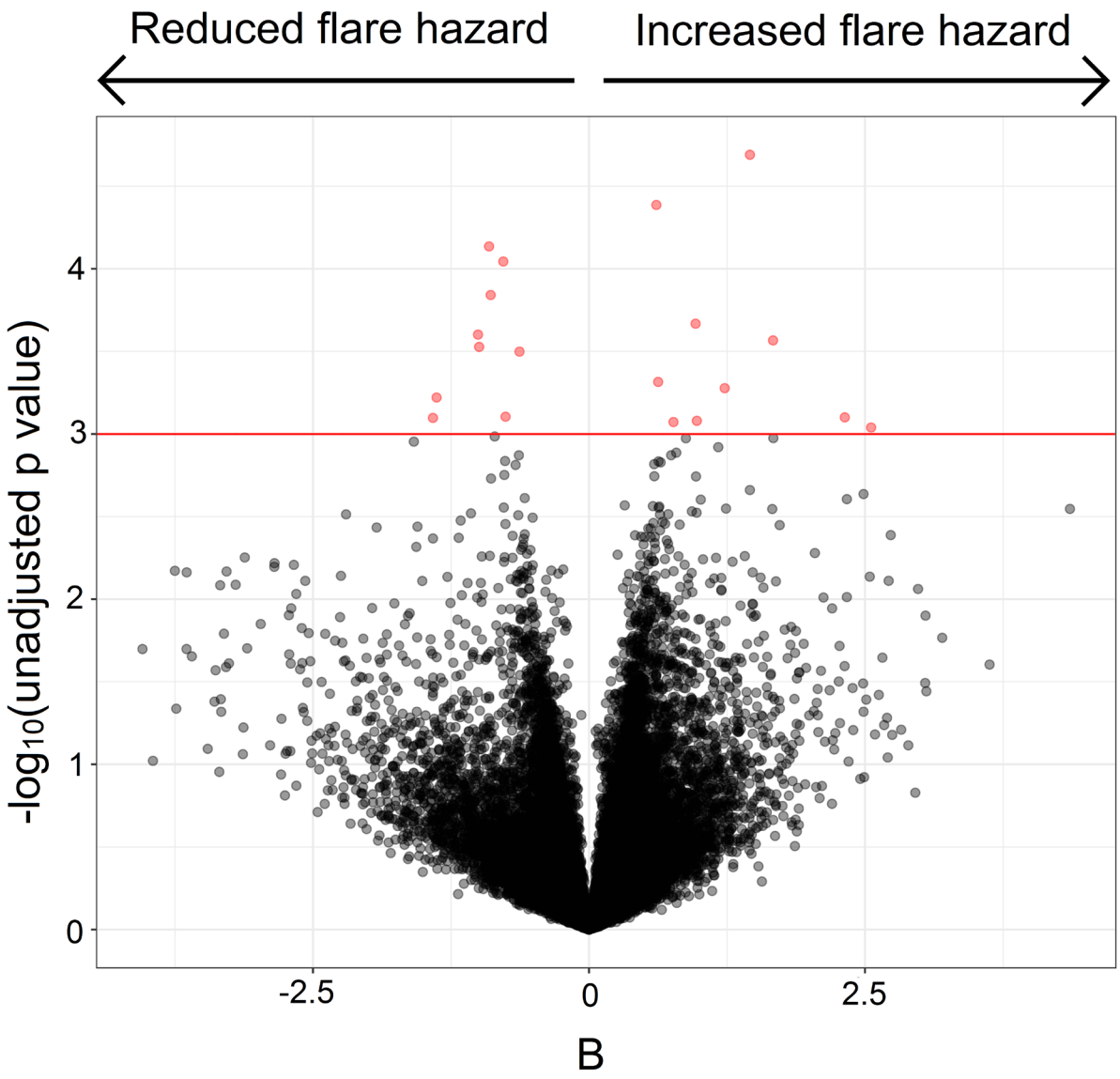


Figure 7.15 - Volcano plot showing baseline gene expression in circulating $CD4^+$ T cells as analysed by univariate Cox regression of time-to-flare following DMARD cessation. The horizontal line shows the unadjusted p-value < 0.001 threshold – genes that exceeded this threshold are highlighted in red. B: univariate Cox regression coefficient.

Table 7.8 – Association between baseline gene expression and time-to-flare following DMARD cessation by univariate Cox regression, using an unadjusted significance threshold of p<0.001. HGNC: HUGO gene nomenclature committee. LincRNA: long intergenic non-coding RNA. P value calculated by the likelihood ratio test.

Ensembl gene ID	HR_{flare}	HR_{flare} 95% CI	B	Unadjusted p-value	Adjusted p-value	HGNC symbol	Description
ENSG00000102362	4.30	1.93 – 9.58	1.46	2.04E-05	0.555	*SYTL4*	synaptotagmin like 4
ENSG00000247033	1.84	1.33 – 2.54	0.61	4.11E-05	0.559		(novel antisense)
ENSG00000276571	0.40	0.25 – 0.65	-0.91	7.33E-05	0.615		(novel antisense)
ENSG00000204965	0.46	0.31 – 0.68	-0.78	9.04E-05	0.615	*PCDHA5*	protocadherin alpha 5
ENSG00000241146	0.41	0.27 – 0.63	-0.89	1.44E-04	0.785	*RPL7P41*	ribosomal protein L7 pseudogene 41
ENSG00000250030	2.63	1.58 – 4.36	0.97	2.15E-04	0.863		(novel processed pseudogene)
ENSG00000213296	0.37	0.20 – 0.66	-1.01	2.50E-04	0.863		(known processed pseudogene)
ENSG00000229619	5.30	2.05 – 13.7	1.67	2.72E-04	0.863	*MBNL1-AS1*	muscleblind-like protein 1 - antisense RNA 1
ENSG00000125046	0.37	0.22 – 0.61	-0.99	2.97E-04	0.863	*SSUH2*	suppressor of stomatin mutant uncoordination (ssu-2) homolog (C. elegans)
ENSG00000182489	0.53	0.38 – 0.75	-0.63	3.17E-04	0.863	*XKRX*	Kell Blood Group Complex Subunit-Related, X-Linked
ENSG00000144366	1.87	1.30 – 2.70	0.63	4.84E-04	1.000	*GULP1*	engulfment adaptor PTB domain containing 1
ENSG00000237473	3.42	1.58 – 7.39	1.23	5.28E-04	1.000		(known lincRNA)
ENSG00000228010	0.25	0.11 – 0.56	-1.38	6.02E-04	1.000		(novel antisense)
ENSG00000250827	0.47	0.30 – 0.74	-0.76	7.86E-04	1.000	*MFSD4BP1*	major facilitator superfamily domain containing 4B pseudogene 1
ENSG00000042286	10.2	2.42 – 42.6	2.32	7.94E-04	1.000	*AIFM2*	apoptosis inducing factor, mitochondria associated 2
ENSG00000231305	0.24	0.11 – 0.56	-1.41	7.99E-04	1.000		(known antisense)
ENSG00000255330	2.66	1.32 – 5.36	0.98	8.33E-04	1.000	*SOGA3*	suppressor of glucose, autophagy associated (SOGA) family member 3
ENSG00000227070	2.15	1.41 – 3.28	0.77	8.47E-04	1.000		(novel antisense)
ENSG00000162636	12.9	2.57 – 64.5	2.56	9.14E-04	1.000	*FAM102B*	family with sequence similarity 102 member B

Table 7.9 – Association of baseline gene expression and time-to-flare following DMARD-cessation in a preliminary 14-variable backward stepwise multivariate Cox regression model. B: Cox regression coefficient. P-value calculated by the Wald test.

Ensembl gene ID	B	HR_{flare}	95% CI HR_{flare}	p
ENSG00000204965	-2.80	0.06	0.01 – 0.58	0.015
ENSG00000241146	-1.90	0.15	0.03 – 0.71	0.017
ENSG00000229619	4.48	88.2	2.05 – 3800	0.020
ENSG00000228010	-7.12	0.00	0.00 – 0.59	0.034
ENSG00000125046	-2.53	0.08	0.01 – 0.88	0.039
ENSG00000162636	13.62	$8.19x10^{5}$	$1.18 – 5.69x10^{11}$	0.047
ENSG00000227070	4.11	60.64	1.03 – 3560	0.048
ENSG00000250827	1.78	5.92	0.95 – 36.8	0.056
ENSG00000042286	2.75	15.6	0.86 – 284	0.063
ENSG00000247033	2.22	9.20	0.88 – 96.4	0.064
ENSG00000276571	-1.46	0.23	0.05 – 1.17	0.076
ENSG00000237473	1.77	5.87	0.72 – 47.9	0.098
ENSG00000182489	0.85	2.33	0.77 – 7.06	0.133
ENSG00000102362	-2.34	0.10	0.002 – 4.30	0.227

Table 7.10 – Association of baseline gene expression and time-to-flare following DMARD-cessation in a final 11-variable backward stepwise multivariate Cox regression model. B: Cox regression coefficient. P-value calculated by the Wald test.

Ensembl gene ID	B	HR_{flare}	95% CI HR_{flare}	p
ENSG00000228010	-4.15	0.02	0.00 – 0.14	2.24E-04
ENSG00000162636	6.97	1060	22.6 – 50000	3.88E-04
ENSG00000227070	1.78	5.94	2.08 – 16.9	8.63E-04
ENSG00000204965	-1.69	0.18	0.07 – 0.52	1.45E-03
ENSG00000229619	2.93	18.7	2.90 – 121	2.08E-03
ENSG00000247033	1.18	3.26	1.53 – 6.98	2.29E-03
ENSG00000125046	-1.49	0.23	0.09 – 0.60	2.62E-03
ENSG00000241146	-1.32	0.27	0.10 – 0.71	7.96E-03
ENSG00000276571	-0.83	0.44	0.18 – 1.07	7.07E-02
ENSG00000250827	0.71	2.04	0.89 – 4.69	9.42E-02
ENSG00000042286	1.75	5.78	0.68 – 49.3	1.09E-01

7.6.3 ROC analysis

Based on a p value threshold of <0.001, three variables were selected from this final stepwise multivariate model, namely: ENSG00000228010, ENSG00000162636 and ENSG00000227070. Candidate genes were explored for their biomarker utility either alone, or in combination with each other – when combined, expression values for each gene were weighted by the respective coefficient in the final stepwise Cox regression model. Patients were dichotomised for each gene score using two thresholds determined by ROC analysis optimised for the prediction of flare and remission, as previously discussed (Methods 3.10.1) (Table 7.11).

The ROC curve for each variable, together with the survival curves for dichotomised groups based on flare and remission biomarker thresholds are presented in Figure 7.16. Based on overall AUC, it is clear to see that the composite scores provided greater predictive utility than any of the single gene expression variables alone. The prognostic performance of the composite scores are detailed further in Table 7.12.

Table 7.11 – Composite scores ranked by area under the receiver operating characteristic curve (ROC_{AUC}). Variables included within each score are indicated in green, and those excluded are indicated in red.

ENSG00000228010	ENSG00000162636	ENSG00000227070	Remission threshold	Flare threshold	ROC_{AUC}
✓	✓	✓	59.19	60.55	0.900
✓	✓	✗	75.86	79.4	0.854
✓	✗	✓	39.33	41.17	0.841
✗	✓	✓	0.18	2.69	0.820
✗	✓	✗	19.21	19.97	0.750
✓	✗	✗	56.38	59.1	0.743
✗	✗	✓	-19.11	-15.96	0.685

Figure 7.16 – Receiver-operating characteristic curves for RNAseq composite biomarker scores for the prediction of flare following DMARD cessation. A: MCP1+IL27+CRP, B: MCP1+IL27, C: MCP1+CRP, D: MCP1, E: IL27+CRP, E: CRP, F: IL27.

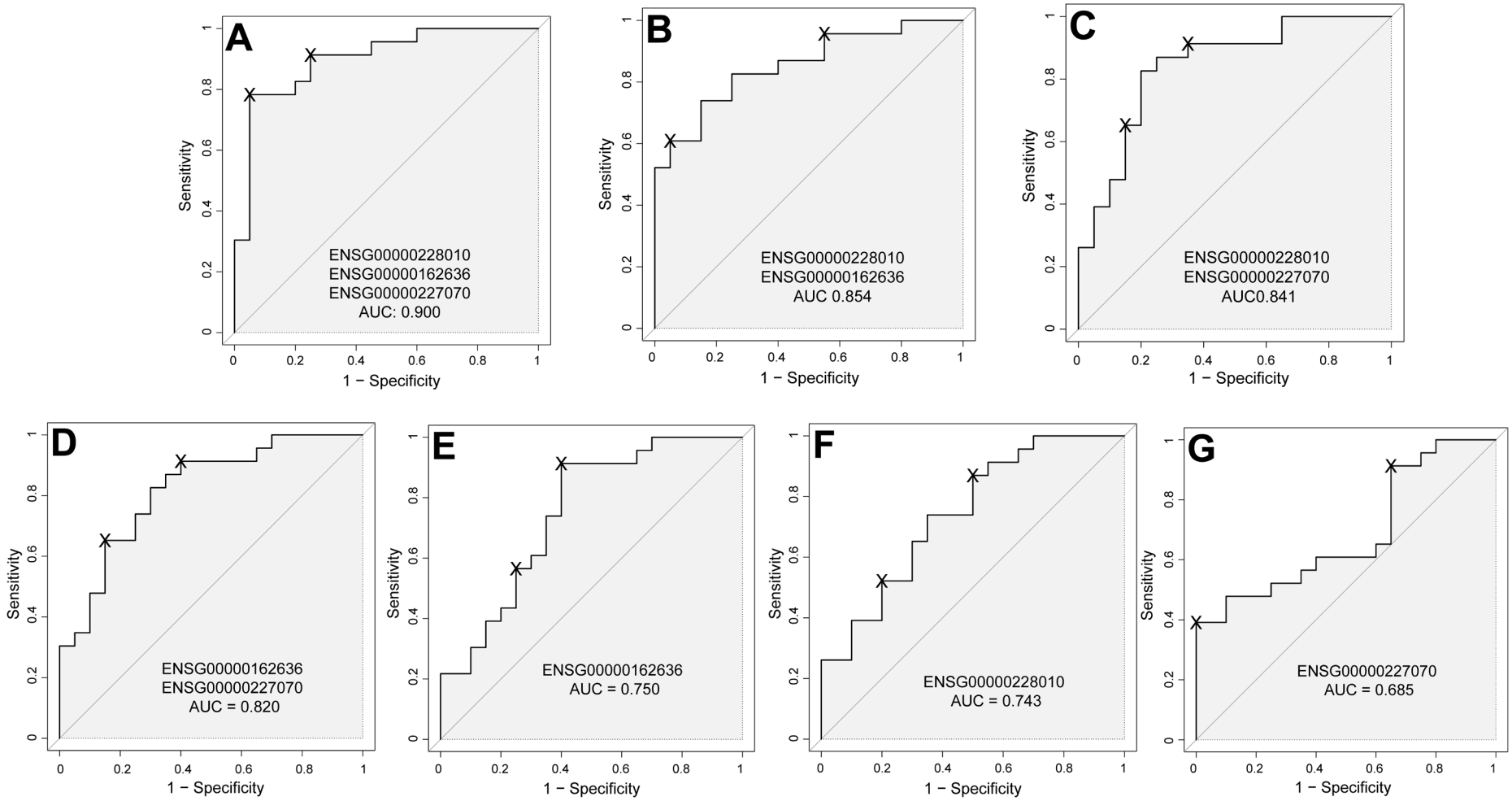


Table 7.12 - Predictive utility of cytokine/chemokine variables in predicting flare following DMARD cessation, with a positive test defined by either flare or remission thresholds. Optimum pairs of predictive metrics are highlighted in **bold**. NPV: negative predictive value; PPV: positive predictive value.

Variable	ROC_{AUC} (95% CI)	Threshold	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
ENSG00000228010 ENSG00000162636 ENSG00000227070	0.90 (0.81 – 0.99)	Flare (60.55)	**0.78** (0.61 – 0.96)	0.95 (0.85 – 1.00)	**0.95** (0.84 – 1.00)	0.79 (0.67 – 0.95)
		Remission (59.19)	0.91 (0.78 – 1.00)	**0.75** (0.55 – 0.90)	0.81 (0.70 – 0.92)	**0.89** (0.74 – 1.00)
ENSG00000228010 ENSG00000162636	0.85 (0.74 – 0.97)	Flare (79.4)	0.61 (0.39 – 0.78)	0.95 (0.85 – 1.00)	0.94 (0.81 – 1.00)	0.68 (0.58 – 0.80)
		Remission (75.86)	0.96 (0.87 – 1.00)	0.45 (0.25 – 0.65)	0.67 (0.59 – 0.77)	0.91 (0.70 – 1.00)
ENSG00000228010 ENSG00000227070	0.84 (0.72 – 0.96)	Flare (41.17)	0.65 (0.43 – 0.83)	0.85 (0.65 – 1.00)	0.83 (0.68 – 1.00)	0.68 (0.56 – 0.82)
		Remission (39.33)	0.91 (0.78 – 1.00)	0.65 (0.45 – 0.85)	0.75 (0.65 – 0.88)	0.88 (0.71 – 1.00)

Overall, the 3-gene composite score performed the best for prediction of both flare and remission and discriminated patients with significantly different flare-free survival times (Formula 7.1 and Figure 7.17).

Formula 7.1 – The 3-gene composite biomarker score, based on log-transformed gene expression values.

Composite score = 6.97(ENSG00000162636) + 1.78(ENSG00000227070)
- 4.15(ENSG00000228010) - 1.22(ACR/EULAR Boolean remission)

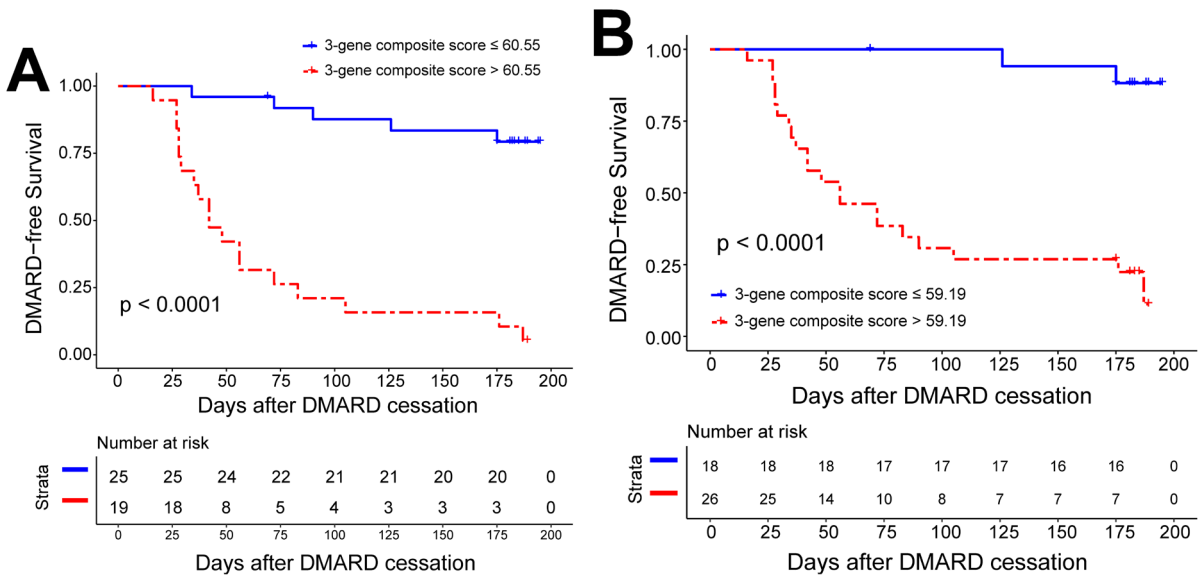


Figure 7.17 - A: Kaplan-Meier plot of DMARD-free survival stratified by 3-gene composite score >60.55 (red) versus ≤ 60.55 (blue). B: Kaplan-Meier plot of DMARD-free survival stratified by 3-gene composite score >59.19 (red) or ≤59.19 (blue). P-values calculated by log-rank test.

7.6.4 Sensitivity analysis incorporating processing time

During the laboratory isolation procedure, $CD4^+$ T cells are alive until the final cell lysis step. The transcriptional profiles of the cells may therefore be influenced by the isolation procedure itself. In order to ascertain the effect of this upon the composite biomarker score, a sensitivity analysis was performed whereby the three genes from the final composite score were entered in to a multivariate Cox regression model with the addition of total time from venepuncture to freezing of $CD4^+$ T cell lysate. Total processing time showed no significant association with time-to-flare, whereas the three genes remained strongly associated independent of processing time (Table 7.13). Proportionality of hazards was demonstrated for all four variables and the model a whole.

Table 7.13 – Sensitivity analysis incorporating genes from the final composite biomarker score, together with total time from venepuncture to freezing of cell lysate, in a multivariate Cox regression model. B: Cox regression coefficient.

Variable	B	HR_{flare}	95% CI HR_{flare}	p
ENSG00000162636	2.863	17.51	3.15	97.31
ENSG00000227070	0.713	2.04	1.32	3.14
ENSG00000228010	-1.785	0.17	0.06	0.44
Total time	-0.004	1.00	0.99	1.00

7.7 Discussion

7.7.1 Baseline comparison of flare versus drug-free remission patients

In the primary analysis of the gene expression data of this study, $CD4^+$ T cell gene expression was compared at baseline between those patients who experienced an arthritis flare versus those who remained in DFR following DMARD cessation. Although substantial number of differentially expressed genes (DEGs) were observed, none of these were robust to multiple test correction. Using *post hoc* unadjusted p-value thresholds of <0.001 and <0.01, 11 and 118 genes respectively were identified as differentially expressed at the pre-specified >1.5 fold-change level. Pathway analysis of the all 118 DEGs identified 12 as functioning within a network of genes involved in cell cycle, cell death and inflammatory response processes, though with no connecting edges between these identified nodes. A review of the published literature for the 11 DEGs at the p<0.001 significance level does however yield some interesting observations, as detailed below.

7.7.1.a Killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 4 (KIR2DS4)

Expression of Killer cell immunoglobulin like receptor, two Ig domains and short cytoplasmic tail 4 (*KIR2DS4*) was up-regulated in flare versus DFR (log_2FC 1.93, unadjusted p = 0.0003). KIRs are a diverse group of transmembrane glycoproteins that belong to the immunoglobulin superfamily, and are expressed on NK cells and T cells (Béziat *et al.*, 2017). Inhibitory KIRs tend to bind class I HLA molecules and are thought to play an important role in viral and tumour surveillance, whereas the ligands for the majority of stimulatory KIRs remain unknown (Colucci and Traherne, 2017). Akin to HLA molecules, KIRs are notable for their vast allelic diversity and are inherited in haplotypes with ethnic and geographical separation (Béziat *et al.*, 2017). A role for KIRs in the development of autoimmune disease has been postulated (Kusnierczyk, 2013), particularly by $CD4^+CD28^-KIR^+$ T cells in RA and vasculitis (Yen *et al.*, 2001; van Bergen and Koning, 2010). Of particular relevance to the findings of my study, KIR2DS4 has been implicated in the pathogenesis of RA. In a case-control Taiwanese genotyping study of 122 RA patients and 96 healthy controls, presence of the *KIR2DS4* gene was significantly greater in RA patients versus controls (OR 1.9, 95% CI 1.1 – 3.4, corrected p<0.01) (Yen *et al.*, 2006). Furthermore, in a Polish study of 312 RA patients

treated with methotrexate, presence of the full-length *KIR2DS4* gene was associated with a lower chance of responding to methotrexate (OR 0.43, 95% CI 0.22 – 0.99, p = 0.033) (Majorczyk *et al.*, 2014). Given the apparent association of KIR2DS4 with both the development of RA and its response to treatment, it is thus conceivable that the higher KIR2DS4 expression by $CD4^+$ T cells observed in this study may be of functional relevance in the predisposition to flare following DMARD cessation.

7.7.1.b Engulfment adaptor PTB domain containing 1 (GULP1)

Expression of engulfment adaptor PTB domain containing 1 (*GULP1*) was up-regulated in flare versus DFR (log_2FC 1.26, unadjusted p = 4.82 x 10^{-4}). GULP1 is an evolutionarily conserved adaptor protein involved in the phagocytosis of apoptotic cells (Kinchen and Ravichandran, 2007). In a small study of four patients with RA and three with OA, Qingchun *et al.* (2008) analysed differential expression of apoptosis-related genes. Included within the eight apoptosis-related DEGs was GULP1, which was down-regulated in RA compared to OA synovium. Exactly how this relates to the observation of up-regulated baseline *GULP1* expression by $CD4^+$ T cells in the flare group of my study is uncertain, though nevertheless may suggest a role for the protein in RA processes. GULP1 can also bind to one of the two NPxY motifs in the cytoplasmic domain of low-density lipoprotein receptor-related protein (LRP) (Su *et al.*, 2002) – a protein with structural homology to low-density lipoprotein receptor (LDLR) – that is thought to be important in various signal transduction pathways (Lin and Hu, 2014). This observation may be of relevance given the up-regulation of LDLR at the time of flare in my study (discussed further below). Nevertheless, although LDLR also possesses an NPxY motif in its cytoplasmic tail (Lillis *et al.*, 2008), there are no reports of GULP1-LDLR interactions in the published literature.

7.7.1.c Synaptotagmin like 4 (SYTL4)

The most significantly up-regulated DEG by p-value in flare vs. DFR patients at baseline was Synaptotagmin like 4 (*SYTL4*, also known as Slp4, or granuphilin) (log_2FC 0.97, unadjusted p value 1.56 x 10^{-4}). SYTL4 interacts with the protein Rab27a to reduce exocytosis of dense-core vesicles in rat PC12 cells (Fukuda *et al.*, 2002). Mutations in Rab27a are the underlying defect in the rare type 2 Griscelli syndrome, where defects in vesicle exocytosis lead to albinism (due to reduced melanin secretion) and immunodeficiency with haemophagocytic lymphohistiocytosis (Anikster *et al.*, 2002). Although no publications relate to the role of

SYL4 in lymphocytes, the structurally related proteins SYTL1 and SYTL2 play a role in secretion of vesicles at the immunological synapse of mouse and human cytotoxic T lymphocytes (Holt *et al.*, 2008). Nevertheless, the significance of up-regulated *SYTL4* expression in the global $CD4^+$ T cell population observed in flare patients at baseline is difficult to interpret based on gene expression data alone, and its functional effect would depend upon the cellular subpopulation of interest.

7.7.2 Baseline comparison of patients with healthy controls

DEGs were compared between patients and healthy controls in exploratory analyses to identify disease-specific genes associated with flare and sustained DFR. A greater number of DEGs were observed in the flare vs. healthy (58) than the remission vs. healthy (39) comparisons, using a pre-specified 1.5 fold-change threshold and a *post hoc* unadjusted significance threshold of $p < 0.001$. Furthermore, DEGs that were robust to multiple-test correction were only observed in the flare vs. healthy comparison: α-fibrinogen (*FGA*), β-fibrinogen (*FGB*), and α1-microglobulin/bikunin precursor (*AMBP*). These three genes, together with other relevant genes up-regulated at the unadjusted $p<0.001$ threshold in the flare vs. healthy analysis, are discussed below.

7.7.2.a Fibrinogen

The expression of both *FGA* (log_2FC 2.42, adjusted $p = 0.003$) and *FGB* (log_2FC 2.00, adjusted p 0.029) were significantly up-regulated in flare patients vs. healthy controls at baseline. Fibrinogen is a large hexameric protein, consisting of two subunits each containing three chains: α, β and γ (Mosesson, 2005). Upon cleavage by thrombin, fibrinogen forms insoluble fibrin – the principle scaffolding substrate of blood clots (Mosesson, 2005). Fibrinogen is mainly synthesised by hepatocytes, and its expression is well-established to increase during times of systemic inflammation as part of the acute-phase response, especially in response to IL-6 (Fish and Neerman-Arbez, 2012). Nevertheless, fibrinogen is also expressed by other cell types. For example, human lung epithelial cells up-regulate expression of γ-fibrinogen in response to exogenous IL-6 *in vitro* (Nguyen and Simpson-Haidaris, 2000). Furthermore, synthesis of β-fibrinogen by circulating $CD4^+$ T cells was observed by Alberio *et al.* (2012), who found lower expression in patients with Parkinson's disease versus healthy controls. However, no reports of T-cell specific expression of fibrinogen in RA have been published.

7.7.2.b α1-microglobulin/bikunin precursor (AMBP)

AMBP was significantly up-regulated in both flare (log_2FC 2.13, unadjusted p 1.21 x 10^{-6}) and DFR (log_2FC 2.05, unadjusted p = 3.31 x 10^{-6}) versus healthy controls, and was robust to multiple test correction in the flare group comparison (adjusted p = 0.016). AMBP is a precursor protein that is cleaved to form two separate secreted proteins: α1-microglobulin and bikunin. Bikunin (also known as ulinastatin) is a Kunitz-type serine protease inhibitor found in blood and urine (Fries and Blom, 2000). Bikunin has been demonstrated to show immunomodulatory properties, such as the inhibition of lipopolysaccharide (LPS)-induced release of TNFα by human PBMCs *in vitro* (Matsuzaki *et al.*, 2004). Bikunin is in current clinical use (outside of Europe and USA) for the treatment of acute pancreatitis and severe sepsis (Moggia *et al.*, 2017). α1-microglobulin (also known as protein HC) is a plasma protein with immunosuppressive actions *in vitro*, including suppression of human PBMC proliferation (Akerstrom and Logdberg, 1984), inhibition of human neutrophil chemotaxis (Mendez *et al.*, 1986), and inhibition of IL-2 production by murine $CD4^+$ T cell hybridomas (Wester *et al.*, 1998). Although AMBP is primarily expressed in hepatocytes, animal studies have demonstrated expression in other tissues including pancreatic lymph nodes (Yip *et al.*, 2009), and renal tubular epithelium (Grewal *et al.*, 2005). Immunohistochemical studies with human tissue have also demonstrated the presence of bikunin in the brain, skin, testes and lungs (Businaro *et al.*, 1992). Circulating AMBP levels have also been demonstrated to discriminate patients with active ulcerative colitis from those with quiescent disease (Wasinger *et al.*, 2016). Given the above observations, it is theoretically possible that up-regulated AMBP expression by $CD4^+$ T cells could play a potentially immunoregulatory role in the context of RA flare, albeit insufficient in flare patients. However, no published studies exist to demonstrate either the expression of AMBP by human $CD4^+$ T cells or the relevance of such cell-specific expression in the context of inflammatory processes.

7.7.2.c Interleukin-15

$CD4^+$ T cell expression of *IL15* was greater in flare patients versus healthy controls (log_2FC 1.25, unadjusted p 1.13 x 10^{-4}). As reviewed by Yang *et al.* (2015), numerous observations implicate IL-15 in the pathogenesis and maintenance of synovitis in RA, notably: SNPs in the *IL15* gene are associated with rate of joint destruction in RA (Knevel *et al.*, 2012); IL-15 levels are elevated in in the sera (Gonzalez-Alvaro *et al.*, 2003) and synovial fluid (McInnes *et al.*, 1996) of RA patients; and circulating IL-15 levels correlate with both RhF/ACPA titres

and clinical disease activity (Pavkova Goldbergova *et al.*, 2012). In mice, exogenous IL-15 can induce a local T cell inflammatory infiltrate (McInnes *et al.*, 1996), whereas blockade of the cytokine can ameliorate synovitis in experimental arthritis (Ferrari-Lacraz *et al.*, 2004) and has been trialled in RA patients (Baslund *et al.*, 2005). IL-15 has several pro-inflammatory effects, including differentiation of Th17 and Th1 cells, stimulation of B cells and NK cells, and promotion of CD8+ memory T cells survival (Brincks and Woodland, 2010; Yang *et al.*, 2015). Nevertheless, IL-15 has also been demonstrated to have immunoregulatory roles in some murine models of inflammatory bowel disease (Tosiek *et al.*, 2016) and experimental autoimmune encephalomyelitis (Yu *et al.*, 2014). IL-15 is expressed by a variety of human cell types including synovial macrophages (Thurkow *et al.*, 1997) and RA synovial fibroblasts (Miranda-Carus *et al.*, 2004), and is produced in an autocrine/juxtacrine manner by both $CD4^+$ and $CD8^+$ T cells (Miranda-Carus *et al.*, 2005). Indeed, it has been observed that both peripheral and synovial T cells from patients with active RA express surface IL-15 and can potentiate osteoclast differentiation in co-culture with autologous monocytes *in vitro* – an effect that was lost once clinical remission was achieved (Miranda-Carus *et al.*, 2006). Given the above observations, it certainly seems plausible that the increased $CD4^+$ T cell expression of IL-15 observed at baseline in flare patients vs. healthy controls may be relevant in the pathogenesis of flare following DMARD cessation. Furthermore, the reported pro-Th17 effects of the cytokine would be in keeping with the increased $CD4^+$ T cell expression of the gene encoding the pro-Th17 transcription factor BATF observed at the time of flare in my study.

7.7.2.d SH3 domain containing RING finger 3 (SH3RF3)

The expression of SH3 domain containing really interesting new gene (RING) finger 3 (*SH3RF3*) was down-regulated in flare patients vs. healthy controls ($\log_2$ FC -1.15, unadjusted $p = 1.13 \times 10^{-4}$). SH3RF3 is a member of the SH3RF family of multi-domain scaffold proteins involved in cell survival and apoptosis (Kim *et al.*, 2014b). The structurally related SH3RF1 is expressed at high levels in RA synovial fibroblasts, where it has an anti-apoptotic effect (Tsuda *et al.*, 2010). Differential exon usage and lower expression of SH3RF has been identified in neutrophils from children with active versus quiescent juvenile idiopathic arthritis (JIA) (Jiang *et al.*, 2015). In contrast, amongst 1168 differentially-methylated genes identified in a genome-wide methylation array study of $CD4^+$ T cells, slight hypomethylation of *SH3RF* was observed in Han Chinese RA patients versus healthy controls

(Δβ -0.007, $p = 1.63 \times 10^{-7}$) (Guo *et al.*, 2017). It is difficult to reconcile the biological significance of these findings with the results of my study.

7.7.2.e Orosomucoid 1 (ORM1)

Orosomucoid 1 (also known as α-1-acid glycoprotein) is an abundant plasma protein secreted by hepatocytes as part of the acute-phase response (Fournier *et al.*, 2000) though is also produced outside of the liver, including by T lymphocytes during activation (Stefanini *et al.*, 1989). ORM1 inhibits lymphocyte proliferation *in vitro* (Pos et al., 1990), and exogenous ORM1 has a protective effect against TNF-α administration in mice (Libert *et al.*, 1994). It has thus been proposed that ORM1 represents a negative-feedback loop to modulate systemic immunity during the acute phase response (Libert *et al.*, 1994). Both circulating (Cylwik *et al.*, 2010) and urinary (Park *et al.*, 2016) ORM1 correlate with disease activity in RA, though it has been suggested based on similar fucosylation patterns that synovial ORM1 likely originates from the circulation rather than the synovium (Havenaar *et al.*, 1997). In my study, $CD4^+$ T cell expression of *ORM1* was higher in flare patients versus healthy controls (log_2 FC 2.72, $p = 1.73 \times 10^{-4}$). This would be in keeping with an activated T cell phenotype described above, though whether ORM1 produced in this setting represents an autocrine negative feedback response or merely increased cellular activity is unclear.

7.7.2.f CD70

CD70 expression was up-regulated in flare patients versus healthy controls (log2 FC 1.70, unadjusted $p = 1.85 \times 10^{-4}$). The binding of surface-expressed CD70 to its ligand, CD27, provides a well-established costimulatory signal important in T and B cell activation (Han *et al.*, 2016a). Expression of CD70 is restricted to activated dendritic cells, T cells and B cells, whereas CD27 is expressed primarily on naïve $CD4^+$ and $CD8^+$ T cells, as well as subsets of NK and B cells (Han *et al.*, 2016a). High levels of surface CD70 expression have been observed in $CD4^+$ T cells from the blood (Park *et al.*, 2014) and synovial fluid (Brugnoni *et al.*, 1997) of RA patients compared to controls. Surface CD70 can identify a subpopulation of $CD4^+$ T cells in RA that express the key Th17 transcription factor RORγt, and produce high levels of IFN-γ and IL-17 after stimulation *in vitro (Park et al., 2014)*. CD70-expressing $CD4^+$ T cells have been implicated in a bystander-effect of lowering the T cell activation threshold in RA (Lee *et al.*, 2007), and blockade of CD27-CD70 interaction with anti-CD70

antibody can ameliorate synovitis in the murine collagen-induced arthritis model (Oflazoglu *et al.*, 2009).

In a study of 54 patients with a range of autoimmune rheumatic diseases (including RA, systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and Sjögren's syndrome (SS)), Strickland *et al.* (2016) used multi-parameter flow cytometry to identify a novel subset of $CD3^{+}CD4^{+}CD28^{+}CD11a^{hi}CD70^{+}KIR2DL4^{+}$ lymphocytes which were more abundant in the blood of patients than healthy controls. Furthermore, the proportion of lymphocytes positive for this combination of markers significantly correlated with disease severity in patients with SLE, RA and SS (Strickland *et al.*, 2016). The observations of this study are of particular relevance given the up-regulation of both *CD70* and the KIR molecule *KIR2DS4* gene expression observed in flare patients in my study, and raise the possibility of a similar $CD4^{+}$ T cell subset at play in the pathogenesis of RA flare. This merits further exploration by flow cytometry using PBMC samples from my study.

7.7.2.g Comparison between contrast groups

Of the abovementioned genes, four were up-regulated in both flare vs. healthy and remission vs. healthy analyses at the unadjusted $p<0.001$ threshold: *AMBP*, *FGA*, *FGB*, and *ORM1*. It is conceivable that the expression of these genes relate to disease-specific processes common to RA patients regardless of their outcome following subsequent DMARD cessation; alternatively, expression of these genes may be a consequence of DMARD cessation itself. However, teasing apart these two possibilities is difficult given the strong link between the proteins encoded by all of these genes and the acute phase response. Although only conjecture, it seems plausible that the expression of these genes may relate to underlying subclinical inflammation, particularly given their established links with the acute-phase response. Indeed, in a longitudinal study of RA patients treated with methotrexate, De Graaf *et al.* (1994) observed a reduction in circulating ORM1 levels, though only in those patients who clinically responded to methotrexate – thus suggesting a disease activity-related fall in ORM1 rather than a direct effect of methotrexate therapy *per se*.

7.7.3 Longitudinal analyses

7.7.3.a Flare patients – flare versus baseline visits

The comparison of $CD4^+$ T cell gene expression between flare and baseline visits revealed two DEGs that were robust to multiple test correction, and 81 DEGs at the unadjusted $p<0.001$ threshold. These DEGs included up-regulation of genes encoding microtubular and centrosomal proteins, together with topoisomerase-IIα, all of which are known to play crucial roles in the cell cycle. The most significantly up-regulated gene by p-value was cell division cycle associated 7 (*CDCA7*), which has been implicated in the growth of lymphoblastoid and solid-organ malignancies (Osthus *et al.*, 2005). Mutations in CDCA7 have also been linked to human immunodeficiency (Thijssen *et al.*, 2015). *MKI67*, which encodes the cell-surface molecule Ki-67, was also up-regulated at the time of flare in these cells. This is a notable observation given the long-established use of this cell-surface molecule as a lymphocyte proliferation marker in immunology studies, especially in flow-cytometry applications (Palutke *et al.*, 1987). Indeed, many of the identified DEGs have well-established roles in cellular proliferation, and paint a picture of a transition from relatively quiescent populations of $CD4^+$ T cells at baseline, to transcriptionally active and proliferating cells at the time of flare. This would be in keeping with evidence to support a proliferative expansion of $CD4^+$ T cells in active RA, including oligoclonal expansion and premature immunosenescence within the $CD4^+$ T cell compartment (see Introduction 1.2.3a).

Several genes not directly involved in the machinery of the cell cycle were also observed to be up-regulated at the time of flare, such as low-density lipoprotein receptor (*LDLR*), thyroid stimulating hormone receptor (*TSHR*), basic leucine zipper ATF-like transcription factor (*BATF*), and *CD109*. Both LDLR and TSHR play important roles in cellular metabolism, and their up-regulation suggests greater metabolic demands of the $CD4^+$ T cells at the time of flare in keeping with a proliferative phenotype. TSHR surface expression has been observed by flow cytometry in a subset of naïve $CD45RB^{hi}$ T cells in mice, and may be important in the modulation of their function (Bagriacik and Klein, 2000). Furthermore, cholesterol metabolism has been implicated in the modulation of T cell function, and may play a role in regulatory versus effector T cell balance. Epidemiological studies have led to the intriguing observation of an apparent inverse relationship between circulating total cholesterol and LDL levels and cardiovascular risk in RA – an effect termed the 'lipid paradox' (Myasoedova *et al.*, 2011). Exogenous cholesterol sulphate has been shown to inhibit T cell receptor (TCR) signalling by murine T cells *in vitro* (Wang et al., 2016), and knock-out mice deficient in apolipoprotein-E – a major constituent of LDL particles – develop exacerbated collagen-

induced arthritis (Postigo *et al.*, 2011; Alvarez *et al.*, 2016). Therefore, the observation of increased LDLR expression by $CD4^+$ T cells at the time of flare may represent a regulatory response that could be perturbed in the patients who experience an arthritis flare. Further studies to confirm an up-regulated surface expression of LDLR, and to examine the effect of this upon lymphocyte function *ex vivo*, would be useful first steps to further investigate this finding.

BATF is a transcription factor that, via binding with interferon regulatory factor 4 (IRF4) and IRF8, plays a key role in Th17 cell differentiation (Glasmacher *et al.*, 2012; Li *et al.*, 2012b). BATF is furthermore involved in the differentiation of follicular helper T (T_{FH}) cells and early effector $CD8^+$ T cells (Murphy *et al.*, 2013). BATF deficient mice have dramatically lower numbers of IL-17 producing Th17 cells, and fail to form germinal centres following antigen immunisation (Betz *et al.*, 2010). Given the above observations, the up-regulation of BATF observed in $CD4^+$ T cells at the time of flare may therefore represent a differentiation of these cells towards a Th17 phenotype. This hypothesis would is further supported by the higher circulating serum levels of the pro-Th17 cytokine IL-6 at the time of flare. These observations merit further study by confirmation of surface expression of Th17 markers by $CD4^+$ T cells at the time of flare.

CD109 is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein found on the surface of activated T cells, platelets and endothelial cells (Lin *et al.*, 2002). CD109 functions as a negative regulator of transforming growth factor-β (TGF-β), itself a predominantly negative regulator of immunity. Blockade by CD109 has been associated with a poor prognostic subset of diffuse large B-cell lymphoma (Yokoyama *et al.*, 2017), and may play a role in reduced TGF-β signalling observed in psoriasis (Litvinov *et al.*, 2011). The longitudinal up-regulation of CD109 observed in my study is thus further potential evidence to support an activated proliferative phenotype of $CD4^+$ T cells at the time of flare.

7.7.3.b DFR patients – month six versus baseline visits

Nineteen DEGs were observed at an unadjusted p threshold of <0.001, with substantially lower mean expression values than the 81 DEGs observed at the same significance threshold in the flare vs. baseline analysis. Furthermore, many of DEGs observed in this remission analysis were pseudogenes of unknown function. Functional analysis suggested downregulation of genes within a network of cell signalling and injury, though with very few edges between the identified nodes. Perplexingly, 3/9 of the protein-coding DEGs were

immunoglobulin genes, which was unexpected in this $CD4^+$ T cell analysis. This may suggest contamination of some samples with B cells, though one would expect this effect to have been at least partly mitigated by the statistical correction for $CD4^+$ T cell purity. Alternatively, this may represent genuine low-level $CD4^+$ T cell expression of immunoglobulin-related genes – indeed, immunoglobulin gene expression has been observed in tissues such as human healthy lung epithelium, human hepatocytes in cirrhosis, and normal mouse neurons (Chen *et al.*, 2009). The functional significance of these observations however remains unclear, and in my study would first require validation of the RNAseq findings with confirmation of immunoglobulin chain expression at the protein level.

Another unexpected observation was the downregulation of *IL10* at month six versus baseline in DFR patients (log_2FC -0.89, unadjusted p 5.14×10^{-4}). This was mirrored by a trend towards higher *IL10* expression at the time of flare vs. baseline in those patients who experienced an arthritis flare (log_2FC 0.68, unadjusted p = 0.004). Given the well-established role of IL-10 as a predominantly immunoregulatory cytokine of crucial importance to the function of Tr1 cells (Pot *et al.*, 2011), it is surprising that its expression by $CD4^+$ T cells would decrease with time in those patients who maintain DFR. Nevertheless, there are two conceivable explanations for this observation. First, IL-10 is not exclusively immunoregulatory, and its role in promoting B cell proliferation is thought to be of importance in the pathogenesis of some autoimmune diseases such as SLE (Peng *et al.*, 2013). Thus reducing IL-10 production by $CD4^+$ T cells could help maintain remission in certain settings. Alternatively, lower expression of IL-10 could represent a gradual shift away from a regulatory Tr1-like phenotype and towards an effector state. However, the absence of any of the DEGs identified in the flare vs. baseline comparison would suggest that if this were occurring, then the $CD4^+$ T cells are unlikely to be following a similar differentiation pathway to that observed in flare patients. Indeed, the functional relevance of this observed reduction in $CD4^+$ T cell IL-10 production with time in the DFR patient population is hard to interpret in the absence of measures of inflammation and gene expression within the synovial compartment.

7.7.4 Predictive biomarker survival analysis

Using a similar approach as previously, univariate followed by multivariate Cox regression was performed using the RNAseq data in order to develop a predictive biomarker of DFR and flare following DMARD cessation. Cox regression was selected as the analysis model of

choice, as this allowed for use of a time-to-event outcome measure, rather than the binary grouping to flare versus DFR described above. This was anticipated to yield greater statistical power – indeed, unadjusted p values were on average 10-fold smaller for the Cox regression analysis compared to the baseline flare vs. DFR comparison. However, standard bioinformatics pipelines for survival analysis using RNAseq data do not currently exist, and adjustment for false-discovery rate using the standard method of Benjamini-Hochberg appeared particularly conservative when applied to the univariate Cox regression results. The issue of apportioning statistical significance in Cox regression analysis when using high dimensionality data has been highlighted in the published literature (Witten and Tibshirani, 2010), and is addressed in detail in the final discussion chapter (see Discussion 9).

Univariate Cox regression identified a similar set of genes associated with time-to-flare at the unadjusted $p<0.001$ threshold as compared to the standard analysis pipeline of DEGs between flare vs. DFR groups, thus providing a degree of internal validation of this approach. After stepwise backward multivariate Cox regression modelling, three genes were identified whose expression significantly associated with time-to-flare at the $p < 0.001$ threshold: ENSG00000228010, ENSG00000162636 and ENSG00000227070. One of these genes (ENSG00000162636) encodes the protein family with sequence similarity 102 member B (FAM102B). Whilst no publications exist concerning the function of FAM102B, the paralogous FAM102A (also known as Early Oestrogen-Induced Gene 1) is known to be involved in oestrogen signalling (Wang *et al.*, 2004) as well as osteoclast differentiation (Choi *et al.*, 2013), and is implicated in cell membrane trafficking (Zhang and Aravind, 2010). Both ENSG00000228010 and ENSG00000227070 are predicted to be novel antisense genes, though no published data relates to their putative targets or physiological function. In a composite score, these three genes predicted flare and sustained DFR following DMARD cessation, with an ROC_{AUC} of 0.90.

There are limitations to the statistical techniques used to generate this 3-gene predictive score, most notably over-fitting of the data and the lack of a validation cohort. These limitations are common to all of the biomarker analyses of this Thesis, and are discussed in detail later (see Discussion 9). A further limitation is the lack of functional annotation of the three identified genes, this making it impossible to assess the face validity of the score based on knowledge of physiological function. Nevertheless, it must be highlighted that the aim of this analysis was to generate a composite score for use as a predictive biomarker, rather than to inform an understanding of underlying biology. Indeed, it is entirely conceivable gene transcription in the late stages of a functional pathway – whose expression represents an integrative and

potentially amplified function of an array of previous signalling events - may have greater utility as a biomarker compared to the initial mediators in the pathway. This analysis should therefore be viewed as complementary to, rather than a replacement for, the previous analyses based on binary contrast groups, the latter of which are more suited to extrapolation to underlying biological function as discussed above.

Only one other study exists in the published literature that utilises $CD4^+$ T cell gene expression to predict DFR in RA. In this study, Teitsma *et al.* (2017) explored gene expression data from fluorescence-activated cell sorting (FACS)-isolated $CD4^+$ T cells from a subset of 60 patients of the U-Act-Early study. In this study, DMARD-naïve patients (n = 317) with active early RA (DAS28-ESR > 2.6) were randomised to receive tocilizumab plus placebo, methotrexate plus placebo, or tocilizumab plus methotrexate in a double-blind design (Bijlsma *et al.*, 2016). If sustained remission was achieved – defined as DAS28-ESR < 2.6 and ≤ 4 swollen joints for ≥ 24 weeks (though 2.6 <DAS28-ESR<3.2 on ≤2 visits during this period was allowed) – then DMARDs were gradually tapered to complete cessation. Sustained DFR was defined as a DAS28-ESR < 2.6 maintained for at least 3 months and until the end of the study 2-year follow-up period (though 2.6<DAS28-ESR<3.2 at a single visit was permitted) (Bijlsma *et al.*, 2016). In an exploratory analysis, $CD4^+$ T cell gene expression by RNAseq at baseline (i.e. before initiation of DMARD treatment) was compared between in 60 patients who successfully achieved sustained DFR versus those who did not (tocilizumab only: 13 DFR and 11 non-DFR; methotrexate only: 10 DFR and 7 non-DFR; tocilizumab plus methotrexate: 14 DFR, 5 non-DFR) (Teitsma *et al.*, 2017). Instead of analysing individual DEGs or utilising Cox regression survival analysis, the authors of this study used a network analysis approach to identify significantly different functional pathway modules between the study arms. The most significant module in each arm identified pathways involved in leukocyte migration and G-protein signalling (tocilizumab only arm), response to bacteria or biotic stimuli (methotrexate only arm), and transcription and translation (methotrexate and tocilizumab arm) (Teitsma *et al.*, 2017). In contrast, RNAseq analysis $CD14^+$ monocytes did not reveal any relevant significant networks. The individual differentially co-expressed genes in the top $CD4^+$ T cell module of each study arm was made publically available by the authors, though the fold-change and statistical significance for each individual gene was not disclosed. Comparison of these with an extended list of DEGs identified in my study (unadjusted $p<0.01$ across all analyses) revealed only a few common genes: *ORM1*, *IL12*, haptoglobin (*HP*) and calpain 8 (*CAPN8*) (Table 7.14). Intriguingly, all of the common genes identified fell within the methotrexate-only arm analysis of the Teitsma *et al.* (2017) study.

Table 7.14 – Differentially expressed genes common to the $CD4^+$ T cell RNAseq analysis of both my study and the study by Teitsma et al. (2017). HC: healthy control.

Gene	Observation in my study	Observation by Teitsma *et al.* (2017)
ORM1	Up-regulated in flare vs. HC (log_2FC 2.72, $p = 1.73x\ 10^{-4}$) Up-regulated in DFR vs. HC (log_2FC 2.43, $p = 8.12 \times 10^{-4}$)	Up-regulated in DFR vs. non-DFR in methotrexate only arm
IL12A	Up-regulated at flare visit vs. baseline in flare patients (log_2FC 0.69, $p = 5.88 \times 10^{-4}$)	Up-regulated in DFR vs. non-DFR in methotrexate only arm
HP	Up-regulated in flare vs. HC (log_2FC 2.28, $p = 2.80 \times 10^{-3}$)	Up-regulated in DFR vs. non-DFR in methotrexate only arm
CAPN8	Up-regulated in flare vs. HC (log_2FC 1.32, $p = 2.66 \times 10^{-3}$)	Up-regulated in DFR vs. non-DFR in methotrexate only arm

Of the proteins encoded by these genes, the functional relevance of ORM1 and IL12 have been discussed previously in this Thesis (see Results 7.7.2.e and Results 6.5.4). Haptoglobin is an abundant circulating protein that functions to bind free haemoglobin. As an acute phase protein, haptoglobin is produced by hepatocytes during episodes of systemic inflammation and has been observed to mediate pro-inflammatory effects in murine skin (Shen *et al.*, 2012) and cardiac (Shen *et al.*, 2015) transplant models. However, as discussed by Huntoon *et al.* (2013), haptoglobin knock-out mice have been observed to exhibit both suppressed (Huntoon *et al.*, 2008) and enhanced (Arredouani *et al.*, 2005) immunity in different studies. Haptoglobin is also expressed by non-hepatic tissues, including human neutrophils (Theilgaard-Monch *et al.*, 2006). Furthermore, bone marrow chimera experiments in mice have demonstrated that haptoglobin expression by B cells is important for a functional immune response (Huntoon *et al.*, 2013). The upregulation of haptoglobin expression in flare versus healthy control in my study may thus reflect an immunostimulatory role of T-cell specific haptoglobin production.

Calpains are a family of evolutionarily conserved calcium-dependent serine proteases, and are known as 'modulator proteases' given their predilection for limited proteolytic modification rather than destruction of their target proteins (Ono *et al.*, 2016). Calpains have been implicated in a broad range of human diseases, and several calpain inhibitors are currently in clinical development (Ono *et al.*, 2016). In human T cells, treatment with the calpain inhibitor E64D *in vitro* has been demonstrated to block IκBα degradation, itself an inhibitor of the pro-inflammatory transcription factor nuclear factor κB (NFκB) (Ponnappan *et al.*, 2005).

Although there are no published reports relating specifically to the expression of CAPN8 by lymphocytes, given the observations above it is theoretically plausible that the up-regulated expression of CAPN8 observed in flare patients vs. healthy controls in my study may be relevant to the susceptibility to flare following DMARD cessation.

Direct comparison of the results of my study with those of Teitsma *et al.* (2017) is however problematic owing to the timing of sampling. Whereas $CD4^+$ T cell samples in my study were collected from patients with established RA on DMARDs in clinical remission, Teitsma *et al.* (2017) collected samples from early DMARD-naïve patients with active RA. It is thus difficult to be certain whether upregulation of gene expression at this early time point is directly linked to subsequent achievement of DFR, or rather is a biomarker of response to DMARD therapy which, in turn, may facilitate a remission status that is permissive for the development of other processes relevant in the subsequent maintenance of DFR following DMARD cessation.

7.7.5 Limitations

Only a few DEGs were robust to multiple test correction. This is likely the result of a combination of both the relatively small size of the sample population, and a small magnitude of differential gene expression between the comparison groups. This latter possibility is further exemplified by the generally low mean expression levels of many of the DEGs. Nevertheless, despite not being robust to multiple test correction, many of these DEGs encode for proteins with biological plausibility for involvement in pro-inflammatory and immune dysregulation processes. It is important however to weigh this against the effect of cell-specific gene expression analysis in this study – indeed, it is perhaps unsurprising that many of the genes identified in this RNAseq analysis of $CD4^+$ T cells have functional roles in $CD4^+$ T cell biology.

A further limitation relevant to the longitudinal analyses is the possible effect of DMARD cessation itself upon $CD4^+$ T cell gene expression, which may not necessarily be mechanistically linked to the pathogenesis of RA flare. This is further compounded by a general lack of understanding of the mechanism of action of most current DMARDs, thus making it hard to account for these effects in downstream analyses.

A more general limitation of RNAseq analysis is the difficulty in ascribing a direct link between gene expression, protein translation and, ultimately, protein function at the cellular and anatomical site of disease. Although whole-genome differential gene expression analysis

provides unparalleled insights in to the transcriptional activity of cell populations, a wide variety of transcriptional regulatory mechanisms mean that mRNA abundance does not necessarily directly reflect protein translation. Add to this the effects of post-translational protein modification, and the complexities of intracellular trafficking combined with cellular migration and dynamic cell-cell interactions, and it becomes clear that transcriptional profiling lies several critical steps upstream of an actual functional response in the diseased tissue. Therefore, whilst observations of RNAseq analysis are undeniably illuminative and ideally suited to a hypothesis-free exploratory approach as adopted in this study, validation of findings at the protein and cellular level at the site of disease is nevertheless mandatory.

7.8 Summary

The most illuminative results from the analysis of $CD4^+$ T cell gene expression have come from the longitudinal comparison of flare visit with baseline. This analysis revealed a strong signature of upregulation of genes involved in cellular proliferation, as well as the pro-Th17 transcription factor BATF. These results provide evidence of activation of $CD4^+$ T cells at the time of arthritis flare, and are in keeping with a phenotype of systemic inflammation in keeping with clinical measures of increased disease activity, and the observed increase in levels of acute-phase and pro-inflammatory cytokines such as IL-6 (see Results 6.5.4). Furthermore, comparison of baseline $CD4^+$ T cell gene expression in flare patients versus healthy controls demonstrated upregulation of several genes encoding proteins known to be correlated with disease activity in RA, such as IL-15 and ORM1. This may suggest greater subclinical levels of inflammation in these patients at baseline, thus predisposing to a greater risk of arthritis flare upon DMARD cessation.

In comparison, relatively little longitudinal change was seen in $CD4^+$ T cell gene expression within patients who remained in DFR, or at baseline between DFR patients versus healthy controls. This is in keeping with the longitudinal cytokine data discussed previously (see Results 6.5.4), which also showed very little longitudinal change within the DFR group. These observations suggest a stable phenotype of remission in these patients, which is characterised by an absence of the inflammatory signature seen in flare patients rather than the presence of a pro-tolerogenic signature that actively maintains DFR. The one possible exception to this is the up-regulated expression of the gene encoding the immunoregulatory protein AMBP in DFR patients versus healthy controls – nevertheless, the same observation

was also seen in flare patients vs. healthy controls, thus making the functional relevance of this observation unclear.

In the primary analysis of flare vs DFR patients at baseline, the comparative dearth of convincing DEGs likely reflects the above-mentioned limitations of the analysis, including small sample size, unpaired rather than paired statistical comparisons, and a smaller magnitude of differential gene expression in comparison to the flare event vs. baseline analysis. Despite these limitations, baseline expression of only three genes performed well in discriminating flare vs. DFR when re-applied to the same test cohort. The predictive performance of these genes in combination with clinical and cytokine/chemokine parameters will be addressed in the next chapter.

Chapter 8. Results 5 – Integrative Analysis

8.1 Introduction

In the previous results chapters of this Thesis, I have presented findings from complementary yet distinct work streams encompassing clinical, ultrasound, cytokine and gene expression data. For each domain, I have used the same approach of systematic variable reduction followed by variable combination to form composite scores, and assessment of their predictive utility in predicting arthritis flare following DMARD cessation. For the purposes of lucidity, analyses have thus far been separated according to the methodology of variable generation. However, this separation is somewhat artificial in real-life clinical settings, when a variety of variables from different domains are available for assessment.

In this final results chapter, I aim to simultaneously analyse results from all variable domains in order to synthesise a global predictive biomarker score to predict RA flare and sustained drug-free remission following DMARD cessation. The motivations for this integrative analysis are two-fold. First, by combining variables from different domains I aim to create a global biomarker score that outperforms any of the single-domain composite scores. Second, the process of variable combination can be expected to lead to variable redundancy, thus allowing for the final variable set to be smaller than the sum of the individual domain variable sets.

In performing this integrative analysis, I use the same process familiar from previous chapters starting with a two-step variable reduction incorporating univariate followed by stepwise backward multivariate Cox regression modelling. Receiver-operating characteristic (ROC) curve analysis is then used to further refine the variable set and select an optimum model for use as a predictive biomarker of flare and sustained remission following DMARD cessation. This process is performed for all variable domains, and then repeated with exclusion of gene expression data – the latter step in recognition of the difficulties inherent to translation of cell-specific gene expression to clinical practice.

The structure of the remainder of this results chapter is as follows:

8.2 Integrative analysis 1: including gene expression data

8.3 Integrative analysis 2: excluding gene expression data

8.4 Sensitivity analyses

8.5 Discussion

8.6 Summary

8.2 Integrative analysis 1: including gene expression data

8.2.1 Variable selection

Before starting the process of integrative analysis, a reduced variable set was defined for exploration. The number of variables was reduced not only to avoid unnecessary and laborious downstream model reduction, but also to minimise over-fitting of the expansive data set to the relatively small study population.

Baseline variables were selected based upon their statistical significance in the domain-specific backward stepwise multivariate Cox regression models described in the previous results chapters of this Thesis. Only those variables which were associated with time-to-flare in their respective multivariate models at an unadjusted significance threshold of $p<0.05$ (or $p<0.001$ in the case of gene expression data) were selected for integrative analysis. Thresholds were not set for the Cox regression coefficients, as these were expected to change with the merging of variable domains in the integrative analyses. No ultrasound variables were included in the integrative analysis, as none were significantly associated with time-to-flare in univariate Cox regression analysis.

In total, 11 variables were selected for integrative analysis as detailed in Table 8.1.

As discussed previously (see Results 6.2.1), cytokine data was unavailable for one patient at baseline. This patient was thus excluded, leaving 43/44 patients for the main integrative analysis. A separate sensitivity analysis was subsequently performed with imputation of the missing data for this patient (see Results 8.4.1).

Table 8.1 – The thirteen baseline variables selected for integrative analysis.

Domain	Variable
Clinical	RhF positive
	ACPA positive
	ACR/EULAR Boolean remission
	Months since last change in DMARDs
	Current methotrexate
Cytokine	ln(MCP1+1)
	ln(IL27+1)
	ln(CRP+1)
$CD4^+$ T cell gene expression	ENSG00000228010
	ENSG00000162636
	ENSG00000227070

8.2.2 Cox regression

The association between baseline variables and time-to-flare following DMARD cessation was analysed by univariate Cox regression for the 43 patients where complete data was available (Table 8.2). No significant deviation from proportionality of hazards was observed for any of the univariate variables. Given the univariate nature of this analysis, the coefficients and statistical significance of these variables mirror those already detailed in their respective results chapters – minor discrepancies reflect the exclusion of the single patient without cytokine data.

Table 8.2 – Association of baseline variables with time-to-flare following DMARD-cessation, as analysed by univariate Cox regression. B: Cox regression coefficient.

Variable	B	HR_{flare}	HR_{flare} 95% CI	Unadjusted p value
ENSG00000228010	-1.49	0.23	0.10 – 0.50	0.0003
ENSG00000227070	0.75	2.13	1.40 – 3.24	0.0004
ENSG00000162636	2.47	11.8	2.33 – 60.2	0.0029
ln(MCP1+1)	2.21	9.13	1.97 – 42.3	0.0047
ln(CRP+1)	0.43	1.53	1.02 – 2.31	0.0421
Months since last DMARD change	-0.02	0.98	0.97 – 1.00	0.0471
ACPA positive	0.82	2.27	0.96 – 5.37	0.0622
RhF positive	0.77	2.15	0.91 – 5.11	0.0824
ln(IL27+1)	0.95	2.58	0.78 – 8.53	0.1203
ACR/EULAR remission	-0.65	0.52	0.23 – 1.19	0.1223
Current methotrexate	1.46	4.31	0.58 – 32.1	0.1535

All 11 baseline variables were then entered in to a multivariate Cox regression model. Stepwise backward selection based on Akaike information criterion (AIC) was then performed to fit a stepwise Cox regression model (see Methods 3.8.1). After four selection steps, 7 variables remained in this stepwise model (Table 8.3).

Proportionality of hazards was again assessed for each variable in the final stepwise multivariate Cox regression model. A significant departure from proportional hazards was observed only for current methotrexate use ($p = 0.006$), though as noted before in Chapter 6, this was only notable for a single outlier with no discernible trend in the remainder of the data (Figure 8.1). The global Schoenfeld test was non-significant ($p = 0.087$), indicating proportionality of hazards for the model as a whole.

Table 8.3 - Association of baseline variables with time-to-flare following DMARD-cessation in a backward stepwise multivariate Cox regression model.

Variable	B	HR_{flare}	HR_{flare} 95% CI	Unadjusted p value
ENSG00000227070	1.14	3.12	1.81 – 5.36	0.00004
ENSG00000228010	-1.98	0.14	0.05 – 0.36	0.00005
ENSG00000162636	2.82	16.72	2.24 – 125	0.00608
ln(IL27+1)	1.92	6.85	1.61 – 29.1	0.00915
ACR/EULAR Boolean remission	-1.22	0.29	0.11 – 0.76	0.01205
RhF positive	0.94	2.56	0.91 – 7.19	0.07470
Current methotrexate	1.66	5.28	0.55 – 50.2	0.14779

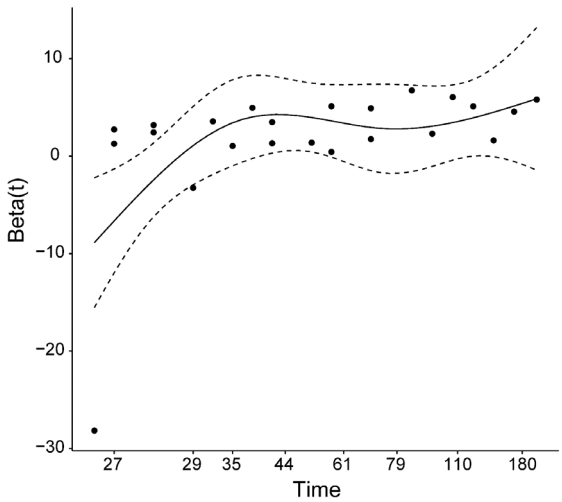


Figure 8.1 – Correlation of scaled Schoenfeld residuals (Beta(t)) against Kaplan-Meier-transformed flare-free survival time for current methotrexate in the stepwise multivariate Cox regression model. Dashed lines indicate ± 2 standard errors of the smoothed spline fit with 4 degrees of freedom (solid line). Discounting the single outlier, there does not appear to be any observable correlation between the scaled residuals and survival time.

8.2.3 Receiver-operating characteristic (ROC) analysis

Five variables were significantly associated with time-to-flare at an unadjusted significance threshold of <0.05 in the multivariate stepwise Cox regression model: ENSG00000227070, ENSG00000228010, ENSG00000162636, ln(IL27+1) and baseline ACR/EULAR Boolean remission. Values of these five variables were multiplied by their respective stepwise multivariate Cox regression coefficient and then summed to create composite scores. The predictive performance of all 31 potential combinations of these variables to predict flare and remission following DMARD cessation was then compared by area under the receiver-operating characteristic curve (ROC_{AUC}). The ten composite scores with the highest ROC_{AUC} are shown in Table 8.4 (for a full listing of all composite scores, see Appendix J).

The composite score with the highest ROC_{AUC} included all variables; notably, the removal of ln(IL27+1) resulted in only a small drop in ROC_{AUC} (Formulae 8.1 – 8.2 and Figure 8.2).

Formula 8.1 – Five-variable composite score

Composite score = 1.14(ENSG00000227070) + 2.82 (ENSG00000162636) +
1.92(ln[IL27+1])
- 1.98(ENSG00000228010) - 1.22(ACR/EULAR Boolean remission)

Formula 8.2 – Four-variable composite score

Composite score = 1.14(ENSG00000227070) + 2.82 (ENSG00000162636)
- 1.98(ENSG00000228010) - 1.22(ACR/EULAR Boolean remission)

Table 8.4 – The top ten integrative composite scores ranked by ROC_{AUC}. Variables included within each score are indicated in green, and those excluded are indicated in red.

ACR/EULAR Boolean remission	ENSG00000162636	ENSG00000227070	ENSG00000228010	ln(IL27+1)	ROC_{AUC}
✓	✓	✓	✓	✓	0.963
✓	✓	✓	✓	✗	0.954
✓	✗	✓	✓	✗	0.927
✓	✗	✓	✓	✓	0.920
✓	✓	✗	✓	✓	0.918
✗	✓	✓	✓	✓	0.908
✗	✓	✓	✓	✗	0.908
✓	✓	✗	✓	✗	0.902
✗	✗	✓	✓	✗	0.874
✓	✓	✓	✗	✓	0.867

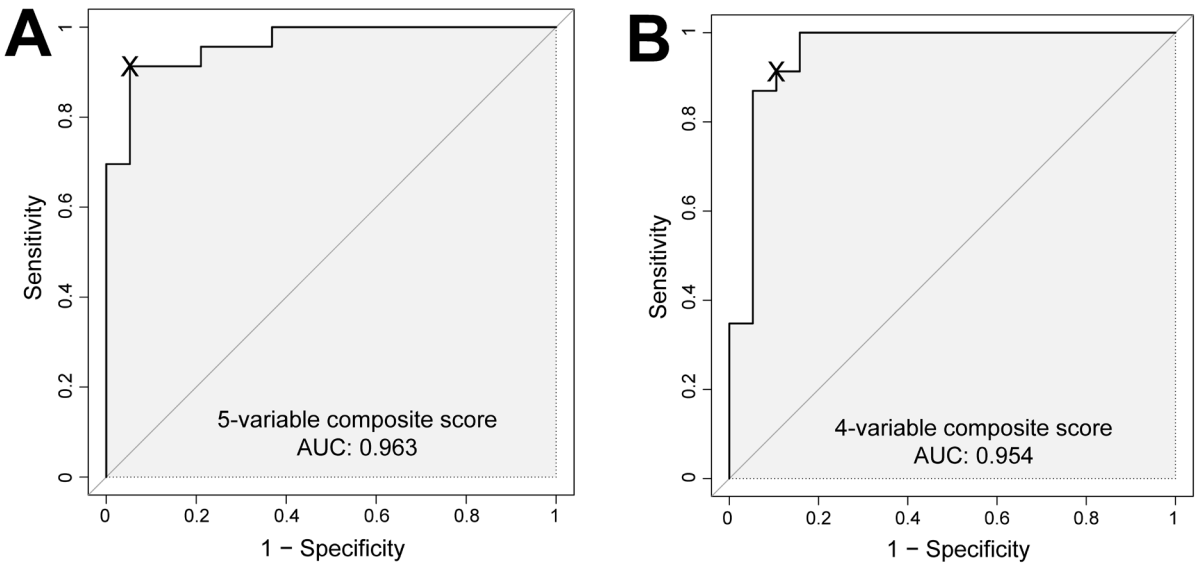


Figure 8.2 – ROC curves for 5-variable (A) and 4-variable (B – dropping ln(IL27+1)) composite scores. Threshold values used for assessment of predictive performance are highlighted by crosses.

8.2.4 Composite score predictive performance

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value of each of the three composite scores detailed above are presented in Table 8.5 and Figure 8.3, using the same threshold values as in the main analysis. A single optimum threshold for each composite score was selected manually based on minimising the ROC coordinate distance from the top left corner of the ROC curve plot.

Table 8.5 – Utility of the two composite scores in predicting arthritis flare following DMARD cessation. PPV: positive predictive value; NPV: negative predictive value.

Composite score	Threshold value	ROC_{AUC} (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
5-variable	37.41	**0.96** (0.92 – 1.00)	**0.91** (0.78 – 1.00)	**0.95** (0.84 – 1.00)	**0.96** (0.87 – 1.00)	**0.90** (0.78 – 1.00)
4-variable	23.16	**0.95** (0.88 – 1.00)	**0.91** (0.78 – 1.00)	**0.89** (0.74 – 1.00)	**0.92** (0.81 – 1.00)	**0.89** (0.77 – 1.00)

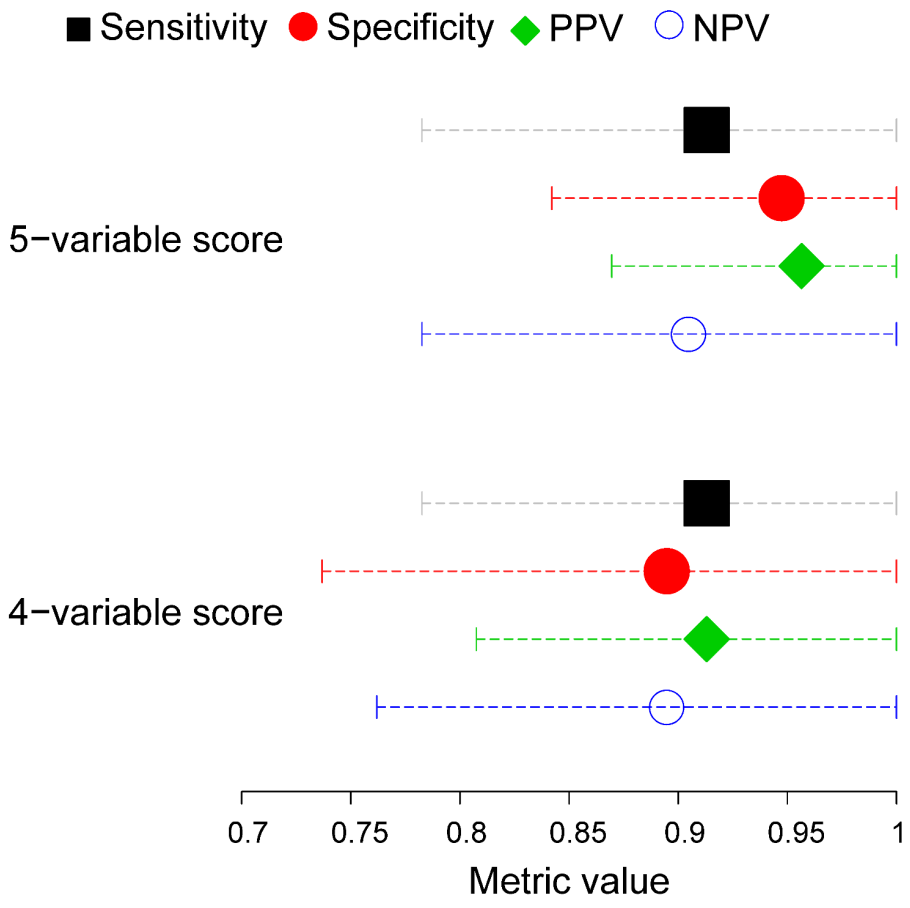


Figure 8.3 – Predictive performance metrics of the two composite scores.

The confidence interval of each metric was relatively wide for both scores, reflecting the small sample size of the cohort. There was a slight trend towards a greater specificity and PPV in the 5-variable score compared to the 4-variable score, with slightly narrower confidence intervals for these two metrics in the 5-variable score. Nevertheless, the differences were modest, suggesting only a minor contribution of ln(IL27+1) to the predictive performance of the model.

8.3 Integrative analysis 2: excluding gene expression data

8.3.1 Variable selection

A substantial limitation to the clinical utility of a predictive score that relies upon $CD4^+$ T cell gene expression is the difficulty faced in translating such a time- and resource-consuming laboratory technique to routine clinical practice. A second set of integrative analyses was therefore performed with the exclusion of gene expression data, using the eight variables listed in Table 8.6. As previously, one patient with missing baseline cytokine data was excluded, leaving 43/44 patients available for the main no-gene analysis.

8.3.2 Cox regression

All eight variables were simultaneously entered in a multivariate Cox regression model followed by backward selection based on AIC to fit a stepwise Cox regression model. After one selection step, seven variables remained in this stepwise model (Table 8.7).

Proportionality of hazards was assessed for each variable in the final stepwise multivariate Cox regression model. A significant departure from proportional hazards was observed only for current methotrexate use ($p = 0.033$) – again this was only notable for a single outlier (Figure 8.4). The global Schoenfeld test was non-significant ($p = 0.138$), indicating proportionality of hazards for the model as a whole.

Table 8.6 – The eight variables included in the no-gene integrative analysis.

Domain	Variable
Clinical	RhF positive
	ACPA positive
	ACR/EULAR Boolean remission
	Months since last change in DMARDs
	Current methotrexate
Cytokine	ln(MCP1+1)
	ln(IL27+1)
	ln(CRP+1)

Table 8.7 - Association of baseline variables with time-to-flare following DMARD-cessation in the no-gene backward stepwise multivariate Cox regression model.

Variable	B	HR_{flare}	HR_{flare} 95% CI	Unadjusted p value
Months since last DMARD change	-0.03	0.97	0.95 – 0.99	0.002
ACR/EULAR Boolean remission	-1.32	0.27	0.11 – 0.66	0.004
ACPA positive	1.27	3.57	1.34 – 9.52	0.011
ln(IL27+1)	1.39	4.03	1.17 – 14.0	0.028
RhF positive	1.14	3.14	1.01 – 9.73	0.047
ln(MCP1+1)	1.62	5.08	1.01 – 25.6	0.049
Current methotrexate	2.13	8.44	0.96 – 74.4	0.055

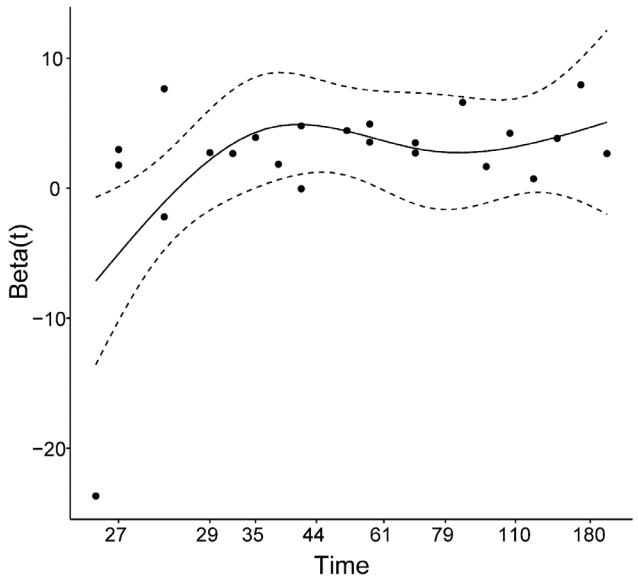


Figure 8.4 – Correlation of scaled Schoenfeld residuals (Beta(t)) against Kaplan-Meier-transformed flare-free survival time for current methotrexate in the no-gene stepwise multivariate Cox regression model. Dashed lines indicate ± 2 standard errors of the smoothed spline fit (solid line). Discounting the single outlier, there does not appear to be any observable correlation between the scaled residuals and survival time.

8.3.3 Receiver-operating characteristic (ROC) analysis

All seven variables from the backwards multivariate Cox regression model were advanced to the composite score fitting stage. Composite scores were calculated by summing the products of variables with their respective regression coefficient for all 127 possible combinations of the seven variables. The composite scores were then ranked in order of ROC_{AUC} – the full ranking is presented in Appendix J, and the ten scores with the highest ROC_{AUC} are listed in Table 8.8.

Exclusion of ln(IL27+1) to create a 6-variable composite score yielded the highest ROC_{AUC}, which was essentially equal to that of the full 7-variable score. Notably, the third highest composite score excluded both cytokine variables to leave the five clinical variables that formed the clinical composite score presented in Chapter 4, albeit with different coefficients to previously. The formulae for these three composite scores are presented in Formulae 8.3 – 8.5.

Formula 8.3 – Seven-variable no-gene composite score

Composite score = 1.39(ln[IL27+1]) + 1.62(ln[MCP1+1]) + 1.14(RhF positive)
+ 1.27(ACPA positive) +2.13(Current methotrexate)
– 1.32(ACR/EULAR Boolean remission)
– 0.03(months since last DMARD change)

Formula 8.4 – Six-variable no-gene composite score

Composite score = 1.62(ln[MCP1+1]) + 1.14(RhF positive)
+ 1.27(ACPA positive) +2.13(Current methotrexate)
– 1.32(ACR/EULAR Boolean remission)
– 0.03(months since last DMARD change)

Formula 8.5 – Five-variable no-gene composite score

Composite score = 1.14(RhF positive) + 1.27(ACPA positive) +2.13(Current methotrexate)
– 1.32(ACR/EULAR Boolean remission)
– 0.03(months since last DMARD change)

Table 8.8 - The top ten composite scores in the no-gene analysis ranked by ROC_{AUC}. Variables included within each score are indicated in green, and those excluded are indicated in red.

ACPA positive	ACR/EULAR Boolean remission	Current methotrexate	Months since last change in DMARDs	ln(IL27+1)	ln(MCP1+1)	RhF positive	ROC_{AUC}
✓	✓	✓	✓	✗	✓	✓	0.957
✓	✓	✓	✓	✓	✓	✓	0.954
✓	✓	✓	✓	✗	✗	✓	0.936
✓	✓	✓	✓	✗	✓	✗	0.931
✗	✓	✓	✓	✓	✓	✓	0.929
✓	✓	✓	✓	✓	✓	✗	0.924
✓	✓	✓	✓	✓	✗	✓	0.920
✗	✓	✓	✓	✓	✗	✓	0.911
✗	✓	✓	✓	✗	✓	✓	0.911
✓	✓	✓	✓	✓	✗	✗	0.904

8.3.4 Composite score predictive performance

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value of each of the three no-gene composite scores detailed above are presented in Table 8.9, Figure 8.5 and Figure 8.6.

Table 8.9 – Utility of the no-gene analysis composite scores in predicting arthritis flare following DMARD cessation. PPV: positive predictive value; NPV: negative predictive value.

Composite score	Threshold value	ROC_{AUC} (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
7-variable	20.28	**0.95** (0.90 – 1.00)	**0.96** (0.87 – 1.00)	**0.84** (0.68 – 1.00)	**0.88** (0.77 – 1.00)	**0.94** (0.83 – 1.00)
6-variable	9.83	**0.96** (0.90 – 1.00)	**0.96** (0.87 – 1.00)	**0.84** (0.68 – 1.00)	**0.88** (0.78 – 1.00)	**0.94** (0.83 – 1.00)
5-variable	0.701	**0.94** (0.87 – 1.00)	**0.91** (0.78 – 1.00)	**0.79** (0.58 – 0.95)	**0.85** (0.72 – 0.96)	**0.89** (0.75 – 1.00)

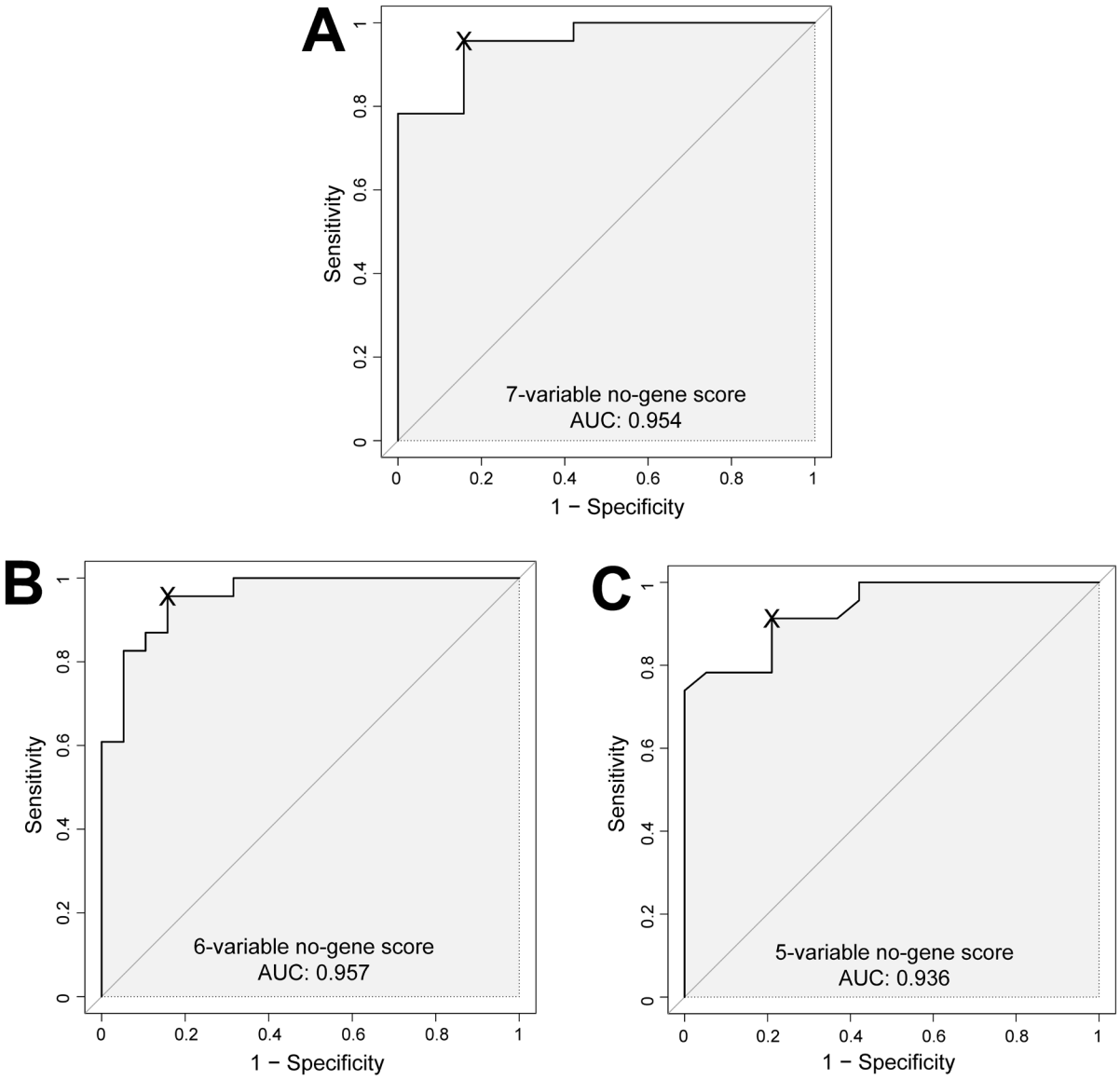


Figure 8.5 – ROC curves for 7-variable (A), 6-variable (B – dropping ln(IL27+1)) and 5-variable (C – dropping ln(IL27+1) and ln(MCP1+1)) no-gene composite scores. Threshold values used for assessment of predictive performance are highlighted by crosses.

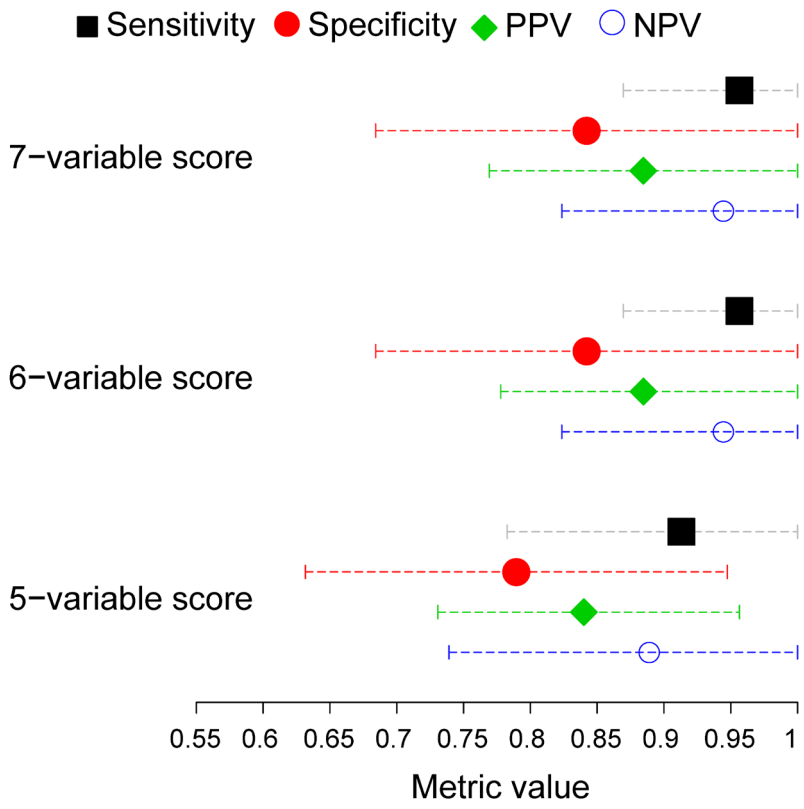


Figure 8.6 – Predictive performance metrics of the three composite scores in the no-gene analysis.

The performance of the 6-variable composite score is virtually indistinguishable from that of the full 7-variable score. The 5-variable score showed lower point estimates for all predictive metrics, though the small magnitude of the differences was nonetheless impressive given its reliance entirely on clinical parameters.

8.4 Sensitivity analyses

8.4.1 Imputation of missing cytokine data

In the above analyses, one patient was excluded owing to lack of baseline cytokine data. In a sensitivity analysis, missing cytokine data was imputed for this patient using the study population median for each cytokine variable. The above analyses were then repeated with the inclusion of gene expression data to ascertain whether the additional clinical and gene expression data gained by inclusion of this patient had any effect upon the results.

The sensitivity analysis used the same five-variable composite score (Formula 8.1), coefficients and threshold values as in the main analysis (Results 8.2). The predictive performance of the composite scores after imputation was indeed very similar to that of the main analysis (Table 8.10)

Table 8.10 – Utility of the composite scores in the cytokine imputation sensitivity analysis for predicting arthritis flare following DMARD cessation. PPV: positive predictive value; NPV: negative predictive value.

Composite score	Threshold value	ROC_{AUC} (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
5-variable no imputation	37.41	**0.96** (0.92 – 1.00)	**0.91** (0.78 – 1.00)	**0.95** (0.84 – 1.00)	**0.96** (0.87 – 1.00)	**0.90** (0.78 – 1.00)
5-variable with imputation	37.41	**0.96** (0.90 – 1.00)	**0.91** (0.78 – 1.00)	**0.90** (0.75 – 1.00)	**0.91** (0.81 – 1.00)	**0.90** (0.78 – 1.00)

8.4.2 Substitution of high-sensitivity CRP (hsCRP) values

The local clinical laboratory assay used for measuring serum CRP concentration has a lower limit of detection (LLOD) of 5mg/L. As specified in the study protocol, CRP values below this LLOD were treated as zero for the purposes of DAS28-CRP calculation. However, this raises the possibility of under-estimation of disease activity, with the possibility of false-classification of low-grade arthritis flare as remission. To address this concern, another sensitivity analysis was performed whereby the CRP concentration was substituted in borderline cases for that measured by the high-sensitivity CRP (hsCRP) electrochemiluminescence assay included on the MSD plates.

Of the 184 study visits, only 7 visits (involving 4 patients) were identified where hsCRP measurement had potential to result in a rise of DAS28-CRP from below to above the remission threshold of 2.4. Substituting hsCRP values for these visits resulted in no change to remission status in 3/7 visits, and a reclassification from remission to flare in 4/7 visits (3 patients). Two patients – who both maintained drug-free remission to six months in the main analysis – were reclassified as DAS28-CRP>2.4 at the baseline visit, and were thus excluded from this sensitivity analysis. The remaining patient – who was classified as arthritis flare at 176 days after DMARD cessation – was reclassified from remission to flare at day 78 in the sensitivity analysis.

Following exclusion/reclassification of these three patients as described above, the original 5 variable composite score (Formula 8.1) was applied with the additional exclusion of the patient with missing baseline cytokine/chemokine data as previously described (Results 8.2). A minor adjustment to the threshold value was necessary to maximise the predictive utility of the sensitivity analysis model. The predictive performance of the composite score after hsCRP substitution was very similar to that of the main analysis (Table 8.10). This suggests that any effect of underestimation of disease activity as a result of the LLOD of the clinical CRP assay had negligible effect upon the predictive utility of the composite biomarker score.

Table 8.11 – Utility of the composite scores in the hsCRP substitution sensitivity analysis for predicting arthritis flare following DMARD cessation. PPV: positive predictive value; NPV: negative predictive value.

Composite score	Threshold value	ROC_{AUC} (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
5-variable main analysis	37.410	**0.96** (0.92 – 1.00)	**0.91** (0.78 – 1.00)	**0.95** (0.84 – 1.00)	**0.96** (0.87 – 1.00)	**0.90** (0.78 – 1.00)
5-variable hsCRP substitution	37.406	**0.96** (0.91 – 1.00)	**0.91** (0.78 – 1.00)	**0.94** (0.82 – 1.00)	**0.96** (0.87 – 1.00)	**0.89** (0.76 – 1.00)

8.5 Discussion

In this integrative analysis, variables from the composite biomarker scores of clinical, cytokine and RNAseq domains were combined together in a multivariate Cox regression model, with backwards stepwise selection used to create a final model with five variables: three gene expression (ENSG00000228010, ENSG00000162636 and ENSG00000227070), one cytokine (IL-27), and one clinical (ACR/EULAR Boolean remission). Whereas little is known regarding the role of the three gene variables, the relevance of IL-27 and ACR/EULAR Boolean remission in the context of DFR has been extensively discussed (see Results 6.5.3 and Results 4.9.4d).

When re-applied to the study population, this 5-variable composite score demonstrated a high predictive utility for outcome following DMARD cessation: ROC_{AUC} 0.96 (95% CI 0.92 – 1.00), sensitivity 0.91 (0.78 – 1.00), specificity 0.95 (0.84 – 1.00), PPV 0.96 (0.87 – 1.00), NPV 0.90 (0.78 – 1.00). Thus, in this study population, patients with a negative test score had a 90% chance of remaining in DFR at the end of the six-month follow-up period, versus only 4% for those with a positive score. Such a score would undoubtedly be of great utility in

helping guide DMARD withdrawal in the clinic, and would represent a quantum leap beyond the approximately even chance of flare that would otherwise be predicted in the absence of a predictive biomarker based on outcome of the entire non-stratified study population. However, potential over-fitting of data as a consequence of dimension reduction (see Discussion 9), together with wide confidence intervals around the point estimates of predictive metrics, mandates the validation of this composite score in an independent patient population to assess its true predictive performance in the clinic. It is also important to note that PPV and NPV are both influenced by the prevalence of flare within the test population. Therefore, different rates of flare in a subsequent validation cohort may substantially influence the PPV and NPV of the composite score.

A pragmatic limitation of this composite score is its reliance on gene expression data, and the inherent difficulties this creates in future translation to clinical practice. The isolation of $CD4^+$ T cells, RNA extraction, and subsequent transcriptional analysis represents a laborious package of laboratory work that requires time, technical expertise and an array of laboratory equipment. Even if only mRNA relating to the three genes of interest were measured using focussed techniques such as reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), there would still be several processing steps required from whole blood to $CD4^+$ T cell-specific gene expression measurement. It would thus be challenging to incorporate these steps within a fully automated system, which would be the preferable form of delivery of the assay if used in clinical laboratory settings.

Despite this limitation, recent technological advances enable novel approaches to the measurement of expression of a limited number of genes within a specific cellular subset, which may be more amenable to future clinical translation. Of particular note is the recently-marketed PrimeFlow™ RNA assay (Affymetrix eBisocience Ltd.), which uses intracellular in situ-hybridisation of fluorescent probes to target genes in a system which is compatible with existing flow-cytometry equipment (Affymetrix eBioscience, 2017). Using this technique, it is possible to measure the intracellular abundance of up to four RNA targets in combination with cell-surface protein markers, thus allowing the measurement of cell-specific gene expression without the need for cell subset isolation or RNA extraction. Such an approach would be more suited to large-scale throughput and automation in clinical applications.

It is particularly striking to note that a 6-variable composite score devoid of any gene expression data performs similarly to composite score including gene-expression data. This raises the possibility of a predictive biomarker based only on clinical autoantibody and cytokine variables, thus circumventing the technical difficulties in measuring cell-specific

gene expression. It is prudent however to note some cautionary aspects of this no-gene model which may have bearing upon its performance in subsequent validation studies. First, the no-gene composite score relies heavily upon the inclusion of current methotrexate, with the greatest coefficient of any of the variables in the model. This is potentially problematic given the limitations of this variable and potential for over-fitting given cofounding factors, as discussed previously (see Results 4.9.4.c). Furthermore, the reliance on a greater number of variables in the no-gene score (6) versus the composite score with gene expression (5) further increases the risk of over-fitting of the data. However, even if the no-gene composite score demonstrates suboptimal performance in validation studies, it may nevertheless still be of use in settings where laboratory resources are limited. Indeed, it is such resource-limited healthcare systems where the economic benefits of DMARD cessation would have arguably the greatest impact.

Visual summaries of the three key composite scores are presented in Figures 8.7 and 8.8. Nevertheless, direct comparison of the scores is complicated by the wide confidence intervals around each metric, a reflection of the small size of the study population.

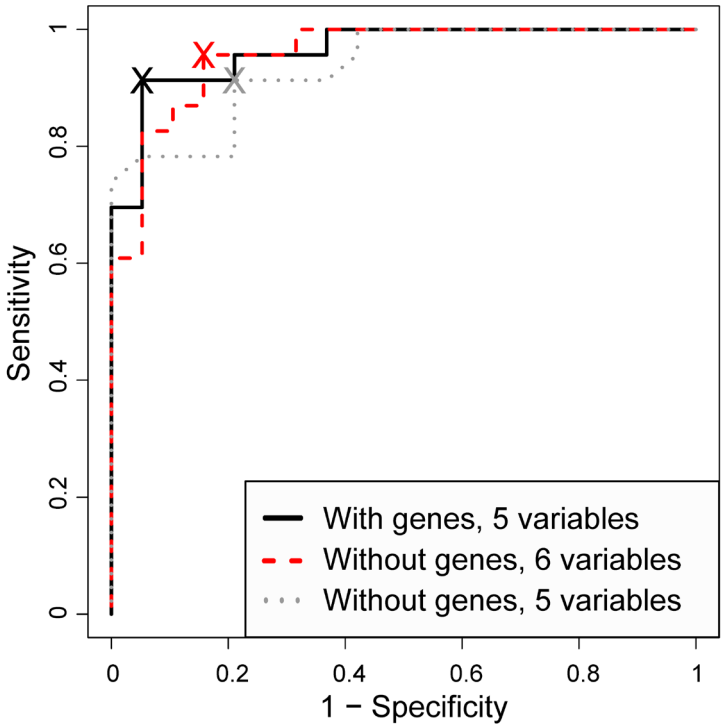


Figure 8.7 – Receiver operating characteristic curves for the three composite scores

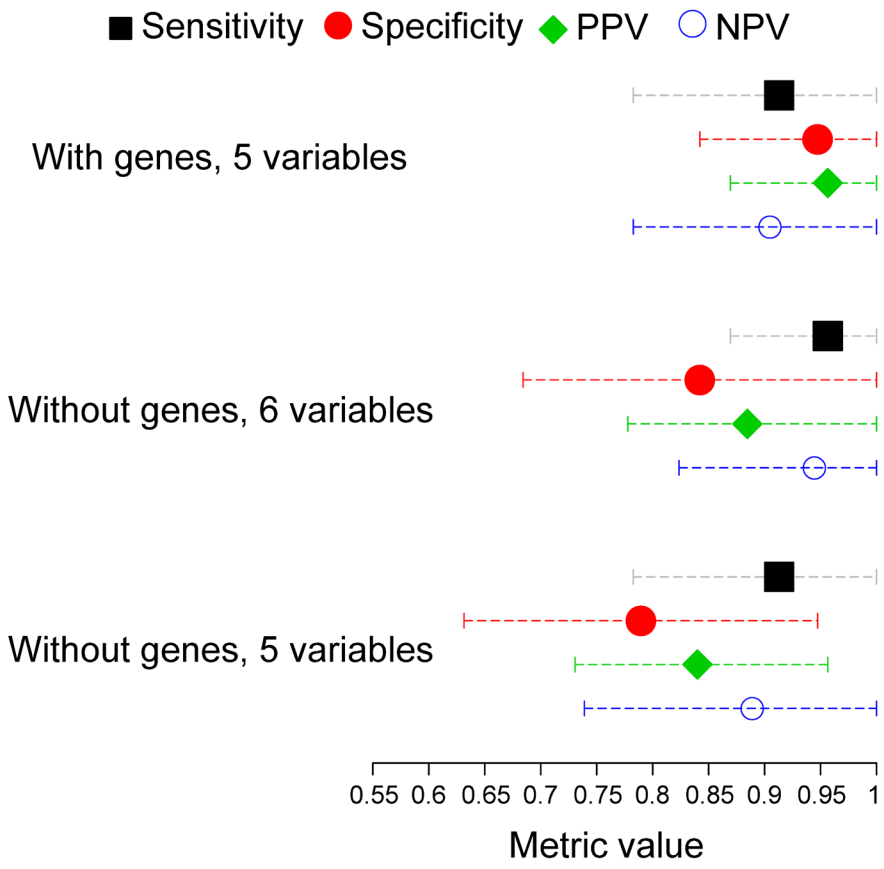


Figure 8.8 - Predictive performance metrics of the three composite scores.

8.6 Summary

In this final results chapter, I have presented an integrative analysis encompassing variables from three complementary domains including clinical, circulating cytokine and $CD4^+$ T cell gene expression data. I have described a systematic approach to variable reduction, followed by combination of variables to form composite scores. Optimum combinations of variables were then identified based on their utility in predicting flare versus drug-free remission following DMARD cessation. This process was performed both with and without gene expression data. Finally, to account for missing cytokine data in one patient and a high LLOD for the clinical CRP assay, sensitivity analyses were performed with median imputation of the missing cytokine data and substitution with hsCRP data respectively. The results of these sensitivity analyses were corroborative of the main analyses.

There are undoubtedly limitations to this analysis, most notably a lack of statistical power and over-fitting of data, which I will address in detail in the next chapter. These results clearly

require further study and validation in an independent cohort before generalisation to wider patient populations can be inferred. Nonetheless, these observations provide a tantalising first insight in to the use of such biomarker composite scores in identifying patients who may benefit from DMARD withdrawal in the setting of RA remission.

Chapter 9. General Discussion

9.1 Study evaluation

There are several notable strengths of this study. First, this study addresses a question of key clinical, economic, scientific, and patient-centred importance – i.e. when is it appropriate to stop DMARD therapy in RA patients in remission? DMARD therapy, whilst effective, carries risks of potentially severe side effects and requires inconvenient and expensive regular blood monitoring. The observation that just under half of the patients in this study were able to maintain DFR for six months after DMARD cessation is further evidence to support a strategy of step-down therapy in these patients, with consequent minimisation of drug-related adverse events and healthcare costs. The insights that this study provides to the immunological events underlying the emergence of RA flare are novel and, if validated, may also be relevant to the study of other immune-mediated inflammatory diseases that follow a relapsing-remitting course. Furthermore, many RA patients would value the opportunity to decrease and stop their DMARD therapy. This study has provided an opportunity for 44 patients to attempt this with close monitoring and clinical support, and provides an evidence base upon which to extend this approach to a larger validation cohort and, ultimately, the wider patient population.

There are several limitations to this study, some of which are specific to each individual domain and have been addressed in detail in the previous sections. However, some limitations are generic and are of relevance to all domains of this study, and are addressed below.

9.1.1 High dimensionality of data

A challenge throughout this study has been the high dimensionality of data relative to the size of the study population. With the inclusion of whole genome RNAseq data, the number of variables is at least 3 log-fold greater than the number of patients in the study. This presents not only a pragmatic difficulty in reducing the number of variables to a manageable number in a logical and robust manner, but also raises substantial problems with over-fitting of data in the predictive models.

The handling of high-dimensional data is not unique to this study, and is commonly encountered in bioinformatics studies of gene expression. Established data pipelines and workflows have been developed to address this issue, and several open-source computer

packages exist to facilitate this, such as those used in the binary group analyses of RNAseq data in this study. Nevertheless, integrative analyses across multiple variable domains brings additional challenges, such as normalisation across different outcome measures and ascribing hierarchical importance to different variable domains. These challenges are difficult to tackle with an 'off-the-shelf' solution, and although a few computer packages have been developed to address these issues a supervised approach is still required to adequately address biological, technical and statistical issues specific to the individual research project (Kristensen *et al.*, 2014).

It was desirable to use Cox regression survival analysis in this study, as such an approach is ideally suited to the outcome event and study design. Rather than defining outcome as flare or remission after a duration of follow-up largely dictated by the available study resources, survival analysis allows for discrimination of patients based on time-to-flare. This more nuanced approach not only yields greater statistical power, but also is arguably more suited to the underlying biological processes as variables that are associated with a shorter time-to-flare are, in effect, given greater weighting in the final analysis. Nevertheless, survival analysis using high-dimensional data is no less challenging than alternative analytical approaches.

In a comprehensive review of the topic, Witten and Tibshirani (2010) identify four main approaches to variable selection when dealing with high-dimensional data in survival analyses: discrete feature selection, shrinkage, clustering and variance-based methods (Table 9.1). The approach I have used in this study is based upon discrete feature selection, whereby variables are first selected based upon their statistical significance in univariate Cox regression, followed by stepwise fitting of a multivariate Cox regression model. This approach has the advantages of relative conceptual simplicity, and allows variables across different domains to be analysed simultaneously for their effect on survival time. However, this approach is also prone to over-fitting and can result in models dominated by closely-correlated variables (Witten and Tibshirani, 2010). This was partly addressed in my analysis by the adoption of a smaller multivariate significance threshold in the gene expression analysis (uncorrected $p < 0.001$ versus <0.05). Nonetheless, the final composite score is dominated by gene expression variables, whereas an alternative no-gene score performs with almost equal predictive value (albeit with one more variable). The true test of predictive utility lies in validation of the biomarker score in an independent patient cohort regardless of the analytical approach used. Indeed, this study has been conducted with a candid exploratory ethos throughout, aiming to identify potential biomarkers of DFR and flare as hypotheses for further research, rather than present the results as a final definitive *fait accomplit*.

Table 9.1 – Approaches to variable selection in survival analysis of high-dimensionality data. Adapted from Witten and Tibshirani (2010). Lasso: least absolute shrinkage and selection operator)

Approach	Typical statistical methods
Discrete feature selection	Univariate threshold selection Stepwise selection
Shrinkage (penalisation) of coefficients	Ridge regression Lasso
Clustering	Hierarchical clustering
Variance-based methods	Principal components analysis Partial least squares analysis

9.1.2 Size and heterogeneity of the patient cohort

A limitation throughout this study has been the small size of the study cohort. Owing to under-recruitment only 44 patients discontinued DMARDs, which was below the target of 60 patients at the design stage. Removal of the requirement for absence of baseline power Doppler signal would have allowed this target to be achieved, and in retrospect perhaps would have been a better study design. However, the decision to exclude PD-positive patients from DMARD withdrawal was made on logical grounds (see Results 5.6.1), and if more patients had discontinued DMARDs then less laboratory processing would have been possible given the limited study budget. For example, longitudinal RNAseq analysis – arguably the most informative aspect of this study with regards to underlying biology – would not have been possible.

There was considerable heterogeneity within the study population across a range of parameters, including disease duration, baseline DMARD therapy and relative stability of remission at baseline (the latter indicated by the surrogate measures of time since last steroid and time since last change in DMARD therapy). It is possible that such variation between patients led to greater variance in the measured variables, and thus served to reduce the statistical power of the analysis. This was unavoidable, and any further restrictions in the study eligibility criteria would resulted in lower study recruitment. In fact, the study population is broadly representative of a standard rheumatology outpatient population, and thus represents a ‘real-world’ study whose findings are more likely to be generalizable to the wider patient population than a study with highly selective recruitment. One exception to this is for younger patients under the age of 50 years, who are relatively under-represented in this

study cohort. Validation of the findings of this study in a younger cohort would therefore be important to determine whether they hold true in this age range.

9.1.3 Immunopathological subpopulations

One area of particular potential weakness in my statistical analysis is seen in the context of immunopathological subpopulations. As discussed above, there was substantial heterogeneity in demographic factors within the study population. It is thus conceivable that distinct subsets exist within the study population, underscored by heterogeneous immunopathological pathways of inflammation in those patients who experience an arthritis flare. Such subgroups of patients may be equally at risk of arthritis flare upon DMARD cessation, though the immunological measures of this may substantially differ between subgroups. Indeed, such subgrouping is perhaps suggested by the observation of a small group of patients who flare at the end of the study period (as opposed to the median of 48 days). If such subgrouping by immunopathology of flare does indeed exist, then this will have had the effect of diluting the magnitude of any observed differences between flare and DFR my analyses, which treat all flare events as equal.

An alternative analytic approach would therefore be to use hierarchical clustering to group patients based on similarities in their predictive variables, and then explore the outcomes observed in each group. If subpopulations exist within the dataset, and if members of these populations were sufficiently similar with regards to their measured variables, then this approach could help identify distinct immunopathological pathways to arthritis flare – particularly if then extended to longitudinal analysis at the individual patient level. However, such an approach would risk being statistically underpowered in this small study, and any results would potentially be of less utility as a biomarker for clinical use, where a universal marker incorporating variables from all major subpopulations would arguably be preferable. Nevertheless, cluster analysis – especially utilising the high-dimensional $CD4^+$ T cell gene expression data – would be a logical extension to the analyses presented in this Thesis.

9.1.4 Remission definition

The use of DAS28-CRP < 2.4 as the clinical remission criterion for this study could be criticised as being too lenient in comparison to alternative measures such as ACR/EULAR Boolean remission. Indeed, the latter criterion was used as the initial definition of remission

when the study was conceived, though was quickly amended to DAS28-CRP < 2.4 when it became apparent that the strict $VAS_{patient}$ < 10/100 threshold was difficult to achieve in practice, an observation made by many independent research groups (see Methods 3.2). The change to DAS28-CRP remission early in the course of the study resulted in the re-classification of only one patient, who exited the study in DAS28-CRP remission before the amendment was enacted. This patient was treated as censored in remission for the purposes of survival analysis, and excluded from analyses using binary outcome measures.

A further problem arose with the change to DAS28-CRP remission criteria in the handling of CRP levels less than 5mg/L, the lower limit of detection of the assay used by the local hospital clinical biochemistry service. To deal with this, a pragmatic decision was taken to use a value of zero for all CRP measurements below this detectable threshold, though with a risk of underestimating disease activity in some patients. However, only 7/184 study visits were potentially affected, and a sensitivity analysis using hsCRP values measured by MSD electrochemiluminescence demonstrated no apparent influence upon the predictive performance of the final composite biomarker score. However, it is important to note that the MSD hsCRP assay is manufactured for diagnostic use only, and was not performed in this study in line with the necessary quality assurance or accreditation required for a diagnostic clinical test. Future studies would benefit from use of a clinical-grade hsCRP assay, which would then be suitable for use in disease activity assessment as part of the primary study outcome measure.

9.1.5 Frequency of follow-up visits

The schedule of visits at 0, 1, 3, and 6 months following DMARD cessation was based on an assumption that the majority of flare events would occur between three and six months of follow-up. The fact that flare events occurred much earlier than anticipated resulted in fewer longitudinal samples available for patients who experienced an arthritis flare. This was further compounded by the lack of collection of research bloods at the month one visit in the original study protocol, which was subsequently amended. The net result was therefore a reduced number of samples available for longitudinal analysis within the flare group, thus prohibiting a meaningful analysis using more powerful longitudinal statistical techniques, such as smoothing splines and multilevel hierarchical modelling. However, the limited study budget prevented the addition of further visits to the study schedule, and an analysis of change in variables from baseline to final study visit was still possible using the existing dataset.

9.1.6 Anatomical compartment

Both cytokine and $CD4^+$ T cell gene expression data were obtained from peripheral blood samples. This represents a convenient and minimally invasive method of biological sampling, which could be easily translated to routine clinical practice. However, it is difficult to directly infer the pathophysiological processes occurring at the disease site by the study of circulating cytokines and lymphocytes. The upregulation of genes by circulating $CD4^+$ T cells does not necessarily mean the same expression profile is present in synovial T cells, nor is the profile of circulating cytokines and chemokines likely to precisely reflect the milieu within the joint capsule. It is even conceivable that, in some situations, a circulating immune parameter may inversely reflect processes within the joint. For example, when a pathological subset of $CD4^+$ T cell is recruited to the synovial compartment, measures of circulating lymphocyte populations may only reveal a reduced proportion of that subset in the circulation. This is clearly a rather extreme scenario, and in most cases it should be possible to draw some conclusions as to the immune processes within the joint by the study of circulating lymphocytes and immune mediators. However, a deeper understanding of the pathophysiological processes in arthritis flare would undoubtedly be gained by examination of synovial fluid and tissue, though this was not feasible within the limited resources of this study.

Perhaps an even more fundamental limitation lies in the choice of cell type for RNA sequencing. It was hoped that by restricting gene expression analysis solely to $CD4^+$ T cells, an increase in signal-to-noise ratio would be achieved, thus increasing sensitivity for the detection of more subtle changes in gene expression. Although the decision to focus on $CD4^+$ T cells was a rational decision based on evidence of the importance of these cells in the pathogenesis of RA (see Introduction 1.2.3), highly relevant changes in gene expression in alternative cell types may have been overlooked. These include – though are not limited to – B cells, $CD8^+$ T cells, NK cells, and antigen-presenting cells such as synovial macrophages and dendritic cells. Furthermore, the use of bulk RNAseq techniques permits only a global view of gene expression, rather than expression at the individual cell level. Single cell RNAseq technology is now becoming more widely available, and in combination with fluorescence-activated cell sorting (FACS) isolation of cells based on surface protein expression would permit the identification of distinct populations of immune cells that may be relevant in RA pathogenesis, which would otherwise be impossible to distinguish using a bulk RNAseq approach.

9.2 Placing this study in context: pathophysiological insights and clinical impact

This Thesis represents the culmination of an ambitious three-year programme of research that aimed to identify previously uncharacterised biomarkers of drug-free remission and flare in RA. To achieve this, a novel experimental model of DMARD cessation was utilised in the setting of a controlled clinical trial design to study the immunological processes both before and after withdrawal of immunosuppression. Data were collected from a range of different domains including clinical, ultrasound, cytokine and $CD4^+$ T cell gene expression, and combined together to create an integrative overview of immune biology. Furthermore, the study design allowed not only for the identification of baseline biomarkers predictive of outcome following DMARD withdrawal, but also provided a unique insight in to the longitudinal changes that occur at the point of flare at an individual patient level.

The key findings of this study that relate to the underlying pathobiology of disease flare in RA are:

1. Circulating $CD4^+$ T cells display an activated phenotype at the time of arthritis flare, characterised by up-regulation of genes involved in the cell cycle.
2. Th17 differentiation may be an important event in the pathogenesis of RA flare, as evidenced by increased circulating IL-6, and the expression of genes encoding IL-15 and the Th17 transcription factor BATF by circulating $CD4^+$ T cells.
3. Gene expression analysis suggests that distinct subpopulations of circulating $CD4^+$ T cells may be discernible by surface marker expression at the time of flare, notably: CD70, CD109, KIR2DS4, LDLR and TSHR.
4. Patients who maintain DFR appear to be characterised by absence of the pro-inflammatory signals that predispose to disease relapse in the flare group, rather than the presence of active pro-tolerogenic mechanisms that maintain remission.

In addition to the above observations, this study also demonstrates for the first time the feasibility of a multi-domain composite biomarker for predicting DFR and flare following DMARD cessation in RA. If successfully validated in an independent cohort, such a composite biomarker holds promise in guiding an individualised approach to withdrawal of DMARD therapy once remission is achieved. This would not only avoid medication side effects and reduce healthcare costs, but would also be greatly valued by patients living with

RA. Furthermore, the combination of a remission biomarker together with a biomarker of DMARD response opens possibilities of new future paradigms for the treatment of RA. For example, a biomarker predictive of response to a particular biologic therapy, combined with a biomarker of remission, would permit the rapid initiation of biologics as a first line therapy in patients with severe RA, followed by a rapid de-escalation of therapy once stable remission is achieved. Such an approach would permit, in suitable patients, the use of biologic agents as short-term therapies to induce remission early in the course of disease, rather than the current paradigm of long-term treatment used at later disease stages.

9.3 Future directions

As discussed previously in this chapter, there are several avenues for future research based on both the existing data and stored biological samples of this study. Further alternative analyses of the $CD4^+$ T cell RNAseq data are possible, which have potential to shed further light on the functional significance of the findings to-date. For example, unsupervised clustering analysis may be illuminative of specific subpopulations of patients with distinct immunopathological pathways to flare. *In silico* analysis of predicted targets for the two uncharacterised antisense genes (ENSG00000228010 and ENSG00000227070) within the final composite score may shed light on their biological role, which may in turn help to provide further internal functional validation of the predictive model.

Using flow cytometry, it should be possible to explore whether up-regulation of genes that encode surface expressed markers is reflected by increased protein expression at the cell surface. By combining these markers with standard phenotypical markers, and contrasting the expression of these at baseline and flare visit, it may be possible to characterise novel lymphocyte subsets that expand and become activated at the time of arthritis flare. If such subsets exist, then FACS sorting combined with single-cell RNAseq technology would allow for the transcriptional characterisation of these cells, thus providing further insight to the immune processes underlying RA flare with an unprecedented level of detail. With careful experimental design, all of this should be technically possible using existing cryopreserved PBMC samples from this study.

Cryopreserved $CD4^+$ T cell DNA also provides the opportunity for epigenetic analysis of circulating CD4+ T cells both as a baseline predictor of flare versus DFR, and to better understand the mechanisms underlying differential gene expression at the time of arthritis

flare. Epigenetic modification affecting the expression of DEGs identified in the $CD4^+$ T cell RNAseq analysis would also provide further internal validation of these observations within the study cohort.

The most important next step for future research is to validate the findings of this study in a larger independent study population. Such validation would allow for an assessment of over-fitting in the composite biomarker score and, if successful, would provide confidence in the predictive performance of the score necessary for translation of the technique to clinical practice. Indeed, such a validation exercise is already underway in the form of the future Bio-FLARE study (Biological Factors that Limit sustAined Remission in rhEumatoid arthritis), a £3.5 million collaborative multi-centre clinical study funded by the Medical Research Council and conducted by Newcastle, Birmingham and Glasgow Universities as constituent partners of the Arthritis Research UK Centre of Excellence in RA Pathogenesis. The Bio-FLARE study, supported by pilot data from my study, will adopt a similar experimental model of DMARD cessation though in a much larger cohort of 160 patients. A more intensive visit schedule in the initial phases of the study will be possible, and synovial biopsies will be taken at baseline and time of flare to provide crucial insight to the pathophysiological events occurring within the joint synovium. The Bio-FLARE study therefore provides an ideal validation cohort, as well as allowing for a far more detailed analysis of the immunological events that presage RA flare than was possible with the limited resources available for my study. If successfully validated, future research efforts will need to focus on the translation of the predictive biomarker to clinical practice. Consideration will need to be given to the technical feasibility of the assays involved, and their amenability to high-throughput automation. Indeed, factors such as cost and assay reliability can be expected to play a significant role in the decision of which variables to include in a clinical test beyond simple predictive performance alone.

In summary, a wide array of future research is possible based on the findings of this study, which have already directly led to a large multi-centre follow-on study. If successful, this comprehensive programme of future research promises to yield exciting and novel insights in to the pathogenesis of RA flare, the maintenance of RA remission, and future possibility of individualised tapering and withdrawal of DMARD therapy for RA patients in remission.

Appendix A. Health Assessment Questionnaire Disability Index (HAQ-DI)

The HAQ-DI questionnaire completed by patients in this study is reproduced below. The questionnaire is protected under copyright by Stanford University, although it is made freely available in the public domain for research (Bruce and Fries, 2003). For discussion, see Methods 3.5.1.

HEALTH ASSESSMENT QUESTIONNAIRE (HAQ-DI)©

Name: _____ Date: _____

Please place an "x" in the box which best describes your abilities OVER THE PAST WEEK:

	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
DRESSING & GROOMING				
Are you able to:				
Dress yourself, including shoelaces and buttons?	☐	☐	☐	☐
Shampoo your hair?	☐	☐	☐	☐
ARISING				
Are you able to:				
Stand up from a straight chair?	☐	☐	☐	☐
Get in and out of bed?	☐	☐	☐	☐
EATING				
Are you able to:				
Cut your own meat?	☐	☐	☐	☐
Lift a full cup or glass to your mouth?	☐	☐	☐	☐
Open a new milk carton?	☐	☐	☐	☐
WALKING				
Are you able to:				
Walk outdoors on flat ground?	☐	☐	☐	☐
Climb up five steps?	☐	☐	☐	☐

Please check any AIDS OR DEVICES that you usually use for any of the above activities:

☐ Devices used for Dressing (button hook, zipper pull, etc.)
☐ Built up or special utensils
☐ Crutches
☐ Cane
☐ Wheelchair
☐ Special or built up chair
☐ Walker

Please check any categories for which you usually need HELP FROM ANOTHER PERSON:

☐ Dressing and grooming ☐ Arising ☐ Eating ☐ Walking

- 1 -

Appendix A (continued)

Please place an "x" in the box which best describes your abilities OVER THE PAST WEEK:

	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
HYGIENE				
Are you able to:				
Wash and dry your body?	☐	☐	☐	☐
Take a tub bath?	☐	☐	☐	☐
Get on and off the toilet?	☐	☐	☐	☐
REACH				
Are you able to:				
Reach and get down a 5 pound object (such as a bag of sugar) from above your head?	☐	☐	☐	☐
Bend down to pick up clothing from the floor?	☐	☐	☐	☐
GRIP				
Are you able to:				
Open car doors?	☐	☐	☐	☐
Open previously opened jars?	☐	☐	☐	☐
Turn faucets on and off?	☐	☐	☐	☐
ACTIVITIES				
Are you able to:				
Run errands and shop?	☐	☐	☐	☐
Get in and out of a car?	☐	☐	☐	☐
Do chores such as vacuuming or yard work?	☐	☐	☐	☐

Please check any AIDS OR DEVICES that you usually use for any of the above activities:

☐ Raised toilet seat ☐ Bathtub bar ☐ Long-handled appliances for reach

☐ Bathtub seat ☐ Long-handled appliances in bathroom ☐ Jar opener (for jars previously opened)

Please check any categories for which you usually need HELP FROM ANOTHER PERSON:

☐ Hygiene ☐ Reach ☐ Gripping and opening things ☐ Errands and chores

Your ACTIVITIES: To what extent are you able to carry out your everyday physical activities such as walking, climbing stairs, carrying groceries, or moving a chair?

COMPLETELY	MOSTLY	MODERATELY	A LITTLE	NOT AT ALL
☐	☐	☐	☐	☐

Your PAIN: How much pain have you had IN THE PAST WEEK?
On a scale of 0 to 100 (where zero represents "no pain" and 100 represents "severe pain"), please record the number below.

☐☐☐

Your HEALTH: Please rate how well you are doing on a scale of 0 to 100 (0 represents "very well" and 100 represents "very poor" health), please record the number below.

☐☐☐

Appendix B. Ultrasound scoring template

The ultrasound scoring template used in this study is reproduced below. For discussion, see Methods 3.6.

Joint	No. of images	Synovitis		Paratenonitis / Tenosynovitis		Erosions
		Greyscale	Power	Greyscale	Power	
Wrist (dorsomedian)		0 1 2 3	0 1 2 3	0 1	0 1 2 3	
Wrist (palmomedian)		0 1 2 3	0 1 2 3	0 1	0 1 2 3	
Wrist (ulnar)		0 1 2 3	0 1 2 3	0 1	0 1 2 3	
MCPJ2 dorsal			0 1 2 3	0 1	0 1 2 3	0 1
MCPJ2 palmar		0 1 2 3	0 1 2 3	0 1	0 1 2 3	0 1
MCPJ2 radial						0 1
MCPJ3 dorsal			0 1 2 3	0 1	0 1 2 3	0 1
MCPJ3 palmar		0 1 2 3	0 1 2 3	0 1	0 1 2 3	0 1
PIPJ2 dorsal			0 1 2 3			0 1
PIPJ2 palmar		0 1 2 3	0 1 2 3			0 1
PIPJ3 dorsal			0 1 2 3			0 1
PIPJ3 palmar		0 1 2 3	0 1 2 3			0 1
MTPJ2 dorsal		0 1 2 3	0 1 2 3			0 1
MTPJ2 plantar						0 1
MTPJ5 dorsal		0 1 2 3	0 1 2 3			0 1
MTPJ5 plantar						0 1
MTPJ5 lateral						0 1
TOTAL						

Appendix C. List of laboratory reagents and equipment

The laboratory reagents and equipment used in this project together with manufacturer and catalogue number details are listed below. For discussion, see Chapter 3.

$CD4^+$ T cell isolation		
RoboSep™ automated cell separator	Stemcell	20000 (discontinued)
Rosettesep™ human monocyte depletion cocktail	Stemcell	15668
HetaSep™	Stemcell	07906
RoboSep™ Human Whole Blood CD4 Positive Selection Kit	Stemcell	18082RF
RoboSep™ buffer	Stemcell	20104
RoboSep™ filter tips	Stemcell	20125
Falcon™ 14ml round bottom polystyrene tubes	Becton Dickinson Biosciences	352057
$CD4^+$ T cell lysis and RNA/DNA extraction		
RNase AWAY™	Molecular BioProducts	7003
Buffer RLT Plus	Qiagen	1053393
β-mercaptoethanol	Sigma-Aldrich	M3148
QIAshredder spin column	Qiagen	79656
Allprep™ DNA/RNA/miRNA Universal Kit	Qiagen	80224
NanoDrop™ ND1000 UV spectrophotometer	Thermo Fisher Scientific	1660
Next-generation RNA sequencing		
Tapestation™ 4200	Agilent	G2991AA
ScreenTape™ tube strips		
TruSeq™ Stranded mRNA HT Sample Prep Kit	Illumina	RS-122-2103
Agencourt™ AMPure™ XP Beads	Beckman Coulter Genomics	A63881
NextSeq™ 500	Illumina	SY-415-1001
NextSeq™ 500 High-Output Kit	Illumina	FC-404-1005
PBMC isolation		
Lymphoprep™	Axis-Shield	1114544
EASYstrainer™ 70µm nylon filter	Breiner Bio-One	542070
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich	D2650

Flow cytometry		
FACSCanto™ II flow cytometer	Becton Dickinson Biosciences	338962
Fluorescein isothiocyanate (FITC) Mouse Anti-Human CD4 v4 antibody (clone L120)	Becton Dickinson Biosciences	340422
R-phycoerythrin (PE) Mouse Anti-Human CD14 antibody (clone M5E2)	Becton Dickinson Biosciences	555398
Allophycocyanin (APC) Mouse Anti-Human CD19 antibody (clone HIB19)	Becton Dickinson Biosciences	555415
Pacific Blue™ (PB) Mouse Anti-Human CD3 antibody (clone UCHT1)	Becton Dickinson Biosciences	558117
Dulbecco's phosphate buffered saline (DPBS), calcium- and magnesium-free	Lonza	BE17-513F
Bovine serum albumin (BSA)	Sigma-Aldrich	A9647
Ethylenediaminetetraacetic acid (EDTA), 0.5mM, endotoxin-free	Thermo Fisher Scientific	11561575
Sodium azide	Sigma-Aldrich	S2002
Flow cytometry buffer	DPBS + 0.5% BSA + 1mM EDTA + 0.01% sodium azide	
Flow cytometry antibody mixture	34.5µL flow cytometry buffer + 2µL human IgG + 5µL CD4v4-FITC + 2.5µL CD14-PE + 5µL CD19-APC + 1µL CD3-PB	
Plasma separation		
cOmplete™ mini protease inhibitor cocktail tablets	Roche	4693124001
Cytokine/chemokine multiplex electrochemiluminescence assays		
V-PLEX™ human cytokine 30-plex kit	Meso Scale Diagnostics	K15054D-1
V-PLEX™ Th17 panel 1 (human) kit	Meso Scale Diagnostics	K15085D-1
V-PLEX™ Plus vascular injury panel 2 (human) kit	Meso Scale Diagnostics	K15198G-1
MESO™ QuickPlex SQ120	Meso Scale Diagnostics	AI0AA-0
General reagents		
Hanks buffered saline solution (HBSS) with Phenol Red, calcium- and magnesium-free	Lonza	BE10-543F
Vacuette™ K3 EDTA 9ml tube	Greiner Bio-One	455036
Vacuette™ Z Serum Separator Clot Activator tube	Greiner Bio-One	455071
TEMPUS™ tube	Applied Biosystems	4342792

Appendix D. Patient demographics for entire study cohort

The demographics of all patients recruited to the study (i.e. including patients who stopped DMARDs and those who were ineligible for DMARD cessation) is presented below. For discussion, see Results 4.4.1.

Demographic	Value
Number of patients recruited	74
Satisfied 2010 ACR/EULAR RA diagnostic criteria: n(%)	70 (95%)
Satisfied 1987 ACR RA diagnostic criteria: n(%)	67 (91%)
Age: median (IQR) [range]	67 (56 – 72) [35 – 86]
Female: n(%)	42 (57%)
Years since RA diagnosis: median (IQR) [range]	6 (3 – 12) [1 – 40]
Symptom duration in months prior to first rheumatology review: median (IQR) [range]	5 (2.5 – 10) [1 – 204]
Months from first rheumatology review to starting first DMARD: median (IQR) [range]	1 (0 – 4) [0 – 210]
Months since last steroid: median (IQR) [range]	30 (13 – 48) [0 – 152]
Months since last change in DMARDs: median (IQR) [range]	24 (13.3 – 49.5) [2 – 132]
Current smoker: n (%)	8 (11%)
Previous smoker: n (%)	36 (49%)
Never smoker: n(%)	30 (41%)
Weekly alcohol unit intake: median (IQR) [range]	4 (0 – 10) [0 – 50]
Total DMARDs since diagnosis: median [range]	2 [1 – 5]
Current MTX monotherapy: n(%)	40 (54%)
Current SFZ monotherapy: n(%)	9 (12%)
Current HCQ monotherapy: n(%)	1 (1%)
Current MTX+SFZ: n(%)	6 (8%)
Current MTX+HCQ: n(%)	11 (15%)
Current SFZ+HCQ: n(%)	3 (4%)
Current MTX+SFZ+HCQ: n(%)	4 (5%)
RhF positive: n(%)	44 (59%)
ACPA positive: n(%)	41 (55%)
RhF or ACPA positive: n(%)	52 (70%)
RhF and ACPA positive: n(%)	33 (45%)
Baseline 28 SJC: median (IQR) [range]	0 (0 – 0) [0 – 3]
Baseline 28 TJC: median (IQR) [range]	0 (0 – 0) [0 – 10]
Baseline patient VAS (mm): median (IQR) [range]	5 (1 – 15) [0 – 35]
Baseline CRP in mg/L: median (IQR) [range]	0 (0 – 0) [0 – 13]
Baseline ESR in mm/hr: median (IQR) [range]	9 (5 – 20) [1 – 77]
Baseline DAS28-CRP: median (IQR) [range]	1.17 (1.00 – 1.81) [0.96 – 3.49]
Baseline DAS28-ESR: median (IQR) [range]	1.85 (1.23 – 2.24) [0.48 – 4.37]
ACR/EULAR Boolean remission: n(%)	40 (54%)
Presence of joint erosion on baseline 7-joint ultrasound scan: n(%)	51 (69%)

Appendix E. Clinical composite score ROC analysis

Composite clinical biomarker scores ranked by area under the receiver-operating characteristic curve (ROC_{AUC}). Variables included within each score are indicated in green, and those excluded are indicated in red. For discussion, see Results 4.7.

RhF positive	ACPA positive	ACR/EULAR Boolean remission	DMARD change (months)	Current methotrexate	DMARD commencement (months)	ROC_{AUC}
✗	✓	✓	✓	✓	✗	0.850
✓	✓	✓	✓	✓	✗	0.848
✗	✗	✓	✓	✓	✗	0.837
✓	✗	✓	✓	✓	✗	0.833
✓	✓	✓	✓	✓	✓	0.805
✗	✓	✓	✓	✓	✓	0.798
✗	✓	✗	✓	✓	✗	0.787
✓	✗	✗	✓	✓	✗	0.786
✓	✗	✓	✓	✓	✓	0.782
✓	✓	✓	✓	✗	✓	0.777
✓	✓	✓	✓	✗	✗	0.774
✓	✗	✓	✓	✗	✗	0.772
✗	✓	✓	✓	✗	✗	0.767
✓	✓	✗	✓	✓	✗	0.762
✗	✗	✗	✓	✓	✗	0.761
✓	✓	✗	✓	✓	✓	0.759
✗	✓	✓	✗	✓	✗	0.758
✗	✓	✗	✓	✓	✓	0.757
✓	✓	✓	✗	✓	✓	0.756
✗	✗	✓	✓	✗	✗	0.746
✗	✓	✓	✓	✗	✓	0.743
✓	✓	✓	✗	✓	✗	0.739
✗	✗	✓	✓	✓	✓	0.739
✓	✓	✗	✓	✗	✓	0.736
✓	✗	✓	✓	✗	✓	0.734
✗	✓	✓	✗	✓	✓	0.733
✗	✗	✓	✗	✓	✗	0.733
✓	✗	✗	✓	✓	✓	0.732
✓	✗	✓	✗	✓	✗	0.732
✓	✓	✓	✗	✗	✓	0.728
✓	✗	✗	✓	✗	✗	0.720
✓	✓	✗	✗	✓	✓	0.717
✗	✓	✗	✓	✗	✓	0.709
✓	✓	✗	✗	✗	✓	0.707
✗	✗	✗	✓	✓	✓	0.707
✓	✗	✓	✗	✓	✓	0.705
✓	✓	✗	✓	✗	✗	0.703
✗	✓	✗	✓	✗	✗	0.702

Appendix E (continued)

RhF positive	ACPA positive	ACR/EULAR Boolean remission	DMARD change (months)	Current methotrexate	DMARD commencement (months)	ROC_{AUC}
✗	✓	✗	✗	✓	✓	0.700
✓	✓	✗	✗	✓	✗	0.695
✗	✓	✗	✗	✓	✗	0.690
✓	✗	✗	✓	✗	✓	0.689
✗	✓	✓	✗	✗	✗	0.679
✗	✓	✓	✗	✗	✓	0.676
✓	✓	✓	✗	✗	✗	0.675
✗	✗	✓	✓	✗	✓	0.675
✓	✗	✓	✗	✗	✗	0.675
✗	✗	✓	✗	✓	✓	0.674
✗	✗	✗	✓	✗	✗	0.672
✓	✗	✗	✗	✓	✗	0.667
✓	✗	✓	✗	✗	✓	0.666
✓	✗	✗	✗	✓	✓	0.665
✗	✓	✗	✗	✗	✓	0.653
✗	✗	✓	✗	✗	✗	0.639
✓	✓	✗	✗	✗	✗	0.638
✓	✗	✗	✗	✗	✓	0.623
✗	✗	✗	✓	✗	✓	0.618
✗	✗	✓	✗	✗	✓	0.611
✗	✗	✗	✗	✓	✓	0.607
✗	✗	✗	✗	✓	✗	0.603
✓	✗	✗	✗	✗	✗	0.601
✗	✓	✗	✗	✗	✗	0.601
✗	✗	✗	✗	✗	✓	0.538

Appendix F. Association of clinical variables with ultrasound findings at baseline.

For discussion of the data presented in these tables, see Results 5.4.

Table F.1 – Association of clinical parameters with the presence of joint GS in the setting of RA clinical remission by multivariate ordinal regression. BH: Benjamini-Hochberg; OR: odds ratio of increase in total joint GS score.

Variable	OR	95% CI	Unadjusted p-value	BH adjusted p-value
Male sex	5.04	1.47 – 17.26	0.010	0.139
ESR (mm/hr)	1.05	1.00 – 1.09	0.038	0.245
Smoking pack years	1.03	1.00 – 1.07	0.052	0.245
CRP (mg/L)	0.84	0.69 – 1.03	0.091	0.264
RhF positive	0.38	0.12 – 1.21	0.101	0.264
Disease duration (years)	1.06	0.99 – 1.15	0.113	0.264
TJC28	0.54	0.21 – 1.40	0.207	0.414
ACPA positive	2.14	0.54 – 8.48	0.278	0.486
Age	0.98	0.94 – 1.03	0.379	0.589
ACR/EULAR Boolean remission	0.57	0.10 – 3.15	0.519	0.727
HAQ-DI score	0.78	0.25 – 2.43	0.664	0.845
Weekly alcohol unit intake	1.01	0.94 – 1.10	0.736	0.859
Patient VAS	1.00	0.93 – 1.09	0.954	0.985
SJC28	1.01	0.33 – 3.09	0.985	0.985

Table F.2 – Association of clinical parameters with the presence of joint PD in the setting of RA clinical remission by multivariate ordinal regression. BH: Benjamini-Hochberg; OR: odds ratio of increase in total joint PD score.

Variable	OR	95% CI	Unadjusted p-value	BH adjusted p-value
Sex	0.24	0.05 – 1.24	0.088	0.907
Age	1.04	0.97 – 1.12	0.228	0.907
Disease duration (years)	1.01	0.91 – 1.11	0.856	0.979
Smoking pack years	0.99	0.93 – 1.04	0.608	0.907
Weekly alcohol unit intake	1.03	0.93 – 1.13	0.623	0.907
RhF positive	1.26	0.26 – 6.06	0.773	0.979
ACPA positive	1.03	0.16 – 6.50	0.979	0.979
ACR/EULAR Boolean remission	0.30	0.03 – 3.11	0.315	0.907
HAQ-DI score	0.66	0.11 – 3.88	0.648	0.907
ESR (mm/hr)	0.98	0.92 – 1.05	0.594	0.907
CRP (mg/L)	0.99	0.78 – 1.26	0.952	0.979
SJC28	0.50	0.11 – 2.18	0.353	0.907
TJC28	0.49	0.11 – 2.16	0.348	0.907
Patient VAS	0.96	0.85 – 1.08	0.460	0.907

Table F.3 – Association of clinical parameters with the presence of tendon GS in the setting of RA clinical remission by multivariate ordinal regression. BH: Benjamini-Hochberg; OR: odds ratio of increase in total tendon GS score.

Variable	OR	95% CI	Unadjusted p-value	BH adjusted p-value
SJC28	5.37	1.46 – 19.72	0.011	0.159
Weekly alcohol unit intake	0.88	0.77 – 1.00	0.044	0.307
TJC28	0.32	0.09 – 1.21	0.094	0.439
ACR/EULAR Boolean remission	3.90	0.40 – 37.96	0.241	0.614
ESR (mm/hr)	0.97	0.92 – 1.02	0.267	0.614
CRP (mg/L)	0.86	0.66 – 1.13	0.293	0.614
Sex	0.51	0.12 – 2.10	0.349	0.614
RhF positive	0.52	0.13 – 2.04	0.351	0.614
Patient VAS	1.05	0.94 – 1.16	0.395	0.614
HAQ-DI score	1.77	0.38 – 8.33	0.468	0.632
Age	1.02	0.96 – 1.09	0.513	0.632
Disease duration (years)	0.97	0.89 – 1.07	0.542	0.632
Smoking pack years	1.01	0.96 – 1.05	0.725	0.781
ACPA positive	1.21	0.24 – 6.11	0.816	0.816

Table F.4 – Association of clinical parameters with the presence of erosions in the setting of RA clinical remission by multivariate ordinal regression. BH: Benjamini-Hochberg; OR: odds ratio of increase in total erosion score.

Variable	OR	95% CI	Unadjusted p-value	BH adjusted p-value
Disease duration (years)	1.16	1.06 – 1.27	0.002	0.024
TJC28	0.17	0.05 – 0.56	0.004	0.025
ESR (mm/hr)	0.92	0.85 – 0.99	0.022	0.101
Weekly alcohol unit intake	0.93	0.85 – 1.02	0.117	0.357
CRP (mg/L)	1.17	0.95 – 1.42	0.134	0.357
RhF positive	0.42	0.12 – 1.48	0.177	0.357
HAQ-DI score	2.19	0.67 – 7.19	0.195	0.357
SJC28	2.20	0.65 – 7.45	0.204	0.357
Patient VAS	1.04	0.95 – 1.15	0.400	0.622
Age	0.98	0.93 – 1.04	0.540	0.740
Smoking pack years	0.99	0.95 – 1.03	0.581	0.740
ACR/EULAR Boolean remission	1.30	0.18 – 9.12	0.793	0.926
Sex	1.06	0.31 – 3.56	0.930	0.973
ACPA positive	1.03	0.24 – 4.47	0.973	0.973

Appendix G. Cytokine/chemokine calibrator variation

The percentage coefficient of variation for the calibrators for each analyte on each electrochemiluminescence plate are presented in the tables below. Seven calibrator solutions of known concentration were provided by the manufacturer, and were ran in duplicate on each plate. The manufacturer states that the %CV is typically less than 20% for repeat measurements – the %CV of calibrator pairs (CV1-7) that exceeded this threshold are highlighted in red. For discussion, see Results 6.2.2.

Plate 1

MSD plate	Assay	CV 1	CV 2	CV 3	CV 4	CV 5	CV 6	CV 7
Cytokine panel 1 (human)	GM-CSF	2.4	3.6	0.6	4.3	1.9	8.1	1.6
	IL-12/IL-23p40 subunit	4.2	2.4	1.9	2.9	0.8	1.3	2.2
	IL-15	3.3	1.5	1.2	0.6	1.7	1.6	1.6
	IL-16	2.1	1.6	4.3	4.8	0.9	6.3	0
	IL-17A	1.7	0.9	2.1	2.3	2.7	1.6	5.1
	IL-1α	16.6	0.5	0.4	4.5	3.7	18.3	12.6
	IL-5	0.6	1.4	2.1	2.8	0.1	1.2	7.9
	IL-7	6.9	2.8	2.6	4.2	3.3	3.1	0
	TNF-β	0.3	2.4	0.5	0.1	4.3	2.7	22.8
	VEGF	3.1	0.6	1	1.4	1.3	3.5	0
Chemokine panel 1 (human)	Eotaxin	1.3	1.4	0.4	7.2	8.4	78.4	40.2
	Eotaxin-3	5.9	3.7	9.3	2.1	6	6.9	32.6
	IL-8(HA)	3.1	2.2	22.1	13.1	19.4	31.8	0
	IP-10	3.7	1.9	3.4	5.8	2.6	2.9	0.9
	MCP-1	0.4	1.1	3.8	1	6	2.8	42
	MCP-4	0.8	1.7	3.1	8.3	13.1	31.2	0
	MDC	4.8	5	1.5	3.4	3.1	5.3	122
	MIP-1α	5.4	1.6	0.5	1.7	2.5	19.6	0
	MIP-1β	3	1.5	3.9	3.3	1.5	7.8	43.8
	TARC	2.6	4.1	27.6	22.5	21.1	22.6	0
Proinflammatory panel 1 (human)	IFN-γ	8	6.3	0.8	3	0.9	35.9	79.9
	IL-10	1.5	2.1	2.7	5.6	1.1	1.7	60.8
	IL-12p70 subunit	28.2	0.3	33.6	5.6	40.4	0	0
	IL-13	7.5	2	12.1	2.8	0	0	0
	IL-1β	3.6	4.7	9.3	5.5	0.6	8.8	20.6
	IL-2	3.9	16.8	16.5	23.1	0.5	20.2	34.9
	IL-4	4.2	64.4	4.3	1.4	3.6	6.6	73.4
	IL-6	5	15.5	41.9	5.2	0.7	14.8	0
	IL-8	16.1	7.6	22	2.6	31.8	2.2	0
	TNF-α	32.2	5.9	65.8	22.3	76.5	0	0
Th17 panel 1 (human)	IL-17A	2.1	0.9	6.1	4.2	3.4	4.4	7.1
	IL-21	3.5	2.6	0.9	2.1	1	6.7	52.6
	IL-22	7	0.7	3.6	0.8	2.4	6.1	9.6
	IL-23	5.5	5.6	3.1	1.6	0.4	1.9	10.5
	IL-27	7.3	4.5	7.8	4	0.9	0.4	8.2
	MIP-3α	3.4	4.7	11.6	7	4.4	15	0
	IL-31	0.7	5.1	6.1	8.3	3.4	7.6	9.5
Vascular injury panel 2 (human)	CRP	0.3	4.9	3.3	10	3.7	4.8	13.3
	ICAM-1	9.1	9	7.9	14.3	7	12.9	16
	SAA	7.8	5.7	5.7	22	9.2	7.6	64
	VCAM-1	10	6.8	4.4	8.3	5.9	6.2	37.9

Plate 2

MSD plate	Assay	CV 1	CV 2	CV 3	CV 4	CV 5	CV 6	CV 7
Cytokine panel 1 (human)	GM-CSF	0.9	10.9	0.3	2	2.5	14.4	7.7
	IL-12/IL-23p40 subunit	1.3	0.2	0.7	0.8	1.2	1.2	5.3
	IL-15	1.4	2.1	0.5	2	0.9	2	6.5
	IL-16	1.2	6	0.4	3.1	8	51.4	0
	IL-17A	3.2	0.5	0.4	1.4	1.1	0.5	0.8
	IL-1α	8.9	13.2	5.1	6.1	26.1	0.4	48.7
	IL-5	3.7	0.7	4.4	3.7	3.5	0.1	13.6
	IL-7	2.7	3.2	1.7	3.9	2	6.1	4.4
	TNF-β	2.3	1.7	1.9	1.9	0.4	3.5	13.9
	VEGF	2.2	0.6	3.6	3	2.1	11.8	0
Chemokine panel 1 (human)	Eotaxin	1.2	2.4	0.3	5.4	9.2	20.2	0
	Eotaxin-3	3.4	4.8	0.8	1.8	7.7	14.1	6.8
	IL-8(HA)	4.5	8.8	1.5	15.4	7.6	32.9	0
	IP-10	10.2	5.1	2.7	0.4	2.8	0.4	0.1
	MCP-1	0.5	3.2	3.1	4.4	3	6.6	2.7
	MCP-4	2.3	2.5	2.8	4.6	4.5	0	0
	MDC	9.1	1.2	5.9	5.1	1.3	2.5	134.8
	MIP-1α	1.6	1.3	0.6	2.5	9.1	2.6	0
	MIP-1β	2.8	1.8	0.1	0.8	1.6	3.2	13.3
	TARC	0.8	11.9	0	0.4	1.4	11.2	0.9
Proinflammatory panel 1 (human)	IFN-γ	46.9	4	40.4	68	1.1	0	88.3
	IL-10	0.2	0.8	0.8	1.3	3.2	0.6	2.5
	IL-12p70 subunit	85.3	7.3	0.8	4.7	16.8	0	45.5
	IL-13	6.3	1.6	4.6	25.6	6.1	0	9.9
	IL-1β	13	2.1	4.9	0.3	3.4	7.3	7.2
	IL-2	9.7	0.7	7.3	0.5	2.9	13.3	1
	IL-4	56	1.2	8	12.5	3.9	19.9	12.6
	IL-6	5.9	6	10.3	4	0.8	3.4	19.2
	IL-8	12.9	6.2	5.5	0.2	33.6	7.6	10.9
	TNF-α	100.2	26.6	40.4	27	5.4	15.8	0
Th17 panel 1 (human)	IL-17A	10	2.2	3.5	2.6	2.3	4.8	5.5
	IL-21	1	5.5	4.7	4.4	1.4	0.3	14.4
	IL-22	1.6	2.5	0.7	0.7	1.5	0.9	35.1
	IL-23	1.5	0.6	0.9	0.7	2.4	5.2	4.8
	IL-27	4.9	1.5	1.7	3	2.8	25.5	0
	MIP-3α	3.3	0.1	0.7	2.2	0.2	10.3	82.7
	IL-31	2	1.8	1.8	1.5	3.7	0.3	9.6
Vascular injury panel 2 (human)	CRP	11.1	1.3	0.7	2.5	3.7	2.9	5
	ICAM-1	0.3	3	4.8	1.8	7.8	14	21.3
	SAA	1.1	2.7	1.1	0.7	0.5	16.9	12.9
	VCAM-1	1.7	0.1	0.5	0.5	0.1	0.8	8.5

Appendix H. Cytokine/chemokine equilibration

Linear regression was used to assess the correlation between cytokine/chemokine concentrations in duplicated samples present on both paired electrochemiluminescence plates, and then applied to equilibrate readings on the two plates. The regression coefficient (m), constant (c), and R^2 values for each analyte are detailed in the table below. For discussion, see Results 6.2.5. NA: regression not possible owing to ≤2 samples available for regression.

Assay	Number of samples available for regression	m	c	R^2
Eotaxin	35	0.992	-0.077	0.961
Eotaxin3	15	1.040	-0.240	0.965
IP10	35	0.997	-0.040	0.985
MCP1	35	0.960	0.140	0.902
MCP4	35	0.922	0.400	0.933
MDC	35	0.925	0.481	0.945
MIP1α	28	0.244	2.162	0.083
MIP1β	35	0.964	0.137	0.978
TARC	35	1.025	-0.293	0.953
GMCSF	0	NA	NA	NA
IL12/23p40	35	0.963	0.226	0.973
IL15	35	0.879	0.209	0.901
IL16	35	0.904	0.610	0.843
IL17A	4	1.027	-0.030	0.968
IL1α	0	NA	NA	NA
IL5	3	1.551	-0.180	0.789
IL7	35	0.861	0.501	0.925
TNFβ	0	NA	NA	NA
VEGF	35	0.978	0.192	0.974
IFNγ	28	0.972	0.285	0.956
IL10	11	0.917	0.142	0.834
IL12p70	0	NA	NA	NA
IL13	0	NA	NA	NA
IL1β	2	NA	NA	NA
IL2	2	NA	NA	NA
IL4	0	NA	NA	NA
IL6	8	0.913	0.069	0.994
IL8	33	0.980	-0.585	0.955
TNFα	33	0.910	0.179	0.800
IL21	0	NA	NA	NA
IL22	19	0.977	0.003	0.964
IL23	0	NA	NA	NA
IL27	19	0.958	0.325	0.852
MIP3α	19	0.919	0.305	0.843
IL31	0	NA	NA	NA
CRP	6	1.096	-0.952	0.969
ICAM1	5	1.099	-1.214	0.979
SAA	5	0.911	1.006	0.749
VCAM1	5	0.999	-0.145	0.994

Appendix I. Differential gene expression supplementary tables

For discussion, see Chapter 7.

Table I.1 – Unabbreviated list of differentially expressed genes at baseline between flare patients and healthy controls, using an unadjusted significance threshold of $p<0.001$. Positive log-fold change indicates higher expression in the patient group, whereas negative log-fold change indicates higher expression in the control group. HGNC: HUGO gene nomenclature committee; lincRNA: long intergenic non-coding RNA; * = significant after FDR adjustment.

Ensembl gene ID	Log_2FC	Unadjusted p-value	HGNC symbol	Description
ENSG00000171560	**2.42**	**1.05E-07**	**FGA**	**fibrinogen alpha chain ***
ENSG00000106927	**2.13**	**1.21E-06**	**AMBP**	**alpha-1-microglobulin/bikunin precursor ***
ENSG00000171564	**2.00**	**3.19E-06**	**FGB**	**fibrinogen beta chain ***
ENSG00000163631	4.07	8.15E-06	ALB	albumin
ENSG00000182489	-2.85	2.50E-05	XKRX	Kell Blood Group Complex Subunit-Related, X-Linked
ENSG00000198538	-1.30	2.89E-05	ZNF28	zinc finger protein 28
ENSG00000223551	1.87	3.22E-05	TMSB4XP4	thymosin beta 4, X-linked pseudogene 4
ENSG00000226029	0.78	8.16E-05	LINC01772	long intergenic non-protein coding RNA 1772
ENSG00000141622	1.47	9.25E-05	RNF165	ring finger protein 165
ENSG00000251411	2.32	1.08E-04		(known processed pseudogene)
ENSG00000197841	-0.93	1.11E-04	ZNF181	zinc finger protein 181
ENSG00000164136	1.25	1.13E-04	IL15	interleukin 15
ENSG00000172985	-1.15	1.13E-04	SH3RF3	SH3 domain containing ring finger 3
ENSG00000247311	1.85	1.18E-04		(novel antisense)
ENSG00000112139	6.09	1.35E-04	MDGA1	MAM domain containing glycosylphosphatidylinositol anchor 1
ENSG00000088538	0.85	1.52E-04	DOCK3	dedicator of cytokinesis 3
ENSG00000131080	1.84	1.68E-04	EDA2R	ectodysplasin A2 receptor
ENSG00000229314	2.72	1.73E-04	ORM1	orosomucoid 1
ENSG00000125726	1.70	1.85E-04	CD70	CD70 molecule
ENSG00000152242	0.60	2.01E-04	C18orf25	chromosome 18 open reading frame 25
ENSG00000261115	1.94	2.31E-04	TMEM178B	transmembrane protein 178B
ENSG00000172349	-0.64	2.52E-04	IL16	interleukin 16
ENSG00000229473	1.63	2.60E-04	RGS17P1	regulator of G-protein signaling 17 pseudogene 1
ENSG00000185010	1.07	2.65E-04	F8	coagulation factor VIII
ENSG00000267939	2.31	2.77E-04		(novel lincRNA)
ENSG00000279148	1.87	2.77E-04		(known TEC)
ENSG00000265293	1.64	2.79E-04	ARGFXP2	arginine-fifty homeobox pseudogene 2
ENSG00000261487	1.59	3.00E-04		(known processed transcript)
ENSG00000197180	1.29	3.01E-04		uncharacterized protein BC009467
ENSG00000169398	-0.82	3.29E-04	PTK2	protein tyrosine kinase 2
ENSG00000082213	-0.70	3.48E-04	C5orf22	chromosome 5 open reading frame 22
ENSG00000072110	-0.76	3.82E-04	ACTN1	actinin alpha 1
ENSG00000228382	1.91	3.96E-04	ITPKB-IT1	Inositol-Trisphosphate 3-Kinase B intronic transcript 1
ENSG00000131969	1.92	3.98E-04	ABHD12B	abhydrolase domain containing 12B
ENSG00000149557	2.77	4.12E-04	FEZ1	fasciculation and elongation protein zeta 1
ENSG00000259657	1.35	4.33E-04	PIGHP1	phosphatidylinositol glycan anchor biosynthesis class H pseudogene 1
ENSG00000165259	1.94	4.42E-04	HDX	highly divergent homeobox
ENSG00000264739	2.16	5.08E-04		(novel antisense)
ENSG00000162892	-0.61	5.55E-04	IL24	interleukin 24
ENSG00000115129	1.15	5.65E-04	TP53I3	tumor protein p53 inducible protein 3
ENSG00000244588	2.25	5.89E-04	RAD21L1	Double-Strand-Break Repair Protein Rad21 cohesin complex component like 1
ENSG00000272329	1.85	6.00E-04		(known lincRNA)
ENSG00000154655	1.66	6.13E-04	L3MBTL4	l(3)mbt-like 4 (Drosophila)

Table I.1 (continued)

Ensembl gene ID	Log_2FC	Unadjusted p-value	HGNC symbol	Description
ENSG00000272630	1.09	6.41E-04		(known lincRNA)
ENSG00000171115	-0.81	6.78E-04	GIMAP8	GTPase, IMAP family member 8
ENSG00000205786	1.13	6.86E-04	LINC01531	long intergenic non-protein coding RNA 1531
ENSG00000278356	1.34	7.01E-04		(known sense intronic)
ENSG00000159882	-0.67	7.02E-04	ZNF230	zinc finger protein 230
ENSG00000246016	1.95	7.53E-04	LINC01513	long intergenic non-protein coding RNA 1513
ENSG00000272086	1.27	7.64E-04		(novel lincRNA)
ENSG00000271447	-1.47	7.73E-04	MMP28	matrix metallopeptidase 28
ENSG00000160318	3.03	8.05E-04	CLDND2	claudin domain containing 2
ENSG00000271680	-1.73	8.45E-04		(novel processed pseudogene)
ENSG00000273598	-1.89	8.69E-04		(novel unprocessed pseudogene)
ENSG00000162946	-0.65	8.87E-04	DISC1	disrupted in schizophrenia 1
ENSG00000228543	2.14	9.03E-04		(known lincRNA)
ENSG00000151729	0.64	9.34E-04	SLC25A4	solute carrier family 25 member 4
ENSG00000219433	-1.71	9.71E-04	BTBD10P2	BTB domain containing 10 pseudogene 2

Table I.2 – Unabbreviated list of differentially expressed genes at baseline between remission patients and healthy controls, using an unadjusted significance threshold of $p<0.001$. Positive log-fold change indicates higher expression in the patient group, whereas negative log-fold change indicates higher expression in the control group. HGNC: HUGO gene nomenclature committee; lincRNA: long intergenic non-coding RNA; TEC: to be experimentally confirmed.

Ensembl gene ID	Log_2FC	Unadjusted p-value	HGNC symbol	Description
ENSG00000106927	2.05	3.31E-06	AMBP	alpha-1-microglobulin/bikunin precursor
ENSG00000226029	0.92	5.02E-06	LINC01772	long intergenic non-protein coding RNA 1772
ENSG00000112139	7.13	1.09E-05	MDGA1	meprin, A-5 protein, and receptor protein-tyrosine phosphatase mu (MAM) domain containing glycosylphosphatidylinositol anchor 1
ENSG00000247311	2.06	2.19E-05		(novel antisense)
ENSG00000198538	-1.33	2.21E-05	ZNF28	zinc finger protein 28
ENSG00000163631	3.88	2.24E-05	ALB	albumin
ENSG00000171560	1.87	2.79E-05	FGA	fibrinogen alpha chain
ENSG00000171564	1.74	4.48E-05	FGB	fibrinogen beta chain
ENSG00000256913	1.67	5.94E-05		(novel processed pseudogene)
ENSG00000259657	1.54	6.92E-05	PIGHP1	phosphatidylinositol glycan anchor biosynthesis class H pseudogene 1
ENSG00000265293	1.75	1.24E-04	ARGFXP2	arginine-fifty homeobox pseudogene 2
ENSG00000204380	2.30	1.55E-04	PKP4-AS1	Plakophilin 4 - antisense RNA 1
ENSG00000228382	2.06	1.59E-04	ITPKB-IT1	Inositol-Trisphosphate 3-Kinase B - intronic transcript 1
ENSG00000279148	1.92	2.10E-04		(known TEC)
ENSG00000214081	-2.58	2.24E-04	CYP4F30P	cytochrome P450 family 4 subfamily F member 30, pseudogene
ENSG00000165259	2.05	2.48E-04	HDX	highly divergent homeobox
ENSG00000251411	2.19	2.70E-04		(known processed pseudogene)
ENSG00000154099	0.98	3.28E-04	DNAAF1	dynein axonemal assembly factor 1
ENSG00000261487	1.58	3.77E-04		(novel processed transcript)
ENSG00000253676	1.79	3.77E-04	TAGLN2P1	transgelin 2 pseudogene 1
ENSG00000267795	2.13	4.60E-04	SMIM22	small integral membrane protein 22
ENSG00000163659	1.04	4.65E-04	TIPARP	2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) inducible poly(ADP-ribose) polymerase
ENSG00000141622	1.32	4.66E-04	RNF165	ring finger protein 165
ENSG00000115129	1.18	4.67E-04	TP53I3	tumor protein p53 inducible protein 3
ENSG00000165202	-1.84	5.22E-04	OR1Q1	olfactory receptor family 1 subfamily Q member 1
ENSG00000246016	2.02	5.28E-04	LINC01513	long intergenic non-protein coding RNA 1513
ENSG00000266992	-3.61	5.64E-04	DHX40P1	DEAH-box helicase 40 pseudogene 1
ENSG00000159882	-0.68	5.68E-04	ZNF230	zinc finger protein 230

Table I.2 (continued)

Ensembl gene ID	Log_2FC	Unadjusted p-value	HGNC symbol	Description
ENSG00000272086	1.31	5.71E-04		(novel lincRNA)
ENSG00000267939	2.17	6.42E-04		(novel lincRNA)
ENSG00000219797	0.81	6.48E-04	PPIAP9	peptidylprolyl isomerase A pseudogene 9
ENSG00000264548	1.75	6.61E-04		(novel antisense)
ENSG00000273598	-1.95	6.67E-04		(novel unprocessed pseudogene)
ENSG00000228918	2.03	6.68E-04	LINC01344	long intergenic non-protein coding RNA 1344
ENSG00000235217	-0.59	7.60E-04	TSPY26P	testis specific protein, Y-linked 26, pseudogene
ENSG00000229314	2.43	8.12E-04	ORM1	orosomucoid 1
ENSG00000205765	-0.88	8.20E-04	C5orf51	chromosome 5 open reading frame 51
ENSG00000250656	2.00	9.24E-04	ST3GAL1P1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1 pseudogene 1
ENSG00000069696	1.97	9.30E-04	DRD4	dopamine receptor D4

Table I.3 – Unabbreviated list of differentially expressed genes between flare versus baseline visits in 17 patients who experienced an arthritis flare, using an unadjusted significance threshold of $p<0.001$. Positive log-fold change indicates higher expression at the flare visit, whereas negative log-fold change indicates higher expression at baseline. HGNC: HUGO gene nomenclature committee; lincRNA: long intergenic non-coding RNA; TEC: to be experimentally confirmed.* = significant after multiple test correction ($p<0.05$).

Ensembl gene ID	Log_2FC	Unadjusted p-value	HGNC symbol	Description
ENSG00000144354	**1.05**	**2.79E-07**	**CDCA7**	**cell division cycle associated 7 ***
ENSG00000130164	**0.79**	**1.47E-06**	**LDLR**	**low density lipoprotein receptor ***
ENSG00000165409	1.19	8.59E-06	TSHR	thyroid stimulating hormone receptor
ENSG00000171533	1.69	9.98E-06	MAP6	microtubule associated protein 6
ENSG00000137474	1.37	1E-05	MYO7A	myosin VIIA
ENSG00000156127	0.78	1.47E-05	BATF	basic leucine zipper ATF-like transcription factor
ENSG00000251537	1.80	1.89E-05		(known protein coding)
ENSG00000088325	1.10	2.07E-05	TPX2	TPX2, microtubule nucleation factor
ENSG00000156535	0.72	2.15E-05	CD109	CD109 molecule
ENSG00000138180	1.36	2.6E-05	CEP55	centrosomal protein 55
ENSG00000137812	0.83	2.97E-05	KNL1	kinetochore scaffold 1
ENSG00000216819	-1.48	3.5E-05	TUBB2BP1	tubulin beta 2B class IIb pseudogene 1
ENSG00000131747	1.21	3.91E-05	TOP2A	topoisomerase (DNA) II alpha
ENSG00000170312	1.02	3.91E-05	CDK1	cyclin dependent kinase 1
ENSG00000148773	1.53	4.2E-05	MKI67	marker of proliferation Ki-67
ENSG00000267496	-0.90	4.34E-05	FAM215A	family with sequence similarity 215 member A (non-protein coding)
ENSG00000263218	1.72	4.62E-05		(known antisense RNA)
ENSG00000237649	1.14	4.98E-05	KIFC1	kinesin family member C1
ENSG00000137804	0.91	5.56E-05	NUSAP1	nucleolar and spindle associated protein 1
ENSG00000175063	1.59	9.31E-05	UBE2C	ubiquitin conjugating enzyme E2 C
ENSG00000176890	1.11	9.61E-05	TYMS	thymidylate synthetase
ENSG00000213297	-2.05	0.000101	ZNF625-ZNF20	ZNF625-ZNF20 readthrough (NMD candidate)
ENSG00000226310	-1.28	0.000124		(known antisense RNA)
ENSG00000060982	0.72	0.000127	BCAT1	branched chain amino acid transaminase 1
ENSG00000024422	-1.18	0.000131	EHD2	EH domain containing 2
ENSG00000186187	0.59	0.000133	ZNRF1	zinc and ring finger 1
ENSG00000184661	1.39	0.000136	CDCA2	cell division cycle associated 2
ENSG00000163808	1.17	0.000137	KIF15	kinesin family member 15
ENSG00000270111	-0.74	0.000144		(known LincRNA)
ENSG00000178999	0.95	0.000153	AURKB	aurora kinase B
ENSG00000114346	0.59	0.000169	ECT2	epithelial cell transforming 2
ENSG00000251211	1.19	0.000177		(processed pseudogene)
ENSG00000066279	1.06	0.000178	ASPM	abnormal spindle microtubule assembly
ENSG00000035499	0.98	0.000235	DEPDC1B	DEP domain containing 1B

Table I.3 (continued)

Ensembl gene ID	Log_2FC	Unadjusted p-value	HGNC symbol	Description
ENSG00000109805	1.12	0.000248	NCAPG	non-SMC condensin I complex subunit G
ENSG00000165304	0.94	0.000259	MELK	maternal embryonic leucine zipper kinase
ENSG00000228792	-1.05	0.000281		(known LincRNA)
ENSG00000230266	1.16	0.000286	XXYLT1-AS2	XXYLT1 antisense RNA 2
ENSG00000271817	-1.21	0.000307		Small nucleolar RNA U3
ENSG00000007968	0.79	0.000307	E2F2	E2F transcription factor 2
ENSG00000126787	1.53	0.000309	DLGAP5	DLG associated protein 5
ENSG00000248564	1.12	0.000338		(processed pseudogene)
ENSG00000175267	0.93	0.000341	VWA3A	von Willebrand factor A domain containing 3A
ENSG00000138160	0.85	0.000342	KIF11	kinesin family member 11
ENSG00000171848	1.35	0.000358	RRM2	ribonucleotide reductase regulatory subunit M2
ENSG00000136982	1.30	0.00036	DSCC1	DNA replication and sister chromatid cohesion 1
ENSG00000177602	0.89	0.000372	GSG2	germ cell associated 2, haspin
ENSG00000101057	1.22	0.000386	MYBL2	MYB proto-oncogene like 2
ENSG00000270116	-1.28	0.000402		(sense intronic)
ENSG00000250091	0.60	0.000415	DNAH10OS	dynein axonemal heavy chain 10 opposite strand
ENSG00000145386	1.06	0.000423	CCNA2	cyclin A2
ENSG00000212663	1.22	0.000424		(known LincRNA)
ENSG00000094804	1.09	0.000425	CDC6	cell division cycle 6
ENSG00000243761	-0.99	0.000429		(processed pseudogene)
ENSG00000156970	0.95	0.00043	BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B
ENSG00000241790	1.79	0.000462	ENO1P4	enolase 1 pseudogene 4
ENSG00000146670	1.14	0.00052	CDCA5	cell division cycle associated 5
ENSG00000117724	0.64	0.000528	CENPF	centromere protein F
ENSG00000160207	-1.01	0.000555	HSF2BP	heat shock transcription factor 2 binding protein
ENSG00000228665	-0.62	0.000557		(processed pseudogene)
ENSG00000168811	0.69	0.000588	IL12A	interleukin 12A
ENSG00000117399	1.41	0.000624	CDC20	cell division cycle 20
ENSG00000093009	1.15	0.000625	CDC45	cell division cycle 45
ENSG00000168078	1.51	0.00065	PBK	PDZ binding kinase
ENSG00000072571	1.15	0.000676	HMMR	hyaluronan mediated motility receptor
ENSG00000121152	0.71	0.000708	NCAPH	non-SMC condensin I complex subunit H
ENSG00000109674	1.17	0.000717	NEIL3	nei like DNA glycosylase 3
ENSG00000089685	1.17	0.000723	BIRC5	baculoviral IAP repeat containing 5
ENSG00000279416	-1.13	0.000761		(TEC)
ENSG00000213885	-0.80	0.000763	RPL13AP7	ribosomal protein L13a pseudogene 7
ENSG00000259212	0.69	0.000766		(known antisense RNA)
ENSG00000138696	1.08	0.00077	BMPR1B	bone morphogenetic protein receptor type 1B
ENSG00000157456	1.20	0.000817	CCNB2	cyclin B2
ENSG00000118193	0.72	0.000835	KIF14	kinesin family member 14
ENSG00000011347	-0.92	0.000844	SYT7	synaptotagmin 7
ENSG00000090889	1.11	0.000858	KIF4A	kinesin family member 4A
ENSG00000138778	0.73	0.000895	CENPE	centromere protein E
ENSG00000188223	-2.39	0.000911		(known protein coding)
ENSG00000169679	0.71	0.000922	BUB1	BUB1 mitotic checkpoint serine/threonine kinase
ENSG00000235688	1.20	0.000926		(known antisense RNA)
ENSG00000186340	-1.03	0.000962	THBS2	thrombospondin 2

Appendix J. ROC analysis for integrative biomarker scores

For discussion, see Chapter 8.

Table J.1 – Area under the receiver operating characteristic curve (ROC_{AUC}) of 31 combinations of the five variables from the stepwise multivariate Cox regression model that included gene expression data. Variables included within each score are indicated in green, and those excluded are indicated in red.

Baseline ACR/EULAR remission	ENSG00000162636	ENSG00000227070	ENSG00000228010	ln(IL27+1)	ROC_{AUC}
✓	✓	✓	✓	✓	0.963
✓	✓	✓	✓	✗	0.954
✓	✗	✓	✓	✗	0.927
✓	✗	✓	✓	✓	0.920
✓	✓	✗	✓	✓	0.918
✗	✓	✓	✓	✓	0.908
✗	✓	✓	✓	✗	0.908
✓	✓	✗	✓	✗	0.902
✗	✗	✓	✓	✗	0.874
✓	✓	✓	✗	✓	0.867
✓	✓	✓	✗	✗	0.867
✗	✓	✗	✓	✗	0.860
✗	✓	✗	✓	✓	0.849
✗	✗	✓	✓	✓	0.844
✗	✓	✓	✗	✗	0.822
✗	✓	✓	✗	✓	0.819
✓	✓	✗	✗	✓	0.812
✓	✗	✓	✗	✓	0.810
✓	✗	✗	✓	✓	0.805
✓	✓	✗	✗	✗	0.801
✓	✗	✗	✓	✗	0.794
✗	✗	✗	✓	✗	0.769
✓	✗	✓	✗	✗	0.767
✗	✗	✓	✗	✓	0.764
✗	✓	✗	✗	✓	0.762
✗	✗	✗	✓	✓	0.744
✗	✓	✗	✗	✗	0.737
✗	✗	✓	✗	✗	0.696
✓	✗	✗	✗	✓	0.691
✓	✗	✗	✗	✗	0.634
✗	✗	✗	✗	✓	0.613

Table J.2 - Area under the receiver operating characteristic curve (ROC_{AUC}) of 31 combinations of the seven variables from the stepwise multivariate Cox regression model that excluded gene expression data. Variables included within each score are indicated in green, and those excluded are indicated in red.

ACPA positive	ACR/EULAR Boolean remission	Current methotrexate	Months since last change in DMARDs	ln(IL27+1)	ln(MCP1)	RhF positive	ROC_{AUC}
✓	✓	✓	✓	×	✓	✓	0.957
✓	✓	✓	✓	✓	✓	✓	0.954
✓	✓	✓	✓	×	×	✓	0.936
✓	✓	✓	✓	×	✓	×	0.931
×	✓	✓	✓	✓	✓	✓	0.929
✓	✓	✓	✓	✓	✓	×	0.924
✓	✓	✓	✓	✓	×	✓	0.920
×	✓	✓	✓	✓	×	✓	0.911
×	✓	✓	✓	×	✓	✓	0.911
✓	✓	✓	✓	✓	×	×	0.904
✓	✓	✓	✓	×	×	×	0.902
×	✓	✓	✓	×	×	✓	0.899
×	✓	✓	✓	✓	✓	×	0.897
✓	✓	×	✓	✓	✓	✓	0.890
✓	✓	×	✓	×	✓	✓	0.888
×	✓	✓	✓	×	✓	×	0.886
✓	✓	✓	×	✓	✓	×	0.883
✓	✓	×	✓	×	✓	×	0.879
✓	×	✓	✓	✓	✓	×	0.876
✓	✓	✓	×	✓	✓	✓	0.874
×	✓	×	✓	✓	✓	✓	0.874
×	✓	×	✓	×	✓	✓	0.870
✓	×	✓	✓	✓	✓	✓	0.867
✓	×	✓	✓	×	✓	✓	0.867
✓	×	✓	✓	×	✓	×	0.863
×	✓	✓	✓	×	×	×	0.863
✓	✓	×	✓	✓	✓	×	0.860
×	×	✓	✓	×	✓	✓	0.860
×	✓	✓	✓	✓	×	×	0.858
×	×	✓	✓	✓	✓	✓	0.856
✓	✓	×	✓	×	×	✓	0.856
✓	✓	✓	×	×	✓	×	0.851
✓	✓	×	✓	✓	×	✓	0.849
×	✓	✓	×	✓	✓	×	0.847
✓	✓	✓	×	✓	×	✓	0.844
×	✓	×	✓	×	✓	×	0.844
✓	✓	✓	×	×	✓	✓	0.842
×	✓	✓	×	✓	✓	✓	0.842
✓	×	✓	✓	×	×	✓	0.842
✓	×	✓	✓	✓	×	✓	0.840
✓	✓	✓	×	×	×	✓	0.840
×	✓	×	✓	✓	×	✓	0.840
✓	×	✓	✓	×	×	×	0.839
×	×	✓	✓	✓	✓	×	0.838
×	×	✓	✓	✓	×	✓	0.838
×	✓	×	✓	✓	✓	×	0.835
✓	✓	✓	×	✓	×	×	0.833
✓	✓	×	×	✓	✓	×	0.833
✓	✓	×	×	✓	✓	✓	0.831
✓	✓	×	✓	✓	×	×	0.831
✓	✓	×	✓	×	×	×	0.831
×	✓	×	✓	×	×	✓	0.831
×	×	✓	✓	×	×	✓	0.828
✓	×	✓	✓	✓	×	×	0.826
✓	×	×	✓	✓	✓	×	0.815
×	✓	✓	×	✓	×	✓	0.815
✓	✓	×	×	×	✓	×	0.815
×	×	✓	✓	×	✓	×	0.812
×	✓	✓	×	×	✓	×	0.810

Table J.2 (continued)

ACPA positive	ACR/EULAR Boolean remission	Current methotrexate	Months since last change in DMARDs	ln(IL27+1)	ln(MCP1)	RhF positive	ROC_{AUC}
✓	×	×	✓	×	✓	×	0.808
✓	✓	✓	×	×	×	×	0.805
✓	×	×	✓	✓	✓	✓	0.803
×	✓	×	×	✓	✓	✓	0.801
✓	×	×	✓	×	✓	✓	0.799
×	✓	✓	×	×	✓	✓	0.799
✓	×	✓	×	✓	✓	×	0.796
×	×	×	✓	✓	✓	✓	0.796
×	×	✓	✓	×	×	×	0.795
×	×	×	✓	×	✓	✓	0.794
✓	✓	×	×	×	✓	✓	0.789
×	×	✓	✓	✓	×	×	0.789
✓	✓	×	×	✓	×	✓	0.787
×	✓	×	×	✓	✓	×	0.785
✓	×	✓	×	×	✓	✓	0.783
✓	×	✓	×	✓	✓	✓	0.780
×	×	×	✓	✓	✓	×	0.780
✓	×	✓	×	×	✓	×	0.778
×	×	✓	×	✓	✓	×	0.778
×	✓	✓	×	✓	×	×	0.776
×	✓	✓	×	×	×	✓	0.771
×	✓	×	✓	✓	×	×	0.771
✓	×	×	✓	✓	×	✓	0.769
✓	✓	×	×	×	×	✓	0.769
×	✓	×	✓	×	×	×	0.769
×	×	×	✓	✓	×	✓	0.764
✓	×	×	✓	×	×	✓	0.762
✓	×	×	✓	✓	×	×	0.760
✓	×	✓	×	✓	×	✓	0.757
×	×	×	✓	×	✓	×	0.757
×	×	✓	×	✓	✓	✓	0.755
×	✓	×	×	✓	×	✓	0.755
×	✓	×	×	×	✓	✓	0.753
×	✓	×	×	×	✓	×	0.751
×	×	×	✓	×	×	✓	0.747
✓	×	×	✓	×	×	×	0.745
✓	✓	×	×	✓	×	×	0.744
✓	×	✓	×	✓	×	×	0.744
✓	×	×	×	✓	✓	×	0.744
×	×	✓	×	×	✓	✓	0.741
×	×	✓	×	✓	×	✓	0.739
×	×	✓	×	×	✓	×	0.739
✓	×	×	×	✓	✓	✓	0.737
×	✓	✓	×	×	×	×	0.733
×	×	×	×	✓	✓	×	0.732
✓	×	✓	×	×	×	✓	0.725
✓	×	×	×	×	✓	✓	0.725
×	×	×	×	✓	✓	✓	0.721
✓	×	×	×	×	✓	×	0.721
✓	✓	×	×	×	×	×	0.720
✓	×	✓	×	×	×	×	0.711
×	×	×	✓	✓	×	×	0.707
×	×	×	✓	×	×	×	0.706
×	×	✓	×	✓	×	×	0.705
×	×	×	×	×	✓	×	0.705
×	×	×	×	×	✓	✓	0.703
×	✓	×	×	×	×	✓	0.695
✓	×	×	×	✓	×	✓	0.693
×	✓	×	×	✓	×	×	0.691
×	×	✓	×	×	×	✓	0.686
×	×	×	×	✓	×	✓	0.682
✓	×	×	×	✓	×	×	0.659
✓	×	×	×	×	×	✓	0.658
×	✓	×	×	×	×	×	0.634

Table J.2 (continued)

ACPA positive	ACR/EULAR Boolean remission	Current methotrexate	Months since last change in DMARDs	ln(IL27+1)	ln(MCP1)	RhF positive	ROC_{AUC}
✓	×	×	×	×	×	×	0.616
×	×	×	×	×	×	✓	0.616
×	×	×	×	✓	×	×	0.613
×	×	✓	×	×	×	×	0.610

References

Abe, A., Ishikawa, H., Nakazono, K., Murasawa, A. and Wakaki, K. (2016) 'A comparison of the ultrasonography images of the joints of patients with rheumatoid arthritis and the corresponding synovial histological findings', *Mod Rheumatol*, 26(4), pp. 534-9.

Adler, R.A., El-Hajj Fuleihan, G., Bauer, D.C., Camacho, P.M., Clarke, B.L., Clines, G.A., Compston, J.E., Drake, M.T., Edwards, B.J., Favus, M.J., Greenspan, S.L., McKinney, R., Jr., Pignolo, R.J. and Sellmeyer, D.E. (2016) 'Managing Osteoporosis in Patients on Long-Term Bisphosphonate Treatment: Report of a Task Force of the American Society for Bone and Mineral Research', *J Bone Miner Res*, 31(1), pp. 16-35.

Affymetrix eBioscience (2017) *PrimeFlow™ RNA Assay Technology Validation Paper* Available at: https://tools.thermofisher.com/content/sfs/brochures/FC04271-1_Prime-FlowRNA-Assay-TechValPaper_v13_102914_web.pdf (Accessed: August 13, 2017).

Aga, A.B., Hammer, H.B., Olsen, I.C., Uhlig, T., Kvien, T.K. and van der Heijde, D. (2016) 'First step in the development of an ultrasound joint inflammation score for rheumatoid arthritis using a data-driven approach', 75(8), pp. 1444-51.

Aga, A.B., Lie, E., Uhlig, T., Olsen, I.C., Wierod, A., Kalstad, S., Rodevand, E., Mikkelsen, K., Kvien, T.K. and Haavardsholm, E.A. (2015) 'Time trends in disease activity, response and remission rates in rheumatoid arthritis during the past decade: results from the NOR-DMARD study 2000-2010', *Ann Rheum Dis*, 74(2), pp. 381-8.

Ahern, M.J., Hall, N.D., Case, K. and Maddison, P.J. (1984) 'D-penicillamine withdrawal in rheumatoid arthritis', *Ann Rheum Dis*, 43(2), pp. 213-7.

Ajeganova, S., van Steenbergen, H.W., van Nies, J.A., Burgers, L.E., Huizinga, T.W. and van der Helm-van Mil, A.H. (2016) 'Disease-modifying antirheumatic drug-free sustained remission in rheumatoid arthritis: an increasingly achievable outcome with subsidence of disease symptoms', *Ann Rheum Dis*, 75(5), pp. 867-73.

Akerstrom, B. and Logdberg, L. (1984) 'Alpha 1-microglobulin glycopeptides inhibit antigen-specific stimulation of human peripheral blood leucocytes', *Scand J Immunol*, 20(6), pp. 559-63.

Alberio, T., Pippione, A.C., Zibetti, M., Olgiati, S., Cecconi, D., Comi, C., Lopiano, L. and Fasano, M. (2012) 'Discovery and verification of panels of T-lymphocyte proteins as biomarkers of Parkinson's disease', *Sci Rep*, 2, p. 953.

Aletaha, D., Nell, V.P., Stamm, T., Uffmann, M., Pflugbeil, S., Machold, K. and Smolen, J.S. (2005) 'Acute phase reactants add little to composite disease activity indices for rheumatoid arthritis: validation of a clinical activity score', *Arthritis Res Ther*, 7(4), pp. R796-806.

Aletaha, D., Neogi, T., Silman, A.J., Funovits, J., Felson, D.T., Bingham, C.O., 3rd, Birnbaum, N.S., Burmester, G.R., Bykerk, V.P., Cohen, M.D., Combe, B., Costenbader, K.H., Dougados, M., Emery, P., Ferraccioli, G., Hazes, J.M., Hobbs, K., Huizinga, T.W., Kavanaugh, A., Kay, J., Kvien, T.K., Laing, T., Mease, P., Menard, H.A., Moreland, L.W., Naden, R.L., Pincus, T., Smolen, J.S., Stanislawska-Biernat, E., Symmons, D., Tak, P.P., Upchurch, K.S., Vencovsky, J., Wolfe, F. and Hawker, G. (2010) '2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative', *Arthritis Rheum*, 62(9), pp. 2569-81.

Aletaha, D. and Smolen, J.S. (2011) 'Joint damage in rheumatoid arthritis progresses in remission according to the Disease Activity Score in 28 joints and is driven by residual swollen joints', *Arthritis Rheum*, 63(12), pp. 3702-11.

Alexander, W.R.M. (1967) 'The Rheumatoid Factor', *Res Medica*, 6(1), pp. 17-19.

Alivernini, S., Peluso, G., Fedele, A.L., Tolusso, B., Gremese, E. and Ferraccioli, G. (2016) 'Tapering and discontinuation of TNF-alpha blockers without disease relapse using ultrasonography as a tool to identify patients with rheumatoid arthritis in clinical and histological remission', *Arthritis Res Ther*, 18(1), p. 39.

Alivernini, S., Tolusso, B., Petricca, L., Bui, L., Di Sante, G., Peluso, G., Benvenuto, R., Fedele, A.L., Federico, F., Ferraccioli, G. and Gremese, E. (2017) 'Synovial features of patients with rheumatoid arthritis and psoriatic arthritis in clinical and ultrasound remission differ under anti-TNF therapy: a clue to interpret different chances of relapse after clinical remission?', *Ann Rheum Dis*, 76(7), pp. 1228-36.

Alvarez, P., Genre, F., Iglesias, M., Augustin, J.J., Tamayo, E., Escola-Gil, J.C., Lavin, B., Blanco-Vaca, F., Merino, R. and Merino, J. (2016) 'Modulation of autoimmune arthritis severity in mice by apolipoprotein E (ApoE) and cholesterol', *Clin Exp Immunol*, 186(3), pp. 292-303.

Andersen, M., Boesen, M., Ellegaard, K., Christensen, R., Soderstrom, K., Soe, N., Spee, P., Morch, U.G., Torp-Pedersen, S., Bartels, E.M., Danneskiold-Samsoe, B., Vendel, N., Karlsson, L. and Bliddal, H. (2014a) 'Synovial explant inflammatory mediator production corresponds to rheumatoid arthritis imaging hallmarks: a cross-sectional study', *Arthritis Res Ther*, 16(3), p. R107.

Andersen, M., Ellegaard, K., Hebsgaard, J.B., Christensen, R., Torp-Pedersen, S., Kvist, P.H., Soe, N., Romer, J., Vendel, N., Bartels, E.M., Danneskiold-Samsoe, B. and Bliddal, H. (2014b) 'Ultrasound colour Doppler is associated with synovial pathology in biopsies from hand joints in rheumatoid arthritis patients: a cross-sectional study', *Ann Rheum Dis*, 73(4), pp. 678-83.

Anderson, A.E., Pratt, A.G., Sedhom, M.A., Doran, J.P., Routledge, C., Hargreaves, B., Brown, P.M., Le Cao, K.A., Isaacs, J.D. and Thomas, R. (2016) 'IL-6-driven STAT signalling in circulating CD4+ lymphocytes is a marker for early anticitrullinated peptide antibody-negative rheumatoid arthritis', *Ann Rheum Dis*, 75(2), pp. 466-73.

Andrews, S. (2016) *FastQC. A quality control tool for high throughput sequence data.* [Computer program]. Available online at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Anikster, Y., Huizing, M., Anderson, P.D., Fitzpatrick, D.L., Klar, A., Gross-Kieselstein, E., Berkun, Y., Shazberg, G., Gahl, W.A. and Hurvitz, H. (2002) 'Evidence that Griscelli Syndrome with Neurological Involvement Is Caused by Mutations in RAB27A, Not MYO5A', *Am J Hum Genet*, 71(2), pp. 407-414.

Antonelli, A., Ferrari, S.M., Giuggioli, D., Ferrannini, E., Ferri, C. and Fallahi, P. (2014) 'Chemokine (C-X-C motif) ligand (CXCL)10 in autoimmune diseases', *Autoimmun Rev*, 13(3), pp. 272-80.

Arend, W.P. (2001) 'Physiology of cytokine pathways in rheumatoid arthritis', *Arthritis Rheum*, 45(1), pp. 101-6.

Arkema, E.V., Lu, B., Malspeis, S., Karlson, E.W. and Costenbader, K.H. (2015) 'Monocyte chemotactic protein-1 elevation prior to the onset of rheumatoid arthritis among women', *Biomark Med*, 9(8), pp. 723-9.

Arnett, F.C., Edworthy, S.M., Bloch, D.A., McShane, D.J., Fries, J.F., Cooper, N.S., Healey, L.A., Kaplan, S.R., Liang, M.H., Luthra, H.S., Medsger Jr, T.A., Mitchell, D.M., Neustadt, D.H., Pinals, R.S., Schaller, J.G., Sharp, J.T., Wilder, R.L. and Hunder, G.G. (1988) 'The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis', *Arthritis Rheum*, 31(3), pp. 315-24.

Arredouani, M.S., Kasran, A., Vanoirbeek, J.A., Berger, F.G., Baumann, H. and Ceuppens, J.L. (2005) 'Haptoglobin dampens endotoxin-induced inflammatory effects both in vitro and in vivo', *Immunology*, 114(2), pp. 263-71.

Awasthi, A., Carrier, Y., Peron, J.P., Bettelli, E., Kamanaka, M., Flavell, R.A., Kuchroo, V.K., Oukka, M. and Weiner, H.L. (2007) 'A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells', *Nat Immunol*, 8(12), pp. 1380-9.

Axelsen, M.B., Eshed, I., Duer-Jensen, A., Moller, J.M., Pedersen, S.J. and Ostergaard, M. (2014) 'Whole-body MRI assessment of disease activity and structural damage in rheumatoid arthritis: first step towards an MRI joint count', *Rheumatology (Oxford)*, 53(5), pp. 845-53.

Azizi, G., Yazdani, R. and Mirshafiey, A. (2015) 'Th22 cells in autoimmunity: a review of current knowledge', *Eur Ann Allergy Clin Immunol*, 47(4), pp. 108-17.

Bache, S.M. and Wickham, H. (2014) *magrittr: A Forward-Pipe Operator for R. R package version 1.5.* [Computer program]. https://CRAN.R-project.org/package=magrittr.

Backhaus, M., Ohrndorf, S., Kellner, H., Strunk, J., Backhaus, T.M., Hartung, W., Sattler, H., Albrecht, K., Kaufmann, J., Becker, K., Sorensen, H., Meier, L., Burmester, G.R. and Schmidt, W.A. (2009) 'Evaluation of a novel 7-joint ultrasound score in daily rheumatologic practice: a pilot project', *Arthritis Rheum*, 61(9), pp. 1194-201.

Bagriacik, E.U. and Klein, J.R. (2000) 'The thyrotropin (thyroid-stimulating hormone) receptor is expressed on murine dendritic cells and on a subset of CD45RBhigh lymph node T cells: functional role for thyroid-stimulating hormone during immune activation', *J Immunol*, 164(12), pp. 6158-65.

Bakdash, J.Z. and Marusich, L.R. (2017) 'Repeated Measures Correlation', *Front Psychol*, 8, p. 456.

Baker, K., Isaacs, J. and Thompson, B. (2015) 'E38. Restoring or Threatening a Normal Life: Withdrawing Medication from Patients with Rheumatoid Arthritis in Remission', *Rheumatology*, 54(suppl 1), p. i187.

Baker, K.F. (2014) *A pilot study of remission in rheumatoid arthritis from clinical and patient perspectives*. Unpublished MRes thesis. Newcastle University.

Baker, K.F. and Isaacs, J.D. (2014) 'Prospects for therapeutic tolerance in humans', *Curr Opin Rheumatol*, 26(2), pp. 219-27.

Baker, K.F. and Isaacs, J.D. (2018) 'Novel therapies for immune-mediated inflammatory diseases: What can we learn from their use in rheumatoid arthritis, spondyloarthritis, systemic lupus erythematosus, psoriasis, Crohn's disease and ulcerative colitis?', *Ann Rheum Dis*, 77(2), pp. 175-87.

Baker, K.F., Pratt, A.G., Thompson, B. and Isaacs, J.D. (2017) 'Let's not fool ourselves. In RA, the ACR/EULAR remission criteria are not perfect!', *Ann Rheum Dis*, 76(6), p. e12.

Bakker, M.F., Jacobs, J.W., Kruize, A.A., van der Veen, M.J., van Booma-Frankfort, C., Vreugdenhil, S.A., Bijlsma, J.W., Lafeber, F.P. and Welsing, P.M. (2012) 'Misclassification of disease activity when assessing individual patients with early rheumatoid arthritis using disease activity indices that do not include joints of feet', *Ann Rheum Dis*, 71(6), pp. 830-5.

Balsa, A., de Miguel, E., Castillo, C., Peiteado, D. and Martin-Mola, E. (2010) 'Superiority of SDAI over DAS-28 in assessment of remission in rheumatoid arthritis patients using power Doppler ultrasonography as a gold standard', *Rheumatology (Oxford)*, 49(4), pp. 683-90.

Baron, D., Ramstein, G., Chesneau, M., Echasseriau, Y., Pallier, A., Paul, C., Degauque, N., Hernandez-Fuentes, M.P., Sanchez-Fueyo, A., Newell, K.A., Giral, M., Soulillou, J.P., Houlgatte, R. and Brouard, S. (2015) 'A common gene signature across multiple studies relate biomarkers and functional regulation in tolerance to renal allograft', *Kidney Int*, 87(5), pp. 984-95.

Bartoli, C., Civatte, M., Pellissier, J. and Figarella-Branger, D. (2001) 'CCR2A and CCR2B, the two isoforms of the monocyte chemoattractant protein-1 receptor are up-regulated and expressed by different cell subsets in idiopathic inflammatory myopathies', *Acta Neuropathologica*, 102(4), pp. 385-392.

Baslund, B., Tvede, N., Danneskiold-Samsoe, B., Larsson, P., Panayi, G., Petersen, J., Petersen, L.J., Beurskens, F.J., Schuurman, J., van de Winkel, J.G., Parren, P.W., Gracie, J.A., Jongbloed, S., Liew, F.Y. and McInnes, I.B. (2005) 'Targeting interleukin-15 in patients with rheumatoid arthritis: a proof-of-concept study', *Arthritis Rheum*, 52(9), pp. 2686-92.

Begovich, A.B., Carlton, V.E., Honigberg, L.A., Schrodi, S.J., Chokkalingam, A.P., Alexander, H.C., Ardlie, K.G., Huang, Q., Smith, A.M., Spoerke, J.M., Conn, M.T., Chang, M., Chang, S.Y., Saiki, R.K., Catanese, J.J., Leong, D.U., Garcia, V.E., McAllister, L.B., Jeffery, D.A., Lee, A.T., Batliwalla, F., Remmers, E., Criswell, L.A., Seldin, M.F., Kastner, D.L., Amos, C.I., Sninsky, J.J. and Gregersen, P.K. (2004) 'A missense single-nucleotide polymorphism in a gene encoding a protein tyrosine phosphatase (PTPN22) is associated with rheumatoid arthritis', *Am J Hum Genet*, 75(2), pp. 330-7.

Bellis, E., Scire, C.A., Carrara, G., Adinolfi, A., Batticciotto, A., Bortoluzzi, A., Cagnotto, G., Caprioli, M., Canzoni, M., Cavatorta, F.P., De Lucia, O., Di Sabatino, V., Draghessi, A., Filippou, G., Farina, I., Focherini, M.C., Gabba, A., Gutierrez, M., Idolazzi, L., Luccioli, F., Macchioni, P., Massarotti, M.S., Mastaglio, C., Menza, L., Muratore, M., Parisi, S., Picerno, V., Piga, M., Ramonda, R., Raffeiner, B., Rossi, D., Rossi, S., Rossini, P., Sakellariou, G., Scioscia, C., Venditti, C., Volpe, A., Matucci-Cerinic, M. and Iagnocco, A. (2016)

'Ultrasound-detected tenosynovitis independently associates with patient-reported flare in patients with rheumatoid arthritis in clinical remission: results from the observational study STARTER of the Italian Society for Rheumatology', *Rheumatology (Oxford)*, 55(10), pp. 1826-36.

Benjamini, Y. and Hochberg, Y. (1995) 'Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing', *Journal of the Royal Statistical Society Series B-Methodological*, 57(1), pp. 289-300.

Berkelaar, M.e.a. (2015) *lpSolve: Interface to 'Lp_solve' v. 5.5 to Solve Linear/Integer Programs. R package version 5.6.13.* [Computer program]. https://CRAN.R-project.org/package=lpSolve.

Betz, B.C., Jordan-Williams, K.L., Wang, C., Kang, S.G., Liao, J., Logan, M.R., Kim, C.H. and Taparowsky, E.J. (2010) 'Batf coordinates multiple aspects of B and T cell function required for normal antibody responses', *J Exp Med*, 207(5), pp. 933-42.

Béziat, V., Hilton, H.G., Norman, P.J. and Traherne, J.A. (2017) 'Deciphering the killer-cell immunoglobulin-like receptor system at super-resolution for natural killer and T-cell biology', *Immunology*, 150(3), pp. 248-264.

Bijlsma, J.W., Welsing, P.M., Woodworth, T.G., Middelink, L.M., Petho-Schramm, A., Bernasconi, C., Borm, M.E., Wortel, C.H., ter Borg, E.J., Jahangier, Z.N., van der Laan, W.H., Bruyn, G.A., Baudoin, P., Wijngaarden, S., Vos, P.A., Bos, R., Starmans, M.J., Griep, E.N., Griep-Wentink, J.R., Allaart, C.F., Heurkens, A.H., Teitsma, X.M., Tekstra, J., Marijnissen, A.C., Lafeber, F.P. and Jacobs, J.W. (2016) 'Early rheumatoid arthritis treated with tocilizumab, methotrexate, or their combination (U-Act-Early): a multicentre, randomised, double-blind, double-dummy, strategy trial', *Lancet*, 388(10042), pp. 343-55.

Bjørn-Helge, M., Wehrens, R. and Hovde Liland, K. (2016) *pls: Partial Least Squares and Principal Component Regression. R package version 2.6-0.* [Computer program]. https://CRAN.R-project.org/package=pls.

Bohne, F., Martinez-Llordella, M., Lozano, J.J., Miquel, R., Benitez, C., Londono, M.C., Manzia, T.M., Angelico, R., Swinkels, D.W., Tjalsma, H., Lopez, M., Abraldes, J.G., Bonaccorsi-Riani, E., Jaeckel, E., Taubert, R., Pirenne, J., Rimola, A., Tisone, G. and Sanchez-Fueyo, A. (2012) 'Intra-graft expression of genes involved in iron homeostasis predicts the development of operational tolerance in human liver transplantation', *J Clin Invest*, 122(1), pp. 368-82.

Bossert, M., Prati, C., Vidal, C., Bongain, S., Toussirot, E. and Wendling, D. (2012) 'Evaluation of self-report questionnaires for assessing rheumatoid arthritis activity: a cross-sectional study of RAPID3 and RADAI5 and flare detection in 200 patients', *Joint Bone Spine*, 79(1), pp. 57-62.

Bottini, N. and Firestein, G.S. (2013) 'Duality of fibroblast-like synoviocytes in RA: passive responders and imprinted aggressors', *Nat Rev Rheumatol*, 9(1), pp. 24-33.

Bouman, C.A.M., van der Maas, A., van Herwaarden, N., Sasso, E.H., van den Hoogen, F.H.J. and den Broeder, A.A. (2017) 'A multi-biomarker score measuring disease activity in rheumatoid arthritis patients tapering adalimumab or etanercept: predictive value for clinical and radiographic outcomes', *Rheumatology (Oxford)*, 56(6), pp. 973-80.

Bozdogan, H. (1987) 'Model selection and Akaike's Information Criterion (AIC): The general theory and its analytical extensions', *Psychometrika*, 52(3), pp. 345-70.

Bray, N.L., Pimentel, H., Melsted, P. and Pachter, L. (2016) 'Near-optimal probabilistic RNA-seq quantification', *Nat Biotech*, 34(5), pp. 525-7.

Breedveld, F. (2011) 'The value of early intervention in RA--a window of opportunity', *Clin Rheumatol*, 30 Suppl 1, pp. S33-9.

Brincks, E.L. and Woodland, D.L. (2010) 'Novel roles for IL-15 in T cell survival', *F1000 Biol Rep*, 2, p. 67.

Brocq, O., Millasseau, E., Albert, C., Grisot, C., Flory, P., Roux, C.H. and Euller-Ziegler, L. (2009) 'Effect of discontinuing TNFalpha antagonist therapy in patients with remission of rheumatoid arthritis', *Joint Bone Spine*, 76(4), pp. 350-5.

Brouard, S., Mansfield, E., Braud, C., Li, L., Giral, M., Hsieh, S.C., Baeten, D., Zhang, M., Ashton-Chess, J., Braudeau, C., Hsieh, F., Dupont, A., Pallier, A., Moreau, A., Louis, S., Ruiz, C., Salvatierra, O., Soulillou, J.P. and Sarwal, M. (2007) 'Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance', *Proc Natl Acad Sci U S A*, 104(39), pp. 15448-53.

Brown, A.K., Conaghan, P.G., Karim, Z., Quinn, M.A., Ikeda, K., Peterfy, C.G., Hensor, E., Wakefield, R.J., O'Connor, P.J. and Emery, P. (2008) 'An explanation for the apparent dissociation between clinical remission and continued structural deterioration in rheumatoid arthritis', *Arthritis Rheum*, 58(10), pp. 2958-67.

Brown, A.K., Quinn, M.A., Karim, Z., Conaghan, P.G., Peterfy, C.G., Hensor, E., Wakefield, R.J., O'Connor, P.J. and Emery, P. (2006) '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*, 54(12), pp. 3761-73.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. and Wiley, D.C. (1993) 'Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1', *Nature*, 364(6432), pp. 33-9.

Bruce, B. and Fries, J.F. (2003) 'The Stanford Health Assessment Questionnaire: dimensions and practical applications', *Health Qual Life Outcomes*, 1, p. 20.

Brugnoni, D., Airo, P. and Cattaneo, R. (1997) 'CD70 (CD27 ligand) expression by synovial fluid CD4+ T lymphocytes in rheumatoid arthritis: comment on the article by Kohem et al', *Arthritis Rheum*, 40(6), pp. 1186-8.

Bugatti, S., Manzo, A., Benaglio, F., Klersy, C., Vitolo, B., Todoerti, M., Sakellariou, G., Montecucco, C. and Caporali, R. (2012) 'Serum levels of CXCL13 are associated with ultrasonographic synovitis and predict power Doppler persistence in early rheumatoid arthritis treated with non-biological disease-modifying anti-rheumatic drugs', *Arthritis Res Ther*, 14(1), p. R34.

Burgoyne, C.H., Field, S.L., Brown, A.K., Hensor, E.M., English, A., Bingham, S.L., Verburg, R., Fearon, U., Lawson, C.A., Hamlin, P.J., Straszynski, L., Veale, D., Conaghan, P., Hull, M.A., van Laar, J.M., Tennant, A., Emery, P., Isaacs, J.D. and Ponchel, F. (2008) 'Abnormal T cell differentiation persists in patients with rheumatoid arthritis in clinical remission and predicts relapse', *Ann Rheum Dis*, 67(6), pp. 750-7.

Businaro, R., Leali, F.M., De Renzis, G., Pompili, E., Pagliari, G., Menghi, G. and Fumagalli, L. (1992) 'Inter-alpha-trypsin inhibitor-related immunoreactivity in human tissues and body fluids', *Cell Mol Biol*, 38(4), pp. 463-71.

Bykerk, V.P., Akhavan, P., Hazlewood, G.S., Schieir, O., Dooley, A., Haraoui, B., Khraishi, M., Leclercq, S.A., Legare, J., Mosher, D.P., Pencharz, J., Pope, J.E., Thomson, J., Thorne, C., Zummer, M. and Bombardier, C. (2012) 'Canadian Rheumatology Association recommendations for pharmacological management of rheumatoid arthritis with traditional and biologic disease-modifying antirheumatic drugs', *J Rheumatol*, 39(8), pp. 1559-82.

Cao, Y., Doodes, P.D., Glant, T.T. and Finnegan, A. (2008) 'IL-27 induces a Th1 immune response and susceptibility to experimental arthritis', *J Immunol*, 180(2), pp. 922-30.

Caporali, R. and Smolen, J.S. (2018) 'Back to the future: forget ultrasound and focus on clinical assessment in rheumatoid arthritis management', *Ann Rheum Dis*, 77(1), pp. 18-20.

Castrejon, I., Dougados, M., Combe, B., Guillemin, F., Fautrel, B. and Pincus, T. (2013) 'Can remission in rheumatoid arthritis be assessed without laboratory tests or a formal joint count? possible remission criteria based on a self-report RAPID3 score and careful joint examination in the ESPOIR cohort', *J Rheumatol*, 40(4), pp. 386-93.

Centola, M., Cavet, G., Shen, Y., Ramanujan, S., Knowlton, N., Swan, K.A., Turner, M., Sutton, C., Smith, D.R., Haney, D.J., Chernoff, D., Hesterberg, L.K., Carulli, J.P., Taylor, P.C., Shadick, N.A., Weinblatt, M.E. and Curtis, J.R. (2013) 'Development of a multi-biomarker disease activity test for rheumatoid arthritis', *PLoS One*, 8(4), p. e60635.

Chatzidionysiou, K., Turesson, C., Teleman, A., Knight, A., Lindqvist, E., Larsson, P., Cöster, L., Forslind, K., van Vollenhoven, R. and Heimbürger, M. (2016) 'A multicentre, randomised, controlled, open-label pilot study

on the feasibility of discontinuation of adalimumab in established patients with rheumatoid arthritis in stable clinical remission', *RMD Open*, 2(1).

Chen, C.Y., Fuh, L.J., Huang, C.C., Hsu, C.J., Su, C.M., Liu, S.C., Lin, Y.M. and Tang, C.H. (2017) 'Enhancement of CCL2 expression and monocyte migration by CCN1 in osteoblasts through inhibiting miR-518a-5p: implication of rheumatoid arthritis therapy', *Sci Rep*, 7(1), p. 421.

Chen, H. (2016) *VennDiagram: Generate High-Resolution Venn and Euler Plots. R package version 1.6.17.* [Computer program]. https://CRAN.R-project.org/package=VennDiagram.

Chen, S., Deng, C., Hu, C., Li, J., Wen, X., Wu, Z., Li, Y., Zhang, F. and Li, Y. (2016) 'Association of MCP-1-2518A/G polymorphism with susceptibility to autoimmune diseases: a meta-analysis', *Clin Rheumatol*, 35(5), pp. 1169-79.

Chen, Z., Qiu, X. and Gu, J. (2009) 'Immunoglobulin expression in non-lymphoid lineage and neoplastic cells', *Am J Pathol*, 174(4), pp. 1139-48.

Cheung, P.P., Mari, K., Devauchelle-Pensec, V., Jousse-Joulin, S., D'Agostino, M.A., Chales, G., Gaudin, P., Mariette, X., Saraux, A. and Dougados, M. (2016) 'Predictive value of tender joints compared to synovitis for structural damage in rheumatoid arthritis', *RMD Open*, 2(1), p. e000205.

Cho, M.L., Yoon, B.Y., Ju, J.H., Jung, Y.O., Jhun, J.Y., Park, M.K., Park, S.H., Cho, C.S. and Kim, H.Y. (2007) 'Expression of CCR2A, an isoform of MCP-1 receptor, is increased by MCP-1, CD40 ligand and TGF-beta in fibroblast like synoviocytes of patients with RA', *Exp Mol Med*, 39(4), pp. 499-507.

Choi, H.K., Kang, H.R., Jung, E., Kim, T.E., Lin, J.J. and Lee, S.Y. (2013) 'Early estrogen-induced gene 1, a novel RANK signaling component, is essential for osteoclastogenesis', *Cell Res*, 23(4), pp. 524-36.

Chong, W.P., van Panhuys, N., Chen, J., Silver, P.B., Jittayasothorn, Y., Mattapallil, M.J., Germain, R.N. and Caspi, R.R. (2015) 'NK-DC crosstalk controls the autopathogenic Th17 response through an innate IFN-gamma-IL-27 axis', *J Exp Med*, 212(10), pp. 1739-52.

Christensen, R.H.B. (2015) 'ordinal - Regression Models for Ordinal Data. R package version 2015.6-28'. http://www.cran.r-project.org/package=ordinal/.

Clemente, J.C., Manasson, J. and Scher, J.U. (2018) 'The role of the gut microbiome in systemic inflammatory disease', *BMJ*, 360, p. j5145.

Cohen, M.D. and Keystone, E. (2015) 'Rituximab for Rheumatoid Arthritis', *Rheumatol Ther*, 2(2), pp. 99-111.

Colucci, F. and Traherne, J. (2017) 'Killer-cell immunoglobulin-like receptors on the cusp of modern immunogenetics', *Immunology*, 152(4), pp. 556-561.

Connolly, M., Rooney, P.R., McGarry, T., Maratha, A.X., McCormick, J., Miggin, S.M., Veale, D.J. and Fearon, U. (2016) 'Acute serum amyloid A is an endogenous TLR2 ligand that mediates inflammatory and angiogenic mechanisms', *Ann Rheum Dis*, 75(7), pp. 1392-8.

Cross, M., Smith, E., Hoy, D., Carmona, L., Wolfe, F., Vos, T., Williams, B., Gabriel, S., Lassere, M., Johns, N., Buchbinder, R., Woolf, A. and March, L. (2014) 'The global burden of rheumatoid arthritis: estimates from the global burden of disease 2010 study', *Ann Rheum Dis*, 73(7), pp. 1316-22.

Curtis, J.R., Brahe, C.H., Ostergaard, M., Hetland, M.L., Hambardzumyan, K., Saevarsdottir, S., Wang, X., Sasso, E.H. and Huizinga, T.W. (2017) 'THU0091 High multi-biomarker disease activity score is associated with high risk of radiographic progression in six studies [abstract]', *Ann Rheum Dis*, 76(Suppl 2), p. 234.

Cylwik, B., Chrostek, L., Gindzienska-Sieskiewicz, E., Sierakowski, S. and Szmitkowski, M. (2010) 'Relationship between serum acute-phase proteins and high disease activity in patients with rheumatoid arthritis', *Adv Med Sci*, 55(1), pp. 80-5.

D'Agostino, M.A., Boers, M., Wakefield, R.J., Berner Hammer, H., Vittecoq, O., Filippou, G., Balint, P., Moller, I., Iagnocco, A., Naredo, E., Ostergaard, M., Gaillez, C. and Le Bars, M. (2016a) 'Exploring a new ultrasound

score as a clinical predictive tool in patients with rheumatoid arthritis starting abatacept: results from the APPRAISE study', *RMD Open*, 2(1), p. e000237.

D'Agostino, M.A., Terslev, L., Wakefield, R., Ostergaard, M., Balint, P., Naredo, E., Iagnocco, A., Backhaus, M., Grassi, W. and Emery, P. (2016b) 'Novel algorithms for the pragmatic use of ultrasound in the management of patients with rheumatoid arthritis: from diagnosis to remission', *Ann Rheum Dis*, 75(11), pp. 1902-8.

D'Agostino, M.A., Wakefield, R.J., Berner-Hammer, H., Vittecoq, O., Filippou, G., Balint, P., Moller, I., Iagnocco, A., Naredo, E., Ostergaard, M., Boers, M., Gaillez, C., Van Holder, K., Le Bars, M. and OMERACT-EULAR-Ultrasound Task Force (2016c) 'Value of ultrasonography as a marker of early response to abatacept in patients with rheumatoid arthritis and an inadequate response to methotrexate: results from the APPRAISE study', *Ann Rheum Dis*, 75(10), pp. 1763-9.

Dadoun, S., Zeboulon-Ktorza, N., Combescure, C., Elhai, M., Rozenberg, S., Gossec, L. and Fautrel, B. (2013) 'Mortality in rheumatoid arthritis over the last fifty years: systematic review and meta-analysis', *Joint Bone Spine*, 80(1), pp. 29-33.

Dale, J., Alcorn, N., Capell, H. and Madhok, R. (2007) 'Combination therapy for rheumatoid arthritis: methotrexate and sulfasalazine together or with other DMARDs', *Nat Clin Pract Rheumatol*, 3(8), pp. 450-8.

Dale, J. and Porter, D. (2010) 'Pharmacotherapy: concepts of pathogenesis and emerging treatments. Optimising the strategy of care in early rheumatoid arthritis', *Best Pract Res Clin Rheumatol*, 24(4), pp. 443-55.

Dale, J., Stirling, A., Zhang, R., Purves, D., Foley, J., Sambrook, M., Conaghan, P.G., van der Heijde, D., McConnachie, A., McInnes, I.B. and Porter, D. (2016) 'Targeting ultrasound remission in early rheumatoid arthritis: the results of the TaSER study, a randomised clinical trial', *Ann Rheum Dis*, 75(6), pp. 1043-50.

Dales, L.G. and Ury, H.K. (1978) 'An improper use of statistical significance testing in studying covariables', *Int J Epidemiol*, 7(4), pp. 373-5.

De Graaf, T.W., Van Ommen, E.C., Van der Stelt, M.E., Kerstens, P.J., Boerbooms, A.M. and Van Dijk, W. (1994) 'Effects of low dose methotrexate therapy on the concentration and the glycosylation of alpha 1-acid glycoprotein in the serum of patients with rheumatoid arthritis: a longitudinal study.', *J Rheumatol*, 21(12), pp. 2209-16.

de Pablo, P., Romaguera, D., Fisk, H.L., Calder, P.C., Quirke, A.M., Cartwright, A.J., Panico, S., Mattiello, A., Gavrila, D., Navarro, C., Sacerdote, C., Vineis, P., Tumino, R., Ollier, W.E., Michaud, D.S., Riboli, E., Venables, P.J. and Fisher, B.A. (2018) 'High erythrocyte levels of the n-6 polyunsaturated fatty acid linoleic acid are associated with lower risk of subsequent rheumatoid arthritis in a southern European nested case-control study', *Ann Rheum Dis*, Published Online First: 07 February 2018. doi: 10.1136/annrheumdis-2017-212274.

De Silva, M. and Hazleman, B.L. (1981) 'Long-term azathioprine in rheumatoid arthritis: a double-blind study', *Ann Rheum Dis*, 40(6), pp. 560-3.

DeLong, E.R., DeLong, D.M. and Clarke-Pearson, D.L. (1988) 'Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach', *Biometrics*, 44(3), pp. 837-45.

Deshmane, S.L., Kremlev, S., Amini, S. and Sawaya, B.E. (2009) 'Monocyte chemoattractant protein-1 (MCP-1): an overview', *J Interferon Cytokine Res*, 29(6), pp. 313-26.

Diogo, D., Okada, Y. and Plenge, R.M. (2014) 'Genome-wide association studies to advance our understanding of critical cell types and pathways in rheumatoid arthritis: recent findings and challenges', *Curr Opin Rheumatol*, 26(1), pp. 85-92.

dOliveira, I., Mensing, W., Figueiredo, C., Englbrecht, M., Haschka, J., Hueber, A., Kleyer, A., Cavallaro, A., Schett, G. and Rech, J. (2016) 'FRI0082 Subclinical MRI Inflammation Does Not Predict Relapse Risk in Rheumatoid Arthritis Patients Tapering Dmards [abstract]', *Ann Rheum Dis*, 75(Suppl 2), p. 456.

Durinck, S., Spellman, P.T., Birney, E. and Huber, W. (2009) 'Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt', *Nat Protoc*, 4(8), pp. 1184-91.

Eastgate, J.A., Symons, J.A., Wood, N.C., Grinlinton, F.M., di Giovine, F.S. and Duff, G.W. (1988) 'Correlation of plasma interleukin 1 levels with disease activity in rheumatoid arthritis', *Lancet*, 2(8613), pp. 706-9.

El Miedany, Y., El Gaafary, M., Youssef, S., Ahmed, I., Bahlas, S., Hegazi, M. and Nasr, A. (2016) 'Optimizing therapy in inflammatory arthritis: prediction of relapse after tapering or stopping treatment for rheumatoid arthritis patients achieving clinical and radiological remission', *Clin Rheumatol*, 35(12), pp. 2915-23.

Elliott, M.J., Maini, R.N., Feldmann, M., Kalden, J.R., Antoni, C., Smolen, J.S., Leeb, B., Breedveld, F.C., Macfarlane, J.D., Bijl, H. and et al. (1994) 'Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (cA2) versus placebo in rheumatoid arthritis', *Lancet*, 344(8930), pp. 1105-10.

Emery, P., Burmester, G.R., Bykerk, V.P., Combe, B.G., Furst, D.E., Barre, E., Karyekar, C.S., Wong, D.A. and Huizinga, T.W. (2015) 'Evaluating drug-free remission with abatacept in early rheumatoid arthritis: results from the phase 3b, multicentre, randomised, active-controlled AVERT study of 24 months, with a 12-month, double-blind treatment period', *Ann Rheum Dis*, 74(1), pp. 19-26.

Emery, P., Hammoudeh, M., FitzGerald, O., Combe, B., Martin-Mola, E., Buch, M.H., Krogulec, M., Williams, T., Gaylord, S., Pedersen, R., Bukowski, J. and Vlahos, B. (2014) 'Sustained remission with etanercept tapering in early rheumatoid arthritis', *N Engl J Med*, 371(19), pp. 1781-92.

Eriksson, J.K., Neovius, M., Ernestam, S., Lindblad, S., Simard, J.F. and Askling, J. (2013) 'Incidence of rheumatoid arthritis in Sweden: a nationwide population-based assessment of incidence, its determinants, and treatment penetration', *Arthritis Care Res (Hoboken)*, 65(6), pp. 870-8.

Espersen, G.T., Vestergaard, M., Ernst, E. and Grunnet, N. (1991) 'Tumour necrosis factor alpha and interleukin-2 in plasma from rheumatoid arthritis patients in relation to disease activity', *Clin Rheumatol*, 10(4), pp. 374-6.

Ewels, P., Magnusson, M., Lundin, S. and Kaller, M. (2016) 'MultiQC: summarize analysis results for multiple tools and samples in a single report', *Bioinformatics*, 32(19), pp. 3047-8.

Falco, M.M., Bleda, M., Carbonell-Caballero, J. and Dopazo, J. (2016) 'The pan-cancer pathological regulatory landscape', *Sci Rep*, 6, p. 39709.

Fan, Q. and Liu, B. (2016) 'Identification of a RNA-Seq Based 8-Long Non-Coding RNA Signature Predicting Survival in Esophageal Cancer', *Med Sci Monit*, 22, pp. 5163-72.

Fautrel, B. and den Broeder, A.A. (2015) 'De-intensifying treatment in established rheumatoid arthritis (RA): Why, how, when and in whom can DMARDs be tapered?', *Best Pract Res Clin Rheumatol*, 29(4-5), pp. 550-65.

Fautrel, B., Morel, J., Berthelot, J.M., Constantin, A., De Bandt, M., Gaudin, P., Maillefert, J.F., Meyer, O., Pham, T., Saraux, A., Solau-Gervais, E., Vittecoq, O., Wendling, D., Erpelding, M.L. and Guillemin, F. (2017) 'Validation of FLARE-RA, a Self-Administered Tool to Detect Recent or Current Rheumatoid Arthritis Flare', *Arthritis Rheumatol*, 69(2), pp. 309-19.

Fautrel, B., Pham, T., Alfaiate, T., Gandjbakhch, F., Foltz, V., Morel, J., Dernis, E., Gaudin, P., Brocq, O., Solau-Gervais, E., Berthelot, J.M., Balblanc, J.C., Mariette, X. and Tubach, F. (2016) 'Step-down strategy of spacing TNF-blocker injections for established rheumatoid arthritis in remission: results of the multicentre non-inferiority randomised open-label controlled trial (STRASS: Spacing of TNF-blocker injections in Rheumatoid ArthritiS Study)', *Ann Rheum Dis*, 75(1), pp. 59-67.

Feist, E. and Burmester, G.R. (2013) 'Rheumatoid arthritis—clinical features ', in Watts, R.A., Conaghan, P.G., Denton, C., Foster, H., Isaacs, J.D. and Müller-Ladner, U. (eds.) *Oxford Textbook of Rheumatology*. 4th edn. Oxford: Oxford University Press.

Felson, D.T., Smolen, J.S., Wells, G., Zhang, B., van Tuyl, L.H., Funovits, J., Aletaha, D., Allaart, C.F., Bathon, J., Bombardieri, S., Brooks, P., Brown, A., Matucci-Cerinic, M., Choi, H., Combe, B., de Wit, M., Dougados, M., Emery, P., Furst, D., Gomez-Reino, J., Hawker, G., Keystone, E., Khanna, D., Kirwan, J., Kvien, T.K., Landewe, R., Listing, J., Michaud, K., Martin-Mola, E., Montie, P., Pincus, T., Richards, P., Siegel, J.N., Simon, L.S., Sokka, T., Strand, V., Tugwell, P., Tyndall, A., van der Heijde, D., Verstappen, S., White, B., Wolfe, F., Zink, A. and Boers, M. (2011) 'American College of Rheumatology/European League Against Rheumatism provisional definition of remission in rheumatoid arthritis for clinical trials', *Arthritis Rheum*, 63(3), pp. 573-86.

Fernando, M.M., Stevens, C.R., Walsh, E.C., De Jager, P.L., Goyette, P., Plenge, R.M., Vyse, T.J. and Rioux, J.D. (2008) 'Defining the role of the MHC in autoimmunity: a review and pooled analysis', *PLoS Genet*, 4(4), p. e1000024.

Ferrari-Lacraz, S., Zanelli, E., Neuberg, M., Donskoy, E., Kim, Y.S., Zheng, X.X., Hancock, W.W., Maslinski, W., Li, X.C., Strom, T.B. and Moll, T. (2004) 'Targeting IL-15 receptor-bearing cells with an antagonist mutant IL-15/Fc protein prevents disease development and progression in murine collagen-induced arthritis', *J Immunol*, 173(9), pp. 5818-26.

Figueiredo, C.P., Bang, H., Cobra, J.F., Englbrecht, M., Hueber, A.J., Haschka, J., Manger, B., Kleyer, A., Reiser, M., Finzel, S., Tony, H.P., Kleinert, S., Wendler, J., Schuch, F., Ronneberger, M., Feuchtenberger, M., Fleck, M., Manger, K., Ochs, W., Schmitt-Haendle, M., Lorenz, H.M., Nuesslein, H., Alten, R., Henes, J., Krueger, K., Rech, J. and Schett, G. (2017) 'Antimodified protein antibody response pattern influences the risk for disease relapse in patients with rheumatoid arthritis tapering disease modifying antirheumatic drugs', *Ann Rheum Dis*, 76(2), pp. 399-407.

Filkova, M., Cope, A., Mant, T. and Galloway, J. (2016) 'Is there a role of synovial biopsy in drug development?', *BMC Musculoskelet Disord*, 17, p. 172.

Finckh, A., Liang, M.H., van Herckenrode, C.M. and de Pablo, P. (2006) 'Long-term impact of early treatment on radiographic progression in rheumatoid arthritis: A meta-analysis', *Arthritis Rheum*, 55(6), pp. 864-72.

Fish, R.J. and Neerman-Arbez, M. (2012) 'Fibrinogen gene regulation', *Thromb Haemost*, 108(3), pp. 419-26.

Fleischmann, R., Connolly, S.E., Maldonado, M.A. and Schiff, M. (2016) 'Brief Report: Estimating Disease Activity Using Multi-Biomarker Disease Activity Scores in Rheumatoid Arthritis Patients Treated With Abatacept or Adalimumab', *Arthritis Rheumatol*, 68(9), pp. 2083-9.

Fleischmann, R., van der Heijde, D., Koenig, A.S., Pedersen, R., Szumski, A., Marshall, L. and Bananis, E. (2015) 'How much does Disease Activity Score in 28 joints ESR and CRP calculations underestimate disease activity compared with the Simplified Disease Activity Index?', *Ann Rheum Dis*, 74(6), pp. 1132-7.

Fleischmann, R.M., van der Heijde, D., Gardiner, P.V., Szumski, A., Marshall, L. and Bananis, E. (2017) 'DAS28-CRP and DAS28-ESR cut-offs for high disease activity in rheumatoid arthritis are not interchangeable', *RMD Open*, 3(1), p. e000382.

Foltz, V., Gandjbakhch, F., Etchepare, F., Rosenberg, C., Tanguy, M.L., Rozenberg, S., Bourgeois, P. and Fautrel, B. (2012) 'Power Doppler ultrasound, but not low-field magnetic resonance imaging, predicts relapse and radiographic disease progression in rheumatoid arthritis patients with low levels of disease activity', *Arthritis Rheum*, 64(1), pp. 67-76.

Forsyth, C., Kouvari, M., D'Cunha, N.M., Georgousopoulou, E.N., Panagiotakos, D.B., Mellor, D.D., Kellett, J. and Naumovski, N. (2017) 'The effects of the Mediterranean diet on rheumatoid arthritis prevention and treatment: a systematic review of human prospective studies', *Rheumatol Int*, Epub ahead of print 18 Dec 2017. doi: 10.1007/s00296-017-3912-1.

Fournier, T., Medjoubi, N.N. and Porquet, D. (2000) 'Alpha-1-acid glycoprotein', *Biochim Biophys Acta*, 1482(1-2), pp. 157-71.

Frank-Bertoncelj, M. and Gay, S. (2014) 'The epigenome of synovial fibroblasts: an underestimated therapeutic target in rheumatoid arthritis', *Arthritis Res Ther*, 16(3), p. 117.

Fransen, J., Creemers, M.C. and Van Riel, P.L. (2004) 'Remission in rheumatoid arthritis: agreement of the disease activity score (DAS28) with the ARA preliminary remission criteria', *Rheumatology (Oxford)*, 43(10), pp. 1252-5.

Fransen, J., Welsing, P.M.J., De Keijzer, R.M.H. and van Riel, P.L.C.M. (2003) 'Development and validation of the DAS28 using CRP [abstract]', *Ann Rheum Dis*, 62(Suppl. 1), p. 10.

Freeston, J.E., Wakefield, R.J., Conaghan, P.G., Hensor, E.M., Stewart, S.P. and Emery, P. (2010) 'A diagnostic algorithm for persistence of very early inflammatory arthritis: the utility of power Doppler ultrasound when added to conventional assessment tools', *Ann Rheum Dis*, 69(2), pp. 417-9.

Fries, E. and Blom, A.M. (2000) 'Bikunin — not just a plasma proteinase inhibitor', *Int J Biochem Cell Biol*, 32(2), pp. 125-37.

Fujii, H., Shao, L., Colmegna, I., Goronzy, J.J. and Weyand, C.M. (2009) 'Telomerase insufficiency in rheumatoid arthritis', *Proc Natl Acad Sci U S A*, 106(11), pp. 4360-5.

Fukae, J., Tanimura, K., Atsumi, T. and Koike, T. (2014) 'Sonographic synovial vascularity of synovitis in rheumatoid arthritis', *Rheumatology (Oxford)*, 53(4), pp. 586-91.

Fukuda, M., Kanno, E., Saegusa, C., Ogata, Y. and Kuroda, T.S. (2002) 'Slp4-a/granuphilin-a regulates dense-core vesicle exocytosis in PC12 cells', *J Biol Chem*, 277(42), pp. 39673-8.

Fullerton, B., Jeitler, K., Seitz, M., Horvath, K., Berghold, A. and Siebenhofer, A. (2014) 'Intensive glucose control versus conventional glucose control for type 1 diabetes mellitus', *Cochrane Database Syst Rev*, 2, p. Cd009122.

Gamer, M., Lemon, J., Fellows, I. and Singh, P. (2012) *irr: Various Coefficients of Interrater Reliability and Agreement. R package version 0.84.* [Computer program]. https://CRAN.R-project.org/package=irr.

Gandjbakhch, F., Foltz, V., Mallet, A., Bourgeois, P. and Fautrel, B. (2011) 'Bone marrow oedema predicts structural progression in a 1-year follow-up of 85 patients with RA in remission or with low disease activity with low-field MRI', *Ann Rheum Dis*, 70(12), pp. 2159-62.

Gandjbakhch, F., Haavardsholm, E.A., Conaghan, P.G., Ejbjerg, B., Foltz, V., Brown, A.K., Dohn, U.M., Lassere, M., Freeston, J.E., Olsen, I.C., Boyesen, P., Bird, P., Fautrel, B., Hetland, M.L., Emery, P., Bourgeois, P., Horslev-Petersen, K., Kvien, T.K., McQueen, F.M. and Ostergaard, M. (2014) 'Determining a magnetic resonance imaging inflammatory activity acceptable state without subsequent radiographic progression in rheumatoid arthritis: results from a followup MRI study of 254 patients in clinical remission or low disease activity', *J Rheumatol*, 41(2), pp. 398-406.

Ganguly, D., Haak, S., Sisirak, V. and Reizis, B. (2013) 'The role of dendritic cells in autoimmunity', *Nat Rev Immunol*, 13(8), pp. 566-77.

Gartner, M., Alasti, F., Supp, G., Mandl, P., Smolen, J.S. and Aletaha, D. (2015) 'Persistence of subclinical sonographic joint activity in rheumatoid arthritis in sustained clinical remission', *Ann Rheum Dis*, 74(11), pp. 2050-3.

Gaujoux-Viala, C., Nam, J., Ramiro, S., Landewe, R., Buch, M.H., Smolen, J.S. and Gossec, L. (2014) 'Efficacy of conventional synthetic disease-modifying antirheumatic drugs, glucocorticoids and tofacitinib: a systematic literature review informing the 2013 update of the EULAR recommendations for management of rheumatoid arthritis', *Ann Rheum Dis*, 73(3), pp. 510-5.

Geng, Y., Han, J., Deng, X. and Zhang, Z. (2014) 'Presence of power Doppler synovitis in rheumatoid arthritis patients with synthetic and/or biological disease-modifying anti-rheumatic drug-induced clinical remission: experience from a Chinese cohort', *Clin Rheumatol*, 33(8), pp. 1061-6.

Geng, Y., Han, J., Deng, X. and Zhang, Z. (2016) 'Deep clinical remission: an optimised target in the management of rheumatoid arthritis? Experience from an ultrasonography study', *Clin Exp Rheumatol*, 34(4), pp. 581-6.

Ghiti Moghadam, M., Vonkeman, H.E., Ten Klooster, P.M., Tekstra, J., van Schaardenburg, D., Starmans-Kool, M., Brouwer, E., Bos, R., Lems, W.F., Colin, E.M., Allaart, C.F., Meek, I.L., Landewe, R., Bernelot Moens, H.J., van Riel, P.L., van de Laar, M.A., Jansen, T.L. and on behalf of the Dutch National POET Collaboration (2016) 'Stopping Tumor Necrosis Factor Inhibitor Treatment in Patients With Established Rheumatoid Arthritis in Remission or With Stable Low Disease Activity: A Pragmatic Multicenter, Open-Label Randomized Controlled Trial', *Arthritis Rheumatol*, 68(8), pp. 1810-7.

Gizinski, A.M. and Fox, D.A. (2014) 'T cell subsets and their role in the pathogenesis of rheumatic disease', *Curr Opin Rheumatol*, 26(2), pp. 204-10.

Glasmacher, E., Agrawal, S., Chang, A.B., Murphy, T.L., Zeng, W., Vander Lugt, B., Khan, A.A., Ciofani, M., Spooner, C.J., Rutz, S., Hackney, J., Nurieva, R., Escalante, C.R., Ouyang, W., Littman, D.R., Murphy, K.M.

and Singh, H. (2012) 'A genomic regulatory element that directs assembly and function of immune-specific AP-1-IRF complexes', *Science*, 338(6109), pp. 975-80.

Goekoop-Ruiterman, Y.P., de Vries-Bouwstra, J.K., Allaart, C.F., van Zeben, D., Kerstens, P.J., Hazes, J.M., Zwinderman, A.H., Ronday, H.K., Han, K.H., Westedt, M.L., Gerards, A.H., van Groenendael, J.H., Lems, W.F., van Krugten, M.V., Breedveld, F.C. and Dijkmans, B.A. (2005) 'Clinical and radiographic outcomes of four different treatment strategies in patients with early rheumatoid arthritis (the BeSt study): a randomized, controlled trial', *Arthritis Rheum*, 52(11), pp. 3381-90.

Gonzalez-Alvaro, I., Ortiz, A.M., Garcia-Vicuna, R., Balsa, A., Pascual-Salcedo, D. and Laffon, A. (2003) 'Increased serum levels of interleukin-15 in rheumatoid arthritis with long- term disease', *Clin Exp Rheumatol*, 21(5), pp. 639-42.

Gonzalez, A., Maradit Kremers, H., Crowson, C.S., Nicola, P.J., Davis, J.M., 3rd, Therneau, T.M., Roger, V.L. and Gabriel, S.E. (2007) 'The widening mortality gap between rheumatoid arthritis patients and the general population', *Arthritis Rheum*, 56(11), pp. 3583-7.

Gordon, M. and Lumley, T. (2017) *forestplot: Advanced Forest Plot Using 'grid' Graphics. R package version 1.7.* [Computer program]. https://CRAN.R-project.org/package=forestplot.

Gotzsche, P.C., Hansen, M., Stoltenberg, M., Svendsen, A., Beier, J., Faarvang, K.L., Wangel, M., Rydgren, L., Halberg, P., Juncker, P., Andersen, V., Hansen, T.M. and Endahl, L. (1996) 'Randomized, placebo controlled trial of withdrawal of slow-acting antirheumatic drugs and of observer bias in rheumatoid arthritis', *Scand J Rheumatol*, 25(4), pp. 194-9.

Grassi, W. and Filippucci, E. (2003) 'Is power Doppler sonography the new frontier in therapy monitoring?', *Clin Exp Rheumatol*, 21(4), pp. 424-8.

Grassi, W., Filippucci, E. and Busilacchi, P. (2004) 'Musculoskeletal ultrasound', *Best Pract Res Clin Rheumatol*, 18(6), pp. 813-26.

Gregersen, P.K., Silver, J. and Winchester, R.J. (1987) 'The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis', *Arthritis Rheum*, 30(11), pp. 1205-13.

Grewal, J.S., Tsai, J.Y. and Khan, S.R. (2005) 'Oxalate-inducible AMBP gene and its regulatory mechanism in renal tubular epithelial cells', *Biochem J*, 387(Pt 3), pp. 609-16.

Grigor, C., Capell, H., Stirling, A., McMahon, A.D., Lock, P., Vallance, R., Kincaid, W. and Porter, D. (2004) 'Effect of a treatment strategy of tight control for rheumatoid arthritis (the TICORA study): a single-blind randomised controlled trial', *Lancet*, 364(9430), pp. 263-9.

Gullick, N.J., Evans, H.G., Church, L.D., Jayaraj, D.M., Filer, A., Kirkham, B.W. and Taams, L.S. (2010) 'Linking power Doppler ultrasound to the presence of th17 cells in the rheumatoid arthritis joint', *PLoS One*, 5(9), p. e12516.

Guo, S., Zhu, Q., Jiang, T., Wang, R., Shen, Y., Zhu, X., Wang, Y., Bai, F., Ding, Q., Zhou, X., Chen, G. and He, D.Y. (2017) 'Genome-wide DNA methylation patterns in CD4+ T cells from Chinese Han patients with rheumatoid arthritis', *Mod Rheumatol*, 27(3), pp. 441-447.

Haavardsholm, E.A., Aga, A.B., Olsen, I.C., Lillegraven, S., Hammer, H.B., Uhlig, T., Fremstad, H., Madland, T.M., Lexberg, A.S., Haukeland, H., Rodevand, E., Hoili, C., Stray, H., Noraas, A., Hansen, I.J., Bakland, G., Nordberg, L.B., van der Heijde, D. and Kvien, T.K. (2016) 'Ultrasound in management of rheumatoid arthritis: ARCTIC randomised controlled strategy trial', *BMJ*, 354, p. i4205.

Haavardsholm, E.A., Lie, E. and Lillegraven, S. (2012) 'Should modern imaging be part of remission criteria in rheumatoid arthritis?', *Best Pract Res Clin Rheumatol*, 26(6), pp. 767-85.

Hall, A.O., Beiting, D.P., Tato, C., John, B., Oldenhove, G., Lombana, C.G., Pritchard, G.H., Silver, J.S., Bouladoux, N., Stumhofer, J.S., Harris, T.H., Grainger, J., Wojno, E.D., Wagage, S., Roos, D.S., Scott, P., Turka, L.A., Cherry, S., Reiner, S.L., Cua, D., Belkaid, Y., Elloso, M.M. and Hunter, C.A. (2012) 'The cytokines

interleukin 27 and interferon-gamma promote distinct Treg cell populations required to limit infection-induced pathology', *Immunity*, 37(3), pp. 511-23.

Hammer, H.B., Bolton-King, P., Bakkeheim, V., Berg, T.H., Sundt, E., Kongtorp, A.K. and Haavardsholm, E.A. (2011) 'Examination of intra and interrater reliability with a new ultrasonographic reference atlas for scoring of synovitis in patients with rheumatoid arthritis', *Ann Rheum Dis*, 70(11), pp. 1995-8.

Hammer, H.B. and Kvien, T.K. (2011) 'Comparisons of 7- to 78-joint ultrasonography scores: all different joint combinations show equal response to adalimumab treatment in patients with rheumatoid arthritis', *Arthritis Res Ther*, 13(3), p. R78.

Hammer, H.B., Sveinsson, M., Kongtorp, A.K. and Kvien, T.K. (2010) 'A 78-joints ultrasonographic assessment is associated with clinical assessments and is highly responsive to improvement in a longitudinal study of patients with rheumatoid arthritis starting adalimumab treatment', *Ann Rheum Dis*, 69(7), pp. 1349-51.

Han, B.K., Olsen, N.J. and Bottaro, A. (2016a) 'The CD27-CD70 pathway and pathogenesis of autoimmune disease', *Semin Arthritis Rheum*, 45(4), pp. 496-501.

Han, J., Geng, Y., Deng, X. and Zhang, Z. (2016b) 'Subclinical Synovitis Assessed by Ultrasound Predicts Flare and Progressive Bone Erosion in Rheumatoid Arthritis Patients with Clinical Remission: A Systematic Review and Metaanalysis', *J Rheumatol*, 43(11), pp. 2010-8.

Harrow, J., Frankish, A., Gonzalez, J.M., Tapanari, E., Diekhans, M., Kokocinski, F., Aken, B.L., Barrell, D., Zadissa, A., Searle, S., Barnes, I., Bignell, A., Boychenko, V., Hunt, T., Kay, M., Mukherjee, G., Rajan, J., Despacio-Reyes, G., Saunders, G., Steward, C., Harte, R., Lin, M., Howald, C., Tanzer, A., Derrien, T., Chrast, J., Walters, N., Balasubramanian, S., Pei, B., Tress, M., Rodriguez, J.M., Ezkurdia, I., van Baren, J., Brent, M., Haussler, D., Kellis, M., Valencia, A., Reymond, A., Gerstein, M., Guigo, R. and Hubbard, T.J. (2012) 'GENCODE: the reference human genome annotation for The ENCODE Project', *Genome Res*, 22(9), pp. 1760-74.

Haschka, J., Englbrecht, M., Hueber, A.J., Manger, B., Kleyer, A., Reiser, M., Finzel, S., Tony, H.P., Kleinert, S., Feuchtenberger, M., Fleck, M., Manger, K., Ochs, W., Schmitt-Haendle, M., Wendler, J., Schuch, F., Ronneberger, M., Lorenz, H.M., Nuesslein, H., Alten, R., Demary, W., Henes, J., Schett, G. and Rech, J. (2016) 'Relapse rates in patients with rheumatoid arthritis in stable remission tapering or stopping antirheumatic therapy: interim results from the prospective randomised controlled RETRO study', *Ann Rheum Dis*, 75(1), pp. 45-51.

Havenaar, E.C., Dolhain, R.J., Turner, G.A., Goodarzi, M.T., van Ommen, E.C., Breedveld, F.C. and van Dijk, W. (1997) 'Do synovial fluid acute phase proteins from patients with rheumatoid arthritis originate from serum?', *Glycoconj J*, 14(4), pp. 457-65.

Heimans, L., Akdemir, G., Boer, K.V., Goekoop-Ruiterman, Y.P., Molenaar, E.T., van Groenendael, J.H., Peeters, A.J., Steup-Beekman, G.M., Lard, L.R., de Sonnaville, P.B., Grillet, B.A., Huizinga, T.W. and Allaart, C.F. (2016) 'Two-year results of disease activity score (DAS)-remission-steered treatment strategies aiming at drug-free remission in early arthritis patients (the IMPROVED-study)', *Arthritis Res Ther*, 18, p. 23.

Hetland, M.L., Stengaard-Pedersen, K., Junker, P., Lottenburger, T., Ellingsen, T., Andersen, L.S., Hansen, I., Skjodt, H., Pedersen, J.K., Lauridsen, U.B., Svendsen, A., Tarp, U., Podenphant, J., Hansen, G., Lindegaard, H., de Carvalho, A., Ostergaard, M. and Horslev-Petersen, K. (2006) 'Combination treatment with methotrexate, cyclosporine, and intraarticular betamethasone compared with methotrexate and intraarticular betamethasone in early active rheumatoid arthritis: an investigator-initiated, multicenter, randomized, double-blind, parallel-group, placebo-controlled study', *Arthritis Rheum*, 54(5), pp. 1401-9.

Hetland, M.L., Stengaard-Pedersen, K., Junker, P., Ostergaard, M., Ejbjerg, B.J., Jacobsen, S., Lottenburger, T., Hansen, I., Tarp, U., Andersen, L.S., Svendsen, A., Pedersen, J.K., Lauridsen, U.B., Ellingsen, T., Lindegaard, H., Podenphant, J., Vestergaard, A., Jurik, A.G. and Horslev-Petersen, K. (2010) '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', *Ann Rheum Dis*, 69(10), pp. 1789-95.

Hill, J.A., Southwood, S., Sette, A., Jevnikar, A.M., Bell, D.A. and Cairns, E. (2003) 'Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule', *J Immunol*, 171(2), pp. 538-41.

Hirahara, K. and Nakayama, T. (2016) 'CD4+ T-cell subsets in inflammatory diseases: beyond the Th1/Th2 paradigm', *Int Immunol*, 28(4), pp. 163-71.

Hirata, S., Dirven, L., Shen, Y., Centola, M., Cavet, G., Lems, W.F., Tanaka, Y., Huizinga, T.W. and Allaart, C.F. (2013) 'A multi-biomarker score measures rheumatoid arthritis disease activity in the BeSt study', *Rheumatology (Oxford)*, 52(7), pp. 1202-7.

Hirata, S., Li, W., Kubo, S., Fukuyo, S., Mizuno, Y., Hanami, K., Sawamukai, N., Yamaoka, K., Saito, K., Defranoux, N.A. and Tanaka, Y. (2016) 'Association of the multi-biomarker disease activity score with joint destruction in patients with rheumatoid arthritis receiving tumor necrosis factor-alpha inhibitor treatment in clinical practice', *Mod Rheumatol*, 26(6), pp. 850-6.

Holoshitz, J. (2010) 'The rheumatoid arthritis HLA-DRB1 shared epitope', *Curr Opin Rheumatol*, 22(3), pp. 293-8.

Holt, O., Kanno, E., Bossi, G., Booth, S., Daniele, T., Santoro, A., Arico, M., Saegusa, C., Fukuda, M. and Griffiths, G.M. (2008) 'Slp1 and Slp2-a localize to the plasma membrane of CTL and contribute to secretion from the immunological synapse', *Traffic*, 9(4), pp. 446-57.

Hoskins, P.R., Martin, K. and Thrush, A. (2010) *Diagnostic ultrasound physics and equipment*. 2 edn. Leiden: Cambridge University Press.

Houssiau, F.A., Devogelaer, J.P., Van Damme, J., de Deuxchaisnes, C.N. and Van Snick, J. (1988) 'Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides', *Arthritis Rheum*, 31(6), pp. 784-8.

Huizinga, T.W., Conaghan, P.G., Martin-Mola, E., Schett, G., Amital, H., Xavier, R.M., Troum, O., Aassi, M., Bernasconi, C. and Dougados, M. (2015) 'Clinical and radiographic outcomes at 2 years and the effect of tocilizumab discontinuation following sustained remission in the second and third year of the ACT-RAY study', *Ann Rheum Dis*, 74(1), pp. 35-43.

Humby, F.C., Al Balushi, F., Lliso, G., Cauli, A. and Pitzalis, C. (2017) 'Can Synovial Pathobiology Integrate with Current Clinical and Imaging Prediction Models to Achieve Personalized Health Care in Rheumatoid Arthritis?', *Front Med (Lausanne)*, 4, p. 41.

Humphreys, J.H., Verstappen, S.M.M., Hyrich, K.L., Chipping, J.R., Marshall, T. and Symmons, D.P.M. (2013) '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*, 72(8), pp. 1315-20.

Humphreys, J.H., Warner, A., Chipping, J., Marshall, T., Lunt, M., Symmons, D.P. and Verstappen, S.M. (2014) 'Mortality trends in patients with early rheumatoid arthritis over 20 years: results from the Norfolk Arthritis Register', *Arthritis Care Res (Hoboken)*, 66(9), pp. 1296-301.

Hunt, L., Hensor, E.M., Nam, J., Burska, A.N., Parmar, R., Emery, P. and Ponchel, F. (2016) 'T cell subsets: an immunological biomarker to predict progression to clinical arthritis in ACPA-positive individuals', *Ann Rheum Dis*, 75(10), pp. 1884-9.

Huntoon, K.M., Russell, L., Tracy, E., Barbour, K.W., Li, Q., Shrikant, P.A., Berger, F.G., Garrett-Sinha, L.A. and Baumann, H. (2013) 'A unique form of haptoglobin produced by murine hematopoietic cells supports B-cell survival, differentiation and immune response', *Mol Immunol*, 55(3-4), pp. 345-54.

Huntoon, K.M., Wang, Y., Eppolito, C.A., Barbour, K.W., Berger, F.G., Shrikant, P.A. and Baumann, H. (2008) 'The acute phase protein haptoglobin regulates host immunity', *J Leukoc Biol*, 84(1), pp. 170-81.

Hwang, Y.G., Balasubramani, G.K., Metes, I.D., Levesque, M.C., Bridges, S.L., Jr. and Moreland, L.W. (2016) 'Differential response of serum amyloid A to different therapies in early rheumatoid arthritis and its potential value as a disease activity biomarker', *Arthritis Res Ther*, 18(1), p. 108.

Iezzi, G., Sonderegger, I., Ampenberger, F., Schmitz, N., Marsland, B.J. and Kopf, M. (2009) 'CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4+ T cells', *Proc Natl Acad Sci U S A*, 106(3), pp. 876-81.

Inui, K., Koike, T., Tada, M., Sugioka, Y., Mamoto, K., Okano, T., Sakawa, A., Fukushima, K. and Nakamura, H. (2014) 'AB0395 Restart for Sustaining Remission of Rheumatoid Arthritis in Use of Etanercept: Resume Study [abstract]', *Ann Rheum Dis*, 73(Suppl 2), p. 936.

Isaacs, J.D. (2010) 'The changing face of rheumatoid arthritis: sustained remission for all?', *Nat Rev Immunol*, 10(8), pp. 605-11.

Ishikawa, H. (2017) 'The latest treatment strategy for the rheumatoid hand deformity', *J Orthop Sci*, 22(4), pp. 583-92.

Iwamoto, T., Ikeda, K., Hosokawa, J., Yamagata, M., Tanaka, S., Norimoto, A., Sanayama, Y., Nakagomi, D., Takahashi, K., Hirose, K., Sugiyama, T., Sueishi, M. and Nakajima, H. (2014) 'Prediction of relapse after discontinuation of biologic agents by ultrasonographic assessment in patients with rheumatoid arthritis in clinical remission: high predictive values of total gray-scale and power Doppler scores that represent residual synovial inflammation before discontinuation', *Arthritis Care Res (Hoboken)*, 66(10), pp. 1576-81.

Iwanaga, T., Shikichi, M., Kitamura, H., Yanase, H. and Nozawa-Inoue, K. (2000) 'Morphology and functional roles of synoviocytes in the joint', *Arch Histol Cytol*, 63(1), pp. 17-31.

Jackman, R.P., Utter, G.H., Heitman, J.W., Hirschkorn, D.F., Law, J.P., Gefter, N., Busch, M.P. and Norris, P.J. (2011) 'Effects of blood sample age at time of separation on measured cytokine concentrations in human plasma', *Clin Vaccine Immunol*, 18(2), pp. 318-26.

Jacob, C. (1960) 'A Coefficient of Agreement for Nominal Scales', *Educational and Psychological Measurement*, 20(1), pp. 37-46.

Jain, M., Attur, M., Furer, V., Todd, J., Ramirez, R., Lock, M., Lu, Q.A., Abramson, S.B. and Greenberg, J.D. (2015) 'Increased plasma IL-17F levels in rheumatoid arthritis patients are responsive to methotrexate, anti-TNF, and T cell costimulatory modulation', *Inflammation*, 38(1), pp. 180-6.

Janta, I., Valor, L., De la Torre, I., Martinez-Estupinan, L., Nieto, J.C., Ovalles-Bonilla, J.G., Martinez-Barrio, J., Bello, N., Hinojosa, M., Montoro, M., Gonzalez, C.M., Lopez-Longo, J., Monteagudo, I., Carreno, L. and Naredo, E. (2016) 'Ultrasound-detected activity in rheumatoid arthritis on methotrexate therapy: Which joints and tendons should be assessed to predict unstable remission?', *Rheumatol Int*, 36(3), pp. 387-96.

Jiang, K., Sun, X., Chen, Y., Shen, Y. and Jarvis, J.N. (2015) 'RNA sequencing from human neutrophils reveals distinct transcriptional differences associated with chronic inflammatory states', *BMC Medical Genomics*, 8, p. 55.

Jones, G.W., Bombardieri, M., Greenhill, C.J., McLeod, L., Nerviani, A., Rocher-Ros, V., Cardus, A., Williams, A.S., Pitzalis, C., Jenkins, B.J. and Jones, S.A. (2015) 'Interleukin-27 inhibits ectopic lymphoid-like structure development in early inflammatory arthritis', *J Exp Med*, 212(11), pp. 1793-802.

June, R.R. and Olsen, N.J. (2016) 'Room for more IL-6 blockade? Sarilumab for the treatment of rheumatoid arthritis', *Expert Opin Biol Ther*, 16(10), pp. 1303-9.

Jung, C.R., Hsieh, H.Y. and Hwang, B.F. (2017) 'Air Pollution as a Potential Determinant of Rheumatoid Arthritis: A Population-based Cohort Study in Taiwan', *Epidemiology*, 28 (Suppl 1), pp. s54-9.

Kallberg, H., Padyukov, L., Plenge, R.M., Ronnelid, J., Gregersen, P.K., van der Helm-van Mil, A.H., Toes, R.E., Huizinga, T.W., Klareskog, L. and Alfredsson, L. (2007) 'Gene-gene and gene-environment interactions involving HLA-DRB1, PTPN22, and smoking in two subsets of rheumatoid arthritis', *Am J Hum Genet*, 80(5), pp. 867-75.

Kassambara, A. (2017) *ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.1.2.* [Computer program]. https://CRAN.R-project.org/package=ggpubr.

Kassambara, A. and Kosinski, M. (2017) *survminer: Drawing Survival Curves using 'ggplot2'. R package version 0.3.1.* [Computer program]. https://CRAN.R-project.org/package=survminer.

Kawashiri, S.Y., Fujikawa, K., Nishino, A., Okada, A., Aramaki, T., Shimizu, T., Umeda, M., Fukui, S., Suzuki, T., Koga, T., Iwamoto, N., Ichinose, K., Tamai, M., Mizokami, A., Nakamura, H., Origuchi, T., Ueki, Y., Aoyagi, K., Maeda, T. and Kawakami, A. (2017) 'Ultrasound-detected bone erosion is a relapse risk factor after discontinuation of biologic disease-modifying antirheumatic drugs in patients with rheumatoid arthritis whose ultrasound power Doppler synovitis activity and clinical disease activity are well controlled', *Arthritis Res Ther*, 19(1), p. 108.

Kawashiri, S.Y., Kawakami, A., Okada, A., Koga, T., Tamai, M., Yamasaki, S., Nakamura, H., Origuchi, T., Ida, H. and Eguchi, K. (2011) 'CD4+CD25(high)CD127(low/-) Treg cell frequency from peripheral blood correlates with disease activity in patients with rheumatoid arthritis', *J Rheumatol*, 38(12), pp. 2517-21.

Kawashiri, S.Y., Suzuki, T., Nakashima, Y., Horai, Y., Okada, A., Iwamoto, N., Ichinose, K., Tamai, M., Arima, K., Nakamura, H., Origuchi, T., Uetani, M., Aoyagi, K., Eguchi, K. and Kawakami, A. (2014) 'Ultrasonographic examination of rheumatoid arthritis patients who are free of physical synovitis: power Doppler subclinical synovitis is associated with bone erosion', *Rheumatology (Oxford)*, 53(3), pp. 562-9.

Keating, G.M. (2013) 'Abatacept: a review of its use in the management of rheumatoid arthritis', *Drugs*, 73(10), pp. 1095-119.

Khazaei, H.A., Lunardi, C. and So, A.K. (1995) 'CD4 T cells in the rheumatoid joint are oligoclonally activated and change during the course of disease', *Annals of the Rheumatic Diseases*, 54(4), pp. 314-317.

Kievit, W., Fransen, J., de Waal Malefijt, M.C., den Broeder, A.A. and van Riel, P.L. (2013) 'Treatment changes and improved outcomes in RA: an overview of a large inception cohort from 1989 to 2009', *Rheumatology (Oxford)*, 52(8), pp. 1500-8.

Kievit, W., van Herwaarden, N., van den Hoogen, F.H., van Vollenhoven, R.F., Bijlsma, J.W., van den Bemt, B.J., van der Maas, A. and den Broeder, A.A. (2016) 'Disease activity-guided dose optimisation of adalimumab and etanercept is a cost-effective strategy compared with non-tapering tight control rheumatoid arthritis care: analyses of the DRESS study', *Ann Rheum Dis*, 75(11), pp. 1939-44.

Kim, G.W., Lee, N.R., Pi, R.H., Lim, Y.S., Lee, Y.M., Lee, J.M., Jeong, H.S. and Chung, S.H. (2015) 'IL-6 inhibitors for treatment of rheumatoid arthritis: past, present, and future', *Arch Pharm Res*, 38(5), pp. 575-84.

Kim, S.K., Park, S.H., Bae, J., Son, J.T. and Choe, J.Y. (2014a) 'Performance of Routine Assessment of Patient Index Data 3 (RAPID3) for assessment of rheumatoid arthritis in clinical practice: differential agreement of RAPID3 according to disease activity categories', *Rheumatol Int*, 34(9), pp. 1311-8.

Kim, T.W., Kang, Y.K., Park, Z.Y., Kim, Y.H., Hong, S.W., Oh, S.J., Sohn, H.A., Yang, S.J., Jang, Y.J., Lee, D.C., Kim, S.Y., Yoo, H.S., Kim, E., Yeom, Y.I. and Park, K.C. (2014b) 'SH3RF2 functions as an oncogene by mediating PAK4 protein stability', *Carcinogenesis*, 35(3), pp. 624-34.

Kinchen, J.M. and Ravichandran, K.S. (2007) 'Journey to the grave: signaling events regulating removal of apoptotic cells', *J Cell Sci*, 120(Pt 13), pp. 2143-9.

Kishimoto, T. (2010) 'IL-6: from its discovery to clinical applications', *Int Immunol*, 22(5), pp. 347-52.

Kita, J., Tamai, M., Arima, K., Nakashima, Y., Suzuki, T., Kawashiri, S.Y., Iwamoto, N., Okada, A., Koga, T., Yamasaki, S., Nakamura, H., Origuchi, T., Ida, H., Aoyagi, K., Uetani, M., Eguchi, K. and Kawakami, A. (2012) 'Treatment discontinuation in patients with very early rheumatoid arthritis in sustained simplified disease activity index remission after synthetic disease-modifying anti-rheumatic drug administration', *Mod Rheumatol*, 22(3), pp. 346-52.

Klarenbeek, N.B., Koevoets, R., van der Heijde, D.M., Gerards, A.H., Ten Wolde, S., Kerstens, P.J., Huizinga, T.W., Dijkmans, B.A. and Allaart, C.F. (2011a) 'Association with joint damage and physical functioning of nine composite indices and the 2011 ACR/EULAR remission criteria in rheumatoid arthritis', *Ann Rheum Dis*, 70(10), pp. 1815-21.

Klarenbeek, N.B., van der Kooij, S.M., Guler-Yuksel, M., van Groenendael, J.H., Han, K.H., Kerstens, P.J., Huizinga, T.W., Dijkmans, B.A. and Allaart, C.F. (2011b) 'Discontinuing treatment in patients with rheumatoid arthritis in sustained clinical remission: exploratory analyses from the BeSt study', *Ann Rheum Dis*, 70(2), pp. 315-9.

Klareskog, L., Stolt, P., Lundberg, K., Kallberg, H., Bengtsson, C., Grunewald, J., Ronnelid, J., Harris, H.E., Ulfgren, A.K., Rantapaa-Dahlqvist, S., Eklund, A., Padyukov, L. and Alfredsson, L. (2006) 'A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination', *Arthritis Rheum*, 54(1), pp. 38-46.

Knevel, R., Krabben, A., Brouwer, E., Posthumus, M.D., Wilson, A.G., Lindqvist, E., Saxne, T., de Rooy, D., Daha, N., van der Linden, M.P., Stoeken, G., van Toorn, L., Koeleman, B., Tsonaka, R., Zhernakoza, A., Houwing-Duistermaat, J.J., Toes, R., Huizinga, T.W. and van der Helm-van Mil, A. (2012) 'Genetic variants in IL15 associate with progression of joint destruction in rheumatoid arthritis: a multicohort study', *Ann Rheum Dis*, 71(10), pp. 1651-7.

Kocijan, R., Harre, U. and Schett, G. (2013) 'ACPA and bone loss in rheumatoid arthritis', *Curr Rheumatol Rep*, 15(10), p. 366.

Kremer, J.M., Rynes, R.I. and Bartholomew, L.E. (1987) 'Severe flare of rheumatoid arthritis after discontinuation of long-term methotrexate therapy. Double-blind study', *Am J Med*, 82(4), pp. 781-6.

Kristensen, V.N., Lingjaerde, O.C., Russnes, H.G., Vollan, H.K., Frigessi, A. and Borresen-Dale, A.L. (2014) 'Principles and methods of integrative genomic analyses in cancer', *Nat Rev Cancer*, 14(5), pp. 299-313.

Kuan, W.P., Tam, L.S., Wong, C.K., Ko, F.W.S., Li, T.N., Zhu, T. and Li, E.K. (2010) 'CXCL 9 and CXCL 10 as Sensitive Markers of Disease Activity in Patients with Rheumatoid Arthritis', *J Rheumatol*, 37(2), pp. 257-64.

Kuijper, T.M., Folmer, R., Stolk, E.A., Hazes, J.M.W. and Luime, J.J. (2017) 'Doctors' preferences in de-escalating DMARDs in rheumatoid arthritis: a discrete choice experiment', *Arthritis Res Ther*, 19(1), p. 78.

Kuijper, T.M., Lamers-Karnebeek, F.B., Jacobs, J.W., Hazes, J.M. and Luime, J.J. (2015) 'Flare Rate in Patients with Rheumatoid Arthritis in Low Disease Activity or Remission When Tapering or Stopping Synthetic or Biologic DMARD: A Systematic Review', *J Rheumatol*, 42(11), pp. 2012-22.

Kuijper, T.M., Luime, J.J., de Jong, P.H., Gerards, A.H., van Zeben, D., Tchetverikov, I., de Sonnaville, P.B., van Krugten, M.V., Grillet, B.A., Hazes, J.M. and Weel, A.E. (2016) 'Tapering conventional synthetic DMARDs in patients with early arthritis in sustained remission: 2-year follow-up of the tREACH trial', *Ann Rheum Dis*, 75(12), pp. 2119-23.

Kuriya, B., Sun, Y., Boire, G., Haraoui, B., Hitchon, C., Pope, J.E., Thorne, J.C., Keystone, E.C. and Bykerk, V.P. (2012) 'Remission in early rheumatoid arthritis -- a comparison of new ACR/EULAR remission criteria to established criteria', *J Rheumatol*, 39(6), pp. 1155-8.

Kusnierczyk, P. (2013) 'Killer cell immunoglobulin-like receptor gene associations with autoimmune and allergic diseases, recurrent spontaneous abortion, and neoplasms', *Front Immunol*, 4, p. 8.

Kwak, H.B., Ha, H., Kim, H.N., Lee, J.H., Kim, H.S., Lee, S., Kim, H.M., Kim, J.Y., Kim, H.H., Song, Y.W. and Lee, Z.H. (2008) 'Reciprocal cross-talk between RANKL and interferon-gamma-inducible protein 10 is responsible for bone-erosive experimental arthritis', *Arthritis Rheum*, 58(5), pp. 1332-42.

Lai, X., Wang, H., Cao, J., Li, Y., Dai, Y., Xiang, Y. and Zhang, L. (2016) 'Circulating IL-27 Is Elevated in Rheumatoid Arthritis Patients', *Molecules*, 21(11), p. E1565.

Lamers-Karnebeek, F., Ghiti Moghadam, M., Vonkeman, H.E., Tekstra, J., Schilder, A., Visser, H., Ten Klooster, P.M., Sasso, E.H., Chernoff, D., Lems, W., Van Schaardenburg, D., Landewe, R., Van Albada, L., Radstake, T.R.D.J., Van Riel, P., van de Laar, M.A.F.J. and Jansen, T. (2015) 'Multi-Biomarker Disease Activity Score As a Predictor of Flare in Patients with Rheumatoid Arthritis Who Stop TNF-Alpha-Inhibitor Therapy [abstract] ', *Arthritis Rheumatol.*, 67(Suppl 10), http://acrabstracts.org/abstract/multi-biomarker-disease-activity-score-as-a-predictor-of-flare-in-patients-with-rheumatoid-arthritis-who-stop-tnf-alpha-inhibitor-therapy/. Accessed August 6, 2017.(Suppl 10).

Lamers-Karnebeek, F.B.G., Luime, J., van Riel, P., Jacobs, J. and Jansen, T. (2016) 'OP0181 Prediction of Flare after Stopping TNF-Inhibitor by Baseline Ultrasonography and Patient Characteristics in Rheumatoid Arthritis Patients with Low Disease Activity: 12-Month Results [abstract]', *Ann Rheum Dis*, 75(Suppl 2), p. 125.

Landis, J.R. and Kock, G.G. (1977) 'The Measurement of Observer Agreement for Categorical Data', *Biometrics*, 33(1), pp. 159-174.

Lang, M. (2016) *checkmate: Fast and Versatile Argument Checks. R package version 1.8.2*. [Computer program]. https://CRAN.R-project.org/package=checkmate.

Law, C.W., Chen, Y., Shi, W. and Smyth, G.K. (2014) 'voom: Precision weights unlock linear model analysis tools for RNA-seq read counts', *Genome Biol*, 15(2), p. R29.

Lazarou, I., D'Agostino, M.A., Naredo, E., Humby, F., Filer, A. and Kelly, S.G. (2015) 'Ultrasound-guided synovial biopsy: a systematic review according to the OMERACT filter and recommendations for minimal reporting standards in clinical studies', *Rheumatology (Oxford)*, 54(10), pp. 1867-75.

Ledingham, J., Gullick, N., Irving, K., Gorodkin, R., Aris, M., Burke, J., Gordon, P., Christidis, D., Galloway, S., Hayes, E., Jeffries, A., Mercer, S., Mooney, J., van Leuven, S. and Galloway, J. (2017) 'BSR and BHPR guideline for the prescription and monitoring of non-biologic disease-modifying anti-rheumatic drugs', *Rheumatology (Oxford)*, 56(6), pp. 865-8.

Lee, J.H., Kim, B., Jin, W.J., Kim, H.H., Ha, H. and Lee, Z.H. (2017) 'Pathogenic roles of CXCL10 signaling through CXCR3 and TLR4 in macrophages and T cells: relevance for arthritis', *Arthritis Res Ther*, 19(1), p. 163.

Lee, W.W., Yang, Z.Z., Li, G., Weyand, C.M. and Goronzy, J.J. (2007) 'Unchecked CD70 expression on T cells lowers threshold for T cell activation in rheumatoid arthritis', *J Immunol*, 179(4), pp. 2609-15.

Lee, Y.H. and Bae, S.C. (2016) 'Monocyte chemoattractant protein-1 promoter -2518 polymorphism and susceptibility to vasculitis, rheumatoid arthritis, and multiple sclerosis: A meta-analysis', *Cell Mol Biol (Noisy-le-grand)*, 62(3), pp. 65-71.

Lefevre, S., Knedla, A., Tennie, C., Kampmann, A., Wunrau, C., Dinser, R., Korb, A., Schnaker, E.M., Tarner, I.H., Robbins, P.D., Evans, C.H., Sturz, H., Steinmeyer, J., Gay, S., Scholmerich, J., Pap, T., Muller-Ladner, U. and Neumann, E. (2009) 'Synovial fibroblasts spread rheumatoid arthritis to unaffected joints', *Nat Med*, 15(12), pp. 1414-20.

Leng, X., Xiao, W., Xu, Z., Zhu, X., Liu, Y., Zhao, D., Xu, H., Chen, G., Yu, W., Lu, J., Wang, J., Xia, X., Li, Y., Zhao, Y., Tang, H., Shi, Y., Bao, J., Chen, L., Lin, L., Zhou, L., Zhang, H. and Zhao, Y. (2016) 'Ultrasound7 versus ultrasound12 in monitoring the response to infliximab in patients with rheumatoid arthritis', *Clin Rheumatol*, 35(3), pp. 587-94.

Li, L., Wozniak, L.J., Rodder, S., Heish, S., Talisetti, A., Wang, Q., Esquivel, C., Cox, K., Chen, R., McDiarmid, S.V. and Sarwal, M.M. (2012a) 'A common peripheral blood gene set for diagnosis of operational tolerance in pediatric and adult liver transplantation', *Am J Transplant*, 12(5), pp. 1218-28.

Li, P., Spolski, R., Liao, W., Wang, L., Murphy, T.L., Murphy, K.M. and Leonard, W.J. (2012b) 'BATF-JUN is critical for IRF4-mediated transcription in T cells', *Nature*, 490(7421), pp. 543-6.

Li, W., Sasso, E.H., van der Helm-van Mil, A.H. and Huizinga, T.W. (2016) 'Relationship of multi-biomarker disease activity score and other risk factors with radiographic progression in an observational study of patients with rheumatoid arthritis', *Rheumatology*, 55(2), pp. 357-66.

Liao, J., Liang, G., Xie, S., Zhao, H., Zuo, X., Li, F., Chen, J., Zhao, M., Chan, T.M. and Lu, Q. (2012) 'CD40L demethylation in CD4(+) T cells from women with rheumatoid arthritis', *Clin Immunol*, 145(1), pp. 13-8.

Libert, C., Brouckaert, P. and Fiers, W. (1994) 'Protection by alpha 1-acid glycoprotein against tumor necrosis factor-induced lethality', *J Exp Med*, 180(4), pp. 1571-5.

Lillegraven, S., Prince, F.H., Shadick, N.A., Bykerk, V.P., Lu, B., Frits, M.L., Iannaccone, C.K., Kvien, T.K., Haavardsholm, E.A., Weinblatt, M.E. and Solomon, D.H. (2012) 'Remission and radiographic outcome in

rheumatoid arthritis: application of the 2011 ACR/EULAR remission criteria in an observational cohort', *Ann Rheum Dis*, 71(5), pp. 681-6.

Lillis, A.P., Van Duyn, L.B., Murphy-Ullrich, J.E. and Strickland, D.K. (2008) 'LDL receptor-related protein 1: unique tissue-specific functions revealed by selective gene knockout studies', *Physiol Rev*, 88(3), pp. 887-918.

Lin, L. and Hu, K. (2014) 'LRP-1: functions, signaling and implications in kidney and other diseases', *Int J Mol Sci*, 15(12), pp. 22887-901.

Lin, M., Sutherland, D.R., Horsfall, W., Totty, N., Yeo, E., Nayar, R., Wu, X.F. and Schuh, A.C. (2002) 'Cell surface antigen CD109 is a novel member of the alpha(2) macroglobulin/C3, C4, C5 family of thioester-containing proteins', *Blood*, 99(5), pp. 1683-91.

Liou, L.B., Tsai, W.P., Chang, C.J., Chao, W.J. and Chen, M.H. (2013) 'Blood monocyte chemotactic protein-1 (MCP-1) and adapted disease activity Score28-MCP-1: favorable indicators for rheumatoid arthritis activity', *PLoS One*, 8(1), p. e55346.

Lisbona, M.P., Pamies, A., Ares, J., Almirall, M., Navallas, M., Solano, A. and Maymo, J. (2014) 'Association of bone edema with the progression of bone erosions quantified by hand magnetic resonance imaging in patients with rheumatoid arthritis in remission', *J Rheumatol*, 41(8), pp. 1623-9.

Lisbona, M.P., Solano, A., Ares, J., Almirall, M., Salman-Monte, T.C. and Maymo, J. (2016) 'ACR/EULAR Definitions of Remission Are Associated with Lower Residual Inflammatory Activity Compared with DAS28 Remission on Hand MRI in Rheumatoid Arthritis', *J Rheumatol*, 43(9), pp. 1631-6.

Litvinov, I.V., Bizet, A.A., Binamer, Y., Jones, D.A., Sasseville, D. and Philip, A. (2011) 'CD109 release from the cell surface in human keratinocytes regulates TGF-beta receptor expression, TGF-beta signalling and STAT3 activation: relevance to psoriasis', *Exp Dermatol*, 20(8), pp. 627-32.

Liu, M., Guo, S., Hibbert, J.M., Jain, V., Singh, N., Wilson, N.O. and Stiles, J.K. (2011) 'CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications', *Cytokine Growth Factor Rev*, 22(3), pp. 121-30.

Liu, Q., Zhu, Y., Yong, W.K., Sze, N.S., Tan, N.S. and Ding, J.L. (2015) 'Cutting Edge: Synchronization of IRF1, JunB, and C/EBPbeta Activities during TLR3-TLR7 Cross-Talk Orchestrates Timely Cytokine Synergy in the Proinflammatory Response', *J Immunol*, 195(3), pp. 801-5.

Ljung, L. and Rantapaa-Dahlqvist, S. (2016) 'Abdominal obesity, gender and the risk of rheumatoid arthritis - a nested case-control study', *Arthritis Res Ther*, 18(1), p. 277.

Lopez-Olivo, M.A., Siddhanamatha, H.R., Shea, B., Tugwell, P., Wells, G.A. and Suarez-Almazor, M.E. (2014) 'Methotrexate for treating rheumatoid arthritis', *Cochrane Database Syst Rev*, (6), p. Cd000957.

Lozano, J.J., Pallier, A., Martinez-Llordella, M., Danger, R., Lopez, M., Giral, M., Londono, M.C., Rimola, A., Soulillou, J.P., Brouard, S. and Sanchez-Fueyo, A. (2011) 'Comparison of transcriptional and blood cell-phenotypic markers between operationally tolerant liver and kidney recipients', *Am J Transplant*, 11(9), pp. 1916-26.

Lu, B., Hiraki, L.T., Sparks, J.A., Malspeis, S., Chen, C.Y., Awosogba, J.A., Arkema, E.V., Costenbader, K.H. and Karlson, E.W. (2014) 'Being overweight or obese and risk of developing rheumatoid arthritis among women: a prospective cohort study', *Ann Rheum Dis*, 73(11), pp. 1914-22.

Lubberts, E., Joosten, L.A., Oppers, B., van den Bersselaar, L., Coenen-de Roo, C.J., Kolls, J.K., Schwarzenberger, P., van de Loo, F.A. and van den Berg, W.B. (2001) 'IL-1-independent role of IL-17 in synovial inflammation and joint destruction during collagen-induced arthritis', *J Immunol*, 167(2), pp. 1004-13.

Ma, M.H., Scott, I.C., Kingsley, G.H. and Scott, D.L. (2010) 'Remission in early rheumatoid arthritis', *J Rheumatol*, 37(7), pp. 1444-53.

Maijer, K.I., Li, W., Sasso, E.H., Gerlag, D.M., Defranoux, N.A. and Tak, P.P. (2015) 'Does the multi-biomarker disease activity score have diagnostic value in early rheumatoid arthritis and unclassified arthritis?', *Ann Rheum Dis*, 74(11), pp. 2097-9.

Maini, R.N. (2012) 'Rheumatoid arthritis', in Warrell, D.A., Cox, T.M. and Firth, J.D. (eds.) *Oxford Textbook of Medicine*. Oxford: Oxford University Press.

Majorczyk, E., Pawlik, A., Gendosz, D. and Kusnierczyk, P. (2014) 'Presence of the full-length KIR2DS4 gene reduces the chance of rheumatoid arthritis patients to respond to methotrexate treatment', *BMC Musculoskelet Disord*, 15, p. 256.

Makinen, H., Kautiainen, H., Hannonen, P. and Sokka, T. (2005) 'Is DAS28 an appropriate tool to assess remission in rheumatoid arthritis?', *Ann Rheum Dis*, 64(10), pp. 1410-3.

Mandrekar, J.N. (2011) 'Measures of interrater agreement', *J Thorac Oncol*, 6(1), pp. 6-7.

Markusse, I.M., Akdemir, G., Huizinga, T.W. and Allaart, C.F. (2014a) 'Drug-free holiday in patients with rheumatoid arthritis: a qualitative study to explore patients' opinion', *Clin Rheumatol*, 33(8), pp. 1155-9.

Markusse, I.M., Dirven, L., van den Broek, M., Bijkerk, C., Han, K.H., Ronday, H.K., Bolce, R., Sasso, E.H., Kerstens, P.J., Lems, W.F., Huizinga, T.W. and Allaart, C.F. (2014b) 'A multibiomarker disease activity score for rheumatoid arthritis predicts radiographic joint damage in the BeSt study', *J Rheumatol*, 41(11), pp. 2114-9.

Martinez-Llordella, M., Lozano, J.J., Puig-Pey, I., Orlando, G., Tisone, G., Lerut, J., Benitez, C., Pons, J.A., Parrilla, P., Ramirez, P., Bruguera, M., Rimola, A. and Sanchez-Fueyo, A. (2008) 'Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients', *J Clin Invest*, 118(8), pp. 2845-57.

Maska, L., Anderson, J. and Michaud, K. (2011) 'Measures of functional status and quality of life in rheumatoid arthritis: Health Assessment Questionnaire Disability Index (HAQ), Modified Health Assessment Questionnaire (MHAQ), Multidimensional Health Assessment Questionnaire (MDHAQ), Health Assessment Questionnaire II (HAQ-II), Improved Health Assessment Questionnaire (Improved HAQ), and Rheumatoid Arthritis Quality of Life (RAQoL)', *Arthritis Care Res (Hoboken)*, 63 Suppl 11(S11), pp. S4-13.

Masri, K.R., Shaver, T.S., Shahouri, S.H., Wang, S., Anderson, J.D., Busch, R.E., Michaud, K., Mikuls, T.R., Caplan, L. and Wolfe, F. (2012) 'Validity and reliability problems with patient global as a component of the ACR/EULAR remission criteria as used in clinical practice', *J Rheumatol*, 39(6), pp. 1139-45.

Massart, A., Ghisdal, L., Abramowicz, M. and Abramowicz, D. (2017) 'Operational tolerance in kidney transplantation and associated biomarkers', *Clin Exp Immunol*, 189(2), pp. 138-57.

Matsumoto, Y., Sugioka, Y., Tada, M., Okano, T., Mamoto, K., Inui, K., Habu, D. and Koike, T. (2018) 'Monounsaturated fatty acids might be key factors in the Mediterranean diet that suppress rheumatoid arthritis disease activity: The TOMORROW study', *Clin Nutr*, 37(2), pp. 675-80.

Matsuzaki, H., Kobayashi, H., Yagyu, T., Wakahara, K., Kondo, T., Kurita, N., Sekino, H., Inagaki, K., Suzuki, M., Kanayama, N. and Terao, T. (2004) 'Bikunin inhibits lipopolysaccharide-induced tumor necrosis factor alpha induction in macrophages', *Clin Diagn Lab Immunol*, 11(6), pp. 1140-7.

McGonagle, D. and Tan, A.L. (2008) 'What magnetic resonance imaging has told us about the pathogenesis of rheumatoid arthritis--the first 50 years', *Arthritis Res Ther*, 10(5), p. 222.

McInnes, I.B., al-Mughales, J., Field, M., Leung, B.P., Huang, F.P., Dixon, R., Sturrock, R.D., Wilkinson, P.C. and Liew, F.Y. (1996) 'The role of interleukin-15 in T-cell migration and activation in rheumatoid arthritis', *Nat Med*, 2(2), pp. 175-82.

McInnes, I.B. and Schett, G. (2011) 'The pathogenesis of rheumatoid arthritis', *N Engl J Med*, 365(23), pp. 2205-19.

McInnes, I.B. and Schett, G. (2017) 'Pathogenetic insights from the treatment of rheumatoid arthritis', *Lancet*, 389(10086), pp. 2328-37.

Meka, R.R., Venkatesha, S.H., Dudics, S., Acharya, B. and Moudgil, K.D. (2015) 'IL-27-induced modulation of autoimmunity and its therapeutic potential', *Autoimmun Rev*, 14(12), pp. 1131-41.

Mendez, E., Fernandez-Luna, J.L., Grubb, A. and Leyva-Cobian, F. (1986) 'Human protein HC and its IgA complex are inhibitors of neutrophil chemotaxis', *Proc Natl Acad Sci U S A*, 83(5), pp. 1472-5.

Mickey, R.M. and Greenland, S. (1989) 'The impact of confounder selection criteria on effect estimation', *Am J Epidemiol*, 129(1), pp. 125-37.

Millot, F., Clavel, G., Etchepare, F., Gandjbakhch, F., Grados, F., Saraux, A., Rat, A.C., Fautrel, B., Bourgeois, P. and Fardellone, P. (2011) 'Musculoskeletal ultrasonography in healthy subjects and ultrasound criteria for early arthritis (the ESPOIR cohort)', *J Rheumatol*, 38(4), pp. 613-20.

Miranda-Carus, M.E., Balsa, A., Benito-Miguel, M., Perez de Ayala, C. and Martin-Mola, E. (2004) 'IL-15 and the initiation of cell contact-dependent synovial fibroblast-T lymphocyte cross-talk in rheumatoid arthritis: effect of methotrexate', *J Immunol*, 173(2), pp. 1463-76.

Miranda-Carus, M.E., Benito-Miguel, M., Balsa, A., Cobo-Ibanez, T., Perez de Ayala, C., Pascual-Salcedo, D. and Martin-Mola, E. (2006) 'Peripheral blood T lymphocytes from patients with early rheumatoid arthritis express RANKL and interleukin-15 on the cell surface and promote osteoclastogenesis in autologous monocytes', *Arthritis Rheum*, 54(4), pp. 1151-64.

Miranda-Carus, M.E., Benito-Miguel, M., Llamas, M.A., Balsa, A. and Martin-Mola, E. (2005) 'Human T cells constitutively express IL-15 that promotes ex vivo T cell homeostatic proliferation through autocrine/juxtacrine loops', *J Immunol*, 175(6), pp. 3656-62.

Mitsios, A., Arampatzioglou, A., Arelaki, S., Mitroulis, I. and Ritis, K. (2016) 'NETopathies? Unraveling the Dark Side of Old Diseases through Neutrophils', *Front Immunol*, 7, p. 678.

Miyara, M., Ito, Y. and Sakaguchi, S. (2014) 'TREG-cell therapies for autoimmune rheumatic diseases', *Nat Rev Rheumatol*, 10(9), pp. 543-51.

Moggia, E., Koti, R., Belgaumkar, A.P., Fazio, F., Pereira, S.P., Davidson, B.R. and Gurusamy, K.S. (2017) 'Pharmacological interventions for acute pancreatitis', *Cochrane Database Syst Rev*, 4(4), p. CD011384.

Mohan, C. and Assassi, S. (2015) 'Biomarkers in rheumatic diseases: how can they facilitate diagnosis and assessment of disease activity?', *BMJ*, 351, p. h5079.

Moller-Bisgaard, S., Horslev-Petersen, K., Ejbjerg, B.J., Boesen, M., Hetland, M.L., Christensen, R., Moller, J., Krogh, N.S., Stengaard-Pedersen, K. and Ostergaard, M. (2015) 'Impact of a magnetic resonance imaging-guided treat-to-target strategy on disease activity and progression in patients with rheumatoid arthritis (the IMAGINE-RA trial): study protocol for a randomized controlled trial', *Trials*, 16, p. 178.

Monaco, A.P. (2004) 'Prospects and strategies for clinical tolerance', *Transplant Proc*, 36(1), pp. 227-31.

Moreland, L.W., Russell, A.S. and Paulus, H.E. (2001) 'Management of rheumatoid arthritis: the historical context', *J Rheumatol*, 28(6), pp. 1431-52.

Mosesson, M.W. (2005) 'Fibrinogen and fibrin structure and functions', *J Thromb Haemost*, 3(8), pp. 1894-904.

Murphy, K.M. and Weaver, C. (2016) *Janeway's immunobiology*. 9 edn. New York, NY: Garland Science/Taylor & Francis Group, LLC.

Murphy, T.L., Tussiwand, R. and Murphy, K.M. (2013) 'Specificity through cooperation: BATF-IRF interactions control immune-regulatory networks', *Nat Rev Immunol*, 13(7), pp. 499-509.

Murugaiyan, G., Mittal, A., Lopez-Diego, R., Maier, L.M., Anderson, D.E. and Weiner, H.L. (2009) 'IL-27 is a key regulator of IL-10 and IL-17 production by human CD4+ T cells', *J Immunol*, 183(4), pp. 2435-43.

Myasoedova, E., Crowson, C.S., Kremers, H.M., Roger, V.L., Fitz-Gibbon, P.D., Therneau, T.M. and Gabriel, S.E. (2011) 'Lipid paradox in rheumatoid arthritis: the impact of serum lipid measures and systemic inflammation on the risk of cardiovascular disease', *Ann Rheum Dis*, 70(3), pp. 482-7.

Nakamura, T. (2011) 'Amyloid A amyloidosis secondary to rheumatoid arthritis: pathophysiology and treatments', *Clin Exp Rheumatol*, 29(5), pp. 850-7.

Nam, J.L., Hensor, E.M., Hunt, L., Conaghan, P.G., Wakefield, R.J. and Emery, P. (2016) 'Ultrasound findings predict progression to inflammatory arthritis in anti-CCP antibody-positive patients without clinical synovitis', *Ann Rheum Dis*, 75(12), pp. 2060-7.

Nam, J.L., Villeneuve, E., Hensor, E.M., Wakefield, R.J., Conaghan, P.G., Green, M.J., Gough, A., Quinn, M., Reece, R., Cox, S.R., Buch, M.H., van der Heijde, D.M. and Emery, P. (2014) 'A randomised controlled trial of etanercept and methotrexate to induce remission in early inflammatory arthritis: the EMPIRE trial', *Ann Rheum Dis*, 73(6), pp. 1027-36.

Naredo, E., Collado, P., Cruz, A., Palop, M.J., Cabero, F., Richi, P., Carmona, L. and Crespo, M. (2007) 'Longitudinal power Doppler ultrasonographic assessment of joint inflammatory activity in early rheumatoid arthritis: predictive value in disease activity and radiologic progression', *Arthritis Rheum*, 57(1), pp. 116-24.

Naredo, E., Valor, L., De la Torre, I., Montoro, M., Bello, N., Martinez-Barrio, J., Martinez-Estupinan, L., Nieto, J.C., Ovalles-Bonilla, J.G., Hernandez-Florez, D., Gonzalez, C.M., Lopez-Longo, F.J., Monteagudo, I. and Carreno, L. (2015) 'Predictive value of Doppler ultrasound-detected synovitis in relation to failed tapering of biologic therapy in patients with rheumatoid arthritis', *Rheumatology (Oxford)*, 54(8), pp. 1408-14.

Naredo, E., Wakefield, R.J., Iagnocco, A., Terslev, L., Filippucci, E., Gandjbakhch, F., Aegerter, P., Aydin, S., Backhaus, M., Balint, P.V., Bruyn, G.A., Collado, P., Finzel, S., Freeston, J.E., Gutierrez, M., Joshua, F., Jousse-Joulin, S., Kane, D., Keen, H.I., Moller, I., Mandl, P., Ohrndorf, S., Pineda, C., Schmidt, W.A., Szkudlarek, M., Conaghan, P.G. and D'Agostino, M.A. (2011) 'The OMERACT ultrasound task force--status and perspectives', *J Rheumatol*, 38(9), pp. 2063-7.

National Audit Office (2009) *Services for people with rheumatoid arthritis*. London: The Stationary Office. [Online]. Available at: https://www.nao.org.uk/wp-content/uploads/2009/07/0809823.pdf (accessed March 31, 2017).

National Institute for Health and Care Excellence (2007) *Adalimumab, etanercept and infliximab for the treatment of rheumatoid arthritis. NICE technology appraisal guidance TA130.* London: National Institute for Health and Care Excellence.

National Institute for Health and Care Excellence (2009) *Rheumatoid arthritis: the management of rheumatoid arthritis in adults. NICE clinical guideline CG79*. London: National Institute for Health and Care Excellence.

Nemoto, T., Ogasawara, M., Matsuki, Y., Murayama, G., Yamada, Y., Sugisaki, N., Ando, S., Minowa, K., Kon, T., Tada, K., Matsushita, M., Yamaji, K., Tamura, N. and Takasaki, Y. (2014) 'Can routine clinical measures predict ultrasound-determined synovitis and remission in rheumatoid arthritis patients?', *Clin Exp Rheumatol*, 32(1), pp. 54-60.

Nguyen, H., Ruyssen-Witrand, A., Gandjbakhch, F., Constantin, A., Foltz, V. and Cantagrel, A. (2014) 'Prevalence of ultrasound-detected residual synovitis and risk of relapse and structural progression in rheumatoid arthritis patients in clinical remission: a systematic review and meta-analysis', *Rheumatology (Oxford)*, 53(11), pp. 2110-8.

Nguyen, M.D. and Simpson-Haidaris, P.J. (2000) 'Cell type-specific regulation of fibrinogen expression in lung epithelial cells by dexamethasone and interleukin-1beta', *Am J Respir Cell Mol Biol*, 22(2), pp. 209-17.

Niedbala, W., Cai, B., Wei, X., Patakas, A., Leung, B.P., McInnes, I.B. and Liew, F.Y. (2008) 'Interleukin 27 attenuates collagen-induced arthritis', *Ann Rheum Dis*, 67(10), pp. 1474-9.

Nishimoto, N., Amano, K., Hirabayashi, Y., Horiuchi, T., Ishii, T., Iwahashi, M., Iwamoto, M., Kohsaka, H., Kondo, M., Matsubara, T., Mimura, T., Miyahara, H., Ohta, S., Saeki, Y., Saito, K., Sano, H., Takasugi, K., Takeuchi, T., Tohma, S., Tsuru, T., Ueki, Y., Yamana, J., Hashimoto, J., Matsutani, T., Murakami, M. and Takagi, N. (2014a) 'Drug free REmission/low disease activity after cessation of tocilizumab (Actemra) Monotherapy (DREAM) study', *Mod Rheumatol*, 24(1), pp. 17-25.

Nishimoto, N., Amano, K., Hirabayashi, Y., Horiuchi, T., Ishii, T., Iwahashi, M., Iwamoto, M., Kohsaka, H., Kondo, M., Matsubara, T., Mimura, T., Miyahara, H., Ohta, S., Saeki, Y., Saito, K., Sano, H., Takasugi, K., Takeuchi, T., Tohma, S., Tsuru, T., Ueki, Y., Yamana, J., Hashimoto, J., Matsutani, T., Murakami, M. and Takagi, N. (2014b) 'Retreatment efficacy and safety of tocilizumab in patients with rheumatoid arthritis in recurrence (RESTORE) study', *Mod Rheumatol*, 24(1), pp. 26-32.

Noack, M. and Miossec, P. (2014) 'Th17 and regulatory T cell balance in autoimmune and inflammatory diseases', *Autoimmun Rev*, 13(6), pp. 668-77.

O'Mahony, R., Richards, A., Deighton, C. and Scott, D. (2010) 'Withdrawal of disease-modifying antirheumatic drugs in patients with rheumatoid arthritis: a systematic review and meta-analysis', *Ann Rheum Dis*, 69(10), pp. 1823-6.

Oflazoglu, E., Boursalian, T.E., Zeng, W., Edwards, A.C., Duniho, S., McEarchern, J.A., Law, C.L., Gerber, H.P. and Grewal, I.S. (2009) 'Blocking of CD27-CD70 pathway by anti-CD70 antibody ameliorates joint disease in murine collagen-induced arthritis', *J Immunol*, 183(6), pp. 3770-7.

Ogishima, H., Tsuboi, H., Umeda, N., Horikoshi, M., Kondo, Y., Sugihara, M., Suzuki, T., Matsumoto, I. and Sumida, T. (2014) 'Analysis of subclinical synovitis detected by ultrasonography and low-field magnetic resonance imaging in patients with rheumatoid arthritis', *Mod Rheumatol*, 24(1), pp. 60-8.

Ohrndorf, S., Werner, S.G., Finzel, S. and Backhaus, M. (2013) 'Musculoskeletal ultrasound and other imaging modalities in rheumatoid arthritis', *Curr Opin Rheumatol*, 25(3), pp. 367-74.

Ono, Y., Saido, T.C. and Sorimachi, H. (2016) 'Calpain research for drug discovery: challenges and potential', *Nat Rev Drug Discov*, 15(12), pp. 854-76.

Orr, C., Sousa, E., Boyle, D.L., Buch, M.H., Buckley, C.D., Canete, J.D., Catrina, A.I., Choy, E.H.S., Emery, P., Fearon, U., Filer, A., Gerlag, D., Humby, F., Isaacs, J.D., Just, S.A., Lauwerys, B.R., Le Goff, B., Manzo, A., McGarry, T., McInnes, I.B., Najm, A., Pitzalis, C., Pratt, A., Smith, M., Tak, P.P., Thurlings, R., Fonseca, J.E. and Veale, D.J. (2017) 'Synovial tissue research: a state-of-the-art review', *Nat Rev Rheumatol*, 13(8), pp. 463-75.

Ostergaard, M. and Moller-Bisgaard, S. (2014) 'Optimal use of MRI in clinical trials, clinical care and clinical registries of patients with rheumatoid arthritis', *Clin Exp Rheumatol*, 32(5 Suppl 85), pp. S-17-22.

Osthus, R.C., Karim, B., Prescott, J.E., Smith, B.D., McDevitt, M., Huso, D.L. and Dang, C.V. (2005) 'The Myc target gene JPO1/CDCA7 is frequently overexpressed in human tumors and has limited transforming activity in vivo', *Cancer Res*, 65(13), pp. 5620-7.

Padovano, I., Costantino, F., Breban, M. and D'Agostino, M.A. (2016) 'Prevalence of ultrasound synovial inflammatory findings in healthy subjects', *Ann Rheum Dis*, 75(10), pp. 1819-23.

Palutke, M., KuKuruga, D. and Tabaczka, P. (1987) 'A flow cytometric method for measuring lymphocyte proliferation directly from tissue culture plates using Ki-67 and propidium iodide', *J Immunol Methods*, 105(1), pp. 97-105.

Pandya, J.M., Lundell, A.C., Andersson, K., Nordstrom, I., Theander, E. and Rudin, A. (2017) 'Blood chemokine profile in untreated early rheumatoid arthritis: CXCL10 as a disease activity marker', *Arthritis Res Ther*, 19(1), p. 20.

Paradowska-Gorycka, A., Raszkiewicz, B., Jurkowska, M., Felis-Giemza, A., Romanowska-Prochnicka, K., Manczak, M. and Olesinska, M. (2014) 'Association of single nucleotide polymorphisms in the IL27 gene with rheumatoid arthritis', *Scand J Immunol*, 80(4), pp. 298-305.

Park, J.K., Han, B.K., Park, J.A., Woo, Y.J., Kim, S.Y., Lee, E.Y., Lee, E.B., Chalan, P., Boots, A.M. and Song, Y.W. (2014) 'CD70-expressing CD4 T cells produce IFN-gamma and IL-17 in rheumatoid arthritis', *Rheumatology (Oxford)*, 53(10), pp. 1896-900.

Park, Y.J., Yoo, S.A., Hwang, D., Cho, C.S. and Kim, W.U. (2016) 'Identification of novel urinary biomarkers for assessing disease activity and prognosis of rheumatoid arthritis', *Exp Mol Med*, 48, p. e211.

Pavkova Goldbergova, M., Pavek, N., Lipkova, J., Jarkovsky, J., Stouracova, M., Gatterova, J., Vasku, A., Soucek, M. and Nemec, P. (2012) 'Circulating cytokine pattern and factors describing rheumatoid arthritis: IL-15 as one of the biomarkers for RA?', *Biomarkers*, 17(7), pp. 655-62.

Peluso, G., Michelutti, A., Bosello, S., Gremese, E., Tolusso, B. and Ferraccioli, G. (2011) 'Clinical and ultrasonographic remission determines different chances of relapse in early and long standing rheumatoid arthritis', *Ann Rheum Dis*, 70(1), pp. 172-5.

Peng, H., Wang, W., Zhou, M., Li, R., Pan, H.F. and Ye, D.Q. (2013) 'Role of interleukin-10 and interleukin-10 receptor in systemic lupus erythematosus', *Clin Rheumatol*, 32(9), pp. 1255-66.

Peterfy, C., Burmester, G.R., Bykerk, V.P., Combe, B.G., DiCarlo, J.C., Furst, D.E., Huizinga, T.W., Wong, D.A., Conaghan, P.G. and Emery, P. (2016) 'Sustained improvements in MRI outcomes with abatacept following the withdrawal of all treatments in patients with early, progressive rheumatoid arthritis', *Ann Rheum Dis*, 75(8), pp. 1501-5.

Pflanz, S., Timans, J.C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W.M., Mattson, J.D., Wagner, J.L., To, W., Zurawski, S., McClanahan, T.K., Gorman, D.M., Bazan, J.F., de Waal Malefyt, R., Rennick, D. and Kastelein, R.A. (2002) 'IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells', *Immunity*, 16(6), pp. 779-90.

Pianta, A., Arvikar, S., Strle, K., Drouin, E.E., Wang, Q., Costello, C.E. and Steere, A.C. (2017) 'Evidence of the Immune Relevance of Prevotella copri, a Gut Microbe, in Patients With Rheumatoid Arthritis', *Arthritis Rheumatol*, 69(5), pp. 964-75.

Pinals, R.S., Masi, A.T. and Larsen, R.A. (1981) 'Preliminary criteria for clinical remission in rheumatoid arthritis', *Arthritis Rheum*, 24(10), pp. 1308-15.

Pincus, T., Bergman, M.J., Yazici, Y., Hines, P., Raghupathi, K. and Maclean, R. (2008) 'An index of only patient-reported outcome measures, routine assessment of patient index data 3 (RAPID3), in two abatacept clinical trials: similar results to disease activity score (DAS28) and other RAPID indices that include physician-reported measures', *Rheumatology (Oxford)*, 47(3), pp. 345-9.

Pincus, T., Block, J.A., Yazici, Y., Bergman, M.J. and Sokka, T. (2017) 'Limited Value of the Multi-Biomarker Disease Activity Assay Compared to the Routine Assessment of Patient Index Data 3 (RAPID3) Score in the Prognosis of Important Clinical Outcomes in Rheumatoid Arthritis: Comment on the Article by Fleischmann et al and Accompanying Editorial by Davis', *Arthritis Rheumatol*, 69(4), pp. 866-7.

Ponchel, F., Morgan, A.W., Bingham, S.J., Quinn, M., Buch, M., Verburg, R.J., Henwood, J., Douglas, S.H., Masurel, A., Conaghan, P., Gesinde, M., Taylor, J., Markham, A.F., Emery, P., van Laar, J.M. and Isaacs, J.D. (2002) 'Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis', *Blood*, 100(13), pp. 4550-6.

Ponnappan, S., Cullen, S.J. and Ponnappan, U. (2005) 'Constitutive degradation of IκBα in human T lymphocytes is mediated by calpain', *Immun Ageing*, 2, p. 15.

Pos, O., Oostendorp, R.A., van der Stelt, M.E., Scheper, R.J. and Van Dijk, W. (1990) 'Con A-nonreactive human alpha 1-acid glycoprotein (AGP) is more effective in modulation of lymphocyte proliferation than Con A-reactive AGP serum variants', *Inflammation*, 14(2), pp. 133-41.

Postigo, J., Genre, F., Iglesias, M., Fernandez-Rey, M., Buelta, L., Carlos Rodriguez-Rey, J., Merino, J. and Merino, R. (2011) 'Exacerbation of type II collagen-induced arthritis in apolipoprotein E-deficient mice in association with the expansion of Th1 and Th17 cells', *Arthritis Rheum*, 63(4), pp. 971-80.

Pot, C., Apetoh, L. and Kuchroo, V.K. (2011) 'Type 1 regulatory T cells (Tr1) in autoimmunity', *Semin Immunol*, 23(3), pp. 202-8.

Pratt, A.G. (2011) *New tools for the diagnosis of early rheumatoid arthritis*. PhD Thesis. Newcastle University.

Pratt, A.G., Swan, D.C., Richardson, S., Wilson, G., Hilkens, C.M., Young, D.A. and Isaacs, J.D. (2012) 'A CD4 T cell gene signature for early rheumatoid arthritis implicates interleukin 6-mediated STAT3 signalling, particularly in anti-citrullinated peptide antibody-negative disease', *Ann Rheum Dis*, 71(8), pp. 1374-81.

Prevoo, M.L., van 't Hof, M.A., Kuper, H.H., van Leeuwen, M.A., van de Putte, L.B. and van Riel, P.L. (1995) 'Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis', *Arthritis Rheum*, 38(1), pp. 44-8.

Qingchun, H., Runyue, H., LiGang, J., Yongliang, C., Song, W. and Shujing, Z. (2008) 'Comparison of the expression profile of apoptosis-associated genes in rheumatoid arthritis and osteoarthritis', *Rheumatol Int*, 28(7), pp. 697-701.

R Core Team (2016) *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Radovits, B.J., Fransen, J., Al Shamma, S., Eijsbouts, A.M., van Riel, P.L. and Laan, R.F. (2010) 'Excess mortality emerges after 10 years in an inception cohort of early rheumatoid arthritis', *Arthritis Care Res (Hoboken)*, 62(3), pp. 362-70.

Raffeiner, B., Sfriso, P., Gutierrez, M., Ravagnami, V., Carpentino, G., Botsios, C., Bernardi, L., Ometto, F. and Punzi, L. (2011) 'Usefulness of power Doppler to predict radiological damage in rheumatoid arthritis patients in clinical remission by TNF blockers [abstract]', *Ann Rheum Dis*, 70(Suppl 3), p. 76.

Raker, V.K., Domogalla, M.P. and Steinbrink, K. (2015) 'Tolerogenic Dendritic Cells for Regulatory T Cell Induction in Man', *Front Immunol*, 6, p. 569.

Ramírez García, J., Ruíz-Esquide, V., Celis, R., Cuervo, A., Cabrera, S., Inciarte-Mundo, J., Sanmartí, R. and Cañete, J.D. (2014) 'AB0254 Sonographic and Clinical Characterization of A Prospective Cohort of Patients with Rheumatoid Arthritis in Clinical Remission. Preliminary Results [abstract]', *Ann Rheum Dis*, 73(Suppl 2), p. 888.

Ramirez, J., Celis, R., Usategui, A., Ruiz-Esquide, V., Fare, R., Cuervo, A., Sanmarti, R., Pablos, J.L. and Canete, J.D. (2015) 'Immunopathologic characterization of ultrasound-defined synovitis in rheumatoid arthritis patients in clinical remission', *Arthritis Res Ther*, 18, p. 74.

Ramirez, J., Ruiz-Esquide, V., Pomes, I., Celis, R., Cuervo, A., Hernandez, M.V., Pomes, J., Pablos, J.L., Sanmarti, R. and Canete, J.D. (2014) 'Patients with rheumatoid arthritis in clinical remission and ultrasound-defined active synovitis exhibit higher disease activity and increased serum levels of angiogenic biomarkers', *Arthritis Res Ther*, 16(1), p. R5.

Ramiro, S., Gaujoux-Viala, C., Nam, J.L., Smolen, J.S., Buch, M., Gossec, L., van der Heijde, D., Winthrop, K. and Landewe, R. (2014) 'Safety of synthetic and biological DMARDs: a systematic literature review informing the 2013 update of the EULAR recommendations for management of rheumatoid arthritis', *Ann Rheum Dis*, 73(3), pp. 529-35.

Ramiro, S., Sepriano, A., Chatzidionysiou, K., Nam, J.L., Smolen, J.S., van der Heijde, D., Dougados, M., van Vollenhoven, R., Bijlsma, J.W., Burmester, G.R., Scholte-Voshaar, M., Falzon, L. and Landewe, R.B.M. (2017) 'Safety of synthetic and biological DMARDs: a systematic literature review informing the 2016 update of the EULAR recommendations for management of rheumatoid arthritis', *Ann Rheum Dis*, 76(6), pp. 1101-36.

Rantapaa-Dahlqvist, S., de Jong, B.A., Berglin, E., Hallmans, G., Wadell, G., Stenlund, H., Sundin, U. and van Venrooij, W.J. (2003) 'Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis', *Arthritis Rheum*, 48(10), pp. 2741-9.

Raza, K. and Filer, A. (2015) 'The therapeutic window of opportunity in rheumatoid arthritis: does it ever close?', *Ann Rheum Dis*, 74(5), pp. 793-4.

Rech, J., Hueber, A.J., Finzel, S., Englbrecht, M., Haschka, J., Manger, B., Kleyer, A., Reiser, M., Cobra, J.F., Figueiredo, C., Tony, H.P., Kleinert, S., Wendler, J., Schuch, F., Ronneberger, M., Feuchtenberger, M., Fleck, M., Manger, K., Ochs, W., Schmitt-Haendle, M., Lorenz, H.M., Nuesslein, H., Alten, R., Henes, J., Krueger, K. and Schett, G. (2016) 'Prediction of disease relapses by multibiomarker disease activity and autoantibody status in patients with rheumatoid arthritis on tapering DMARD treatment', *Ann Rheum Dis*, 75(9), pp. 1637-44.

Rintelen, B., Sautner, J., Haindl, P., Mai, H., Brezinschek, H.P. and Leeb, B.F. (2013) 'Remission in rheumatoid arthritis: a comparison of the 2 newly proposed ACR/EULAR remission criteria with the rheumatoid arthritis disease activity index-5, a patient self-report disease activity index', *J Rheumatol*, 40(4), pp. 394-400.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W. and Smyth, G.K. (2015) 'limma powers differential expression analyses for RNA-sequencing and microarray studies', *Nucleic Acids Res*, 43(7), p. e47.

Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J.C. and Muller, M. (2011) 'pROC: an open-source package for R and S+ to analyze and compare ROC curves', *BMC Bioinformatics*, 12, p. 77.

Ropes, M.W., Bennett, G.A., Cobb, S., Jacox, R. and Jessar, R.A. (1958) '1958 Revision of diagnostic criteria for rheumatoid arthritis', *Bull Rheum Dis*, 9(4), pp. 175-6.

Rosengren, S., Wei, N., Kalunian, K.C., Kavanaugh, A. and Boyle, D.L. (2011) 'CXCL13: a novel biomarker of B-cell return following rituximab treatment and synovitis in patients with rheumatoid arthritis', *Rheumatology (Oxford)*, 50(3), pp. 603-10.

Rossini, M., Rossi, E., Bernardi, D., Viapiana, O., Gatti, D., Idolazzi, L., Caimmi, C., Derosa, M. and Adami, S. (2014) 'Prevalence and incidence of rheumatoid arthritis in Italy', *Rheumatol Int*, 34(5), pp. 659-64.

Rowe, B.L.Y. (2016) *futile.logger: A Logging Utility for R. R package version 1.4.3.* [Computer program]. https://CRAN.R-project.org/package=futile.logger.

Sagoo, P., Perucha, E., Sawitzki, B., Tomiuk, S., Stephens, D.A., Miqueu, P., Chapman, S., Craciun, L., Sergeant, R., Brouard, S., Rovis, F., Jimenez, E., Ballow, A., Giral, M., Rebollo-Mesa, I., Le Moine, A., Braudeau, C., Hilton, R., Gerstmayer, B., Bourcier, K., Sharif, A., Krajewska, M., Lord, G.M., Roberts, I., Goldman, M., Wood, K.J., Newell, K., Seyfert-Margolis, V., Warrens, A.N., Janssen, U., Volk, H.D., Soulillou, J.P., Hernandez-Fuentes, M.P. and Lechler, R.I. (2010) 'Development of a cross-platform biomarker signature to detect renal transplant tolerance in humans', *J Clin Invest*, 120(6), pp. 1848-61.

Sakellariou, G., Scire, C.A., Verstappen, S.M., Montecucco, C. and Caporali, R. (2013) 'In patients with early rheumatoid arthritis, the new ACR/EULAR definition of remission identifies patients with persistent absence of functional disability and suppression of ultrasonographic synovitis', *Ann Rheum Dis*, 72(2), pp. 245-9.

Saleem, B., Brown, A.K., Keen, H., Nizam, S., Freeston, J., Wakefield, R., Karim, Z., Quinn, M., Hensor, E., Conaghan, P.G. and Emery, P. (2011) 'Should imaging be a component of rheumatoid arthritis remission criteria? A comparison between traditional and modified composite remission scores and imaging assessments', *Ann Rheum Dis*, 70(5), pp. 792-8.

Saleem, B., Brown, A.K., Quinn, M., Karim, Z., Hensor, E.M., Conaghan, P., Peterfy, C., Wakefield, R.J. and Emery, P. (2012) 'Can flare be predicted in DMARD treated RA patients in remission, and is it important? A cohort study', *Ann Rheum Dis*, 71(8), pp. 1316-21.

Saleem, B., Keen, H., Goeb, V., Parmar, R., Nizam, S., Hensor, E.M., Churchman, S.M., Quinn, M., Wakefield, R., Conaghan, P.G., Ponchel, F. and Emery, P. (2010) 'Patients with RA in remission on TNF blockers: when and in whom can TNF blocker therapy be stopped?', *Ann Rheum Dis*, 69(9), pp. 1636-42.

Saravanan, V. and Kelly, C.A. (2004) 'Reducing the risk of methotrexate pneumonitis in rheumatoid arthritis', *Rheumatology (Oxford)*, 43(2), pp. 143-7.

Scheel, A.K., Hermann, K.G., Kahler, E., Pasewaldt, D., Fritz, J., Hamm, B., Brunner, E., Muller, G.A., Burmester, G.R. and Backhaus, M. (2005) 'A novel ultrasonographic synovitis scoring system suitable for analyzing finger joint inflammation in rheumatoid arthritis', *Arthritis Rheum*, 52(3), pp. 733-43.

Scher, J.U., Sczesnak, A., Longman, R.S., Segata, N., Ubeda, C., Bielski, C., Rostron, T., Cerundolo, V., Pamer, E.G., Abramson, S.B., Huttenhower, C. and Littman, D.R. (2013) 'Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis', *Elife*, 2, p. e01202.

Schett, G., Emery, P., Tanaka, Y., Burmester, G., Pisetsky, D.S., Naredo, E., Fautrel, B. and van Vollenhoven, R. (2016) 'Tapering biologic and conventional DMARD therapy in rheumatoid arthritis: current evidence and future directions', *Ann Rheum Dis*, 75(8), pp. 1428-37.

Schmidt, W.A. (2004) 'Doppler sonography in rheumatology', *Best Pract Res Clin Rheumatol*, 18(6), pp. 827-46.

Schneider, R., Yaneva, T., Beauseigle, D., El-Khoury, L. and Arbour, N. (2011) 'IL-27 increases the proliferation and effector functions of human naive CD8+ T lymphocytes and promotes their development into Tc1 cells', *Eur J Immunol*, 41(1), pp. 47-59.

Schoels, M., Knevel, R., Aletaha, D., Bijlsma, J.W., Breedveld, F.C., Boumpas, D.T., Burmester, G., Combe, B., Cutolo, M., Dougados, M., Emery, P., van der Heijde, D., Huizinga, T.W., Kalden, J., Keystone, E.C., Kvien, T.K., Martin-Mola, E., Montecucco, C., de Wit, M. and Smolen, J.S. (2010) 'Evidence for treating rheumatoid arthritis to target: results of a systematic literature search', *Ann Rheum Dis*, 69(4), pp. 638-43.

Schoenfeld, D. (1982) 'Partial residuals for the proportional hazards regression model', *Biometrika*, 69(1), pp. 239-41.

Scire, C.A., Montecucco, C., Codullo, V., Epis, O., Todoerti, M. and Caporali, R. (2009) 'Ultrasonographic evaluation of joint involvement in early rheumatoid arthritis in clinical remission: power Doppler signal predicts short-term relapse', *Rheumatology (Oxford)*, 48(9), pp. 1092-7.

Scott, D.L., Wolfe, F. and Huizinga, T.W. (2010) 'Rheumatoid arthritis', *Lancet*, 376(9746), pp. 1094-108.

Scott, I.C., Kingsley, G.H. and Scott, D.L. (2013a) 'Can we discontinue synthetic disease-modifying anti-rheumatic drugs in rheumatoid arthritis?', *Clin Exp Rheumatol*, 31(4 Suppl 78), pp. S4-8.

Scott, I.C., Tan, R., Stahl, D., Steer, S., Lewis, C.M. and Cope, A.P. (2013b) 'The protective effect of alcohol on developing rheumatoid arthritis: a systematic review and meta-analysis', *Rheumatology (Oxford)*, 52(5), pp. 856-67.

Scottish Intercollegiate Guidelines Network (2011) *Management of early rheumatoid arthritis (SIGN publication no. 123).* Edinburgh: Scottish Intercollegiate Guidelines Network

Sempere-Ortells, J.M., Perez-Garcia, V., Marin-Alberca, G., Peris-Pertusa, A., Benito, J.M., Marco, F.M., Zubcoff, J.J. and Navarro-Blasco, F.J. (2009) 'Quantification and phenotype of regulatory T cells in rheumatoid arthritis according to disease activity score-28', *Autoimmunity*, 42(8), pp. 636-45.

Sender, L.Y., Gibbert, K., Suezer, Y., Radeke, H.H., Kalinke, U. and Waibler, Z. (2010) 'CD40 ligand-triggered human dendritic cells mount interleukin-23 responses that are further enhanced by danger signals', *Mol Immunol*, 47(6), pp. 1255-61.

Sharp, J.T., Van Der Heijde, D., Boers, M., Boonen, A., Bruynesteyn, K., Emery, P., Genant, H.K., Herborn, G., Jurik, A., Lassere, M., McQueen, F., Ostergaard, M., Peterfy, C., Rau, R., Strand, V., Wassenberg, S. and Weissman, B. (2003) 'Repair of erosions in rheumatoid arthritis does occur. Results from 2 studies by the OMERACT Subcommittee on Healing of Erosions', *J Rheumatol*, 30(5), pp. 1102-7.

Shen, H., Heuzey, E., Mori, D.N., Wong, C.K., Colangelo, C.M., Chung, L.M., Bruce, C., Slizovskiy, I.B., Booth, C.J., Kreisel, D. and Goldstein, D.R. (2015) 'Haptoglobin enhances cardiac transplant rejection', *Circ Res*, 116(10), pp. 1670-9.

Shen, H., Song, Y., Colangelo, C.M., Wu, T., Bruce, C., Scabia, G., Galan, A., Maffei, M. and Goldstein, D.R. (2012) 'Haptoglobin activates innate immunity to enhance acute transplant rejection in mice', *J Clin Invest*, 122(1), pp. 383-7.

Shen, H., Xia, L., Xiao, W. and Lu, J. (2011) 'Increased levels of interleukin-27 in patients with rheumatoid arthritis', *Arthritis Rheum*, 63(3), pp. 860-1.

Singh, J.A., Saag, K.G., Bridges, S.L., Jr., Akl, E.A., Bannuru, R.R., Sullivan, M.C., Vaysbrot, E., McNaughton, C., Osani, M., Shmerling, R.H., Curtis, J.R., Furst, D.E., Parks, D., Kavanaugh, A., O'Dell, J., King, C., Leong, A., Matteson, E.L., Schousboe, J.T., Drevlow, B., Ginsberg, S., Grober, J., St Clair, E.W., Tindall, E., Miller, A.S. and McAlindon, T. (2016) '2015 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis', *Arthritis Rheumatol*, 68(1), pp. 1-26.

Smolen, J.S., Aletaha, D. and McInnes, I.B. (2016a) 'Rheumatoid arthritis', *Lancet*, 388(10055), pp. 2023-38.

Smolen, J.S., Breedveld, F.C., Burmester, G.R., Bykerk, V., Dougados, M., Emery, P., Kvien, T.K., Navarro-Compan, M.V., Oliver, S., Schoels, M., Scholte-Voshaar, M., Stamm, T., Stoffer, M., Takeuchi, T., Aletaha, D.,

Andreu, J.L., Aringer, M., Bergman, M., Betteridge, N., Bijlsma, H., Burkhardt, H., Cardiel, M., Combe, B., Durez, P., Fonseca, J.E., Gibofsky, A., Gomez-Reino, J.J., Graninger, W., Hannonen, P., Haraoui, B., Kouloumas, M., Landewe, R., Martin-Mola, E., Nash, P., Ostergaard, M., Ostor, A., Richards, P., Sokka-Isler, T., Thorne, C., Tzioufas, A.G., van Vollenhoven, R., de Wit, M. and van der Heijde, D. (2016b) 'Treating rheumatoid arthritis to target: 2014 update of the recommendations of an international task force', *Ann Rheum Dis*, 75(1), pp. 3-15.

Smolen, J.S., Breedveld, F.C., Schiff, M.H., Kalden, J.R., Emery, P., Eberl, G., van Riel, P.L. and Tugwell, P. (2003) 'A simplified disease activity index for rheumatoid arthritis for use in clinical practice', *Rheumatology (Oxford)*, 42(2), pp. 244-57.

Smolen, J.S., Emery, P., Ferraccioli, G.F., Samborski, W., Berenbaum, F., Davies, O.R., Koetse, W., Purcaru, O., Bennett, B. and Burkhardt, H. (2015) 'Certolizumab pegol in rheumatoid arthritis patients with low to moderate activity: the CERTAIN double-blind, randomised, placebo-controlled trial', *Ann Rheum Dis*, 74(5), pp. 843-50.

Smolen, J.S., Emery, P., Fleischmann, R., van Vollenhoven, R.F., Pavelka, K., Durez, P., Guerette, B., Kupper, H., Redden, L., Arora, V. and Kavanaugh, A. (2014) 'Adjustment of therapy in rheumatoid arthritis on the basis of achievement of stable low disease activity with adalimumab plus methotrexate or methotrexate alone: the randomised controlled OPTIMA trial', *Lancet*, 383(9914), pp. 321-32.

Smolen, J.S., Landewe, R., Bijlsma, J., Burmester, G., Chatzidionysiou, K., Dougados, M., Nam, J., Ramiro, S., Voshaar, M., van Vollenhoven, R., Aletaha, D., Aringer, M., Boers, M., Buckley, C.D., Buttgereit, F., Bykerk, V., Cardiel, M., Combe, B., Cutolo, M., van Eijk-Hustings, Y., Emery, P., Finckh, A., Gabay, C., Gomez-Reino, J., Gossec, L., Gottenberg, J.E., Hazes, J.M.W., Huizinga, T., Jani, M., Karateev, D., Kouloumas, M., Kvien, T., Li, Z., Mariette, X., McInnes, I., Mysler, E., Nash, P., Pavelka, K., Poor, G., Richez, C., van Riel, P., Rubbert-Roth, A., Saag, K., da Silva, J., Stamm, T., Takeuchi, T., Westhovens, R., de Wit, M. and van der Heijde, D. (2017) 'EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2016 update', *Ann Rheum Dis*, 76(6), pp. 960-977.

Smolen, J.S., Nash, P., Durez, P., Hall, S., Ilivanova, E., Irazoque-Palazuelos, F., Miranda, P., Park, M.C., Pavelka, K., Pedersen, R., Szumski, A., Hammond, C., Koenig, A.S. and Vlahos, B. (2013) 'Maintenance, reduction, or withdrawal of etanercept after treatment with etanercept and methotrexate in patients with moderate rheumatoid arthritis (PRESERVE): a randomised controlled trial', *Lancet*, 381(9870), pp. 918-29.

Soneson, C., Love, M.I. and Robinson, M.D. (2015) 'Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences', *F1000Res*, 4, p. 1521.

Stefanini, G.F., Mazzetti, M., Piccinini, G.C., Capelli, S., Baraldini, M. and Gasbarrini, G. (1989) 'Lymphocyte membrane alpha-1-acid glycoprotein: a cellular synthesis during lymphocyte activation', *Biochem Int*, 19(2), pp. 397-403.

Steiner, G., Tohidast-Akrad, M., Witzmann, G., Vesely, M., Studnicka-Benke, A., Gal, A., Kunaver, M., Zenz, P. and Smolen, J.S. (1999) 'Cytokine production by synovial T cells in rheumatoid arthritis', *Rheumatology (Oxford)*, 38(3), pp. 202-13.

Stolt, P., Kallberg, H., Lundberg, I., Sjogren, B., Klareskog, L. and Alfredsson, L. (2005) 'Silica exposure is associated with increased risk of developing rheumatoid arthritis: results from the Swedish EIRA study', *Ann Rheum Dis*, 64(4), pp. 582-6.

Strickland, F.M., Patel, D., Khanna, D., Somers, E., Robida, A.M., Pihalja, M., Swartz, R., Marder, W. and Richardson, B. (2016) 'Characterisation of an epigenetically altered CD4(+) CD28(+) Kir(+) T cell subset in autoimmune rheumatic diseases by multiparameter flow cytometry', *Lupus Sci Med*, 3(1), p. e000147.

Stucki, G., Liang, M.H., Stucki, S., Bruhlmann, P. and Michel, B.A. (1995) 'A self-administered rheumatoid arthritis disease activity index (RADAI) for epidemiologic research. Psychometric properties and correlation with parameters of disease activity', *Arthritis Rheum*, 38(6), pp. 795-8.

Studenic, P., Smolen, J.S. and Aletaha, D. (2012) 'Near misses of ACR/EULAR criteria for remission: effects of patient global assessment in Boolean and index-based definitions', *Ann Rheum Dis*, 71(10), pp. 1702-5.

Su, H.P., Nakada-Tsukui, K., Tosello-Trampont, A.C., Li, Y., Bu, G., Henson, P.M. and Ravichandran, K.S. (2002) 'Interaction of CED-6/GULP, an adapter protein involved in engulfment of apoptotic cells with CED-1 and CD91/low density lipoprotein receptor-related protein (LRP)', *J Biol Chem*, 277(14), pp. 11772-9.

Svensson, B., Andersson, M.L., Bala, S.V., Forslind, K. and Hafstrom, I. (2013) 'Long-term sustained remission in a cohort study of patients with rheumatoid arthritis: choice of remission criteria', *BMJ Open*, 3(9), p. e003554.

Symmons, D., Turner, G., Webb, R., Asten, P., Barrett, E., Lunt, M., Scott, D. and Silman, A. (2002) 'The prevalence of rheumatoid arthritis in the United Kingdom: new estimates for a new century', *Rheumatology (Oxford)*, 41(7), pp. 793-800.

Szkudlarek, M., Court-Payen, M., Jacobsen, S., Klarlund, M., Thomsen, H.S. and Ostergaard, M. (2003) 'Interobserver agreement in ultrasonography of the finger and toe joints in rheumatoid arthritis', *Arthritis Rheum*, 48(4), pp. 955-62.

Takase, K., Ohno, S., Takeno, M., Hama, M., Kirino, Y., Ihata, A., Ideguchi, H., Mochida, Y., Tateishi, U., Shizukuishi, K., Nagashima, Y., Aoki, I. and Ishigatsubo, Y. (2012) 'Simultaneous evaluation of long-lasting knee synovitis in patients undergoing arthroplasty by power Doppler ultrasonography and contrast-enhanced MRI in comparison with histopathology', *Clin Exp Rheumatol*, 30(1), pp. 85-92.

Takeuchi, T., Matsubara, T., Ohta, S., Mukai, M., Amano, K., Tohma, S., Tanaka, Y., Yamanaka, H. and Miyasaka, N. (2015) 'Biologic-free remission of established rheumatoid arthritis after discontinuation of abatacept: a prospective, multicentre, observational study in Japan', *Rheumatology (Oxford)*, 54(4), pp. 683-91.

Talbot, J., Bianchini, F.J., Nascimento, D.C., Oliveira, R.D., Souto, F.O., Pinto, L.G., Peres, R.S., Silva, J.R., Almeida, S.C., Louzada-Junior, P., Cunha, T.M., Cunha, F.Q. and Alves-Filho, J.C. (2015) 'CCR2 Expression in Neutrophils Plays a Critical Role in Their Migration Into the Joints in Rheumatoid Arthritis', *Arthritis Rheumatol*, 67(7), pp. 1751-9.

Tan, Y.K., Ostergaard, M. and Conaghan, P.G. (2012) 'Imaging tools in rheumatoid arthritis: ultrasound vs magnetic resonance imaging', *Rheumatology (Oxford)*, 51 Suppl 7, pp. vii36-42.

Tanaka, Y. and Hirata, S. (2014) 'Intensive intervention can lead to a treatment holiday from biological DMARDs in patients with rheumatoid arthritis', *Drugs*, 74(18), pp. 2129-39.

Tanaka, Y., Hirata, S., Kubo, S., Fukuyo, S., Hanami, K., Sawamukai, N., Nakano, K., Nakayamada, S., Yamaoka, K., Sawamura, F. and Saito, K. (2015) 'Discontinuation of adalimumab after achieving remission in patients with established rheumatoid arthritis: 1-year outcome of the HONOR study', *Ann Rheum Dis*, 74(2), pp. 389-95.

Tanaka, Y., Takeuchi, T., Mimori, T., Saito, K., Nawata, M., Kameda, H., Nojima, T., Miyasaka, N., Koike, T. and for the R. R. R. study investigators (2010) 'Discontinuation of infliximab after attaining low disease activity in patients with rheumatoid arthritis: RRR (remission induction by Remicade in RA) study', *Ann Rheum Dis*, 69(7), pp. 1286-91.

Tanida, S., Yoshitomi, H., Ishikawa, M., Kasahara, T., Murata, K., Shibuya, H., Ito, H. and Nakamura, T. (2011) 'IL-27-producing CD14(+) cells infiltrate inflamed joints of rheumatoid arthritis and regulate inflammation and chemotactic migration', *Cytokine*, 55(2), pp. 237-44.

Teitsma, X.M., Jacobs, J.W.G., Mokry, M., Borm, M.E.A., Petho-Schramm, A., van Laar, J.M., Bijlsma, J.W.J. and Lafeber, F.P.J. (2017) 'Identification of differential co-expressed gene networks in early rheumatoid arthritis achieving sustained drug-free remission after treatment with a tocilizumab-based or methotrexate-based strategy', *Arthritis Res Ther*, 19(1), p. 170.

ten Wolde, S., Breedveld, F.C., Hermans, J., Vandenbroucke, J.P., van de Laar, M.A., Markusse, H.M., Janssen, M., van den Brink, H.R. and Dijkmans, B.A. (1996) 'Randomised placebo-controlled study of stopping second-line drugs in rheumatoid arthritis', *Lancet*, 347(8998), pp. 347-52.

ten Wolde, S., Hermans, J., Breedveld, F.C. and Dijkmans, B.A. (1997) 'Effect of resumption of second line drugs in patients with rheumatoid arthritis that flared up after treatment discontinuation', *Ann Rheum Dis*, 56(4), pp. 235-9.

Theilgaard-Monch, K., Jacobsen, L.C., Nielsen, M.J., Rasmussen, T., Udby, L., Gharib, M., Arkwright, P.D., Gombart, A.F., Calafat, J., Moestrup, S.K., Porse, B.T. and Borregaard, N. (2006) 'Haptoglobin is synthesized during granulocyte differentiation, stored in specific granules, and released by neutrophils in response to activation', *Blood*, 108(1), pp. 353-61.

Therneau, T. (2015) *A Package for Survival Analysis in S, version 2.38* [Computer program]. https://CRAN.R-project.org/package=survival.

Thiele, K., Huscher, D., Bischoff, S., Spathling-Mestekemper, S., Backhaus, M., Aringer, M., Kohlmann, T., Zink, A. and German Collaborative Arthritis, C. (2013) 'Performance of the 2011 ACR/EULAR preliminary remission criteria compared with DAS28 remission in unselected patients with rheumatoid arthritis', *Ann Rheum Dis*, 72(7), pp. 1194-9.

Thijssen, P.E., Ito, Y., Grillo, G., Wang, J., Velasco, G., Nitta, H., Unoki, M., Yoshihara, M., Suyama, M., Sun, Y., Lemmers, R.J., de Greef, J.C., Gennery, A., Picco, P., Kloeckener-Gruissem, B., Gungor, T., Reisli, I., Picard, C., Kebaili, K., Roquelaure, B., Iwai, T., Kondo, I., Kubota, T., van Ostaijen-Ten Dam, M.M., van Tol, M.J., Weemaes, C., Francastel, C., van der Maarel, S.M. and Sasaki, H. (2015) 'Mutations in CDCA7 and HELLS cause immunodeficiency-centromeric instability-facial anomalies syndrome', *Nat Commun*, 6, p. 7870.

Thurkow, E.W., van der Heijden, I.M., Breedveld, F.C., Smeets, T.J., Daha, M.R., Kluin, P.M., Meinders, A.E. and Tak, P.P. (1997) 'Increased expression of IL-15 in the synovium of patients with rheumatoid arthritis compared with patients with Yersinia-induced arthritis and osteoarthritis', *J Pathol*, 181(4), pp. 444-50.

Tiippana-Kinnunen, T., Paimela, L., Kautiainen, H., Laasonen, L. and Leirisalo-Repo, M. (2010) 'Can disease-modifying anti-rheumatic drugs be discontinued in long-standing rheumatoid arthritis? A 15-year follow-up', *Scand J Rheumatol*, 39(1), pp. 12-8.

Tosh, J.C., Wailoo, A.J., Scott, D.L. and Deighton, C.M. (2011) 'Cost-effectiveness of combination nonbiologic disease-modifying antirheumatic drug strategies in patients with early rheumatoid arthritis', *J Rheumatol*, 38(8), pp. 1593-600.

Tosiek, M.J., Fiette, L., El Daker, S., Eberl, G. and Freitas, A.A. (2016) 'IL-15-dependent balance between Foxp3 and RORgammat expression impacts inflammatory bowel disease', *Nat Commun*, 7, p. 10888.

Trynka, G., Sandor, C., Han, B., Xu, H., Stranger, B.E., Liu, X.S. and Raychaudhuri, S. (2013) 'Chromatin marks identify critical cell types for fine mapping complex trait variants', *Nat Genet*, 45(2), pp. 124-30.

Tsuda, M., Kawaida, R., Kobayashi, K., Shinagawa, A., Sawada, T., Yamada, R., Yamamoto, K. and Aigaki, T. (2010) 'POSH promotes cell survival in Drosophila and in human RASF cells', *FEBS Lett*, 584(22), pp. 4689-94.

Turesson, C. (2013) 'Extra-articular rheumatoid arthritis', *Curr Opin Rheumatol*, 25(3), pp. 360-6.

Turesson, C., Bergstrom, U., Pikwer, M., Nilsson, J.A. and Jacobsson, L.T. (2016) 'A high body mass index is associated with reduced risk of rheumatoid arthritis in men, but not in women', *Rheumatology (Oxford)*, 55(2), pp. 307-14.

Tweehuysen, L., van den Ende, C.H., Beeren, F.M., Been, E.M., van den Hoogen, F.H. and den Broeder, A.A. (2017) 'Little Evidence for Usefulness of Biomarkers for Predicting Successful Dose Reduction or Discontinuation of a Biologic Agent in Rheumatoid Arthritis: A Systematic Review', *Arthritis Rheumatol*, 69(2), pp. 301-8.

van Aken, J., Heimans, L., Gillet-van Dongen, H., Visser, K., Ronday, H.K., Speyer, I., Peeters, A.J., Huizinga, T.W. and Allaart, C.F. (2014) 'Five-year outcomes of probable rheumatoid arthritis treated with methotrexate or placebo during the first year (the PROMPT study)', *Ann Rheum Dis*, 73(2), pp. 396-400.

van Bergen, J. and Koning, F. (2010) 'The tortoise and the hare: slowly evolving T-cell responses take hastily evolving KIR', *Immunology*, 131(3), pp. 301-9.

van den Broek, M., Huizinga, T.W., Dijkmans, B.A. and Allaart, C.F. (2011) 'Drug-free remission: is it already possible?', *Curr Opin Rheumatol*, 23(3), pp. 266-72.

van der Heijde, D.M., van 't Hof, M.A., van Riel, P.L., Theunisse, L.A., Lubberts, E.W., van Leeuwen, M.A., van Rijswijk, M.H. and van de Putte, L.B. (1990) 'Judging disease activity in clinical practice in rheumatoid arthritis: first step in the development of a disease activity score', *Ann Rheum Dis*, 49(11), pp. 916-20.

Van der Leeden, H., Dijkmans, B.A., Hermans, J. and Cats, A. (1986) 'A double-blind study on the effect of discontinuation of gold therapy in patients with rheumatoid arthritis', *Clin Rheumatol*, 5(1), pp. 56-61.

van der Ven, M., Kuijper, T.M., Gerards, A.H., Tchetverikov, I., Weel, A.E.a.M., van Zeben, D., Hazes, J.M.W. and Luime, J.J. (2014) 'Can We Use Ultrasound to Identify Rheumatoid Arthritis Patients in Remission Who Cannot Taper Their Medication? [abstract]', *Arthritis Rheumatol*, 73(S10), pp. S51. Available at: http://acrabstracts.org/abstract/can-we-use-ultrasound-to-identify-rheumatoid-arthritis-patients-in-remission-who-cannot-taper-their-medication/ (Accessed: February 08, 2017).

van der Woude, D., Rantapaa-Dahlqvist, S., Ioan-Facsinay, A., Onnekink, C., Schwarte, C.M., Verpoort, K.N., Drijfhout, J.W., Huizinga, T.W., Toes, R.E. and Pruijn, G.J. (2010) '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*, 69(8), pp. 1554-61.

van der Woude, D., Young, A., Jayakumar, K., Mertens, B.J., Toes, R.E., van der Heijde, D., Huizinga, T.W. and van der Helm-van Mil, A.H. (2009) 'Prevalence of and predictive factors for sustained disease-modifying antirheumatic drug-free remission in rheumatoid arthritis: results from two large early arthritis cohorts', *Arthritis Rheum*, 60(8), pp. 2262-71.

van Gestel, A.M., Prevoo, M.L., van 't Hof, M.A., van Rijswijk, M.H., van de Putte, L.B. and van Riel, P.L. (1996) '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*, 39(1), pp. 34-40.

van Hamburg, J.P., Asmawidjaja, P.S., Davelaar, N., Mus, A.M., Colin, E.M., Hazes, J.M., Dolhain, R.J. and Lubberts, E. (2011) 'Th17 cells, but not Th1 cells, from patients with early rheumatoid arthritis are potent inducers of matrix metalloproteinases and proinflammatory cytokines upon synovial fibroblast interaction, including autocrine interleukin-17A production', *Arthritis Rheum*, 63(1), pp. 73-83.

van Herwaarden, N., van der Maas, A., Minten, M.J., van den Hoogen, F.H., Kievit, W., van Vollenhoven, R.F., Bijlsma, J.W., van den Bemt, B.J. and den Broeder, A.A. (2015) 'Disease activity guided dose reduction and withdrawal of adalimumab or etanercept compared with usual care in rheumatoid arthritis: open label, randomised controlled, non-inferiority trial', *BMJ*, 350, p. h1389.

van Nies, J.A., Krabben, A., Schoones, J.W., Huizinga, T.W., Kloppenburg, M. and van der Helm-van Mil, A.H. (2014) 'What is the evidence for the presence of a therapeutic window of opportunity in rheumatoid arthritis? A systematic literature review', *Ann Rheum Dis*, 73(5), pp. 861-70.

van Onna, M. and Boonen, A. (2016) 'The challenging interplay between rheumatoid arthritis, ageing and comorbidities', *BMC Musculoskeletal Disorders*, 17, p. 184.

van Riel, P.L. (2014) 'The development of the disease activity score (DAS) and the disease activity score using 28 joint counts (DAS28)', *Clin Exp Rheumatol*, 32(5 Suppl 85), pp. S-65-74.

van Steenbergen, H.W., Mangnus, L., Reijnierse, M., Huizinga, T.W. and van der Helm-van Mil, A.H. (2016) 'Clinical factors, anticitrullinated peptide antibodies and MRI-detected subclinical inflammation in relation to progression from clinically suspect arthralgia to arthritis', *Ann Rheum Dis*, 75(10), pp. 1824-30.

van Tuyl, L.H., Vlad, S.C., Felson, D.T., Wells, G. and Boers, M. (2009) 'Defining remission in rheumatoid arthritis: results of an initial American College of Rheumatology/European League Against Rheumatism consensus conference', *Arthritis Rheum*, 61(5), pp. 704-10.

van Vollenhoven, R.F., Ostergaard, M., Leirisalo-Repo, M., Uhlig, T., Jansson, M., Larsson, E., Brock, F. and Franck-Larsson, K. (2016) 'Full dose, reduced dose or discontinuation of etanercept in rheumatoid arthritis', *Ann Rheum Dis*, 75(1), pp. 52-8.

Venables, W.N. and Ripley, B.D. (2002) *Modern Applied Statistics with S.* Fourth edn. New York: Springer.

Verhoef, L.M., Tweehuysen, L., Hulscher, M.E., Fautrel, B. and den Broeder, A.A. (2017) 'bDMARD Dose Reduction in Rheumatoid Arthritis: A Narrative Review with Systematic Literature Search', *Rheumatol Ther*, 4(1), pp. 1-24.

Vermeer, M., Kuper, H.H., van der Bijl, A.E., Baan, H., Posthumus, M.D., Brus, H.L., van Riel, P.L. and van de Laar, M.A. (2012) 'The provisional ACR/EULAR definition of remission in RA: a comment on the patient global assessment criterion', *Rheumatology (Oxford)*, 51(6), pp. 1076-80.

Vreugdenhil, G., Lowenberg, B., Van Eijk, H.G. and Swaak, A.J. (1992) 'Tumor necrosis factor alpha is associated with disease activity and the degree of anemia in patients with rheumatoid arthritis', *Eur J Clin Invest*, 22(7), pp. 488-93.

Wagner, U.G., Koetz, K., Weyand, C.M. and Goronzy, J.J. (1998) 'Perturbation of the T cell repertoire in rheumatoid arthritis', *Proc Natl Acad Sci U S A*, 95(24), pp. 14447-52.

Wakefield, R.J., D'Agostino, M.A., Naredo, E., Buch, M.H., Iagnocco, A., Terslev, L., Ostergaard, M., Backhaus, M., Grassi, W., Dougados, M., Burmester, G.R., Saleem, B., de Miguel, E., Estrach, C., Ikeda, K., Gutierrez, M., Thompson, R., Balint, P. and Emery, P. (2012) 'After treat-to-target: can a targeted ultrasound initiative improve RA outcomes?', *Ann Rheum Dis*, 71(6), pp. 799-803.

Wang, D.Y., Fulthorpe, R., Liss, S.N. and Edwards, E.A. (2004) 'Identification of estrogen-responsive genes by complementary deoxyribonucleic acid microarray and characterization of a novel early estrogen-induced gene: EEIG1', *Mol Endocrinol*, 18(2), pp. 402-11.

Wang, F., Beck-Garcia, K., Zorzin, C., Schamel, W.W. and Davis, M.M. (2016) 'Inhibition of T cell receptor signaling by cholesterol sulfate, a naturally occurring derivative of membrane cholesterol', *Nat Immunol*, 17(7), pp. 844-50.

Ward, M.M. (2004) 'Relative sensitivity to change of the erythrocyte sedimentation rate and serum C-reactive protein concentration in rheumatoid arthritis', *J Rheumatol*, 31(5), pp. 884-95.

Ward, M.M., Guthrie, L.C. and Dasgupta, A. (2017) 'Direct and Indirect Determinants of the Patient Global Assessment in Rheumatoid Arthritis: Differences by Level of Disease Activity', *Arthritis Care Res (Hoboken)*, 69(3), pp. 323-9.

Wasinger, V.C., Yau, Y., Duo, X., Zeng, M., Campbell, B., Shin, S., Luber, R., Redmond, D. and Leong, R.W. (2016) 'Low Mass Blood Peptides Discriminative of Inflammatory Bowel Disease (IBD) Severity: A Quantitative Proteomic Perspective', *Mol Cell Proteomics*, 15(1), pp. 256-65.

Wegner, N., Wait, R., Sroka, A., Eick, S., Nguyen, K.A., Lundberg, K., Kinloch, A., Culshaw, S., Potempa, J. and Venables, P.J. (2010) 'Peptidylarginine deiminase from Porphyromonas gingivalis citrullinates human fibrinogen and alpha-enolase: implications for autoimmunity in rheumatoid arthritis', *Arthritis Rheum*, 62(9), pp. 2662-72.

Wester, L., Michaelsson, E., Holmdahl, R., Olofsson, T. and Akerstrom, B. (1998) 'Receptor for alpha1-microglobulin on T lymphocytes: inhibition of antigen-induced interleukin-2 production', *Scand J Immunol*, 48(1), pp. 1-7.

Weyand, C.M., Yang, Z. and Goronzy, J.J. (2014) 'T-cell aging in rheumatoid arthritis', *Curr Opin Rheumatol*, 26(1), pp. 93-100.

Whiting-O'Keefe, Q.E., Fye, K.H. and Sack, K.D. (1991) 'Methotrexate and histologic hepatic abnormalities: a meta-analysis', *Am J Med*, 90(6), pp. 711-6.

Wickham, H. (2009) *ggplot2: elegant Graphics for Data Analysis*. New York: Springer-Verlag.

Wickham, H., Francois, R., Henry, L. and Müller, K. (2017) *dplyr: A Grammar of Data Manipulation, version 0.7.2* [Computer program]. https://cran.r-project.org/package=dplyr.

Widdifield, J., Paterson, J.M., Bernatsky, S., Tu, K., Tomlinson, G., Kuriya, B., Thorne, J.C. and Bombardier, C. (2014) 'The epidemiology of rheumatoid arthritis in Ontario, Canada', *Arthritis Rheumatol*, 66(4), pp. 786-93.

Witten, D.M. and Tibshirani, R. (2010) 'Survival analysis with high-dimensional covariates', *Stat Methods Med Res*, 19(1), pp. 29-51.

Wong, C.K., Chen, D.P., Tam, L.S., Li, E.K., Yin, Y.B. and Lam, C.W. (2010) 'Effects of inflammatory cytokine IL-27 on the activation of fibroblast-like synoviocytes in rheumatoid arthritis', *Arthritis Res Ther*, 12(4), p. R129.

World Medical Association (2013) 'World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects', *JAMA*, 310(20), pp. 2191-4.

Wozniak, M.B., Le Calvez-Kelm, F., Abedi-Ardekani, B., Byrnes, G., Durand, G., Carreira, C., Michelon, J., Janout, V., Holcatova, I., Foretova, L., Brisuda, A., Lesueur, F., McKay, J., Brennan, P. and Scelo, G. (2013) 'Integrative genome-wide gene expression profiling of clear cell renal cell carcinoma in Czech Republic and in the United States', *PLoS One*, 8(3), p. e57886.

Wu, H.J., Ivanov, II, Darce, J., Hattori, K., Shima, T., Umesaki, Y., Littman, D.R., Benoist, C. and Mathis, D. (2010) 'Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells', *Immunity*, 32(6), pp. 815-27.

Xu, W.D., Firestein, G.S., Taetle, R., Kaushansky, K. and Zvaifler, N.J. (1989) 'Cytokines in chronic inflammatory arthritis. II. Granulocyte-macrophage colony-stimulating factor in rheumatoid synovial effusions', *J Clin Invest*, 83(3), pp. 876-82.

Yamanaka, H., Nagaoka, S., Lee, S.K., Bae, S.C., Kasama, T., Kobayashi, H., Nishioka, Y., Ueki, Y., Seto, Y., Nishinarita, M., Tamura, N., Kimura, N., Saito, K., Tomita, T., Nawata, Y., Suzuki, S., Ishigatsubo, Y., Munakata, Y., Makino, Y., Inoue, E., Tanaka, Y. and Takeuchi, T. (2016) 'Discontinuation of etanercept after achievement of sustained remission in patients with rheumatoid arthritis who initially had moderate disease activity-results from the ENCOURAGE study, a prospective, international, multicenter randomized study', *Mod Rheumatol*, 26(5), pp. 651-61.

Yang, L., Anderson, D.E., Baecher-Allan, C., Hastings, W.D., Bettelli, E., Oukka, M., Kuchroo, V.K. and Hafler, D.A. (2008) 'IL-21 and TGF-β are required for differentiation of human T(H)17 cells', *Nature*, 454(7202), pp. 350-2.

Yang, X.K., Xu, W.D., Leng, R.X., Liang, Y., Liu, Y.Y., Fang, X.Y., Feng, C.C., Li, R., Cen, H., Pan, H.F. and Ye, D.Q. (2015) 'Therapeutic potential of IL-15 in rheumatoid arthritis', *Hum Immunol*, 76(11), pp. 812-8.

Yates, A., Akanni, W., Amode, M.R., Barrell, D., Billis, K., Carvalho-Silva, D., Cummins, C., Clapham, P., Fitzgerald, S., Gil, L., Giron, C.G., Gordon, L., Hourlier, T., Hunt, S.E., Janacek, S.H., Johnson, N., Juettemann, T., Keenan, S., Lavidas, I., Martin, F.J., Maurel, T., McLaren, W., Murphy, D.N., Nag, R., Nuhn, M., Parker, A., Patricio, M., Pignatelli, M., Rahtz, M., Riat, H.S., Sheppard, D., Taylor, K., Thormann, A., Vullo, A., Wilder, S.P., Zadissa, A., Birney, E., Harrow, J., Muffato, M., Perry, E., Ruffier, M., Spudich, G., Trevanion, S.J., Cunningham, F., Aken, B.L., Zerbino, D.R. and Flicek, P. (2016) 'Ensembl 2016', *Nucleic Acids Res*, 44(D1), pp. D710-6.

Yazici, Y. and Swearingen, C.J. (2014) 'MBDA: what is it good for?', *Ann Rheum Dis*, 73(11), p. e72.

Yen, J.H., Lin, C.H., Tsai, W.C., Wu, C.C., Ou, T.T., Hu, C.J. and Liu, H.W. (2006) 'Killer cell immunoglobulin-like receptor gene's repertoire in rheumatoid arthritis', *Scand J Rheumatol*, 35(2), pp. 124-7.

Yen, J.H., Moore, B.E., Nakajima, T., Scholl, D., Schaid, D.J., Weyand, C.M. and Goronzy, J.J. (2001) 'Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis', *J Exp Med*, 193(10), pp. 1159-67.

Yip, L., Su, L., Sheng, D., Chang, P., Atkinson, M., Czesak, M., Albert, P.R., Collier, A.R., Turley, S.J., Fathman, C.G. and Creusot, R.J. (2009) 'Deaf1 isoforms control the expression of genes encoding peripheral tissue antigens in the pancreatic lymph nodes during type 1 diabetes', *Nat Immunol*, 10(9), pp. 1026-33.

Yokoyama, M., Ichinoe, M., Okina, S., Sakurai, Y., Nakada, N., Yanagisawa, N., Jiang, S.X., Numata, Y., Umezawa, A., Miyazaki, K., Higashihara, M. and Murakumo, Y. (2017) 'CD109, a negative regulator of TGF-beta signaling, is a putative risk marker in diffuse large B-cell lymphoma', *Int J Hematol*, 105(5), pp. 614-22.

Yoshida, H. and Hunter, C.A. (2015) 'The immunobiology of interleukin-27', *Annu Rev Immunol*, 33(1), pp. 417-43.

Yoshimi, R., Hama, M., Takase, K., Ihata, A., Kishimoto, D., Terauchi, K., Watanabe, R., Uehara, T., Samukawa, S., Ueda, A., Takeno, M. and Ishigatsubo, Y. (2013) 'Ultrasonography is a potent tool for the prediction of progressive joint destruction during clinical remission of rheumatoid arthritis', *Mod Rheumatol*, 23(3), pp. 456-65.

Yu, P., Bamford, R.N. and Waldmann, T.A. (2014) 'IL-15-dependent CD8+ CD122+ T cells ameliorate experimental autoimmune encephalomyelitis by modulating IL-17 production by CD4+ T cells', *Eur J Immunol*, 44(11), pp. 3330-41.

Zhang, D. and Aravind, L. (2010) 'Identification of novel families and classification of the C2 domain superfamily elucidate the origin and evolution of membrane targeting activities in eukaryotes', *Gene*, 469(1-2), pp. 18-30.

Zhang, L., Li, J.M., Liu, X.G., Ma, D.X., Hu, N.W., Li, Y.G., Li, W., Hu, Y., Yu, S., Qu, X., Yang, M.X., Feng, A.L. and Wang, G.H. (2011) 'Elevated Th22 cells correlated with Th17 cells in patients with rheumatoid arthritis', *J Clin Immunol*, 31(4), pp. 606-14.

Zhang, L., Li, Y.G., Li, Y.H., Qi, L., Liu, X.G., Yuan, C.Z., Hu, N.W., Ma, D.X., Li, Z.F., Yang, Q., Li, W. and Li, J.M. (2012) 'Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis', *PLoS One*, 7(4), p. e31000.

Zhang, Z. and Zhang, R. (2015) 'Epigenetics in autoimmune diseases: Pathogenesis and prospects for therapy', *Autoimmun Rev*, 14(10), pp. 854-63.

Zhao, L., Jiang, Z., Jiang, Y., Ma, N., Zhang, Y., Feng, L. and Wang, K. (2013) 'IL-22+ CD4+ T cells in patients with rheumatoid arthritis', *Int J Rheum Dis*, 16(5), pp. 518-26.