An experimental and clinical investigation of the brain response in inflammatory arthritis

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Abstract

Rheumatoid arthritis (RA) and other inflammatory disorders are associated with a significant burden of mental disorder, including fatigue, cognitive problems, depression, and an enhanced risk of developing dementia in later life. The mechanisms underlying these associations are unclear, though inflammatory effects on the brain are thought to play an important role. Increasing evidence supports a role for communication of immune signals from the periphery to the brain, with structural and functional consequences. There is a lack of data from disease-relevant tissue-specific inflammatory animal models with translational potential.

This thesis sets out to explore the brain response to inflammatory arthritis using a translational approach, spanning measurement of the neuroinflammatory response in the collagen induced arthritis (CIA) mouse model, to the role of inflammatory variables in sickness behaviour in early RA, particularly fatigue, using a clinical cohort study.

CIA was found to be associated with a brain inflammatory response involving upregulated brain transcription of the pro-inflammatory cytokine interleukin-1-beta (Il1ß) and the associated P2x7 receptor. An increased density of IBA1+ cells were demonstrated in the thalamus of arthritic mice, but not elsewhere in the brain. There was no indication of a consistent pattern of chemokine transcriptional changes, leukocyte recruitment to the brain or alterations in markers of plasticity such as hippocampal neurogenesis or neuronal density.

In the clinical cohort drawn from the Scottish Early Rheumatoid Arthritis (SERA) study, fatigue was examined in its relationship with variables relevant to the burden of peripheral inflammation at baseline, 6 and 12 months. While overall levels of fatigue improved markedly within the cohort over time, univariable and multivariable analysis did not reveal a consistent relationship between inflammatory or disease activity variables and fatigue. Fatigue was more closely related to pain, mood, anxiety and subjective patient reported outcome measures.

The experimental and clinical investigation presented in this thesis highlights the nuanced and complex relationship between peripheral inflammation and the

brain response in the context of inflammatory arthritis. Further work is needed to broaden the field of knowledge using tractable and disease-faithful animal models and translational approaches. Further work in this area is vital to the endeavour to enhance our understanding and treatment of mental disorder in the context of inflammatory disease and may yield insights relevant to the role of inflammation in psychiatric disorders more broadly.

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Author's declaration

I declare that the work described in this thesis is original and, except where explicit reference is made to the contribution of others, represents entirely my own efforts. None of the data included in this thesis has been submitted for any other degree at the University of Glasgow or any other institution.

Signature REDACTED FROM ONLINE VERSION

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Definitions/Abbreviations

A	
ACC	Anterior cingulate cortex
ACh	Acetyl choline
ACPA	Anti-citrullinated peptide antibody
ACR	American College of Rheumatology
AGA-VAS	Assessor global assessment VAS
AJ	Adherent junction
AIA	Adjuvant-induced arthritis
ANA	Anti-nuclear antibody
ANOVA	Analysis of variance
APC	Antigen presenting cells
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
B	
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
BMI	Body mass index
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
C	
CAIA	Collagen antibody induced arthritis
cDNA	Complementary DNA
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CNS	Central nervous system
COX	Cyclo-oxygenase
CRP	C-reactive protein
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CV	Coefficient of variation
CVO	Circumventricular organs
D	
DAB	3,3'-diaminobenzidine substrate-chromogen
DAMP	Damage associated molecular pattern
DAS	Disease activity score
DC	Dendritic cell
DCX	Doublecortin
DG	Dentate gyrus
DMARD	Disease modifying anti-rheumatic drug
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
E	
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EQ-VAS	EuroQol VAS
ESR	Erythrocyte sedimentation rate
EtBr	Ethidium bromide

EtOH	Ethanol
EULAR	European League Against Rheumatism

F

FCS	Foetal calf serum
FFPE	Formalin fixed paraffin embedded
FMO	Fluorescence minus one
fMRI	Functional magnetic resonance imaging

G

G-CSF	Granulocyte colony stimulating factor
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte-monocyte colony stimulating factor
GPCR	G-protein coupled receptor
GWAS	Genome wide association study

H

HADS	Hospital anxiety and depression scale
HAQ	Health Assessment Questionnaire
HBSS	Hank's balanced salt solution
HDL	High-density lipoprotein
H&E	Haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HPA	Hypothalamic-pituitary-adrenal
hTNFtg	Transgenic human TNFα

I

IBA1	Ionised calcium binding adaptor molecule 1
IBD	Inflammatory bowel disease
ICAM1	Intracellular adhesion molecule 1
ICD	International Classification of Disease
ID	Intra-dermal
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
IVC	Inferior vena cava

J

JAK	Janus kinase enzyme

L

LPS	Lipopolysaccharide

M

MHC	Major histo-compatibility complex
MS	Multiple sclerosis

N

Na-F	Sodium-fluoroscein dye
NFκß	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National Health Service
NK	Natural killer cell
NLRP3	Nod-like receptor family pyrin domain containing 3 protein
NMDA	N-methyl-D-aspartate
NO	Nitric oxide

O

OCT	Optimal cutting medium

P

PAMP	Pathogen associated molecular pattern
PBL	Peripheral blood leukocyte
PBS	Phosphate buffered saline
PBST	PBS with 1% Tween-20
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGA-VAS	Patient Global Assessment VAS
Poly(I:C)	Polyinosinic-polycytidylic acid
PRR	Pattern recognition receptor
PS1	Presenilin-1

Q

qPCR	Quantitative PCR
qRT-PCR	Quantitative real-time PCR

R

RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
RT	Reverse transcriptase

S

SERA	Scottish Early Rheumatoid Arthritis cohort
SGZ	Subgranular zone
SJC	Swollen joint count
SIMD	Scottish Index of Multiple Deprivation
SLE	Systemic lupus erythematosus
SSRI	Selective serotonin reuptake inhibitor
STW	Scott's tap water

T

T2C	Type II collagen
TAE	Tris-acetate-EDTA
Tbp	TATA-binding protein
TBS	Tris-buffered saline
Tc	Cytotoxic T cell
TC	Total serum cholesterol
Th	Helper T cell
TJ	Tight junction
TJC	Tender joint count
TLR	Toll-like receptors
Tm	Annealing temperature

TMEM119	Transmembrane protein 119
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSPO	translocator protein

U

UA	Undifferentiated arthritis

V

VAS	Visual analogue scale
VEGF2	Vascular endothelial growth factor 2
VIF	Variable inflation factor

W

WCC	White cell count

Note on nomenclature

In keeping with convention, when referring to mRNA transcript, a capital letter followed by lower case will be used e.g. Il6. When referring to protein, all capitals will be used e.g. IL6.

Chapter 1

Introduction

1 Introduction

1.1 Overview and general introduction

Rheumatoid arthritis (RA) is a relatively common inflammatory autoimmune disorder, affecting around 0.5-0.8% of the population (Myasoedova et al., 2010) (Symmons et al., 2002). RA is associated with a significant risk of developing mental disorder, in particular depression and symptoms such as persistent fatigue and cognitive difficulties (Dickens et al., 2002) (Hewlett et al., 2011). Conservative estimates suggest a prevalence of depression in RA at around 18.8% (Matcham et al., 2013). Fatigue is even more common, affecting up to 80% of RA patients (Pollard et al., 2006), and represents a particularly problematic symptom that has gained increasing attention as a research and therapeutic priority. Mental disorder, including depression and fatigue, can compound disability and quality of life impairment, and undermine the effectiveness of RA treatment (Nerurkar et al., 2019). The causal relationships and underlying mechanisms are complex and yet to be properly understood.

Inflammatory disorders, such as RA, have the potential to involve communication of inflammatory signals from the periphery to the brain and provoke a brain inflammatory response, which may be an important step in the production of neuropsychiatric manifestations of inflammatory disease (Capuron and Miller, 2011). Significant advances have been made in further understanding how inflammatory mechanisms may contribute to the emergence of 'sickness behaviour' and mental health disorders such as depression (Dantzer et al., 2006). Sickness behaviour describes a constellation of behavioural responses that are associated with systemic illness and inflammation, including dysphoria, anxiety, fatigue, loss of appetite, amotivation and sleep disturbance (Kelley et al., 2003). Sickness behaviour and depression, whilst not interchangeable, share a considerable conceptual overlap and may share important aetiological pathways (Dantzer et al., 2008). Exploring the mechanisms that underpin sickness behaviour and depression in the context of inflammation may provide important insights into the pathogenesis of these problematic phenomena and may to some extent inform a broader aetiological understanding of depression in the absence of overt inflammatory disease.

This thesis explores this subject using a translational approach by first examining the neuroinflammatory response to peripheral inflammation at the cellular and molecular level using a mouse model of arthritis. Following on from this, moving from the molecular level to the clinical context, an exploration is set out of the relationship between inflammation and the key sickness behaviour symptom of fatigue in a human cohort of early RA patients.

1.2 Overview of the immune system

Mammals have evolved a complex and sophisticated repertoire of cellular and molecular defences to protect against environmental pathogens, foreign antigens and sources of infection. All organisms exist in a dynamic relationship with the environment, including sources of infection and disease, with success and survival depending on the physiological fitness and integrity of the host defences. The immune system comprises an interconnected arrangement of lymphoid organs, cells, humoral factors and cytokines (Parkin and Cohen, 2001). The significance of the immune system becomes most apparent in dysfunction, including underactivity with deficient immune responses and emergence of infection, or overactivity and autoimmune responses directed towards the host and emergence of inflammatory disease. In addition, many parts of the immune system, for example chemokine networks and macrophages, play important roles in tissue development and homeostasis (Klose and Artis, 2016) (Le et al., 2004).

The constituent parts of the immune system can roughly be divided into 3 main categories:-

1. Structural barriers e.g. skin, mucous membranes, the blood-brain barrier (BBB). These barriers are integral to preventing invasion of foreign antigen and potential infectious pathogens.
2. The innate immune system – a network of rapid acting non-specific host defences primed to recognise molecular danger signals and non-self antigens and react to eliminate these threats. However, immunity and protection from this arm of the immune system can be incomplete and the non-specific nature of innate immunity can trigger damage to host tissues

and compromise physiological integrity. The innate immune system also plays an important role in homeostasis by recognising, and either repairing or disposing of, senescent or apoptotic cells (Parkin and Cohen, 2001). This ancient component of the immune system includes danger-signal sensing Toll-like receptors (TLRs), the complement system and phagocytic cells (Janeway Jr and Medzhitov, 2002).

3. The adaptive immune system – a slower-acting specific network of sophisticated defences that recognise pathogens and manufacture bespoke targeted and precise immune responses. The adaptive immune system evolved later and is unique to jawed vertebrates (Cooper and Alder, 2006). This arm of the immune system includes T and B lymphocytes.

The innate and adaptive immune systems operate in concert, and are linked principally by antigen presenting cells (APCs) such as dendritic cells (DCs) which process and package foreign antigens into major histo-compatibility complex proteins (MHC) which are presented to T cells carrying T cell receptors with the highest degree of specificity and affinity for the MHC (Parham, 2005). DCs release co-stimulatory factors that activate and promote proliferation of T cells. T cells can then effect a selective humoral response to eliminate the pathogen. APCs activate B cells which are able to differentiate and manufacture antigen-specific antibodies that 'tag' foreign material for destruction by cytotoxic and phagocytic cells (Murphy and Weaver, 2016).

This same repertoire of cells and molecules, responsible to host-defence, can in dysfunction be responsible for destructive autoimmune inflammatory disease, when recognition and tolerance of self-antigens becomes impaired.

1.2.1 The innate immune system

The innate immune system exists as a first line of defence against pathogens, noxious stimuli and infectious organisms once structural barriers are breached. Innate immune activation involves an inflammatory response characterised by vasodilation, allowing influx of leukocytes to deal with pathogens or tissue damage, often accompanied clinically by the 4 classical indicators of inflammation - rubor, tumor, calor and dolor (MacNeill, 2016). Activation of the innate immune system depends primarily upon pattern recognition receptors (PRRs) such as TLRs, and other receptors that initiate signalling cascades leading

to upregulated transcription of inflammatory mediators in order to eliminate pathogens and clear infected tissues (Takeuchi and Akira, 2010). The ligands for PRRs are molecular signatures of damage and infection – damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs) (Kono and Rock, 2008).

The effector cells of the innate immune system are mainly of myeloid lineage, originating from multipotent haematopoietic stem cells within bone marrow. These cells include DCs, macrophages, and neutrophils. Natural killer (NK) cells however are of lymphoid lineage, arising from common lymphoid progenitor cells (Kondo, 2010).

An early feature of the innate immune response is recruitment and activation of neutrophils (Witko-Sarsat et al., 2000). In the initial stages of the immune response to an area of damage or infection, macrophages become activated and release cytokines including granulocyte colony stimulating factor (G-CSF) and granulocyte-monocyte colony stimulating factor (GM-CSF). These cytokines upregulate division of myeloid precursor cells and stimulate release into circulation of immune cells resulting in characteristic neutrophilia. Neutrophils are attracted to sites of inflammation by chemotaxis, including via chemokines, where they can effect a range of responses including phagocytosis, degranulation and cytokine production (McDonald and Kubes, 2011).

Quiescent monocytes reside in bone marrow, spleen and circulating blood. Upon sensing DAMPS or PAMPS via PRRs, monocytes will migrate to the site of injury/infection and differentiate into macrophages or DCs to sustain the immune response in inflammation (Swirski et al., 2009).

Macrophages are resident within tissues and organs throughout the body, replenished as noted by migrating monocytes. Macrophages are activated by sensing of DAMPS and PAMPS by PRRs. They are phagocytic and play a key role in clearing dead cells, cellular debris, foreign material and pathogens, in addition to presenting foreign antigenic material to cells of the adaptive immune system (Perdiguero and Geissmann, 2016).

Dendritic cells (DCs) are so named due to their intricately branching dendrites (from the Greek *dendron* for tree) that sample the local environment in order to

detect pathogens, and form a key link between the innate and adaptive immune systems (Cabeza-Cabrerizo et al., 2021). DCs can take up pathogens and then migrate to local lymphoid tissues where the antigens are presented to T cells. Dendritic cells are also potent stimulators of T cell responses.

NK cells function mainly to target and destroy altered or pathogen-infected host cells by release of cytolytic granules. They are recruited via chemotaxis to invade and accumulate within sites of injury, inflammation, infection and tumour growth. NK cells release perforins that create pores in targeted cells, allowing cytolytic enzymes to penetrate the cell, thus destroying the cell from inside, thereby playing an important role in anti-viral responses by limiting release of intracellular virions (Paolini et al., 2015). NK cells are both activated by inflammatory cytokines and interferons, in addition to being potent producers of cytokines and chemokines (Biron et al., 1999).

Whilst innate immunity is a key and indispensable defence, aberrant or dysregulated activation of this system can lead to either deficient immune responses, excessive responses such as in septic shock or induction of autoimmunity (Takeuchi and Akira, 2010).

1.2.2 The adaptive immune system

The hallmark of adaptive immunity is the expression of antigen-specific receptors on T and B lymphocytes to coordinate a pathogen-targeted response. Due to the specific nature of the adaptive immune response, it takes several days to develop, but has ‘memory’ and is therefore able to mount a more rapid response in future or in some cases sustain immunity to re-encountered pathogens.

B cells and T cells both originate from bone marrow progenitor cells. Whilst B cells remain within the marrow during development, T cells migrate at an early stage to the thymus. Cell activation must be carefully calibrated to ensure that only damaging antigens prompt a reaction. Selection of T cells that can adequately detect and respond to pathogenic antigen bound to self-MHC, whilst excluding excessively self-MHC reactive cells, occurs during T cell maturation within the thymus.

As noted, APCs such as DCs play an important role in detecting and transporting antigens to lymphoid tissues such as lymph nodes where the antigen is exposed to passing T lymphocytes that then activate over 2-3 days. DCs are stimulated by TLR activation to release important cytokine stimulators of naïve T cells (Parkin and Cohen, 2001). Upon presentation of pathogenic antigen in association with MHC, T cells differentiate into different subtypes of effector T cells.

The family of T cells includes naïve T cells, cytotoxic T cells (Tc), regulatory T cells (Treg), memory T cells and helper T cells (Th). The main effector T cells involved in adaptive immune responses are Th cells (CD4+) and Tc cells (CD8+). Th cells recognise foreign antigen, activate cell mediated responses and activate B cells. Tc cells are important antiviral and antitumour cells, that operate similarly to NK cells but in an antigen specific nature (Parkin and Cohen, 2001).

B cells mainly reside within lymphoid organs where foreign antigen can be recognised and bound by the B cell which then processes with antigen and re-expresses the antigen on the MHC class II molecule of the cell to present to a primed specific T cell. The T cell then produces various cytokines that stimulate B cell transformation into antibody-producing cells. Antibodies serve to neutralise pathogen binding sites, activate the complement system, opsonize bacteria for phagocytosis and sensitise infected cells to antibody-dependent destruction by Tc cells. B cells can exist as short-lived active antibody secreting cells or long-lived plasma cells and memory B cells are responsible for lasting immunity and rapid proliferation and antibody secretion upon re-encountering the same pathogen.

As with the innate immune system, dysregulation of the adaptive immune system can give rise to immunodeficiency or autoimmunity. The process of creating antigen-specific binding sites in T cells and B cells involves random rearrangement and splicing of multiple segments of antigen-binding receptor DNA. This process allows a vast repertoire of receptor and antibody types, however, an inevitable consequence is the development of some autoreactive receptors (Rosenblum et al., 2015). Generally, these will be excluded during maturation within the thymus or bone marrow. Why some autoreactive cells emerge remains to be fully understood. Another important aspect is the balance

between Th cells an Treg cells is thought to be important in the emergence of autoimmunity, with Th cells generally promoting inflammation and loss of self-tolerance, whilst Treg cells maintain immune homeostasis (Lee, 2018).

1.2.3 Cytokines

Cytokines are a group of signalling protein molecules that regulate a broad range of inflammatory processes that are secreted by, and interact with, a broad range of cells involved in inflammation and immunity. These include both immune cells such as leukocytes, and non-immune cells such as endothelial cells and fibroblasts. Cytokines can be pro-inflammatory, or anti-inflammatory and immunoregulatory, sometimes with the same cytokine signalling in different ways depending on the context (Murphy and Weaver, 2016) (Male et al., 2020). Cytokines have activating properties on leukocytes and induce production and release of more inflammatory proteins, enhancing localised and systemic immune responses.

Cytokines can be divided into several different classes. Interferons (IFNs) tend to be particularly important in anti-viral responses, with type 1 IFNs (IFNα and IFNß) being released typically by virus infected cells, and type 2 IFNs (IFNγ) being released by Th1 cells. IFNs form a key part of the early innate response, delaying spread of virus whilst the adaptive response is mounted.

Interleukins (ILs) are signalling cytokines typically produced by T cells, in addition to phagocytes and tissue cells such as endothelium. Many are classically proinflammatory, such as IL1ß and IL6, and induce other cells to proliferate and differentiate. Others such as IL10 are potent anti-inflammatory signallers that restrain inflammation. Tumour necrosis factors (TNFs), including TNFα and TNFß, are important pro-inflammatory cytokines involved in promoting inflammation and cytotoxic immune reactions.

Colony stimulating factors, as mentioned above including G-CSF and GM-CSF, play an important role in stimulating the division and differentiation of precursor cells in the bone marrow, as part of the innate immune response.

The classical pro-inflammatory cytokines IL1ß, IL6 and TNFα are produced by a diverse array of cells, primarily via activation of the nuclear factor kappa-light-

chain-enhancer of activated B cells (NFκB) pathway (Baeuerle and Henkel, 1994). NFκB is a general transcription factor in the immune system which is activated by PRRs to regulate immune function (Liang et al., 2004). Although the function of the classically pro-inflammatory cytokines can be pleiotropic depending on the inflammatory context, they tend to promote T cell and B cell activation and proliferation. IL1ß and TNFα are pyrogens that can act to stimulate prostaglandin synthesis and release causing a fever response (Saper and Breder, 1994). IL6 meanwhile is a potent inducer of acute-phase proteins such as C-reactive protein (CRP), responsible for maintaining inflammatory responses and enhancing pathogen elimination.

1.2.3.1 Cytokines and sickness behaviour

Inflammatory cytokines are known to induce changes in behaviour and subjective experience in the context of inflammation – so called 'sickness behaviour' (Kent et al., 1992) (Dantzer et al., 1998) (Kelley et al., 2003). Symptoms include general malaise, fatigue, reduced motivation, loss of appetite, social withdrawal, anxiety, depressed mood, anhedonia, poor concentration, altered sleep pattern and increased pain sensitivity. The evolutionary function of this behavioural (and in humans psychological) response is thought likely to be to optimise and maximise the utilisation of bodily resources in mounting a life-preserving immune response to injury, illness or infection (Hart, 1988) (Dantzer, 2001). Sickness behaviour symptoms, in particular fatigue, are a recognised phenomenon in the context of inflammatory disorders such as RA (Arnett and Clark, 2012).

Numerous studies in humans and animals have shown that this pattern of sickness behaviour type symptoms can be elicited by systemic administration of cytokines or their induction by instruments such as typhoid vaccination (Wright et al., 2005). Sickness behaviour appears to be induced by the innate immune response involving activation of PRRs by PAMPS and DAMPS leading to pro-inflammatory cytokine release (Kelley et al., 2003). Whilst these events (except in the context of a primary CNS injury/infection) occur in the body periphery, sickness behaviour is mediated within the CNS, likely propagated by CNS response to cytokines, either locally derived or traversing through gaps in the

BBB from the periphery. This raises the topic of immune-to-brain communication which will be explored in more detail in section 1.4.2.

An examination of the principal symptoms of sickness behaviour will demonstrate significant overlap with the psychiatric clinical syndrome of depression. Indeed many of the key symptoms of sickness behaviour form core symptoms in the manualised diagnostic guidelines used in clinical practice such as the International Classification of Disease (ICD)-10 (Organization, 1992) and the Diagnostic and Statistical Manual of Mental Disorders (DSM)-5 (Edition, 2013). This led to an emergence of a new field of research, termed 'immunopsychiatry', in which researchers have sought to explore whether inflammatory and immune mechanisms may be important in the pathogenesis of various mental disorders, in particular depression (Krishnadas and Cavanagh, 2012) (Gibney and Drexhage, 2013). Conditions such as RA, in which inflammation associates with sickness behaviours and psychiatric disorders, can provide a paradigm for investigation in this area.

1.2.3.2 Cytokines and RA

Cytokines are key enablers and promoters in each phase of the pathogenesis of RA, promoting autoimmunity, maintaining chronic inflammation within the joint synovium and potentiating destruction of adjacent tissues such as cartilage and bone (McInnes and Schett, 2007). As such anti-cytokine therapies have proven to be important tools in the treatment of RA. Cytokines are also implicated in the non-articular and systemic manifestations of RA, including IL6 mediated released of acute phase proteins (Nishimoto and Kishimoto, 2006), sickness behaviours, and increased metabolic and cardiovascular disease (Sattar et al., 2003). Anti-cytokine therapies such as anti-TNF have proven to be highly effective biologic anti-rheumatics (Singh et al., 2009).

T cells play a key role in synovitis by production of inflammatory cytokines. Synovial IL1 and IL6, among other cytokines are important in promoting T cell activation within the joint. Activated Th cells release IL17 which sustains inflammation around the joint (Lubberts et al., 2004).

The role of B cells in the development of synovitis is mediated by antigen presentation and cytokine release, whilst synovial B cell differentiation and

proliferation is partly driven by IL6 and IL10 (McInnes and Schett, 2007). The biologic drug Rituximab is an anti-CD20 monoclonal antibody that acts to deplete B cells, and can have substantial and lasting clinical benefit in RA (Edwards et al., 2004).

Macrophages, which represent the major effector cells in RA joint disease, are potent releasors of TNF, IL1, IL6, IL15 and IL18, driving various inflammatory pathways involved in synovitis (Feldmann et al., 1996).

Osteoclast maturation and activation is an important step in the development of articular destruction and erosive arthritis (Schett, 2007). M-CSF, IL1, TNF and IL17 released by Th1 and Th17 cells are important promoters of osteoclast maturation and activation (Lam et al., 2000) (Wei et al., 2005) (Kim et al., 2015), in addition to IL17 released by synovial fibroblasts (Burger et al., 2001). Meanwhile IL4 and IL10 released by Th2 cells, and IFNγ and GM-CSF released by Th1 cells, are inhibitory of osteoclast differentiation (Feldmann et al., 1996).

The pathogenesis of RA is discussed at greater length in section 1.6.2.

1.2.4 Chemokines

Chemokines, so named due to the early observation of their function as chemotactic cytokines, are a group of cytokines that are characterised by a highly conserved cysteine motif. Chemokines are divided into 4 major classes according to their cysteine motif – CCL, CXCL, XCL and CX3CL (Zlotnik and Yoshie, 2000). Chemokines signal via interactions with G-protein couple receptors (GPCRs). Chemokines play a key role in cell migration and recruitment in development, homeostasis, and inflammation. In addition, chemokines play important roles in diverse functions such as angiogenesis (Dimberg, 2010), neurogenesis (Senf et al., 2021) (Williamson and Bilbo, 2013), neuroendocrine function (Callewaere et al., 2007), and in cancer biology including tumorigenesis and metastasis (Vilgelm and Richmond, 2019) (Kakinuma and Hwang, 2006).

1.2.4.1 Chemokines in RA

Chemokines play a central role in the pathogenesis of RA through actions such as leukocyte recruitment to synovial tissue, stimulating release of inflammatory

mediators, promoting cell proliferation and mediating angiogenesis (Iwamoto et al., 2008). Leukocyte infiltration into synovium is a cardinal feature of RA and is dependent on the function of various chemokines. These chemokines are secreted by a variety of cells, in particular synovial fibroblasts and leukocytes (Koch, 2005).

CXCL8, best characterised in regard to its role in neutrophil recruitment, is found in elevated quantities within the synovium and synovial fluid of inflamed RA joints and in the serum of RA patients (Iwamoto et al., 2008). CCL2, a chemoattractant of monocytes, NK cells, T cells and basophils, is also expressed to excess in RA synovium and synovial fluid (Koch et al., 1992). CXCL10, important in T cell recruitment, is elevated in synovial fluid and tissue, released mainly by macrophages and fibroblasts, but also chondrocytes when stimulated by proinflammatory cytokines (Hanaoka et al., 2003). CX3CL1 and its receptor CX3CR1 are expressed in excess in synovial tissue and fluid, and monocytes in joint and peripheral blood show elevated expression CX3CL1 and its receptor in RA (Ruth et al., 2001). CX3CL1 may have an important role in angiogenesis during the formation of RA pannus (Volin et al., 2001). CXCL13, CCL19 and CCL21 are important chemokines in the formation of lymphoid tissue within the synovial tissue (Manzo et al., 2005). Lymphoid neogenesis is thought to be a driver of antigen dependent B cell development in synovial tissue and subsequent autoantibody production (Takemura et al., 2001).

1.3 The CNS immune system

The central nervous system (CNS), composed of the brain and spinal cord, has historically been understood as an area of ‘immune privilege’, isolated from the periphery by the BBB, where humoral immunity may even be absent. However, this view has been revised over recent years in light of numerous discoveries that have led to a clearer understanding of CNS immunity. Whilst the CNS is certainly immune-specialised, it is now clear that a complex and dynamic array of interactions occur between the peripheral and CNS immune system during health and disease (Louveau et al., 2015a).

1.3.1 Cells of the CNS immune system

The cell types of the CNS can broadly be broken down into neurons, glial cells and endothelial cells, that exist throughout the brain in varying ratios and in an interrelated relationship as neurovascular units that regulate function of the brain and through which neuron, glial cell, BBB and vascular system cross-talk can occur (Zlokovic, 2008) (Koehler et al., 2009).

The chief immunologically active cells of the CNS are the glial cells which are summarised below. These cells express TLRs and chemokine receptors, and themselves secrete chemokines and cytokines, and can respond to infective, inflammatory, or noxious stimuli and orchestrating the CNS immune response.

1.3.1.1 Microglia

Microglia are a specialised CNS immune cell that can be conceptualised as brain tissue-resident macrophages. Microglia exist throughout the brain parenchyma, and also in close association with blood vessels (perivascular microglia), and are the only brain cell of myeloid lineage. The majority of microglia are generated after birth and the establishment of the BBB – maintenance and expansion of microglial populations within the brain is thought to be either by self-replication or by division of brain resident progenitors (Ginhoux et al., 2010). Intriguingly, infiltration into brain of circulating progenitors has been shown experimentally in mouse studies of neurodegeneration (Simard et al., 2006). Circulating monocytes may be a more important source of microglia at areas of interface with the periphery such as around the choroid plexus, meninges and perivascular spaces (Simard and Rivest, 2004).

Microglia serve various immune and supportive functions and are key cells of the innate immune response within the CNS. Microglia are highly ramified with extensive processes rich in PRRs, such as TLRs, that allow them to constantly survey the CNS environment for signs of damage or infection (Jack et al., 2005). Upon detection of signals associated with cell damage or infection, microglia can respond in a highly plastic fashion and secrete various cytokines to help eliminate pathogens and recruit other microglia. Upon activation, microglial phenotype can be either M1 proinflammatory state or M2 tissue repair state (Boche et al., 2013). Classically pro-inflammatory activated microglia are

marked by high expression of Ly6C and secrete proinflammatory cytokines including IL1ß and TNFα. They show high phagocytic and proteolytic activity to clear potential pathogens and clear debris associated with injury (Martinez et al., 2008). IL10 appears to promote an M2 anti-inflammatory tissue remodelling state (Banchereau et al., 2012). Current theory suggests however, that rather than dichotomous activation states, microglia may exist in a spectrum of activation states with a high degree of cellular plasticity (Ransohoff, 2016).

Activation of microglia via PRRs leads to activation of the NFκB pathway, inducing a signalling cascade with expression of pro-inflammatory cytokines, in particular IL1ß and also nod-like receptor family pyrin domain containing 3 protein (NLRP3). NLRP3 inflammasome activation further potentiates maturation and release of IL1ß (Leemans et al., 2011). Inflammasomes are polyprotein complexes induced by PAMPs or DAMPS, that are an important facet of innate immune responses by promoting pro-inflammatory cytokine release and also regulating inflammatory cytotoxicity (Gross et al., 2011). P2X7 receptor activation by excess cellular adenosine triphosphate (ATP) is an upstream step in the inflammasome activation, and is a key step in the processing and release of IL1ß by microglia (Campagno and Mitchell, 2021). This process of NLRP3 inflammasome activation appears to be key to the microglial response to peripheral inflammatory stimuli (Garaschuk, 2021)

Inducible nitric oxide (NO) synthase (iNOS) is expressed by microglia, astrocytes and possibly neurons in the context of neuroinflammation (Heneka and Feinstein, 2001). Activation of iNOS produces high levels of NO which is neurotoxic at high concentrations resulting in neuronal death by mechanisms including excitotoxicity via the N-methyl-D-aspartate (NMDA) receptor (Brown and Neher, 2010).

The complement system acts within the CNS to opsonise synapses to guide synaptic pruning by microglia. Though a necessary part of neurodevelopment and learning during homeostasis (Schafer et al., 2012), it can become dysregulated in the context of chronic inflammatory conditions and form part of neurodegenerative processes (Schartz and Tenner, 2020).

The chemokine CX3CL1 and its receptor CX3CR1, expressed by neurons and microglia, plays an important role in microglia-neuron cross-talk and is important in mediating synaptic plasticity, neurogenesis, learning and cognition (Rogers et al., 2011). However, when dysregulated in the context of neuroinflammation, this can potentiate neurotoxicity (Williams et al., 2014).

1.3.1.2 Astrocytes

Astrocytes have various important homeostatic functions including providing structural support to neurons, maintaining a stable chemical environment, regulation of blood flow by inducing vasodilation/vasoconstriction response, and regulation of neurotransmission (Volterra and Meldolesi, 2005).

Astrocytic end-feet are in direct contact with 90% of the capillary network of the brain and play a crucial role in regulating the BBB and modulate endothelial function (Lampron et al., 2013). Blood flow and energy exchange can be modulated by astrocytes via production of prostaglandins and NO (Takano et al., 2006). This intimate relationship with endothelial cells allows interactions within the neurovascular unit to lead to release of various signalling proteins, including proinflammatory cytokines, to loosen the cells of the BBB under certain conditions such as in neuroinflammation (Abbott et al., 2006) (Argaw et al., 2012).

In the context of CNS injury, disease or infection, astrocytes can undergo phenotypic change in a process called reactive astrogliosis, characterised by increased expression of glial fibrillary acidic protein (GFAP) (Jang et al., 2013). This can be provoked by inflammatory cytokines, TLR ligands, hypoxia, and products of neurodegeneration such as amyloid-ß (Carpentier et al., 2005) (Johann et al., 2008) (Perez et al., 2010). Astrocyte support and direct innate immune responses within the CNS by releasing various proinflammatory mediators including IL1ß, IL6, TNFα, CXCL1, CXCL10, CCL2, CCL3 and CCL5 (Choi et al., 2014).

1.3.1.3 Pericytes

Pericytes are derived from vascular smooth muscle cell lineage and cover up to a third of the abluminal surface of endothelial cells in the CNS microcirculation

(Armulik et al., 2011). Like astrocytes, pericytes play a role in modulating cerebral blood flow as part of the concert of CNS immune activity (Bell et al., 2010). In addition, pericytes appear to respond to proinflammatory signals to secrete inflammatory mediators such as iNOS and cyclooxygenase-2 (COX-2), and phagocytose in a macrophage-like manner (Pieper et al., 2014).

1.3.2 CNS immune-privilege and the BBB

As mentioned above, the CNS exists within a specialised immune environment, in which immune activity is modified in order to maintain the delicate homeostatic balance required for brain functioning (Engelhardt et al., 2017). The chief mechanism that maintains this modified immune environment is the BBB. Increasing evidence has shown that the BBB is not a passive, absolute barrier, but rather a dynamic modifiable barrier that actively contributes to the CNS immune response (Muldoon et al., 2013).

The vascular BBB exists along the endothelial lining of the brain's capillaries and includes astrocyte end-feet, pericytes and two layers of basement membrane (Daneman and Prat, 2015). The endothelial cells are connected by tight junctions (TJs) adherent junctions (AJs) to form adhesive contact between the endothelial cells (Correale and Villa, 2009). This dense structural arrangement acts to limit intercellular passage of molecules.

In homeostasis the BBB can be traversed either transcellularly or paracellularly in a tightly regulated manner. Ions and small molecular weight solutes can diffuse paracellularly (Stevenson and Keon, 1998). Passive transmembrane diffusion is possible for small neutral lipophilic molecules (Banks, 2009). Specialised endothelial cells of the BBB possess efflux transporters and nutrient transporters allowing selective active transport of ions, nutrients and other important molecules transcellularly (Daneman and Prat, 2015). Although the majority of immune surveillance within the brain is maintained by microglia, a few leukocytes can traverse the BBB in a chemokine-dependent process by rolling adhesion, firm adhesion and extravasation via leukocyte adhesion molecules. In the context of neuroinflammation, leukocyte recruitment can be significantly increased (Engelhardt, 2008).

In addition to the vascular BBB, there is also the blood-cerebrospinal fluid (CSF) barrier and the circumventricular organs (CVOs) located at the choroid plexus and arachnoid membrane, allowing production and reabsorption of CSF, in addition to free diffusion of small molecules and facilitated diffusion/active transport (Engelhardt and Sorokin, 2009). CVOs act as important transducers of information between blood, CSF and brain (Cottrell and Ferguson, 2004).

In the context of systemic inflammation, various changes can occur resulting in disruption of the BBB and increased permeability. These can broadly be thought of as either disruptive or non-disruptive. Disruptive changes are characterised by alterations to the architecture of the BBB resulting in increased general diffusibility, including TJ changes, denudation of glycocalyx, damage to endothelium and basement membranes. Non-disruptive changes tend not to manifest with architectural alterations, but include endothelial cytokine production, upregulation of receptors and transport mechanisms, and modulation of astrocyte function (Varatharaj and Galea, 2017).

1.4 The brain response to peripheral inflammatory stimuli

Knowledge of the more nuanced status of CNS immune privilege, in addition to observations relating to increased rates of psychiatric and neurological disorders in the presence of inflammatory disease, has led to increasing research focus on the mechanisms by which immune and inflammatory stimuli in the periphery may affect brain function. As noted earlier, systemic inflammation is often accompanied by a distinctive repertoire of behavioural, mood and cognitive responses, known as sickness behaviour.

1.4.1 Models of the brain response to peripheral inflammation

Lipopolysaccharide (LPS) bacterial endotoxin has been extensively used as a peripheral immune inflammatory stimulus in animal models seeking to investigate the brain response to peripheral inflammation. LPS provokes a potent inflammatory response via TLR4 agonism and does not cross the BBB to enter the brain parenchyma to any meaningful extent (Banks and Robinson, 2010). Peripheral administration of LPS has been shown stimulate a

neuroinflammatory response with upregulation of pro-inflammatory cytokines within the brain such as IL1ß, IL6 and TNFα (Qin et al., 2007) (Layé et al., 1994) (Noh et al., 2014) and chemokines including CCL2 (Cazareth et al., 2014) (Erickson and Banks, 2011).

Peripheral LPS stimulation activates brain NLRP3 inflammasome, involved in IL1ß release from microglia, enhancing brain innate immune activity (Garaschuk, 2021) and induces glial cell activation (Bian et al., 2013) (Qin et al., 2007) (Hoogland et al., 2015).

Systemic LPS administration also appears to associate with leukocyte recruitment across the BBB of neutrophils, monocytes and NK cells (Cazareth et al., 2014) (He et al., 2016).

Modified LPS studies have been conducted in humans and LPS has been found to evoke sickness behaviour associated with elevated serum cytokine levels (Schedlowski et al., 2014) (Hannestad et al., 2011). Peripheral LPS studies in humans have also shown significantly increased expression of translocator protein (TSPO) using a TSPO-specific radiotracer and imaging with positron emission tomography (Sandiego et al., 2015). TSPO is a marker for microglial activation (Bae et al., 2014).

In humans, it has long been noted that interferon therapy, as is used sometimes to treat certain cancers and hepatitis C virus, is associated with clinical manifestations of sickness behaviour and a significant risk of major depressive disorder (Renault et al., 1987) (Sockalingam et al., 2011). More recent findings suggest that interferon therapy also increases the risk of subsequent recurrent episodes of depression, suggesting that exposure to high levels of peripheral interferon could effect lasting changes that in some way prime or sensitize individuals to depressive episodes (Chiu et al., 2017).

1.4.2 Periphery-to-CNS immune communication pathways

In recent years, research has progressed exploring some of the mechanisms underlying the way in which peripheral inflammatory stimuli can generate a brain response. There are various ways in which signals, molecules and cells can traverse the BBB and enter the CNS environment and the brain parenchyma.

These include (1.) the humoral pathway: passage through leaky parts of the BBB such as CVOs and also brain endothelial cell activation and release of second messengers into CNS; (2.) the cellular pathway: active transport across the BBB of cytokines and active leukocyte recruitment across the BBB; (3.) the neural pathway: neural reflex communication involving activation of peripheral vagus nerve afferents signalling to the brain (Chavan et al., 2017) (Capuron and Miller, 2011). A summary of the main pathways is described below.

1.4.2.1 The humoral pathway

Leaky or fenestrated regions of the BBB such as CVOs or the choroid plexus allow some proteins, including cytokines and hormones, to enter and exit the CNS environment in a limited fashion (Quan and Banks, 2007). These regions are inherently more permeable due to an altered arrangement of the tight junction composition (Morita et al., 2016). Cytokines that cross the BBB at CVOs may then activate neurons and microglia, propagating a response that can spread into the brain parenchyma (Saijo and Glass, 2011). CVOs may also have an important role in generating hypothalamic-pituitary-adrenal (HPA) axis responses to peripheral inflammation (Buller, 2001), including immune-suppressant or anti-inflammatory top-down signalling (Saperstein et al., 1992).

In addition to CVOs, communication can occur by circulating cytokines activating cells of the BBB such as endothelial cells and perivascular macrophages. Once activated, these cells can release inflammatory mediators including prostaglandins and NO that can then enter the brain as secondary messengers (Serrats et al., 2010) (Lin et al., 2014). By this means, inflammation can be communicated from the peripheral circulation to the neurovascular unit, mediating changes in blood flow, activity, and inflammation in the brain.

The CSF is host to a limited number of effector memory T cells, which express receptors that facilitate homing towards sites of inflammation (Kivisäkk et al., 2006). These cells are believed to access the brain via the choroid plexus (Reboldi et al., 2009).

It was previously supposed that a facet of CNS immune privilege included absence of lymphatic drainage from the CNS (Louveau et al., 2015a). However, a network of dural lymphatics has been discovered that is thought to drain the

immune cells of peripheral origin that may survey the brain, thereby offering a route of brain to periphery communication, relevant to mounting humoral immune responses (Louveau et al., 2015b) (Prinz and Priller, 2017).

1.4.2.2 The cellular pathway

Several studies have shown that inflammatory cytokines can be actively transported across the BBB in a manner which occurs in the absence of BBB disruption. These include TNFα, which appears to bind to a specific TNF-receptor in order to migrate across (Pan and Kastin, 2002). IL1ß, IL6 and IFNs have also been shown to migrate across the BBB by studying migration with radiolabelled cytokines (Banks et al., 1994) (Banks et al., 1991) (Pan et al., 1997). Cytokine transporters at the BBB appear to be dynamic, changing in expression and activity according to the context and in response to disease or inflammation (Quan and Banks, 2007).

Another key aspect of the cellular pathway of immune-to-brain communication, involves trafficking of leukocytes across the BBB and into the CSF and brain parenchyma. Extravasation of leukocytes and tissue infiltration is a key immune defence and hallmark of inflammatory reactions. At the site of injury or infection, leukocytes can enhance and expand the inflammatory reaction. This process is best characterised in regard to neutrophil and monocyte recruitment in peripheral tissues, which occurs in a multi-step cascade involving leukocyte adhesion to the endothelium followed by active transmigration through capillary walls (Ley et al., 2007).

This process is more complicated in the context of the brain due to the less permeable nature of the endothelium of the BBB, which is modified by junctional complexes of TJs and adherens, preventing paracellular migration. Limited transcellular migration may occur, but significant leukocyte trafficking tends to require disruption of the BBB as is seen in the context of significant neuroinflammation, for example in neurodegenerative disease, multiple sclerosis, stroke or encephalitis (Stamatovic et al., 2008). However, evidence now shows that peripheral inflammatory stimuli can be associated with brain recruitment of leukocytes. Mouse models of hepatic inflammation, with elevated systemic TNFα levels, has shown chemokine-mediated recruitment of

monocytes into the brain, in response to elevated microglial secretion of CCL2 (D'Mello et al., 2009) (Kerfoot et al., 2006). There is also emerging evidence for brain monocyte recruitment in the context of stress-induced chronic low-grade peripheral inflammation, again likely mediated by microglial expression of chemokines (Wohleb et al., 2015) (Wohleb et al., 2013). The Aldara model of inflammation, which uses the TLR7-receptor agonist imiquimod applied topically to generate a potent IFN-type inflammatory response, has been shown to associate with substantial upregulation of brain chemokine expression and accumulation of leukocytes within the brain parenchyma (McColl et al., 2016). However, imiquimod has been shown to extremely effectively cross the BBB and it is likely that the immune response within the brain is as a result of direct brain TLR7 agonism rather than a response to peripheral inflammation (Nerurkar et al., 2017).

Leukocyte recruitment to the brain, in particular monocytes, is hypothesised the be an important mechanistic step in the pathogenesis of sickness behaviour in association with inflammation (D'Mello and Swain, 2016). However, in the context of inflammatory diseases such as RA, research exploring the recruitment of leukocytes to the brain is at an early stage in animal models and more research is required to elucidate this process. Even less is known in the human context, in part due to the technical difficulties of imaging brain monocytes in vivo.

1.4.2.3 The neural pathway

Processes of reciprocal bi-directional neuro-immune interaction have been demonstrated, including the expression of TLRs and cytokine receptors on neurons, providing a mechanism for neural modulation in response to immune stimuli (Steinberg et al., 2016) (Hosoi et al., 2005). In addition, immune cells such as T cells, DCs and macrophages have been shown to express neurotransmitter receptors, such as acetylcholine (ACh) and adrenergic receptors (Wang et al., 2003) (Kawashima et al., 2012) (Kawashima et al., 2015). Work exploring this relationship suggests that neuro-immune circuits exist that act to communicate and regulate inflammatory stimuli (Pavlov and Tracey, 2017) (Tracey, 2002) (Tracey, 2009) (Chavan et al., 2017).

In brief, the neuro-immune reflex is thought to be initiated by stimulation of afferent vagus nerve fibres in the periphery by cytokines such as IL1ß. These then signal to the brain, eliciting an efferent vagus nerve response with release of ACh at peripheral lymphoid organs, down-regulating inflammation (Tracey, 2009).

Afferent vagal signalling to the brain is thought to be important in establishing a bespoke brain neuroinflammatory response and in the production of fever and sickness-behaviour (Capuron and Miller, 2011). This has been explored in numerous animal models of peripheral inflammation as summarised elegantly by Chavan et. al (2017).

Pain is a cardinal feature of inflammation, particularly so in the context of RA. Nociceptors project to the brainstem and thalamus, and neural networks with other regions coordinate both modulation of pain perception and physiological and cognitive responses relevant to sickness behaviour (Heinricher et al., 2009). Nociceptors have bidirectional axons. Nociceptors can be activated by cytokines and other inflammatory signalling proteins, whilst efferent signals can promote or suppress inflammation (Pinho-Ribeiro et al., 2017). Small clinical studies examining vagus nerve stimulation in RA have shown promising effects on attenuating inflammation and disease severity (Koopman et al., 2016) (Drewes et al., 2021).

1.4.3 Mechanisms of brain response to peripheral inflammation

As has been discussed, peripheral inflammatory stimuli communicate with the brain via a variety of pathways and can elicit a brain neuroinflammatory response. The functional consequences of this within the brain and what is known about the mechanisms is briefly summarised here.

1.4.3.1 Neural signalling

Serotonin is a key neurotransmitter that can become depleted in the context of inflammation, a process which may be involved in the pathogenesis of depressive symptoms in the context of inflammation and sickness behaviour. Indoleamine 2,3 dioxygenase (IDO) can be stimulated by inflammatory cytokines and inflammatory signalling pathways to breakdown tryptophan, the amino acid

precursor to serotonin, into kynurenine (Schwarcz and Pellicciari, 2002). This results in depleted serotonin availability. The increased levels of kynurenine act to dysregulate glutamate transmission which can give rise to CNS excitotoxicity (McNally et al., 2008).

Inflammatory cytokines also inhibit synthesis, release and reuptake of dopamine, a key neurotransmitter in reward and motivational circuits (Morón et al., 2003). Inflammation, including presence of cytokines and inflammatory mediators such as NO, skews the pathways involved in dopamine synthesis and transport (Kitagami et al., 2003).

1.4.3.2 Neuroendocrine function

Administration of inflammatory cytokines and cytokine inducers such as LPS has been shown to activate the HPA axis, stimulating elevated expression of corticotrophin releasing hormone, adrenocorticotrophic hormone and cortisol (Pace et al., 2007). Chronic levels of inflammation however appear to show an attenuation of HPA axis activation, and the emergence of a flattening of the diurnal variation in activity with deleterious effects including depression, fatigue and increased propensity to cardiovascular disease (Harbuz et al., 2003) (Pariante and Miller, 2001) (Raison et al., 2010) (Matthews et al., 2006).

1.4.3.3 Neural plasticity and hippocampal neurogenesis

Adult neurogenesis is defined as the process of generating functional neurons from precursor cells in the mature brain and is highly evolutionarily conserved. Adult neurogenesis occurs in two distinct regions – the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Ming and Song, 2005). It is estimated that under healthy conditions approximately 700 new neurons are added to the adult human hippocampus daily (Spalding et al., 2013). The process of neurogenesis is tightly controlled by various signals within the neurogenic niche of the SGZ (Van Praag et al., 1999). Given that the neurogenic niche is in close proximity to a rich vascular supply around the DG, there is significant potential for communication from the periphery (Shen et al., 2004).

Excess levels of cytokines and inflammatory mediators within the CNS can undermine neuronal integrity and impair adult neurogenesis through disruption of trophic factors such as brain derived neurotrophic factor (BDNF) and the complex balance of signalling proteins required to support maturation of new neurons (Duman and Monteggia, 2006). Activated microglia release of pro-inflammatory cytokines creating an environment that is hostile to proliferation, differentiation and new cell survival and maturation within the neurogenic niche (Kohman and Rhodes, 2013). A number of animal studies have implicated elevated brain levels of IL1β, IL6, TNFα and IFNα as having inhibitory effects of neurogenesis (Yirmiya and Goshen, 2011) (Monje et al., 2003) (Goshen et al., 2008) (Ben-Hur et al., 2003) (Borsini et al., 2018).

It is thought that impaired hippocampal neurogenesis may be an important factor in the emergence of depression, anxiety, and cognitive problems in the context of inflammatory disease. Indeed, the neurogenic hypothesis of depression posits that impairment and restoration of hippocampal neurogenesis are key respectively to the genesis of, and recovery from depression (Jacobs et al., 2000). However, the evidence supporting the role for hippocampal neurogenesis in the pathophysiology of depression is mixed and merits further investigation. There are 3 main observations that support this theory. Firstly, stress inhibits neurogenesis via various mechanisms and increases vulnerability to depression. Secondly, the time course of the neurogenic process appears to mirror that of the lag to efficacy in antidepressant treatment. Thirdly, antidepressant treatments have been shown to increase the rate of hippocampal neurogenesis (Schoenfeld and Cameron, 2015).

There is also a suggestion that active neurogenesis is important as a buffer to stress responses and depressive behaviour. In a series of experiments exploring the relationship between endocrine and behavioural components of the stress response, Snyder et al (2011) showed that mice in whom neurogenesis had been inhibited showed slower recovery of glucocorticoid levels after acute stress. Additionally, mice showed increased food avoidance in novel environment, increased despair in the forced swim test, and reduced sucrose preference (hedonic response/ reward seeking) following exposure to acute stress (Snyder et al., 2011).

Whether the relationship between hippocampal neurogenesis and depression (and its recovery) is causal, reciprocal or merely a bystander is unclear.

A number of studies using rodents have reported a relationship between enhanced adult hippocampal neurogenesis and improved performance in hippocampal-dependent task experiments (Gould et al., 1999) (Van Praag et al., 1999) (Thuret et al., 2009). However, some studies have contradicted this showing impaired performance (Ambrogini et al., 2004) or no correlation (Jaholkowski et al., 2009).

Various experiments have been reported in which neurogenesis has been manipulated or ablated either by antimitotic agents, focal irradiation and transgenic animals, but the results have often proved conflicting or non-significant (Kohman and Rhodes, 2013). There is evidence to suggest however, that hippocampal neurogenesis may be important for pattern separation – the ability to form distinct representations from similar sensory inputs (Sahay et al., 2011). New neurons may be important in bestowing cognitive flexibility (Burghardt et al., 2012). Whilst all neurons alter their morphology and synapses in response to experiences and stimuli, it is thought that newly generated neurons demonstrate enhanced plasticity and do this much more extensively as they are not yet integrated into existing circuitry, with potential implications for learning. It is interesting to observe that neurogenesis appears to be at its highest rate in infancy, declining gradually thereafter (Spalding et al., 2013).

1.4.3.4 Neural circuitry and connectivity

Effects of inflammation on neural circuits and connectivity has begun to be elucidated in human studies using neuroimaging techniques. Typhoid (*Salmonella typhi*) vaccine inoculation has been used experimentally in humans as a safe model of peripheral inflammation (Reichenberg et al., 2001). Typhoid vaccine is associated with acute 4 to 6 fold increases in serum IL6 concentrations (Hingorani et al., 2000). Typhoid vaccine inoculation is associated with transiently lowered mood (Strike et al., 2004) which has been shown to correlate closely with serum IL6 levels (Wright et al., 2005).

Further study of the brain response to typhoid vaccine has demonstrated activation of the interoceptive pathway, with increased thalamic and insula

activity notable on brain functional magnetic resonance imaging (fMRI) (Harrison et al., 2009). Insula activity in response to typhoid vaccine has also been shown to associate with elevated fatigue, with mediation analysis suggesting that this was a mediating pathway from inflammation (Stefanov et al., 2020).

The mechanism underlying these phenomena is thought to involve activation of the neural pathway of periphery-to-brain communication with signals integrated in the thalamus and brainstem and then relayed to the insular cortex (Critchley and Harrison, 2013). The insula may act as an important centre for evoking visceral reflexes that mediate sickness behaviours by monitoring for errors in 'predictions' of visceral signals and generating efferent signals onwards to the hypothalamus, brainstem and spinal cord to maintain homeostasis (Harrison et al., 2009) (Stefanov et al., 2020).

1.5 Peripheral inflammation, sickness behaviour and depression

So far, we have explored various mechanisms by which peripheral inflammatory stimuli communicate with the brain, discussed some of the resultant neuroinflammatory responses, and touched upon the concept of sickness behaviour. In the following section I will set out the concept of sickness behaviour in greater detail, and in particular the aspects of depressed mood and fatigue which are relevant to this thesis.

Inflammation is known to cause a distinct syndrome of behavioural, emotional, and cognitive effects, collectively termed sickness behaviour. This constellation of symptoms includes many of the core symptoms of the psychiatric syndrome of depression (Dantzer et al., 2006). These symptoms include depressed mood, fatigue, anhedonia, poor concentration, altered sleep pattern, reduced motivation, loss of appetite, social withdrawal, and anxiety.

Depression is an extremely common disorder, representing one of the leading causes of disease burden globally (Whiteford et al., 2013). A complete mechanistic understanding of the pathogenesis of depression continues to be elusive. Greater knowledge of the mechanisms underlying depression is badly

needed in order to facilitated the development of better treatments; up to 60% of sufferers are estimated to experience treatment resistance or incomplete response to antidepressant therapy (Rush et al., 2008). Associations between inflammation and depression have been observed and are an area of ongoing research. A subset of depressed patients are found to have signals of inflammation in peripheral blood (Eller et al., 2008). In addition, depression is notably more common in individuals suffering from inflammatory disorders such as RA (Dickens et al., 2002). Inflammation is a marker for increased risk of poor treatment response/ treatment resistance (Yang et al., 2019) (Strawbridge et al., 2015), with elevated plasma cytokine levels associating with lack of response to selective serotonin reuptake inhibitor (SSRI) medication (O'Brien et al., 2007).

Whilst there is a clear overlap in the basis and manifestation of both sickness behaviour and depressed mood, we still do not fully understand the relationship between the two syndromes. Whilst sickness behaviour is an adaptive response in the context of acute injury/infection and inflammation, it may be that in the context of a chronic stimulus, it becomes a maladaptive response or one that becomes 'hard-wired' within the brain. Some evidence to support this hypothesis may be inferred from Chiu et al (2017), who demonstrated that IFNα therapy is associated both with acute episodes of depression during treatment, but also an increased risk of future recurrent episodes of depression long after ceasing treatment (Chiu et al., 2017).

A clearer understanding of the mechanisms underlying the relationship between inflammation, sickness behaviour and depression may allow both better more stratified diagnosis using biomarkers (for example inflammatory depression vs. non-inflammatory depression), in addition to offering new therapeutic targets. Depression is a highly heterogeneous disorder that is diagnosed on the basis of a categorical symptoms-based classification system, without recourse to biomarkers. A diagnostic stratification system using a biomarker panels, including cytokine levels has been proposed to predict treatment response (Schmidt et al., 2011) (Chan et al., 2016) (Nobis et al., 2020), but as yet no biomarkers have been transformative or shown the utility required to be integrated into real-world clinical practice.

1.5.1 Inflammation and depression

The observations that link depression to immune dysfunction, though many, remain to be resolved and integrated into a unified theory of aetiopathogenesis. Tying the various strands of immunological mechanisms together, not least integrating this with non-immunological factors, remains a major challenge. Anything other than a brief review falls outwith the scope of this chapter.

As noted earlier, among the earliest indicators that depression may be related to immune dysfunction arose from studies examining psychiatric complications in patients treated for hepatitis C infection with interferon therapy, a subset of whom developed a depressive illness (Renault et al., 1987). Systematic review puts the prevalence of IFN-α induced depression in the range of 10-40% for this group (Sockalingam et al., 2011).

Features of inflammation and immune activation are reported in both medically ill and medically healthy patients with depression. These features include elevated levels of cytokines and their soluble receptors, elevations in acute phase proteins, chemokines, adhesion molecules, and inflammatory mediators such as prostaglandins. Systemic inflammatory disorders, such as inflammatory arthropathies, systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD) among others, are strongly associated with an increased risk of developing depression (Dickens et al., 2002) (Matcham et al., 2013) (Wu et al., 2017) (Zhang et al., 2017) (Geiss et al., 2018). Interestingly, psychosocial stress of the sort that is classically considered a major precipitant for depression has been convincingly shown to bring about inflammatory changes and immune activation in its own right, in part through activation of the HPA axis among other mechanisms (Miller et al., 2009). The relationship between inflammatory disease and depression may also be bi-directional. Some evidence from cohort studies suggests that incident depression can increase the risk of developing IBD, whilst antidepressant treatment can be protective (Frolkis et al., 2019). This phenomenon has been observed in other inflammatory disorders, including RA (Vallerand et al., 2019). It is not known whether these observations reflect a causal pathway in which depression has a dysregulating effect on the immune system predisposing to inflammatory disease. Alternatively, depression and

inflammatory disorders could have a shared inflammatory pathway, or shared genetic risks.

In the absence of inflammatory disorders, antidepressant medications such as SSRIs have been shown to reduce levels of circulating inflammatory markers, further strengthening the possibility of a bi-directional relationship (Gałecki et al., 2018). These anti-inflammatory effects have been observed in human studies exploring peripheral markers of inflammation, in addition to effects within the brain in animal studies, including reduction of inflammasome and microglia activation (Dionisie et al., 2021). Drugs that precisely target inflammation such as anti-cytokine therapies have also shown promise in treating depressive symptoms in the context of inflammatory disease (Kappelmann et al., 2018).

The mechanisms by which inflammation may affect the brain and give rise to depressive symptomatology have been set out earlier in section 1.4.3. In essence, prolonged or excessive activation of brain cytokine networks may facilitate a range of mechanisms that act to undermine healthy plasticity via reduced neurotrophic support, inhibition of neurogenesis, glutamatergic toxicity and oxidative stress, disruption of neurotransmitter systems and connectivity.

1.5.2 Inflammation and fatigue

This thesis concerns brain effects of inflammation including sickness behaviour, a central feature of which is fatigue. Fatigue is a normal physiological phenomenon in the context of physical or mental exertion and/or lack of rest. Pathological fatigue by contrast tends to be persistent, exacerbated by minimal effort and not ameliorated by rest. There are few universal definitions of fatigue, in part because it is an inherently subjective phenomenon.

Fatigue is a common and debilitating symptom that is highly prevalent among patients with inflammatory disease, in particular rheumatic disorders (Nikolaus et al., 2013) (Bergman et al., 2009). A theoretical explanatory framework of fatigue in the context of inflammatory disease has yet to be established and research is ongoing. Fatigue is a complex, multidimensional and multicausal phenomenon involving a dynamic interplay of illness-related phenomena, cognitive and emotional components, and personal/environmental factors

(Louati and Berenbaum, 2015). Some of these factors in relation to RA are discussed in Chapter 4. A useful conceptual model put forward by Hewlett et al (2011) is simplified and reproduced below in figure 1.1 (Hewlett et al., 2011). This model highlights the multidimensional nature of fatigue. Although fatigue is not purely explained by inflammation, it is thought to potentially be a key variable.

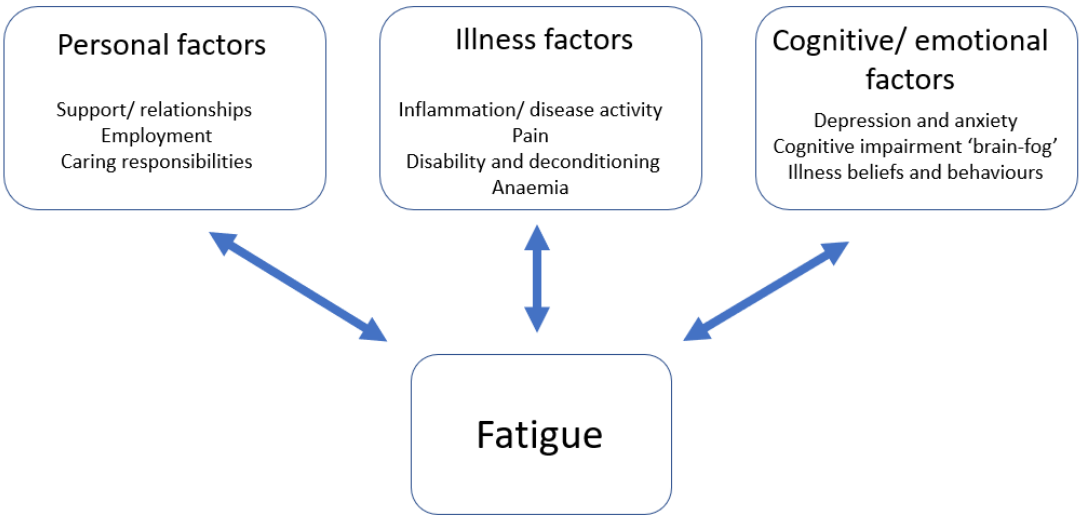

Figure 1.1 The multidimensional nature of fatigue in RA
A dynamic interplay of factors is relevant to the emergence of fatigue in RA. Adapted from Hewlet, et al (2011).

The clearest factors overlapping with inflammatory fatigue have been shown to be pain and depression (Garip et al., 2011) (Pollard et al., 2006) (Lee and Giuliani, 2019b). Fatigue, pain and mood disturbance can be conceptualised as overlapping manifestations of innate immune stimulation with inflammatory cytokines and the brain effects arising via immune-brain communication as discussed earlier. A simplified conceptual model illustrating this is adapted from Louati & Berenbaum (2015) in figure 1.2 (Louati and Berenbaum, 2015). Fatigue, depression and pain have been found to be strongly associated in the context of RA (Nikolaus et al., 2013), whilst biologic drugs which specifically target cytokine activity have been shown to have beneficial effects on fatigue and depressed mood (Chauffier et al., 2012) (Matcham et al., 2018) (Almeida et al., 2016).

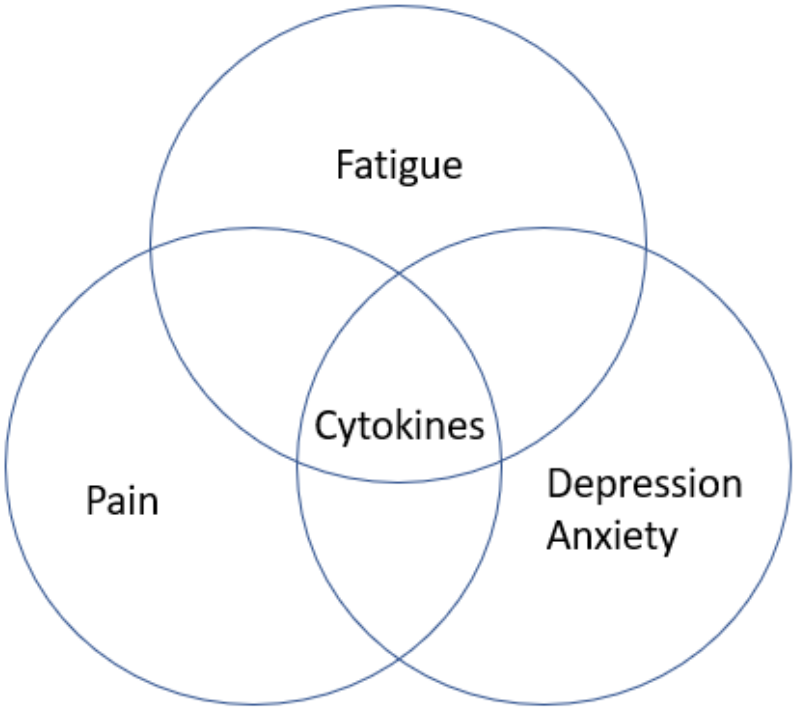

Figure 1.2 The overlapping roles of cytokines in fatigue, mood changes, and pain in RA Innate immune stimulation in the periphery with elevated pro-inflammatory cytokines signals the brain via immune-brain communication pathways, resulting in sickness behaviour syndrome with increased pain sensitivity, depressed mood, and fatigue. Mechanisms implicated include HPA axis activation, oxidative stress, impaired neurogenesis/plasticity, dysregulated neurotransmission, and altered connectivity circuits. Adapted from Louati and Berenbaum (2015).

A key feature of fatigue in the context of sickness behaviour is alteration of motivational states which contribute to inflammatory malaise, and is also a characteristic feature of depression. Early observations by Miller et al. (1964) found that rats injected with endotoxin would not press a bar to obtain food and water, but when given it they would eat and drink, albeit less than normally (Miller, 1964). Hedonic responses were also altered in these experiments (Dantzer et al., 2006). More recent animal models have demonstrated reduced social exploration, hypersomnia and cachexia in response to peripheral injection of IL1 that can be alleviated by administration of IL1-receptor antagonist or the anti-inflammatory cytokine IL10 (Bluthé et al., 1995) (Arnett and Clark, 2012).

Motivational and hedonic responses are traditionally associated with dopaminergic activity within the brain (Schultz et al., 1992). In human neuroimaging studies, administration of IFNα and endotoxin are associated with reduced ventral striatum activity in response to reward stimuli (Capuron et al.,

2012) (Eisenberger et al., 2010). Rodent LPS studies have shown altered dopamine release within the nucleus accumbens, another key part of the reward system (Borowski et al., 1998). Mechanisms by which dopaminergic transmission may be disrupted by inflammation include cytokine mediated disruption of dopamine synthesis (Kitagami et al., 2003) and enhanced synaptic reuptake (Felger and Miller, 2012).

Psychomotor retardation is another symptom that overlaps in fatigue and altered mood. Rodent models of LPS stimulation or administration of IL1β or IL6 show marked reductions in locomotion and motivational behaviours (Dantzer et al., 2006). Reduced movement and increased motor latency is also observed in human studies involving mild inflammatory stimuli such as typhoid vaccination, a phenomenon which may also be related to altered dopaminergic transmission within the basal ganglia (Brydon et al., 2008).

Cognitive changes in the context of inflammation are another aspect of inflammatory fatigue and depression. Impairments in sustained attention, verbal memory, reduced cognitive speed and impaired executive functions, sometimes termed inflammatory 'brain-fog', are commonly seen in inflammatory disorders (Mackay, 2015). Impairment in tasks such as response-time measures and executive function tests such as the Stroop test are seen in human experimental studies of mild inflammation (Capuron et al., 2012) (Harrison et al., 2009). Cognitive fatigue symptoms have been hypothesised to emerge from increased cognitive effort to maintain performance in a task, a hypothesis that may be supported by evidence showing increase anterior cingulate cortex (ACC) activation during mental tasks on fMRI studies of patients with MS (Filippi et al., 2002). ACC activation has been shown during Stroop testing in subjects administered typhoid vaccine (Harrison et al., 2009). Previous research has implicated ACC activity as a signal of modulation of cognitive resources in response to cognitive demand (Shenhav et al., 2013).

Insula activity also appears to correlate with fatigue in the context of inflammation. In a recent typhoid vaccine stimulus study, elevated IL6 was shown to associate with elevated fatigue (Stefanov et al., 2020). Similar findings have been reported previously (Harrison et al., 2009) (Harrison et al., 2015). The insula is significant as it is thought to be an important structure in

respect of 'human awareness', acting as a conduit for information relating to bodily status in order to process and output signals that effect behavioural responses aimed at restoring homeostasis – the conscious experience of fatigue promotes resting behaviours (Karshikoff et al., 2017). Increased insula activity is observed in various studies the context of inflammation (Hannestad et al., 2012) (Benson et al., 2015) (Harrison et al., 2015), and it is suggested that inflammation may sensitize the insula to interoceptive signals, thereby increasing propensity to fatigue (Karshikoff et al., 2017).

1.6 Rheumatoid arthritis

RA is a chronic inflammatory disorder characterised by multi-joint synovitis, autoantibody production, cartilage and bone destruction, and systemic inflammatory sequelae. The prevalence of RA is estimated at around 5 per 1000 adults globally, with incidence peaking in the 5th and 6th decade of life, and is 2 to 3 times more common among females compared to males (Myasoedova et al., 2010). RA is a disorder that is associated with significant morbidity and mortality, in terms of progressive disability, socioeconomic burden, systemic complications and early death. Historically RA was a diagnosis that signalled an inevitable and irreversible physical decline, largely due to progressive joint destruction and systemic inflammation. However, advances in the biological understanding of the pathogenic mechanisms involved in RA have led to discovery of new disease modifying therapies and targeted 'biologic' therapies that have greatly improved outcomes (Aletaha and Smolen, 2018). However, sickness behaviour type symptoms such as fatigue and altered mood, and associated mental health problems such as depression, remain a major source of unmet clinical need.

1.6.1 Diagnosis and clinical presentation

Diagnosis of RA is made on the basis of a clinical assessment of symptoms, radiological investigations, and blood measures of inflammation and autoantibodies. Gold-standard diagnosis involves scoring tools such as the American College of Rheumatology/European League Against Rheumatism 2010

(ACR/EULAR-2010) score (Aletaha et al., 2010), though in recent years there has been increasing interest in diagnosis involving measurement of stratifying molecular biomarkers with prognostic and therapeutic significance (Klareskog et al., 2008) (McInnes and Schett, 2011).

Disease activity is monitored by a combination of clinical assessment of joints, patient report of symptoms and blood measures of inflammation such as erythrocyte sedimentation rate (ESR) and CRP. This is often combined into a clinical score using the disease activity score (DAS)-28 (Van Riel and Renskers, 2016). See section 4.4.4.1 for more detail.

RA tends to affect multiple joints, often in a symmetrical pattern and typically presents with pain and swelling of the hands and feet, particularly wrists, metacarpophalangeal, metatarsophalangeal and proximal interphalangeal joints. Arthritis is typified by tender, painful, 'boggy' swelling arising from inflammatory synovitis and effusion. Larger joints including the knees, shoulders and elbows can also be affected (Aletaha and Smolen, 2018). Extra-articular manifestations can occur, more commonly in untreated/under-treated patients, including rheumatoid vasculitis – a serious necrotizing inflammatory process affecting small and medium calibre arteries in skin and organs (Genta et al., 2006). Without appropriate treatment, RA joint disease leads to progressive and irreversible cartilage and peri-articular bone destruction that causes permanent disability and pain.

RA is associated with various comorbidities, some of which may be explained by the presence of chronic inflammation. Higher rates of cardiovascular disease are though to partly represent the effects of systemic inflammation on the propensity towards atherosclerosis, with cardiovascular disease being closely related to disease activity (Crowson et al., 2018). Higher rates of mental illness, in particular depression, have long been noted in association with RA (see section 1.6.4) (Dickens et al., 2002). An elevated risk of dementia is also noted in association with RA, including relatively recent meta-analysis data (Ungprasert et al., 2016). Interestingly, this relationship appears to be declining over time, and it is thought that this may be a feature of improved inflammation-suppressing therapeutic interventions in RA, filtering through over

time to reduce the strength of the association (Kronzer et al., 2021) (Judge et al., 2017).

The combination of joint disease, and systemic physical and psychological manifestations, can lead to significant impacts on functioning and quality of life.

1.6.2 The Pathogenesis of RA

The pathogenesis of RA is complex and multi-causal, involving an interaction of various host and environmental factors that are thought to give rise to progressive immune system remodelling and autoimmunity that may arise many years prior to clinically detectable arthritis (Weyand and Goronzy, 2021). The key mechanisms involve the malfunctioning of multiple immune cells and signalling networks giving rise to immune-mediated tissue damage, particularly within the joints, but also involving the vascular system and organs. Many key issues and questions regarding the pathogenesis remain unresolved.

The best characterised genetic risk factor for development of RA is the association with certain alleles of human leukocyte antigen (HLA)-DRB1 region within the MHC, which confer particular susceptibility to RA and account for up to 30% of the variance of genetic risk (Gregersen et al., 1987) (Deighton et al., 1989). These genes are responsible for antigen presentation to T lymphocytes. Genome wide association studies (GWAS) have since identified more than 100 risk loci, a great number of which are relevant to inflammation and immune regulation (Okada et al., 2014).

Epigenetic modifications including deoxyribonucleic acid (DNA) methylation and histone acetylation are understood to play an important contributory role in promoting inflammation and breaching immune tolerance in RA. The effects of this include the production of autoantibodies such as anti-citrullinated peptide antibodies (ACPA), anti-nuclear antibodies (ANA), and antibodies to immunoglobulin G (IgG) including rheumatoid factor (RF) (Ospelt et al., 2017). Epigenetic modifications can often occur through environmental influences.

Environmental factors involved in the pathogenesis of RA include smoking (possibly through bronchial stress) (Symmons et al., 1997), mouth and gut microbiome (Wegner et al., 2010), and exposure to viruses is implicated

including Epstein-Barr virus, cytomegalovirus and parvovirus (Tan and Smolen, 2016) (Kozireva et al., 2008) (Halenius and Hengel, 2014). Certain bacterial infections are implicated in the production of autoantibodies including *Eschericia coli* and *Proteus spp. (Auger and Roudier, 1997).* The onset of RA is associated with adverse life events and psychological stress. It is thought that this may be linked to HPA axis activation and consequent immune dysregulation and excess cytokine production, thereby promoting penetrance in a susceptible individual (Capellino et al., 2010).

Recent conceptualisations of RA suggest that the process of RA pathogenesis is likely to begin years or even decades prior to clinical manifestation and involve multiple steps or 'check-points' along the path towards loss of self-tolerance, the emergence of autoimmunity and persistence of chronic inflammation (Weyand and Goronzy, 2021). Steps in this process include the production of autoantibodies, alteration of T cell populations to promote tissue-invasive effector cells and synovial stromal cell population changes that facilitate a chronically inflamed synovium.

The hallmark of RA – synovitis – occurs in the context of leukocyte migration into the synovial membrane, particularly T cells, B cells, neutrophils, and monocytes. In a process orchestrated by cytokines and chemokines there is activation of endothelial cells, neovascularisation and inadequate lymphangiogenesis creating a microenvironment in which intense inflammation can occur with limited cellular egress (Szekanecz et al., 2009) (Polzer et al., 2008). Proliferation of synovial fibroblasts and macrophages leads to synovial hyperplasia, forming a thickened and inflammatory membrane called pannus. The pannus extends over bone and cartilage in an invasive manner effecting cartilage destruction and bony erosions (McInnes and Schett, 2011). The cytokines and chemokines involved in this process are discussed in more detail in sections 1.2.3.2 and 1.2.4.1.

Associations with systemic disease are not fully explained by shared genetic risk and lifestyle factors or by RA treatments. Chronic inflammatory processes and circulating inflammatory mediators are implicated in the emergence of cardiovascular disease, in part via their effects on the stability of atherosclerotic plaques (Sattar and McInnes, 2005). Chronic inflammation also gives rise to

insulin-resistance and dysregulated lipid metabolism (Rho et al., 2010). Chronic inflammation is also implicated in the emergence of fatigue, cognitive changes and depression as discussed elsewhere in this introductory chapter.

1.6.3 Treatment of RA

Trends in the progression, severity and associated disability of RA have shown significant and sustained improvements over recent decades (Welsing et al., 2005) (Minichiello et al., 2016). These improvements are largely attributable to better disease modifying anti-rheumatic drugs (DMARDs), the development of targeted biologic-type drugs, and latterly an aggressive approach to the treatment of early-stage RA – the so called 'treat-to-target' approach (Finckh et al., 2006) (Krishnan et al., 2012) (Xie et al., 2018).

DMARDs form the foundation of modern RA treatment. Some authors include both conventional DMARDs and newer biologic-type drugs under the umbrella of DMARD, however for the purposes of this thesis I will restrict DMARD to the conventional category for simplicity and refer to biologic-type drugs as biologics. DMARDs not only treat the signs and symptoms of RA, but also interfere with the progression of joint damage, and in some cases help achieve effective remission and prevent permanent damage. As a class, DMARDs act by interfering with steps in inflammatory signalling pathways such as adenosine release, or by anti-proliferative properties (Wessels et al., 2008). DMARDs may be supplemented by short-term courses of glucocorticoids to achieve rapid remission in symptoms and disease activity.

Methotrexate is among the most effective and extensively used DMARDs, despite having a number of adverse effects that include fatigue, depressed mood, nausea and hepatotoxicity (Van Ede et al., 2001). Methotrexate can be highly effective as a monotherapy in achieving remission in early RA (25-40%) (Aletaha and Smolen, 2018), or can be given in combination with other DMARDs such as sulfasalazine, leflunomide and hydroxychloroquine.

Patients who fail to show adequate treatment response to conventional DMARD therapies may be stratified using prognostic markers such as auto-antibody status, early radiological evidence of erosive arthritis, and high disease activity. Tools such as Visser scoring (see section 4.4.1) can help guide this assessment

(Visser et al., 2002) though can be unreliable in accurately predicting response to biologics. Biologics are precisely targeted therapies that interfere with cytokines or steps in inflammatory pathways. These drugs include anti-TNF therapies such as Etanercept and Adalimumab, IL6 receptor antibodies such as Sarilumab and Tocilizumab, and Janus kinase enzyme (JAK) inhibitors such as Barcitinib and Tofacitinib. For best efficacy, biologics tend to be given in combination with a conventional DMARD such as methotrexate (Smolen et al., 2020).

A treat-to-target approach became established as the guiding principle of RA treatment in 2010 following the publication of recommendations by an international taskforce of influential experts in the field (Smolen et al., 2010). This approach has been widely confirmed to demonstrate substantial improvements in prognosis, disease progression and levels of disability (Smolen et al., 2016) (Xie et al., 2018). The aim of treat-to-target is to achieve sustained remission in disease activity preventing irreversible joint destruction and involves early robust treatment and regular monitoring. As a result, modern cohorts recruited after 2010 tend to show a different clinical expression of RA in comparison to more historical cohorts. It is not yet entirely clear what effect more recent trends in treatment may have on extra-articular manifestations of RA such as fatigue, depressed mood and sickness behaviour.

1.6.4 Depression in RA

The association between inflammation and depression and the associated mechanisms have been discussed in some detail already within this chapter. Therefore, just a few important points will be recapitulated here.

As has been noted, depression is common in RA, affecting up to between 14% and 48% of cases, and exists in a dynamic relationship with symptoms such as fatigue and pain (Matcham et al., 2013). This relatively wide disparity in prevalence reflects diagnostic heterogeneity within studies, but conservative estimates remain substantial at around 18.8% (Matcham et al., 2013). Depression is a disorder of multifactorial aetiology, and this is also the case in RA, with multiple factors including psychological reactions to disability, quality of life impacts, and impaired psychosocial functioning all contributing to the

emergence of depression (Margaretten et al., 2011). Multiple strands of evidence demonstrate a clear relationship between inflammation and depression in the context of RA (Nerurkar et al., 2019). There is also some evidence of a two-way relationship between RA and depression, possibly mediated via chronic HPA axis activation and increased susceptibility to immune dysregulation and inflammatory disease (Lu et al., 2016) (Vallerand et al., 2019).

The presence of comorbid depression in RA is associated with deleterious effects on RA outcomes including functional progression and response to anti-rheumatic therapies (Sturgeon et al., 2016). Depression has been shown to increase the likelihood of persistent and poorly controlled arthritis symptoms (Michelsen et al., 2017) (Kekow et al., 2011) (Rathbun et al., 2013). Some evidence suggests that disease activity scores such as DAS28 are less responsive to treatment, including with biologics (Matcham et al., 2018). Comorbid depression also increases the functional impact of RA and increases disability (Löwe et al., 2004). Cardiovascular risks may be enhanced and depression is associated with increased all-cause mortality in RA (Ang et al., 2005). Increased rates of DMARD treatment discontinuation are noted among depressed patients with RA (Mattey et al., 2010), though this relationship may be weak and other studies have not shown an association (Wong and Mulherin, 2007). Therefore, the observations described above are unlikely to be solely attributable to poor treatment concordance. Depression remains a major source of morbidity and mortality in RA and an important research and clinical priority.

Depression and anxiety symptoms have been shown in cohort studies to associate with measures of disease activity and inflammation in the context of RA, and appear to improve in the context of active treatment (Fragoulis et al., 2020) (Bacconnier et al., 2015) (Overman et al., 2012). Treatment with anti-TNFα therapies is associated with improvements in depressive symptoms (Deb et al., 2019). Although these results are drawn from observational studies in which causality cannot be inferred, these data add further weight to the inflammatory hypothesis of mental disorder, particularly when considered in the context of the wealth of evidence from animal and human studies exploring brain responses to inflammatory stimuli as discussed above in section 1.4. Some experimental work in the specific context of RA, has suggested altered patterns of

connectivity in brain networks relevant to mood and cognition that may play a role in transducing inflammatory signals into centrally mediated behavioural responses in RA (Schrepf et al., 2018). Further exploration is required in order to gain a greater mechanistic understanding.

1.6.5 Fatigue in RA

Fatigue in relation to inflammation and some of what is known regarding the mechanism of this phenomenon is dealt with earlier in this thesis in section 1.5.2. Therefore, I will focus here on some important points that are particularly relevant to RA and this thesis.

Fatigue is extremely common in RA, affecting up to 80% of patients prior to commencing DMARD treatment (Pollard et al., 2006). It may be that fatigue was historically considered an unimportant, even benign symptom by clinicians, but increasing evidence has emerged of the significance of this symptom. Fatigue is now considered one of the most debilitating symptoms of RA and is consistently rated by patients as among the most burdensome symptoms along with pain and disease activity (Kirwan and Hewlett, 2007). Fatigue can be a problematic symptom, associated with increased clinical care costs, and is a major determinant of functioning and ability to work (Michaud et al., 2003) (Hewlett et al., 2005). Data from the EPIC-Norfolk (pre- treat-to-target era) large population based prospective study, with long-term follow-up, has shown fatigue to be associated with an increased risk of premature mortality (Basu et al., 2016). In acknowledgement of the increasingly recognised importance of fatigue, it is now among the key criteria that should be measured in clinical trials for RA therapies (Kirwan et al., 2005).

As discussed in section 1.5.2, fatigue is a multi-causal and multi-dimensional phenomenon. In the context of RA, fatigue is at least partially thought to be related to disease activity and inflammation. Various studies have reported associations between fatigue and disease activity (Bergman et al., 2009) (Thyberg et al., 2009), and improvements in fatigue have been noted in studies of inflammation and disease-activity targeted treatments (Druce et al., 2015b) (Minnock et al., 2015). However, this relationship is inconsistent and several studies have reported differing results or no association with disease severity

(Pollard et al., 2006) (Stebbings et al., 2010) (Nikolaus et al., 2013). Few if any studies show a relationship between ESR or CRP and fatigue (Druce et al., 2015a) (Druce and Basu, 2019). A significant proportion of RA patients appear to experience persistent fatigue despite achieving remission of disease activity (Druce et al., 2015c) (Druce et al., 2016) (Olsen et al., 2016). Ifesemen et al (2022) explored fatigue over a long follow-up period of up to 3 years using the Early Rheumatoid Arthritis Network (ERAN) cohort that was recruited between 2002 and 2012. They reported high baseline and persistent relatively stable levels of fatigue over time, despite intercurrent changes to disease activity activity and inflammatory variables. High fatigue was consistently associated with pain, poor mental health, female sex and reduced functional capacity (Ifesemen et al., 2022).

Ultimately the relationship may be more nuanced than previously thought and requires further investigation. Significantly, there is very limited study of fatigue from more recent cohorts comprising patients recruited within the era of the treat-to-target approach. A recent study by Holten et al. (2022) reported on fatigue in a modern cohort undergoing treat-to-target therapy and found that early disease remission was associated with marked reductions in fatigue symptoms (Holten et al., 2022).

Various other factors have been found to be of importance in the context of fatigue in RA, in particular pain. Pain is strongly associated with fatigue (Druce et al., 2015c) (Davis et al., 2010) (Van Dartel et al., 2013b) (Feldthusen et al., 2016). Pain may in part explain some of the association with disease activity and there is even some evidence of a dose-effect relationship, with magnitude of pain improvements directly related to fatigue improvements (Pollard et al., 2006) (Druce et al., 2015a). It remains unclear what mechanism underlies the association between pain and fatigue, for example rather than a simple cause-effect relationship, there may exist a shared causal factor such as brain sensitization to both fatigue and pain perception (Druce and McBeth, 2019). This may speak to neuroimaging research as discussed in section 1.5.2, in which fatigue has been shown to associate with greater activity in interoceptive networks (Karshikoff et al., 2017).

Along with pain, mental health disturbance – particularly depression - is also consistently associated with increased susceptibility to, and elevated levels of, fatigue in RA (Fifield et al., 1998) (Jump et al., 2004) (Druce et al., 2015c) (Rat et al., 2012). Persistent depressive symptoms in RA may hinder improvements in fatigue (Druce et al., 2015a) (Fifield et al., 2001).

Treatment of fatigue, particularly in the subset of patients with persistent symptoms despite remission in disease activity, remains a major challenge in clinical practice and more research is required to deepen our understanding of the underlying mechanisms.

1.7 Animal models of RA

Despite their limitations, animal models of human disease remain a key tool in furthering our understanding of the basic biological mechanisms underlying disease pathogenesis and are indispensable in supporting the development of new therapeutic approaches.

The earliest reported animal model of RA was adjuvant-induced arthritis (AIA), in which complete Freund's adjuvant (CFA) is injected intradermally in the paw foot-pads of rats to produce an arthritic response (Pearson, 1956). CFA, which contains Mycobacterium, is a potent stimulator of immune responses, particularly APC activity and T cell mediated responses (Warren et al., 1986). This approach has been modified and refined over the years, but the general principles remain the same (Holmdahl et al., 1992).

AIA was largely superseded by the development of the collagen-induced arthritis (CIA) model, which appeared to show a more RA-faithful immunological and pathological response (Holmdahl et al., 1989). CIA was first described by Trentham et al. (1977) using rats (Trentham et al., 1977), and then in mice by Courtenay et al (Courtenay et al., 1980). CIA is induced by immunizing genetically susceptible mice (usually DBA1 males) with heterologous (usually bovine) type II collagen provoking an immune mediated polyarthritis (Brand et al., 2003) (protocol is described in detail in methods section 2.1.3). CIA is now a well characterised model and has proven to be highly tractable in the development of new therapeutic advances in RA, including in the advancement

of anti-TNF therapies (Holmdahl et al., 2002). CIA is discussed in more detail below.

Collagen antibody induced arthritis (CAIA) is a more recently developed model that shares many similarities to CIA, but bypasses the steps of auto-antibody production by relying on administration of a cocktail of ready-made monoclonal antibodies followed by injection of LPS (Khachigian, 2006). Although a quicker model than CIA, CAIA relies on injection of endotoxin and therefore when studying the effects of arthritis systemically, it is not possible to determine effects of arthritis vs. LPS. Furthermore, in bypassing the steps involved in formation of autoantibodies, important steps in the pathogenesis relevant to human RA may be missed. Although anti-collagen antibodies are by no means a uniform feature of RA, they may be associated with more severe disease (Mullazehi et al., 2012).

Latterly, the advent of transgenic technologies in cytokine biology has heralded novel RA models, including mice that over-express human TNFα gene (Keffer et al., 1991). These mice develop an erosive polyarthritis in response to a TNFα-driven inflammatory cascade, with 100% penetrance and a phenotype that shows chronic and progressive disease (Li and Schwarz, 2003).

1.7.1 Brain response in animal models of RA

Whilst there is now vast literature that explore the brain response to acute discreet peripheral inflammatory stimuli such as injection with LPS or polyinosinic-polycytidylic acid (Poly(I:C)), there has been very little translation of this research into more disease-faithful peripheral chronic inflammatory models (Süß et al., 2020b). This may in part be due to a culture within biological research in which there is a desire to minimise variance of input variables in order to seek purity in output variables, something which can be more difficult in the context of heterogeneous and unpredictable disease models. There exists a relatively limited body of work exploring the neuroinflammatory response to arthritis in animal models.

As discussed in section 1.4.2, periphery-to-brain communication is an important step in the conveyance of inflammatory signals to the brain. Nishioku et al. (2010) explored the integrity of the BBB in the context of CIA and found that CIA

weakened the BBB and demonstrated penetrance of sodium fluorescein dye into the brain parenchyma. They also reported reduced expression of occludin, a key BBB protein (Nishioku et al., 2010). Park et al. (2011) induced CIA in transgenic mice that over-express chimeric mouse/human amyloid precursor protein (APP) and mutant human presenilin 1 (PS1). They also reported BBB disruption and microglial proliferation, findings that may be relevant to the association between RA and an elevated risk of dementia (Park et al., 2011).

Lang et al. (2017) further explored the relationship between neurodegeneration and arthritis by inducing CIA in tau-transgenic mice that express mutant human tau-protein within the CNS. They found that neurodegenerative mice showed enhanced susceptibility and severity of arthritis, perhaps evidence of a two-way immune-brain communication network, relevant to arthritis (Lang et al., 2017). They also found elevated brain CCR2 expression, potentially implicating brain monocyte recruitment. However, somewhat unexpectedly they also reported that the presence of arthritis appeared to slow neurodegeneration (Lang et al., 2017), something echoed by Park et al's (2010) earlier study in APP/PS1 mice that found reduced hippocampal amyloid deposition in arthritic mice (Park et al., 2011). Park et al (2017) hypothesised that this could represent enhanced tau clearance by activated microglia (Park et al., 2011), a process perhaps not sustained in the more chronic time-course of human RA.

As discussed in section 1.4.2 regarding routes of periphery-to-brain communication, endothelial cells within the brain can serve as conduit for inflammatory signals. AIA has been shown to demonstrate vascular endothelial growth factor 2 (VEGF2)-dependent upregulation of intracellular adhesion molecule 1 (ICAM1) by endothelial cells resulting in perivascular microgliosis and astrogliosis in the spinal cord, thought to be an important mediator of pain (Beazley-Long et al., 2018). Both ICAM1 and vascular cell adhesion molecule 1 (VCAM1) have been found to be induced in the hTNFtg model (Süß et al., 2020a). These findings are significant as the suggest that arthritis may be associated with upregulated leukocyte adhesion markers, and by implication, potentially recruitment of leukocytes across the BBB.

Neural pathways are also key to neuroimmune communication. Hess et al. (2011) reported enhanced nociceptive brain activity and limbic system activation

using fMRI in the hTNFtg model, that was ameliorated within 24 hours of TNFα blockade, well before any improvements in arthritis were effected, supportive of an inflammatory neural signalling mechanism (Hess et al., 2011).

Relatively few studies have directly explored neuroinflammation in RA animal models. In the hTNFtg model Süß et al. (2020) recently reported microglial activation and proliferation in the cortex, thalamus and striatum, but not the hippocampus or cerebellum that was reversible with TNFα inhibition (Süß et al., 2020a). Interestingly, hTNFtg mice are not prone to displaying sickness-behaviours linked to depression such as loss of hedonic responses and do not show impaired hippocampal neurogenesis, which may reflect a peculiarity of the model in terms of relatively spared hippocampal response to inflammation (Süß et al., 2015).

In a study by Andersson et al. (2018), CIA has been shown to be associated with increased hippocampal transcription of Il-1ß and increased hippocampal ionised calcium binding adaptor molecule 1 (IBA1) cell density indicating microglial proliferation. This inflammatory signal was also associated with reduced doublecortin (DCX) staining in the dentate gyrus indicating impaired hippocampal neurogenesis (Andersson et al., 2018). CAIA has also been shown to be associated with hippocampal neuroinflammation with elevated levels of TNFα, CCL2 and CXCL1, and with signs of depression-like sickness behaviour (Lopes et al., 2020).

The evidence exploring the neuroinflammatory response in animal models of RA remains limited and further investigation is required to confirm published findings to date and to explore unanswered questions.

1.7.2 The pathogenesis and disease manifestations of CIA

Induction of CIA involves immunisation with bovine type II collagen (T2C) emulsified in CFA and injected intradermally into genetically susceptible adult mice, followed by booster immunisation of T2C injected intraperitoneally (IP) at 21 days, after which disease manifestations tend to occur (see methods section 2.1.3 for full protocol).

CIA relies on both innate immune activation and an adaptive immune response to generate disease with both T cells and B cells playing an important role in the pathogenesis. A strong priming of the innate immune system and a proinflammatory environment is effected by the use of CFA as adjuvant (Billiau and Matthys, 2011). This in turn supports a robust adaptive immune response to T2C. T cells support B cell production anti-T2C antibodies that react and bind with joint cartilage (Terato et al., 1992). In addition, T cells synergistically facilitate joint inflammation by activating synovial macrophages and other immune cells (Holmdahl et al., 2002). Initial immune priming occurs within a few days of initial injection, whilst the earliest signs of joint immune activation are seen after 1 to 2 weeks, with clinically detectable arthritis manifesting not earlier than 14 days and usually soon after the IP T2C injection at 21 days. Joint disease is characterised by marked synovial infiltration by T cells, B cells, macrophages and neutrophils, leading to formation of a hyperplastic pannus much like in human RA. Pannus formation leads to production of enzymes that destroy joint cartilage, whilst proliferation and upregulation of osteoclasts lead to bone erosion (Billiau and Matthys, 2011).

In addition to the shared microscopic pathological features, the resulting arthritis in CIA shares some macroscopic and clinical features with human RA. Much like the predilection for small joints of the hands and feet in human RA, CIA predominantly affects the fore- and hind-paws, rather than large joints. However, whilst RA tends to be a symmetric polyarthritis, CIA is non-symmetric. In addition, RA untreated will lead to chronic and progressive joint disease, whereas CIA shows only transient joint inflammation lasting several weeks, before eventually abating leaving permanently painful and damaged but non-inflamed joints (Billiau and Matthys, 2011).

Like human RA, disease penetrance is not 100% for reasons that remain to some extent unknown. Male mice are more susceptible to CIA than female mice, which is thought to reflect a protective effect of female sex hormones (Jansson and Holmdahl, 2001). Although human RA is more common in females, peak age of onset is in post-menopausal years of the 5^{th} and 6^{th} decade. Stress responses are also thought to play a role, and male susceptibility in CIA may reflect inter-

male aggression and dominance struggles within cage cohorts (Holmdahl et al., 2002).

1.8 The Scottish Early Rheumatoid Arthritis (SERA) cohort

This thesis comprises study exploring the brain response to RA that spans animal research as well as human cohort data. Whilst animal studies may offer insights into the molecular pathogenesis of disease, cohort studies offer a broad perspective from real-life clinical data, which may allow generation of new hypotheses and insights, and contextualise biological findings.

The SERA study is an inception cohort of RA and undifferentiated arthritis (UA) patients that was established to generate data on the contemporary phenotype and clinical course of the disease, and provide an avenue of discovery for new biomarkers (Dale et al., 2016).

Recruitment to SERA began in September 2011, with patients enrolled from National Health Service (NHS) health boards across Scotland within 6 months of first diagnosis, confirmed using ACR/EULAR-2010 criteria. Data collection included routinely collected data regarding demographics, medication prescriptions and comorbid diagnoses. In addition, cases underwent 6 monthly reviews with research nurses to collect data on RA treatment including DMARD therapy, disease-activity and clinic outcome data, and questionnaires relevant to mood, fatigue, general health, pain, and quality of life. Blood samples were also collected at baseline, 6 months and 12 months.

The SERA cohort represents a valuable tool in understanding the evolution of RA in the era of the treat-to-target approach in rheumatology. The SERA cohort integrates a wide range of clinical and non-clinical variables allowing a greater depth of understanding of disease associated variables such as depression or fatigue. In addition, interesting comparisons may be drawn with results from older cohorts, in exploring what effect aggressive treat-to-target therapy has had on extra-articular manifestations of RA such as sickness behaviour type phenomena.

1.9 Aims and justification of thesis

A wealth of evidence indicates that peripheral immune stimuli communicate with the brain and can elicit neuroinflammatory responses. The mechanisms that underpin this process have begun to be more clearly delineated. The functional consequences of immune-to-brain signalling is more uncertain, though a mounting body of research suggests that these processes are important in the emergence of psychiatric comorbidity and in the related syndrome of sickness behaviour. Whilst the association between sickness behaviour, or psychiatric illnesses such as depression, and inflammatory disease is well established, the molecular and cellular mechanisms that underlie these associations is uncertain. A better understanding of these processes remains a major research priority to facilitate the development of new and better therapeutic approaches in the treatment of symptoms such as fatigue and depressive disorders in inflammatory disease. Findings may be translatable to a broader context, given that there may be common aetiological pathways relevant to mental disorder in the absence of overt inflammatory disease.

Animal models remain an indispensable tool in the investigation of disease mechanism. Limited research has explored the relationship between inflammation and brain responses in disease-relevant tissue-specific animal models of inflammatory disorders such as arthritis. By contrast, the field of exploration of brain inflammatory responses to more specific inflammatory stimuli such as endotoxin or cytokines is relatively mature. Investigation of more disease-relevant models, such as CIA in the context of inflammatory arthritis, may be an important stepping-stone in terms of moving forward on the translational journey towards establishing meaningful neurobiological findings for human disease.

The first hypothesis examined in this thesis is:

"Peripheral inflammation in CIA will be associated with a neuroinflammatory response with measurable consequences".

From this broad initial hypothesis, a series of more specific research questions arose and were sequentially investigated.

CIA is an animal model in which an innate and adaptive immune response emerges of time. A second supplementary hypothesis was also explored in relation to this model:

"The brain inflammatory transcriptional response will evolve and vary over time during the model".

To attempt to answer these hypotheses a number of experiments presented in Chapter 3 were completed in order to bring forward the current understanding of the neuroinflammatory consequences of arthritis-associated inflammation.

Understanding the impact of peripheral inflammatory stimuli on the brain in the human disease context is an ongoing challenge, with the available methodologies very different to the field of animal models. Answering complex questions often requires examination of the problem from multiple angles and using varied methodologies and approaches. Having identified indications of a neuroinflammatory response in CIA in Chapter 3, the following chapter goes on to explore the effects of peripheral inflammation in RA in a human cohort with a specific focus on the sickness behaviour-related symptom of fatigue. Fatigue is a complex multidimensional symptom, in which inflammation is implicated. However, in the context of RA, it remains unclear whether fatigue arises as an inflammatory consequence. The available data from modern cohorts is limited. A hypothesis related to this was postulated:

"In early RA, fatigue will be explained by inflammatory variables such as inflammatory markers, in addition to proxy-markers of inflammation such as disease activity. In addition, fatigue will be predicted by demographic and lifestyle variables that are related to inflammation."

To answer this hypothesis, the SERA cohort was harnessed to investigate the factors associated with fatigue. The results of this analysis are presented in Chapter 4.

A synthesis of the findings from the animal and human work is presented in Chapter 5 along with discussion of future directions of study.

Chapter 2

Materials and methods

2 Materials and Methods

2.1 'Wet-lab' experimental work

All 'wet-lab' laboratory experiments were carried out using tissues generated from the CIA mouse model. The materials and methods are described below.

2.1.1 Companies

A full list of companies and suppliers of laboratory materials listed within this chapter and their contact details are noted in Appendix 1.

2.1.2 General materials and reagents

2.1.2.1 Plastics

Unless stated otherwise, all plastics including pipette sterile filter tips, 0.2ml PCR tubes and 2ml Eppendorf tubes were purchased from Starlab. All 1.5ml Eppendorf tubes, 15ml and 50ml centrifuge tubes, 7ml Bijou, and 30ml universal containers were purchased from Greiner.

2.1.2.2 Buffers and reagents

Ethylene-diamine-tetra-acetic acid (EDTA) 0.5M (100ml)

- 18.61g EDTA
- 80ml double distilled water (ddH_2O)
- pH to 8.0
- Add ddH_2O up to 100ml

10x Tris-buffered saline (TBS) (1L)

- 78.8g Tris-HCl
- 87.6g NaCl
- 800ml ddH_2O
- pH to 7.5
- Add ddH_2O up to 1L

1x TBS (1L)

- 100ml 10x TBS

- 900ml ddH_2O

1x TBS with 0.3% triton-x (TBX)

- 100ml 10x TBS
- 900ml ddH2O
- 0.3ml triton-x

10x Sodium citrate buffer

- 29.4g sodium citrate trihydrate (0.1M)
- 800ml ddH2O
- pH to 6.0
- Add ddH2O up to 1L

1x Sodium citrate buffer (antigen retrieval solution)

- 100ml 10x sodium citrate buffer
- 900ml ddH2O
- pH to 6.0

Eosin stain

- 500ml Putt's eosin stain (CellPath)
- 500ml tap water

Scott's tap water (STW)

- 3.5g sodium bicarbonate
- 20g magnesium sulphate heptahydrate
- 1L tap water

1% acid alcohol

- 990ml 70% ethanol (EtOH)
- 10ml 37% HCl

50x Tris-acetate-EDTA (TAE) buffer

- 252g tris-base (2M)
- 650ml ddH2O, mix until tris-base entirely dissolved
- 57.1ml acetic acid, added slowly
- 100ml 0.5M EDTA pH 8.0 (0.05M)
- Add ddH2O up to 1L

1x TAE buffer

- 100ml 50x TAE buffer
- 4900ml ddH2O

2% agarose gel

- 100ml TAE buffer
- 2g agarose
- Heat in microwave until fully dissolved (~2 mins)
- 5ul ethidium bromide

10x Phosphate buffered saline (PBS)

- 80.1g NaCl
- 2g KCl
- 14.4g Na2HPO4
- 2.7g KH2PO4
- 800ml ddH2O

- pH to 7.4
- Add ddH2O up to 1L

1x PBS

- 100ml 10x PBS
- 900ml ddH2O

PBST

- 990ml PBS
- 10ml Tween-20

Flow cytometry rinsing buffer

- Hank's balanced salt solution (HBSS) without Ca+/Mg+ (Gibco - ThermoFisher)
- 0.5% foetal calf serum (FCS)

Flow cytometry digestion buffer:

- 9.6ml HBSS without Ca+/Mg+ (Gibco - ThermoFisher)
- 0.25 ml HEPES 1M (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Gibco ThermoFisher)
- 200 ul DNase I (dilute 5ug/ml) (Sigma)
- 350 ul Liberase TM (2.5mg/ml or 13 U/ml stock) (Sigma)

FACS buffer

- 50 ml PBS without Ca+/Mg+
- 0.5g FCS (0.5%) (Gibco - ThermoFisher)
- 400ul 0.5mM EDTA (Sigma)

Flow cytometry running buffer

- 0.5l PBS without Ca+/Mg+
- 2.5g Bovine serum albumin (BSA) (0.5%) (Gibco - ThermoFisher)
- 2ml 0.5mM EDTA (Sigma)

2.1.3 In vivo procedures

2.1.3.1 Animals

DBA/1 WT mice were purchased from Envigo and were used for CIA model experiments. Only male mice were used due to higher susceptibility to CIA. Mice were purchased aged 6-8 weeks and were initiated on procedure aged 8-10 weeks.

All mice were housed in the Central Research Facility at the University of Glasgow. Mice were given 1-2 weeks to acclimatise and were handled regularly during this period to habituate to handling. Mice were housed in cages of up to 5 mice per cage, temperature controlled at 20-22°C. Diet was standard chow.

All experiments were conducted under the authority of UK Home Office approved project licences.

2.1.3.2 Induction of CIA

CIA was induced by intra-dermal (ID) injection of 100µg of bovine type II collagen (T2C) (MD Biosciences) emulsified in 200µg of CFA (MD Biosciences). This was administered to the shaved skin on either side of the tail base in two 0.05ml injections on day 0. Mice were anaesthetized with isoflurane-oxygen mix to administer injections. Preparation of injection emulsion was as described by Bevaart et al (Bevaart et al., 2010). On day 21 of the model, mice were administered an intra-peritoneal (IP) injection of 100µg T2C in 0.1ml of sterile PBS. Control mice were administered sterile PBS (ThermoFisher) injections on day 0 and day 21.

Mice were monitored throughout the model every 1 to 3 days, including weight monitoring, paw thickness measurements with callipers, and clinical scoring of arthritis.

Clinical scoring was completed according to a standardised clinical scoring system (Brand et al., 2007). Each paw is scored individually on a scale of 0 to 4 as set out in table 2.1, to derive a total severity score of between 0 and 16.

Table 2.1 Clinical scoring of CIA articular severity

Severity score	Degree of inflammation
0	No evidence of erythema or swelling
1	Erythema and mild swelling confined to the tarsals or ankle joint
2	Erythema and mild swelling extending from the ankle to the tarsals
3	Erythema and moderate swelling extending from the ankle to metatarsal joints
4	Erythema and severe swelling encompass the ankle, foot and digits, or ankylosis of the limb

Table adapted from *Collagen-induced arthritis; Nature Protocols (Brand et al., 2007)*

Mice were culled at day 42 or earlier if one of the severity-thresholds, as set out in the project licence, were breached. These included:-

- Weight loss of >20%
- All 4 paws affected
- Total clinical score of >10
- Injection site ulceration of >5mm

Mice were culled by exposure to rising concentration of CO_2 followed by femoral artery dissection in line with UK Home Office Schedule 1 guidance (Britain, 2000).

2.1.3.3 CIA time-point model

The CIA stimulus was administered as described in 2.1.3.2. There was an additional group of mice administered ID injections of sterile PBS emulsified in CFA (i.e. no T2C). Mice were culled by rising concentration of CO_2 and femoral artery dissection at 24 hours, 7 days, 21 days and 28 days.

2.1.4 Tissue collection and processing

2.1.3.1 Retrieval of blood

Immediately after death was confirmed, the mouse abdomen was dissected to reveal the inferior vena cava (IVC). Blood was collected from the IVC using a 1ml syringe flushed with 0.5M EDTA and transferred into a 1.5ml eppendorf tube containing 100µl 0.5M EDTA on ice.

2.1.4.2 Perfusion

After the blood sample was retrieved from the IVC, the dissection was extended to the thoracic cavity where the right atrium was cut to allow remaining blood to drain. Perfusion was then performed by injecting 20ml of ice-cold PBS through the circulation using a 23G needle introduced via the apex of the left ventricle. Characteristic clearing of the liver was observed to ensure adequate blood clearance from systemic circulation.

2.1.4.3 Brain dissection

Immediately following perfusion, mice were decapitated and skin dissected from the skull vault. The vault was opened using scissors to carefully separate the skull along the sagittal suture. Brain, including cerebellum and brainstem was carefully removed and halved longitudinally using a scalpel blade to cut down through the interhemispheric fissure. The right hemisphere was dropped into chilled RNAlater (ThermoFisher) and stored at 4°C for future ribonucleic acid (RNA) extraction whilst the left hemisphere was dropped into either formalin at room temperature or 4% paraformaldehyde (PFA) (SantaCruz) at 4°C for processing for immunohistochemistry (IHC). Where the hippocampus was separated from the rest of the hemisphere, this was done using a desktop magnifier to assist with blunt dissection with forceps to dissect out the hippocampus.

2.1.4.4 Spleen dissection

Spleens were blunt-dissected from the retroperitoneal space, being careful to remove the spleen intact, and dropped into chilled PBS prior to dabbing dry on blue-roll and weighing.

2.1.4.5 Isolation of plasma and peripheral blood leukocytes (PBLs) from blood

Plasma was isolated by centrifugation at 300G for 10 minutes, transferred into a fresh 1.5ml Eppendorf and spun for a further 5 minutes at 10,000G. All centrifugation was conducted at 4°C throughout. The supernatant of platelet free plasma was drawn off and stored in a fresh 1.5ml Eppendorf and stored immediately at -80°C.

The blood pellet within the 1.5ml Eppendorf following the initial 10-minute spin at 300G was set on ice. 1ml of RBC lysis buffer and pellet gently broken up before being added to 4ml of RBC lysis buffer in a 15ml falcon tube for a total of 10 minutes prior to centrifugation at 300G for a further 10 minutes. Supernatant was removed and 10ml of PBS added gently vortexed and centrifuged at 300G for 10 minutes. After supernatant removed the pellet was resuspended in 500µl of PBS and transferred into 1.5ml Eppendorf tube and centrifuged at 600G for 3 minutes. Supernatant was removed and pellet resuspended in 350µl of RLT buffer and immediately frozen at -80°C.

2.1.4.6 Brain RNA extraction

All plastics and solutions used were RNase-free as far as possible.

PBS perfused brain right hemispheres were stored in RNAlater immediately after dissection and stored for no more than 5 days at 4°C prior to processing.

Brain tissues were homogenised in a 2ml tube with 1ml of Qiazol (Qiagen) with a 5mm steel ball (Qiagen) in the TissueLyser LT (Qiagen) at 50 oscillations/second for 10 minutes. They were then rested at room temperature for 5 minutes before adding 200µl of chloroform and left to stand for a further 2 minutes. The homogenised samples were then transferred to fresh 1.5ml Eppendorf tubes and spun at 12,000G for 15 minutes at 4°C. The supernatant was then drawn off and further purified using the RNeasy mini kit (Qiagen) according to manufacturer instructions. Genomic DNA was removed during the process by application of DNase I (Qiagen) to the purification columns for 15 minutes. The purification process involved binding of RNA to RNeasy silica membranes with sequential washes using RW1 and RPE buffers and centrifugation at 10,000rpm for intervals fo 30 seconds to 2 minutes. The RNA was eluted into 50µl of quantitative polymerase chain reaction (qPCR)-grade RNase-free water (ThermoFisher) and placed on ice. The quantity of RNA in each sample was measured using a nanodrop spectrophotometer (Denovix).

2.1.4.7 PBL RNA extraction

Tubes containing the PBLs suspended in RLT buffer were defrosted on ice before being applied to Qiashredder columns (Qiagen) and centrifuged at 10,000rpm for

2 minutes. An equal volume of 70% ethanol was added to the eluent. The subsequent RNA purification, and removal of genomic DNA contamination steps, were completed using the RNeasy minikit following the steps as detailed above for brain RNA purification. RNA was eluted into 35µl of qPCR-grade RNase-free water (ThermoFisher) and placed on ice. The quantity of RNA in each sample was measured using a nanodrop spectrophotometer (Denovix).

2.1.4.8 Conversion of RNA to complementary DNA (cDNA)

Synthesis of cDNA from RNA was completed using the High-Capacity RNA-to-cDNA Kit (ThermoFisher) as per manufacturer instructions. The quantity of RNA used was normalised across samples. For each batch of cDNA synthesised, a –RT (no reverse transcriptase in the PCR reaction) control was created to control for genomic DNA contamination in subsequent qRT-PCR experiments. Each PCR tube contained 10µl of 2X RT Buffer Mix, 1µl of 20X RT Enzyme Mix, up to 9µl of RNA (depending on concentration) and RNase free water to bring total volume up to 20µl per reaction. The samples were incubated in a PCR reaction in a Veriti 96 well thermal cycler (ThermoFisher) at 37°C for 60 minutes and then 95°C for 5 minutes. Each cDNA sample was then diluted 1:5 and stored at -20°C.

2.1.4.9 Brain processing for frozen section immunohistochemistry

Immediately after mice were culled and perfused, brain was dissected as described above. The left hemisphere was dropped into 4% PFA for fixation for 24 hours at 4°C. Brains were then transferred into 30% sucrose for cryoprotection at 4°C for a further 24-48 hours. Brains were then flash-frozen into block of optimal cutting medium (OCT) (TissueTek). This was done by placing brains into OCT in a foil mould which was then semi-immersed in a container of liquid iso-pentane that was super-chilled within a liquid nitrogen container. These tissue blocks were then immediately stored at -80°C until they were sectioned.

2.1.4.10 Cutting of frozen sections

Blocks were sectioned using a Cryostat Model OTF (Bright Instruments Ltd.). Sections were cut in a sagittal orientation at 10µm thickness and mounted onto SuperfrostPlus Slides (VWR). Slides were then stored at -20°C prior to staining.

2.1.4.11 Brain processing for formalin fixed paraffin embedded (FFPE) samples

Following perfusion and dissection, brain left hemispheres were dropped into 10% neutral buffered formalin at room temperature for 24 hours and then transferred into 70% EtOH until processing. All FFPE samples were sent to the University of Glasgow Histology Research Service for processing. The tissue processing protocol was as follows:

1. 70% Ethanol for 1 hour
2. 90% Ethanol for 1 hour
3. 95% Ethanol for 1 hour
4. 100% Ethanol for 1 hour
5. 100% Ethanol for 2 hours
6. 100% Ethanol for 1.5 hours
7. Xylene for 2x 1 hour and 1x 1.5 hours
8. Molten paraffin wax for 2x 4 hours (60-65◦C)

Samples were then embedded in paraffin on a cutting cassette. Wax blocks were cooled on a cold plate until set and then stored at room temperature.

2.1.5 Gene transcription analysis

2.1.5.1 Primer design

Forward and reverse primers were designed for the genes of interest tested. Whilst some primers had been designed by previous research group colleagues, others were designed from scratch using the primer3 software (https://primer3.org/) (URL active as of 20/7/22) and the following conditions:

- Primer length 18-23 base pairs (bp); 20bp optimal
- 40-65% guanine/cytosine (GC) content; 50% optimal
- Primer annealing temperature (Tm) 59.5°C - 61°C; 60°C optimal
- Maximum self-complimentarity of 2
- Maximum 3' self-complimentarity of 1
- Amplicon size 50-150bp

- No more than 2 G or C bases in the last 5 at the 3' end of the primer sequence
- No stretches of G or C exceeding 4 bps
- Use of the rodent mispriming library

Primers for capturing sequences for standards were designed also using the above criteria, with the following modifications:

- Sequence region defined as qRT-PCR region +20bps at the forward and reverse ends.
- Amplicon size 100-1000bps

Where possible primers were selected to be exon spanning to minimise amplification of genomic DNA.

Primer specificity was determined by checking the sequences with NCBI Primer Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (URL active as of 20/7/22).

Primer sequences were manufactured by Integrated DNA Technologies and reconstituted in qPCR grade RNase free water (Thermofisher) before being stored at -20°C.

Primer sequences used are specified in Table 2.2.

2.1.5.2 Synthesis of standards for standard curve generation

Phusion High-Fidelity PCR Mastermix (New England Biolabs) was used as per manufacturer instructions to generate standard templates from cDNA samples positive for the gene of interest. Reactions were prepared in PCR tubes to which the following was added:-

- 2.5µl STD1 primer
- 2.5µl STD2 primer
- 1µl of template cDNA
- 0.5µl Phusion DNA polymerase
- 10µl GC buffer
- 1µl 10mM dNTPs

- 22.5µl nuclease free water

The optimal annealing temperature (Tm) was calculated using the New England Biolabs Tm Calculator (https://tmcalculator.neb.com/) (URL active as of 20/7/22). The samples then underwent PCR cycling in a Veriti 96 well thermal cycler (ThermoFisher) as described in table 2.3.

The standard was then analysed by gel electrophoresis. This was performed using a 1.5 - 2% agarose gel containing 0.005% ethidium bromide (EtBr) with 100bp ladder (New England Biolabs). Samples were run at 120V for 45 minutes and then examined under an ultraviolet lamp. Amplicon bands of the appropriate bp length were then cut out and product extracted using the PureLink Gel Extraction Kit (ThermoFisher) as per manufacturer instructions. The gel segment was immersed in solubilisation buffer and incubated at 50°C for 10 minutes and mixed until dissolved. The contents were then centrifuged through a PureLink column at 12,000G for 1 minute. Wash buffer was then applied to column and centrifuged again at 12,000G for 1 minute. Finally, the column was eluted into 50µl if nuclease free water. The concentration of standard was then measured using spectrophotometry (Denovix). The standard was then stored at -20°C.

An estimate of the quantity of DNA molecules present within the standard was calculated with reference to the sample concentration and molecular weight of the standard sequence using OligoCalc Oligonucleotides Properties Calculator (http://mcb.berkeley.edu/labs/krantz/tools/oligocalc.html). The absolute number of amplicon copies was determined using the following formula:

Copies (per µl) = (Conc. (g/µl) X Avogadro's Constant) / Molecular weight of amplicon

This was used later in quantitative real time PCR (qRT-PCR) experiments to calculate absolute quantification with reference to standard curve serial dilutions quantification.

Table 2.2 Primer sequences for qRT-PCR experiments and synthesis of standards

Gene of interest	qPCR1 (FWD)	qPCR2 (REV)	STD1	STD2
Il1ß	CGCTCAGGGTCACAAGAAAC	GAGGCAAGGAGGAAAACACA	TGGGCTGGACTGTTTCTAATGC	CCACACGTTGACAGCTAGGT
P2x7r	TGGGTCTTGCACATGATCGT	CTCCGTCACCTCTGCTATGC	AGCTGGAACGATGTCTTGCA	GGACACCAGGCAGAGACTTC
Il5	TCCTGCCTCCTCTTCCTGAA	GGGTTCCATCTCCAGCACTT	AGAAGTGTGGCGAGGAGAGA	GGCTGGCTCTCATTCACACT
Il6	CGGCCTTCCCTACTTCACAA	TCATTTCCACGATTTCCCAGAGA	GGAGCCCACCAAGAACGAT	TGGTCCTTAGCCACTCCTTCT
Il10	CAGAGAAGCATGGCCCAGAA	GCTCCACTGCCTTGCTCTTA	TGCTAACCGACTCCTAATGCA	GGCCTTGTAGACACCTTGGT
Tnfα	CACCACCATCAAGGACTCAA	GAGGCAACCTGACCACTCTC	TCTGTGAAGGGAATGGGTGT	GGCTGGCTCTGTGAGGAA
Ifnα	AGAGCCCTGTCTTCCTCAGT	TCACTCCTTCTCCTCACTCAGT	CTCCCAGCTGGCTATGAAGA	CCAGAATTTGCACAGTGAGGT
Ifnß	CACAGCCCTCTCCATCAACT	GCATCTTCTCCGTCATCTCC	GGCTTCCATCATGAACAACA	TCCCACGTCAATCTTTCCTC
Ifnγ	TGCTGATGGGAGGAGATGTCT	AGGGACAGCCTGTTACTACCT	GGGGTGGGGAAGAGATTGTC	TTTGTCATTCGGGTGTAGTCA
Ccl2	GCAGGTGTCCCAAAGAAGCT	TCAGCACAGACCTCTCTCTTGA	AGGTCCCTGTCATGCTTCTG	TGCTTGAGGTGGTTGTGGAA
Ccl3	CAGCCAGGTGTCATTTTCCT	CAGGCATTCAGTTCCAGGTC	CCACGCCAATTCATCGTT	TATGCAGGTGGCAGGAATGT
Ccl4	TGACCAAAAGAGGCAGACAGAT	GCTGTGCCACATCTCTTGGT	CTAACCCCGAGCAACACCAT	CTGAACGTGAGGAGCAAGGA
Ccl5	CTGCTGCTTTGCCTACCTCT	ACACACTTGGCGGTTCCTT	CCCTCACCATCATCCTCACT	TCAGAATCAAGAGGCCCTCTATCC
Ccl11	GCACCCTGAAAGCCATAGTCT	TGGGGTCAGCACAGATCTCT	TGCTGCTCACGGTCACTTC	CTTAGGCTCTGGGTTAGTGTCAA
Ccl19	GTGCCTGCTGTTGTGTTCAC	CAAGACACAGGGCTCCTTCTG	GCCTTCCGCTACCTTCTTAATGA	GCACAGACTTGGCTGGGTTA
Ccl20	ATGGCCGATGAAGCTTGTGA	TGGATCAGCGCACACAGATT	CGACTGTTGCCTCTCGTACA	CCTTCAACCCCAGCTGTGAT
Cxcl1	CCGAAGTCATAGCCACACTCA	AGGTGCCATCAGAGCAGTCT	ACACTCCAACACAGCACCAT	AGCAGAACTGAACTACCATCGA
Cxcl10	GCTCAAGTGGCTGGGATG	GAGGACAAGGAGGGTGTGG	CGATGGATGGACAGCAGAGAGCCT	GACAAGGAGGGTGTGGGGAGCA
Cxcl13	CATACCCAACCCACATCCTT	GCCTGTTCTCAAATAGCCTTTC	AACGCTGCTTCTCCTCCTG	CCATCTCGCAAACCTCTTGT
Cx3cl1	CAACTTCCGAGGCACAGGAT	AGATGTCAGCCGCCTCAAAA	GCCTGAATCCGCCACATTG	CCTGAGGAGATGGGGCTGTA
Cx3cr1	GCTCACGACTGCCTTCTTCT	TGCACTGTCCGGTTGTTCAT	CGTTCGGTCTGGTGGGAAAT	CCCATCTCCCTCGCTTGTG
Ccr2	GAGGTCTCGGTTGGGTTGTAA	GAGGTCTCGGTTGGGTTGTAA	CCACACCCTGTTTCGCTGTA	GCCTCATGCCCTCCTTTCTT
Ccr5	GTCCTCCTCCTGACCACCTT	CAGGGTTTAGGCAGCAGTGT	CCACACCCTGTTTCGCTGTA	CCCAACCTCCCCATTCAGTT
Bdnf	CACTCCACTGCCCATGATGT	GGAGGAGGGAGGGAAAGAGT	TCAAGGTGCTGTTGTCATTGC	TCCCCACCTCCATCCTAGAC
Dcx	GGCCCTTCCTCCTCACTTTC	GGTGCTCAACGTCTCAATGC	GGTCATGTTGCACCCTGGAT	GAGTGGAGTTCAGCGAGCTAT
Aif1 (Iba1)	CTGCCAGCCTAAGACAACCA	ATCCCCTCCAGCCTCTCTTC	GTGTGAGAACGGTCCCAGAA	GACTCTGGCTCACGACTGTT
Tmem119	TGCACCCCAGGAAACATCTC	AGTGGTGCGTTAGGGTGAAG	GAGGGAGCAAAGCCTGTGAA	GCAGAGTGACAGCGACATTG
Gfap	GAGCGTGCAGAGATGATGGA	CGCCTTGTTTTGCTGTTCCA	ATGCCACGCTTCTCCTTGTC	TCTCCTCCTCCAGCGATTCA

Table 2.3 PCR cycling for generation of standards

Cycle step	Cycles	Temperature	Time
Initial denaturing	1	98°C	30 seconds
Denaturation Annealing Extension	30	98°C Variable (~60°C) 72°C	10 seconds 10 seconds 50 seconds
Final extension	1	72°C	7 minutes
Hold	1	4°C	hold

2.1.5.3 Primer validation

To ensure that primers were specific they were validated using PCR and gel electrophoresis. Primers were diluted 1 in 10 to minimise primer-dimerisation. The following was added to PCR tubes per reaction:-

- 0.3µl of 1:10 diluted FWD primer
- 0.3µl of 1:10 diluted REV primer
- 1µl template cDNA
- 3.85µl RNase-free water
- 5µl SYBR Green PerfeCTa FastMix ROX reference dye (VWR)

A PCR reaction was then performed in a Veriti 96 well thermal cycler (ThermoFisher) as shown in table 2.4.

The PCR was then analysed by gel electrophoresis. This was performed using a 2% agarose gel containing 0.005% ethidium bromide (EtBr) with with 100bp ladder (New England Biolabs). Samples were run at 120V for 45 minutes and then examined under an ultraviolet lamp to check for presence of bands of appropriate bp length.

Table 2.4 PCR cycling for validation of primers

Cycle step	Cycles	Temperature	Time
Initial denaturing	1	95°C	3 minutes
Denaturation Annealing	40	95°C 60°C	3 seconds 30 seconds
Denaturation	1	95°C	1 minute
Hold	1	4°C	hold

2.1.5.4 Quantitative real time PCR (qRT-PCR)

qRT-PCR experiments were conducted using the QuantStudio7 (Thermofisher) qRT-PCR machine. Samples were run in technical triplicates on 384 well qPCR plates (Starlab) and calibrated using a standard curve for absolute quantification for each experiment. SYBR Green PerfeCTa FastMix ROX reference dye (VWR) was used. Standard curves were generated from standard dilutions ranging from 10^{-4} to 10^{-9}, allowing 6-point curves. A minimum of 5 points was considered necessary for reliable analysis.

Each well received 5µl SYBR Green PerfeCTa FastMix, 3.85µl RNase-free water, 0.15µl primer pair (1:10 dilution), and 1µl cDNA sample. The thermal cycling was as shown in table 2.5.

Table 2.5 qRT-PCR thermal cycling protocol

Cycle step	Cycles	Temperature	Time
Initial denaturation	1	95°C	20 seconds
Denaturation Amplification	40	95°C 60°C	1 second 20 seconds
Melt curve generation	1	95°C 60°C 95°C	15 seconds 60 seconds 15 seconds

2.1.5.5 qRT-PCR analysis

Quality assessment of the data output from the qRT-PCR reaction was conducted using the QuantStudio7 software. Amplification melt curves were checked for homogeneity. Negative controls were checked for amplification indicating contamination. Standard curves were used to determine efficiency of reactions with a minimum standard of 90-110% required for data to be deemed reliably interpretable. Data was then exported and further analysed using Excel 2016 (Microsoft). Statistical analysis and graphical illustration of results was performed using GraphPad Prism v8 or v9 (GraphPad Software). The data was analysed according to the following steps:-

1. Calculate coefficient of variation (CV) of cycle threshold (Ct) values of triplicates. Triplicates in which CV exceeded 2.5% had an outlier excluded if it was >1.5 from the median value.

2. Calculation of means from quantities calculated with reference to standard curve.
3. Background fluorescence substracted (if present) based on no-template control/ -RT cDNA sample control wells.
4. Absolute quantification of gene expression was calculated relative to TATA-binding protein (Tbp), which was used as the reference gene for all samples. Tbp is a widely used reference gene and has previously successfully been used within the group.

2.1.6 Flow cytometry of brain tissue

Flow cytometry was utilised to determine immune cell populations within the brain, including leukocytes.

2.1.6.1 Preparation of antibody cocktails

Antibody cocktails were prepared the night before the experiment and kept at 4°C in darkness until used. The antibody panel with fluorophores as shown in table 2.6 was used. Antibodies and labelled fluorophores were selected to ensure good delineation of excitation at varying wavelengths in order to maximise cell population delineation. Fluorescence minus one (FMO) control samples were stained with all fluorophores in the panel, minus one, for each target in order to calibrate the experiment and set boundaries for background signal and set gating.

Table 2.6 Flow cytometry antibodies, fluorophores and cocktail concentrations

Antibody name	Target	Fluorophore	Concentration
Zombie Aqua, Biolegend cat# 423101	Live / Dead	V405	1:1000
Anti-mouse CD45, BDBiosciences cat# 550994	CD45	PerCP-Cy5.5	1:1000
Anti-mouse CD3, Biolegend cat# 100206	CD3	PE	1:1000
Anti-mouse F4/80, Biolegend cat# 157306	F4/80	APC	1:1000
Anti-TMEM119, Abcam cat# ab225495	Tmem119	FITC	1:1000
Anti-mouse Ly6g, Biolegend cat# 127621	Ly6g	AF700	1:1000
Anti-mouse CD19, Biolegend cat# 115520	CD19	PeCy7	1:1000
Anti-mouse NK1.1, Biolegend cat# 108731	NK1.1	BV421	1:1000

2.1.6.2 Tissue preparation

Bloodless perfused brain left hemispheres were dropped in HBSS and stored on ice until processed. Each brain was minced on a petri dish using a scalpel and then dropped into 15ml tube containing digestion buffer. Samples were then placed in a shaker for 1 hour at 37°C. The reaction was then quenched with rinsing buffer and the sample mashed through a 70µm strainer into a 50ml tube. Samples were then centrifuged at 400G for 5 minutes at 4°C into a pellet and the supernatant discarded. The pellet was then resuspended in MACS buffer (Miltenyi Biotech) and centrifuged 2x 10 minutes at 300G at 4°C.

2.1.6.3 Myelin removal

Flow cytometry of brain tissue requires removal of myelin, which would otherwise render the tissue too sticky to pass through the cytometer. Cell pellets were resuspended in MACS buffer and 200µl of Myelin Removal Beads (Miltenyi Biotech) was added per sample and incubated at 4°C for 15 minutes. Samples were then centrifuged at 300G for 10 minutes and resuspended. The samples then underwent a myelin depletion cycle using an AutoMACS ProSeparator machine (Miltenyi Biotech).

The remaining sample was then centrifuged at 400G at 4°C for 5 minutes then resuspended in PBS. A 9µl fraction was then taken from the samples and mixed with 1µl of Acridine Orange dye (Sigma-Aldrich) and pipetted onto a Luna cell counting slide (Westburg Life Sciences). Cell counts were measured using the Luna-II automated cell counter (Westburg Life Sciences). Cell count per ml and viability was recorded.

2.1.6.4 Plating and staining

A 96 well round bottom plate was used for plating and staining of samples. 200µl of each sample was pipetted into each sample well. Each antibody/ stain was added to a final concentration of 1:1000. The plate was then incubated in darkness at room temperature for 20 minutes. The plate was then centrifuged at 400G at 4°C for 5 minutes, liquid discarded then cell pellets resuspended in 200µl FACS buffer and repeated. 100µl Fixation Buffer (ThermoFisher) was then added to each sample and incubated for 20 minutes in darkness at 4°C. FACS

buffer was then added, spun at 400G at 4°C for 5 minutes, liquid discarded x2, then resuspended in FACS buffer and stored overnight at 4°C in darkness.

2.1.6.5 Flow cytometry

A Fortessa LSRII flow cytometer (BD Biosciences) was used to run all samples.

UltraComp eBeads compensation beads (Invitrogen) were run through the machine in order to determine optimum voltages for the spectra of interest and minimise spill-over. The voltages are shown in table 2.7. Each sample was then run through the flow cytometer, with cleaning in between each sample. Data was retrieved and exported for analysis using FlowJo software v9 (BD Biosciences).

Table 2.7 Flow cytometer fluorophore spectra voltages

FSC	310V
SSC	265V
FITC	550V
PerCP-Cy5.5	590V
APC	550V
AF700	470V
BV421	390V
V500/BV510	410V
PE	500V
PE-Cy7	537V

2.1.6.6 Flow data analysis

Gating was completed to determine cell populations using FlowJo software. Gating strategy is shown in figure 2.1. Absolute quantification of cell number and percentage of CD45-positive cells was calculated using FlowJo software.

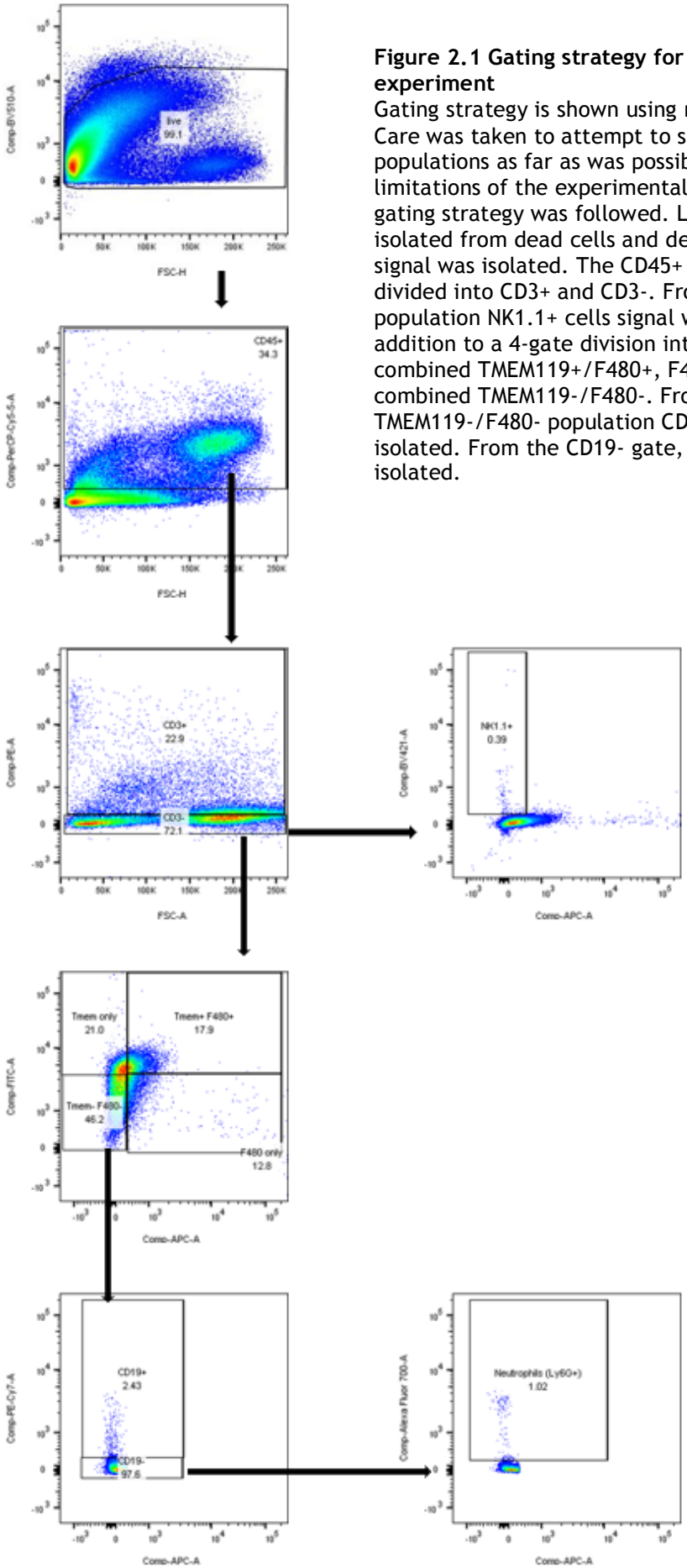

Figure 2.1 Gating strategy for brain flow cytometry experiment
Gating strategy is shown using representative images. Care was taken to attempt to separate distinct cell populations as far as was possible within the limitations of the experimental process. A sequential gating strategy was followed. Live cell signal was isolated from dead cells and debris. Then CD45+ cells signal was isolated. The CD45+ population was then divided into CD3+ and CD3-. From the CD3- population NK1.1+ cells signal was isolated, in addition to a 4-gate division into TMEM119+ only, combined TMEM119+/F480+, F480+ only and combined TMEM119-/F480-. From the combined TMEM119-/F480- population CD19+ cells were isolated. From the CD19- gate, Ly6G+ cell signal was isolated.

2.1.7 Immunohistochemistry (IHC)

2.1.7.1 Haematoxylin and eosin (H&E) staining

H&E staining was used to identify and localize specific brain regions prior to further staining of adjacent sections. Brain left hemispheres were dissected, processed and sectioned as described in section 2.1.3 and 2.1.4. For frozen sections, slides were removed from -20°C storage and defrosted on a slide warmer (Fisher Scientific) set at 50°C for 10 minutes. Slides were then immersed in ice-cold acetone for 5 minutes and air-dried at room temperature for 30 minutes. FFPE sections were instead immersed in xylene for 5 mins. Slides were then rehydrated by immersion/dipping in progressively reducing concentrations of EtOH (absolute – 90% - 70%). They were then briefly washed in running tap water and then immersed in haematoxylin Z stain (CellPath) for 7 minutes. After washing in running tap-water they were dipped in 1% acid alcohol, washed again and immersed in Putt's eosin (CellPath) (1:2 dilution in water) for 4 minutes. They were then washed in running tap water and dehydrated in progressively increasing concentrations of EtOH (70% - 90% - absolute). They were then immersed in 3 separate containers of xylene for 1 minute each before coverslips were mounted with DPX (CellPath). Slides were then dried overnight prior to visualisation under light microscopy.

2.1.7.2 Immunofluorescent IHC (frozen sections)

Brain left hemispheres were perfused, dissected, processed, and sectioned as described earlier. A summary of primary antibodies, secondary antibodies and antibody buffers and blocking serums used is detailed in table 2.8. A negative control (no primary antibody) was always used to ensure valid staining.

Slide mounted sections were brought from storage at either -20°C or -80°C and placed on a slide heater at 50°C for 10 minutes. A hydrophobic barrier was drawn round each tissue section to keep solutions in place using an ImmEdge Hydrophobic PAP pen (Vectorlabs). Slides were then submerged in two consecutive solutions of PBS for 5 minutes each. Block buffer (see table 2.8) was then applied to each tissue section (~50-75µl per section) in a humidified chamber for 1 hour at room temperature. Block buffer was then gently tapped off and slides were immersed in 3 consecutive washes of PBST for 5 minutes

each on a gentle shaker. Primary antibody in antibody buffer was then applied to each section, with negative controls receiving only antibody buffer. They were then incubated overnight in a humidified chamber at 4°C. The following day the antibody solution was tapped off then immersed in 3 consecutive washes of PBST for 5 minutes each on a gentle shaker. Secondary antibody in antibody buffer was then applied to all tissue sections and incubated in a dark humidified chamber at room temperature for 2 hours. The slides were then immersed in 4 consecutive washes of PBST for 5 minutes each on a gentle shaker. They were then tapped dry and 15µl of 1:1000 DAPI dilactate in Mowiol mounting medium (Sigma) was applied to each section prior to attachment of coverslips. Slides were then stored in a dark container at 4°C until they were imaged.

Table 2.8 Antibodies, buffers and antibody concentrations of frozen section immunofluorescent IHC

Target	Primary Antibody	Conc.	Secondary antibody	Conc.	Antibody buffer	Block buffer
IBA1	Anti-IBA1 goat polyclonal (Novus NB100-1028)	1:500	AF568 donkey anti-goat (Abcam ab175704)	1:500	1% donkey serum (ThermoFisher) in PBST	1% horse serum (Gibco/ ThermoFisher) and 1% BSA (Sigma) in PBST
GFAP	Anti-GFAP rabbit polyclonal (Abcam ab7260)	1:1000	AF488 donkey anti-rabbit (ThermoFisher A32790)	1:1000	1% donkey serum in PBST	1% horse serum and 1% BSA in PBST
iNOS	Anti-iNOS rabbit polyclonal (Abcam ab15323)	1:100	AF488 donkey anti-rabbit (ThermoFisher A32790)	1:200	PBST	1% BSA (Sigma) in PBST
DCX	Anti-DCX rabbit polyclonal (Abcam ab18723)	1:1000	AF488 donkey anti-rabbit (ThermoFisher A32790)	1:500	1% donkey serum in PBST	10% donkey serum in PBST

2.1.7.3 Chromogenic IHC (FFPE sections)

Chromogenic staining of NeuN was completed by the Veterinary Diagnostic Services Core Facility at the University of Glasgow. A description of the methods used is listed here. Mounted sections were deparaffinised in several changes of Histo-Clear Histology Clearing Agent (National Diagnostics) and re-hydrated in ethanol solutions of decreasing concentration. The sections were then rinsed in water.

Antigen retrieval was optimised to unmask the specific epitopes for each antibody and facilitate staining. For heat-induced epitope retrieval, sections were treated at full pressure with Access Retrieval Unit (Menarini) in Sodium Citrate buffer (pH 6) for 90 seconds at 125^{o}C. The sections were then rinsed in Tris Tween buffer (pH 7.5).

The sections were treated for 5 minutes at room temperature with 3% hydrogen peroxide in phosphate buffered saline to quench endogenous peroxidase activity. After washing twice with TRIS Tween buffer (pH 7.5), the sections were incubated for 30 minutes at room temperature with the primary antibody anti-NeuN, ab104225 (Abcam) in a 1:15000 dilution. The sections were then washed with TRIS Tween buffer (pH 7.5) to remove the excess primary antibody.

For primary antibody detection, the sections were incubated with EnVision+ System HRP Labelled Polymer Anti-Rabbit Secondary Antibody (Agilent) for 30 minutes at room temperature. The sections were washed with TBS with 0.3% triton-x buffer to remove excess labelled polymer from the sections. This was followed by two 5-minute incubations with 3,3'-diaminobenzidine (DAB) substrate-chromogen (EnVision+ System, Dako). Sections where then rinsed twice with distilled water for 5 minutes each time. Tissues were counterstained using Gill's haematoxylin and mounted using clear resin and coverslips for long-term storage. Details of antibodies used can be found in table 2.9.

Table 2.9 Antibodies and concentrations for chromogenic FFPE section staining

Antibody	Dilution (final concentration)	Species	Catalogue No.
Mouse anti-NeuN	1:15000	Rabbit	Abcam ab177487

2.1.7.4 Image analysis and quantification

Slides were imaged using a PC-controlled Axio Imager M2 (Zeiss) microscope at various levels of magnification. Images were processed using Zen 3.0 Blue Edition software (Zeiss). Cell counts were performed on images using ImageJ software (Fiji - https://imagej.net/software/fiji/) and its cell counter extension.

Images of hippocampus for iNOS staining, IBA1 staining and GFAP staining were imaged at 5x zoom. Cortex images were imaged at 10x zoom. Images of dentate gyrus for DCX staining were imaged at 10x zoom.

All images were blinded by an independent researcher prior to counting and analysis. To ensure reliability of counts, 25% of images were recounted after an interval of 2-3 days to ensure intra-rater consistency. Counting rules were not considered reliable if confidence interval (95-105% calculated based on geometric mean) exceeded.

2.1.8 Enzyme-linked immunosorbent assay (ELISA)

Levels of IL6 in plasma were measured using an IL6 mouse uncoated ELISA kit (ThermoFisher) as per manufacturer instructions. Plate wells were coated with coating buffer and primary antibody and sealed and left overnight at 4 ^{o}C in darkness. This was then discarded and wash buffer (PBS, 0.05% tween) was applied x3 and blotted off. Assay diluent was added for blocking for 1 hour at room temperature in darkness then washed with wash buffer x1. Five-fold serial dilutions were employed to generate standards for standard curve. All samples and standards were added in technical duplicates along with assay diluent and incubated at room temperature in darkness for 2 hours. Wash buffer was then applied and blotted off x3. Detection antibody was then added to each well and the plate was then incubated at room temperature in darkness for 1 hour. Wash buffer was then applied and blotted off x3. Avidin-HRP was applied to each well and incubated at room temperature in darkness for 30 minutes. Wash buffer was then applied and blotted off x4. TMB solution was then added to each well and incubated at room temperature for 15 minutes. Stop solution was then added to arrest the reaction. The plate was then read at 450nm using a TECAN plate reader (Agilent) to determine optical density measurements which were converted to protein concentration values with respect to standard curve measurements of known quantities. Results were included in analysis if variation between technical duplicates was <15%.

2.1.9 Multiplex array

Levels of multiple cytokines and chemokines in plasma were measured using a Multiplex/Luminex system (Merck) as per manufacturer instructions. The assay plate was washed with gentle shaking for 10 minutes at room temperature whilst quality control samples, master mix and standards were reconstituted to the recommended concentrations. Five-fold serial dilutions were employed to generate standards for standard curve. All samples and standards were added in technical duplicates along with matrix solution and assay buffer. Pre-mixed beads were added to each well having been sonicated by vortex mixing. The plate was incubated overnight with gentle agitation. The following day a magnetic plate washer was used to allow the beads to bind to the magnet and the well contents were removed and the plate washed with wash buffer. Detection antibodies were added and incubated for one hour with gentle agitation at room temperature in darkness. Streptavidin-phycoerythrin was then added and the plate incubated to 30 minutes with gentle agitation at room temperature in darkness. Washing with the magnetic plate washer was repeated. Sheath fluid was then added for 5 minutes to arrest the reaction.

Measurements were obtained using the LUMINEX 200 system, using Bio-Plex software version 6.1 (Bio-Rad). Results were included in analysis if variation between technical duplicates was <15%.

2.1.10 Wet-lab statistical methods

2.1.10.1 Power calculations

Power calculations were undertaken using R software v3.5.1 (The R Project - https://www.r-project.org/) to determine the appropriate number of animals to adequately power animal models. Data was used from pilot experiments. Power calculations were undertaken based on data in which the p-value for a 2-group comparison (e.g. CIA and controls) was approaching significance and it was suspected that a biological difference had face validity. Power calculations based on various measures were conducted to enhance pragmatic decision-making regarding powering of experiments.

Each group data was converted into vectors x and y. Mean, standard deviation (SD) and square root of variance of x and y was calculated. A permutation test was conducted by generating 10,000 sets of random numbers within the SD for x and y. The number of sets was determined by the number of mice in x and y (n. value). By setting the alpha to 0.95 (significance threshold p-value 0.05) and the beta to 0.8 (power 80%), it was possible to determine the n. value required to meet these criteria by conducting 10,000 Mann-Whitney U tests on the randomly generated values. The n. value in x and y could be increased and the calculation re-performed until the significance threshold met.

2.1.10.2 Group comparisons

All statistical analysis for animal model work was conducted using GraphPad Prism software 9.3.1 (GraphPad). The threshold for significance was determined to be a p-value of <0.05. In 2 group comparisons where the n. value in either group was <8, statistical significance was determined using Mann-Whitney U test. Where the n. in both groups was ≥ 8, normality testing was performed using D'Agostino & Pearson test, Shapiro-Wilk test, Anderson-Starling test, and Kolmogorov-Smirnov test. QQ plots were also generated and eyeballed for normality. Homoscedasticity was determined by Brown-Forsythe test and by eyeballing the error bars to examine equality of variance between groups. In a 2-group analysis with normally distributed data with equal variance the student's t test was used. Where the data was normally distributed but unequal variance the student's t-test with Welch's correction was used. Where data was not normally distributed Mann-Whitney U test was used.

In ≥ 3-group comparisons the same normality testing was undertaken. In ≥ 3-group comparisons with n. value of <8 per group, Kruskal Wallis with multiple comparisons was used. In groups of ≥ 8 where normality testing determined normal distribution then analysis of variance (ANOVA) with multiple comparisons was used. Abnormally distributed data was analysed using Kruskal Wallis with multiple comparisons.

2.1.10.3 Outliers

In general, removal of outliers was avoided. In cases where there was clear suspicion of an outlier, Grubb's test was used to determine if this was statistically valid.

2.1.10.4 Regression analysis

Simple univariable regression analyses were conducted using GraphPad Prism version 9.3.1 (GraphPad). Best fit values, standard error, 95% confidence intervals, r^2 and significance of deviation of slope from zero were all calculated. A p-value of <0.05 was considered to be the threshold for significance.

Multivariable linear regression was completed using GraphPad Prism version 9.3.1 (GraphPad). One dependent variable and two independent variables were assessed. The ratio of number of dependent variable values per independent variables included was significantly less than the generally accepted minimum of 10 and therefore these analyses were purely exploratory.

Adjusted R^2 was calculated. Post regression diagnostics were completed to check for multi-collinearity and parameter covariance. Normality of residuals was determined by creating QQ plots and checking that distribution was Gaussian.

2.2 'Dry-lab' cohort study analysis

The human data included for analysis in this thesis was drawn from the Scottish Early Rheumatoid Arthritis (SERA) study. The SERA database is drawn from an inception cohort of prospectively recruited patients with newly diagnosed RA, meeting the ACR/EULAR-2010 criteria, or UA. The cohort was established with a view to providing improved RA phenotypic description and to encourage the discovery of prognostic biomarkers. The SERA project was created by the Scottish Collaborative Arthritis Research (SCAR – www.scarnetwork.org) (URL active as of 2/07/22) through a collaboration between the Universities of Aberdeen, Dundee, Edinburgh and Glasgow, NHS Scotland, Healthcare Improvement Scotland, The Chief Scientist's Office Scotland and Pfizer LTD.

Ethical approval for the study protocol and procedures was granted by the West of Scotland Research Ethics Committee.

Patients within the SERA cohort are drawn from cases presenting to NHS secondary care rheumatology centres across Scotland between 2011 and 2015 (Dale et al., 2016). Cases were traced and recruited by the SERA team within 6 months of their initial diagnosis, having continued with standard clinical care with their usual rheumatology team. Treatment decisions and clinical follow-up decisions remained with the patient's usual rheumatology team.

Patients were excluded from the database if their symptoms could be explained by an alternative cause (e.g. an alternative inflammatory arthropathy) or if they were carriers of a blood borne virus.

All participants were consented by the SERA team to provide generic enduring consent for collection of demographic and outcome data, retrieval and linkage of routine healthcare data and long-term storage of data and samples for future research.

A range of demographic data and clinical data, assessments and laboratory tests were completed at the baseline SERA visit, 6 and 12 months. These include:-

- Demographic data at baseline – age at baseline, sex, race, marital status, employment status, Scottish Index of Multiple Deprivation (SIMD) quintile and SIMD data zone rank (see - https://www.data.gov.uk/dataset/d9a46acf-ae72-4dc4-b17c-af76222fd6f6/scottish-index-of-multiple-deprivation-simd-2012) (accessed 20/7/22).
- Lifestyle data at baseline – weekly unitary alcohol intake, smoking status, body mass index (BMI)
- Comorbidities at baseline and medical history.
- Medication treatments at baseline – including whether prescribed DMARDs, biologics and antidepressant medications.
- Clinical assessments, symptom and disease scores measured at each follow-up interval – including DAS28, Visser score (Visser et al., 2002), ACR/EULAR-2010 score (Kay and Upchurch, 2012), EuroQol-visual analogue scale (EQ-VAS) and EQ-5D (Rabin et al., 2011), Patient Global Assessment

visual analogue scale (PGA-VAS) and Assessor Global Assessment VAS (AGA-VAS) (Nikiphorou et al., 2016), Pain-VAS (Joos et al., 1991), Health Assessment Questionnaire (HAQ) score (Bruce and Fries, 2003).

- Fatigue rating scale – measured at each SERA follow-up interval visit using the Patient Reported Outcome Measures Information System (PROMIS) Item Bank v1.0 – Fatigue – Short Form 8a questionnaire (see appendix 2.) (Bingham III et al., 2019).
- Depression rating scale – measured at each SERA follow-up interval visit using the Hospital Anxiety and Depression Rating Scale (HADS) (Zigmond and Snaith, 1983).
- Bloods measured at each SERA follow-up interval including – CRP, ESR, Full Blood Count, Lipid profile, Urea & Electrolytes.

Access to the SERA database was granted following application submission to the SERA Access Committee which is comprised of a scientific steering committee and patient representatives.

2.2.1 Data preparation

SERA data was obtained in the form of Microsoft Excel files which were merged to create a master file using R software (R Core Team).

Fatigue scores were in raw format and were transposed into T-score metrics as previously validated (Bingham III et al., 2019). T-scores are generated to create the equivalent of a percentile score relative to the US population using data from the United States Food and Drug Administration (FDA). A T-score of 50 in PROMIS tools generally indicate the median in the US population – to which these rating scales are calibrated. This conversion system is shown in table 2.10 and is accessible via the FDA - https://www.fda.gov/media/137977/download – accessed 13/04/2022 (URL accessed 13/04/2022).

Table 2.10 PROMIS Fatigue 8a - Adult v1.0 Short Form Conversion Table

Table Raw Score	T -score	Standard Error on T - score
8	33.1	4.8
9	38.5	2.7
10	41.0	2.2
11	42.8	2.0
12	44.3	1.9
13	45.6	1.8
14	46.9	1.8
15	48.1	1.8
16	49.2	1.8
17	50.4	1.8
18	51.5	1.7
19	52.5	1.7
20	53.6	1.7
21	54.6	1.7
22	55.6	1.7
23	56.6	1.7
24	57.5	1.7
25	58.5	1.7
26	59.4	1.7
27	60.4	1.7
28	61.3	1.7
29	62.3	1.7
30	63.3	1.7
31	64.3	1.7
32	65.3	1.7
33	66.4	1.7
34	67.5	1.7
35	68.6	1.7
36	69.8	1.8
37	71.0	1.8
38	72.4	2.0
39	74.2	2.4
40	77.8	3.7

2.2.2 Statistical methods

2.2.2.1 Group comparisons

Group comparisons were conducted using Graphpad Prism version 9.3.1 (GraphPad).

The threshold for significance was determined to be a p-value of <0.05. In 2-group comparisons normality testing was performed using D'Agostino & Pearson test, Shapiro-Wilk test, Anderson-Starling test, and Kolmogorov-Smirnov test. QQ plots were also generated and eyeballed for normality. Homoscedasticity was determined by Brown-Forsythe test and by eyeballing the error bars to examine equality of variance between groups. In a 2-group comparison with normally distributed data with equal variance the student's t test was used. Where the data was normally distributed but unequal variance the student's t-test with Welch's correction was used. Where data was not normally distributed Mann-Whitney U test was used.

In 3 or more group comparisons the same normality testing was undertaken. Where normality testing determined normal distribution then ANOVA with multiple comparisons was used. Abnormally distributed data was analysed using Kruskal-Wallis with multiple comparisons.

2.2.2.2 Simple linear regression

Univariable simple linear regression analyses were conducted using Graphpad Prism version 9.3.1 (GraphPad).

Best fit values, standard error, 95% confidence intervals, r^2 and significance of deviation of slope from zero were all calculated. A p-value of <0.05 was considered the threshold for significance.

2.2.2.3 Multiple linear regression

Multiple linear regression analyses were conducted using Graphpad Prism version 9.3.1 (GraphPad).

Adjusted R^2 was calculated to avoid artefactual increase of R^2 by chance occurring with increasing variables. This also to allowed comparison between regression models.

Post-regression diagnostics were completed to check for multi-collinearity with calculation of variable inflation factor (VIF) and R^2 with other variables. Normality of residuals was determined by creating QQ plots and checking that distribution was Gaussian and homoscedasticity plotted.

To avoid over-fitting the model, the number of variables was limited to at least 10 subjects per independent variable, in keeping with generally accepted standards of multivariable analysis.

Chapter 3

The brain inflammatory response in a mouse model of RA

3 The brain inflammatory response in a mouse model of RA

3.1 Introduction

As discussed in Chapter 1, RA is an inflammatory disorder that is associated with a major burden of sickness behaviour and enhanced risk of neuropsychiatric disease. The brain response to systemic inflammation is thought to play an important role in the genesis of mental disorder in RA, though the mechanisms involved remain to be characterised (Nerurkar et al., 2019). The experiments presented within this chapter were undertaken with the aim to explore the brain inflammatory response in the context of inflammatory arthritis.

Many mouse models of inflammation, such as the LPS model, use very specific inflammatory stimuli which can provide detailed information about specific inflammatory pathways. However, this may mean that the results are less translatable to a more complex, disease-faithful context. Therefore, it was decided to use a tissue-specific inflammatory model that would generate an arthritic inflammatory stimulus.

CIA is a mouse model of inflammatory arthritis which has been used extensively to investigate the pathogenesis of joint pathology in RA (Brand et al., 2007) (Asquith et al., 2009). It is known that CIA generates a general systemic inflammatory response in addition to the joint-focused inflammation (Teixeira et al., 2019), but little is known about the brain response.

I hypothesised that the systemic inflammatory response associated with CIA would provoke a neuroinflammatory response. From this initial hypothesis, a series of research questions sequentially arose. This chapter aims to address the following research questions:-

1. Are there measurable changes in the brain transcription of a range of inflammatory cytokines and chemokines in response to an arthritic inflammatory stimulus?
2. Is there evidence of leucocyte recruitment across the BBB?
3. Does any inflammatory transcriptional response associate with glial cell changes?

4. Is there evidence of inflammasome activation or neurotoxic processes such as altered iNOS expression?
5. Are there changes related to neuroplasticity, such as hippocampal neurogenesis?

The results from the experiments undertaken are presented hereafter and discussed in greater depth in section 3.5.

3.2 Results: The peripheral response to CIA

Prior to investigating the brain response in CIA, it was determined to first briefly characterise the disease response in the joints and the peripheral inflammatory response. The response in terms of arthritis and systemic markers of sickness and inflammation such as weight loss and splenomegaly were analysed. In addition, inflammatory transcription in PBLs and the plasma levels of cytokines and chemokines was investigated.

These markers were chosen for their relevance in determining the extent of the systemic inflammatory response in the CIA model, with a view to then exploring and contextualising the brain inflammatory response.

3.2.1 The arthritic response to the CIA-stimulus

As described in section 1.7.2, the CIA-stimulus involves priming the murine immune system to generate a response against type II collagen and thereby generate an arthritic response, affecting one or more joints, particularly around the fore- and hind-paws.

Typically arthritis develops in around 80% or more of inoculated mice (Brand et al., 2007). However, the timing and severity of arthritis can vary. The factors underlying the arthritic response and its variability are not fully understood, though can include environmental factors, colony infection and technical/protocol issues (Williams, 2004).

A total of 42 mice were exposed to the CIA experimental stimulus over 3 separate animal models (not including controls – injected with sterile saline). Of

this 42, 30 mice developed arthritis of varying severity, suggesting an overall incidence in these models of 71.4% (model 1= 7/8 (87.5%), model 2= 9/16 (56.25%), model 3= 8/10 (80%), model 4= 6/8 (75%).

In the mice that did develop arthritis, there was a significant degree of variability in mouse response. This was manifest in terms of severity, paws affected, timing of development and progression of arthritis.

The range of scores is shown in figure 3.1. The number of paws affected was also varied and is shown in figure 3.2. The number of days that each mouse had detectable arthritis is shown in figure 3.3. The number of days was influenced by the timing of arthritis onset in addition to the severity and progression of arthritis. For example, some mice had to be culled early (prior to day 42) due to rapidly breaching the severity threshold (articular severity score of >10), whilst others developed only mild arthritis and were not culled until the predetermined endpoint at day 42. No mice developed arthritis which subsequent remitted.

Whilst presenting challenges in interpretation, one advantage of this variability was that it was possible to subject the data to regression analysis to determine the impact of severity and chronicity of arthritis.

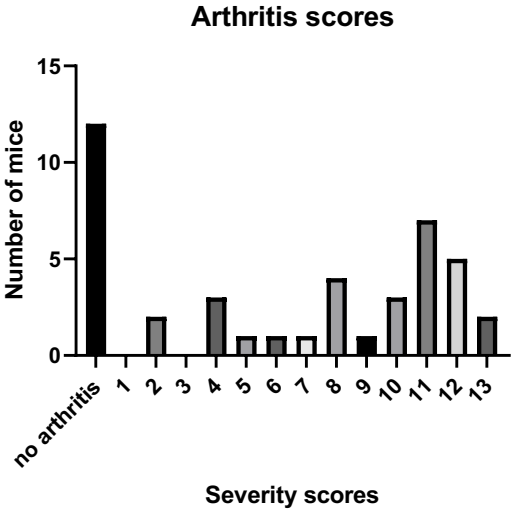

Figure 3.1 Variation in arthritis scores across all models
Graph shows the number of mice administered the CIA-stimulus demonstrating different arthritis scores All mice shown were administered the standard CIA protocol. Scores indicate disease severity at time of culling. All mice were culled at day 42 or earlier if they breached severity threshold.

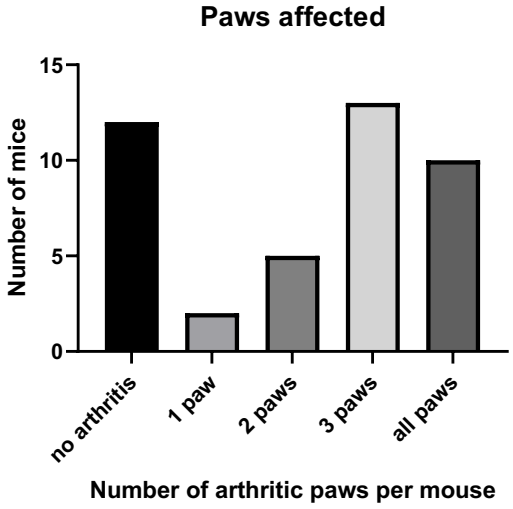

Figure 3.2 Number of paws affected per mouse across all models
All mice shown were administered the standard CIA protocol. Arthritis was monitored every 2-3 days. Graph shows number of paws affected by arthritis at time of culling.

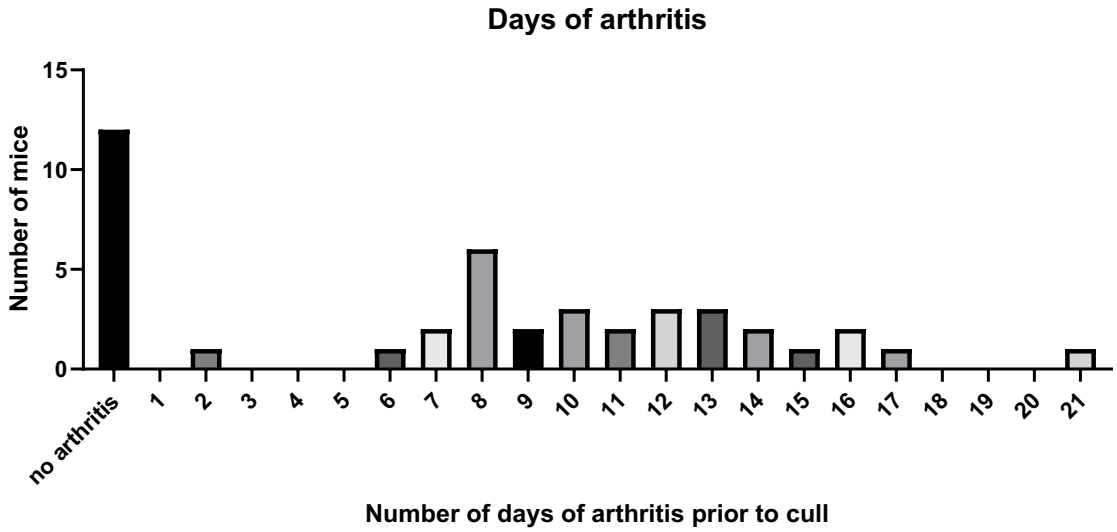

Figure 3.3 Number of days of arthritis prior to culling across all
All mice shown were administered the standard CIA protocol. Arthritis was monitored every 2-3 days. Graph shows the range of days of arthritis at time of culling which was either day 42 or earlier if severity threshold breached. 12 mice did not develop any arthritic response.

3.2.2 Body weight in response to the CIA-stimulus

Mice were weighed every 1-3 days throughout each animal model that was conducted. It was observed that CIA was associated with statistically significant weight-loss following development of clinically detectable arthritis (figure 3.4). Prior to development of arthritis there was no significant weight loss detectable. There was no significant weight loss in mice that were administered the CIA protocol but did not develop arthritis.

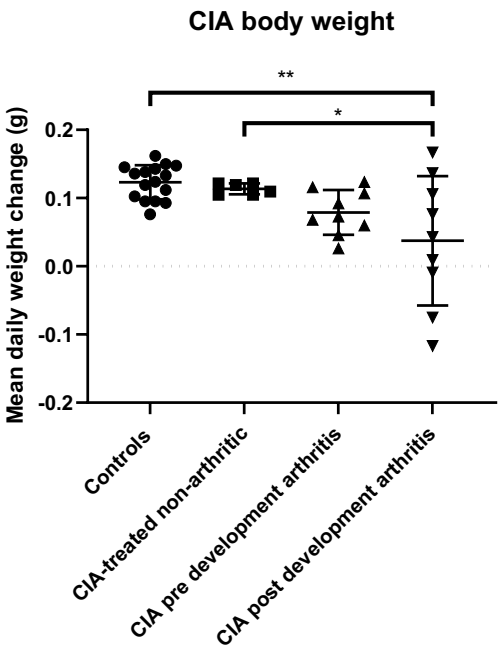

Figure 3.4 Mean daily weight change in CIA Graph shows the mean daily weight change over the course of the model. Data taken from the largest single model conducted. Control mice were administered sham injections only and were all culled at day 42. CIA-protocol mice were administered standard CIA protocol and were culled either at day 42 or earlier if severity threshold breached. Mice were weighed and assessed for arthritis at regular intervals throughout the model. The graph shows mean daily weight change for controls, mice who did not ever develop arthritis in addition arthritic mice pre- and post-development of arthritis. The mean daily weight change for mice that developed arthritis is graphed to show change prior to development of arthritis and post-development of arthritis. Statistical significance was determined using ANOVA with multiple comparisons after normality testing with QQ plot and Shapiro-Wilk test.

3.2.3 Spleen weight in response to the CIA-stimulus

The spleen is a secondary lymphoid organ with functions that include erythrocyte homeostasis, pathogen/antigen filtration and regulation of systemic immune responses (Bronte and Pittet, 2013). Systemic inflammation is known to represent a causal factor in mammalian splenomegaly (Loukov et al., 2016).

The spleens of control and CIA mice were dissected and weighed, and spleen weight normalised to body weight was calculated. There was a clear difference in spleen weight relative to body weight between control mice and CIA arthritic mice, and interestingly also with CIA non-arthritic mice (figure 3.5). There was also a significant difference between CIA-treated arthritic and non-arthritic mice.

These results suggest that the CIA stimulus of CFA+type II collagen causes splenomegaly, most likely due to immune stimulation/ inflammation.

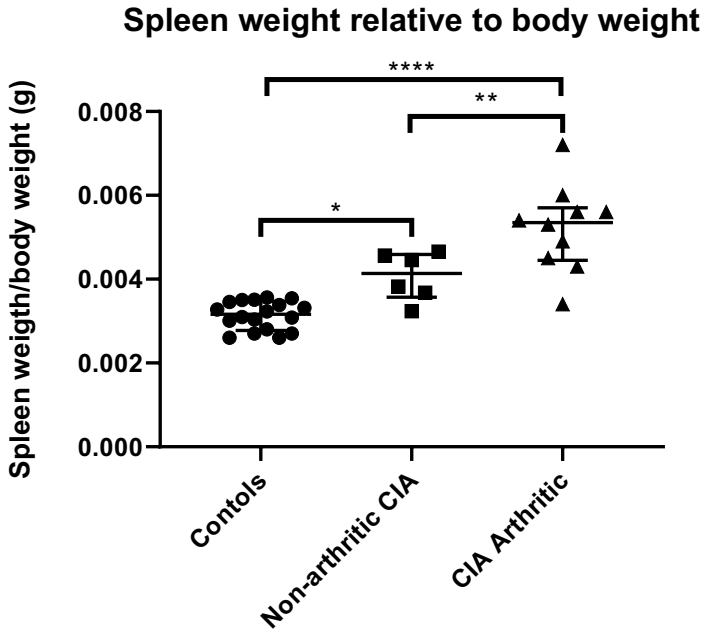

Figure 3.5 Spleen weight relative to body weight in control, CIA protocol non-arthritic and CIA arthritic mice
Spleens were dissected at the time of culling and weighed. Graphs show spleen weight divided by body weight. Following normality testing, statistical significance was determined using one-way ANOVA.

3.2.4 Plasma cytokine levels

Plasma was tested for protein levels of IL6, which is a key cytokine involved in the pathogenesis of arthritis in CIA (Alonzi et al., 1998). A clear increase in IL6 was detected in arthritic mice compared to saline injected controls was identified (figure 3.6). IL6 was elevated in all arthritic mice tested and undetectable in the CIA-stimulus administered non-arthritic mouse and in all saline-injected controls.

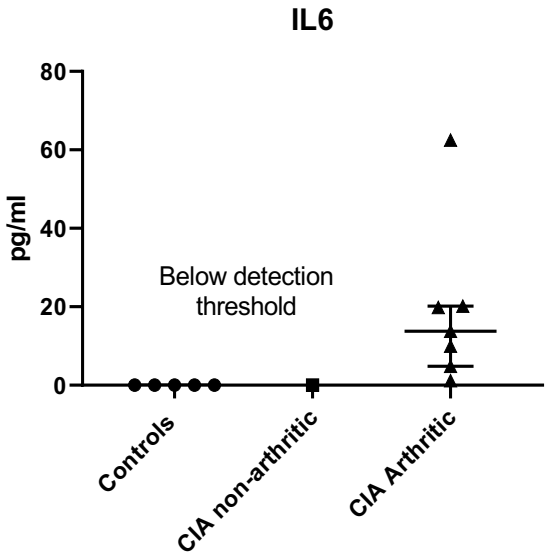

Figure 3.6 Plasma IL6 concentration in CIA
A total of 5 control mice and 8 CIA-protocol mice. Seven mice developed arthritis with disease scores ranging from 2 to 12. One mouse did not develop arthritis. Mice were culled at day 42 or when severity threshold breached. Blood was extracted via IVC and processed to obtain platelet-free plasma. Cytokine levels were measured using pre-coated ELISA kit. Quantification was determined by generating standard curve.

3.2.5 Plasma chemokine and cytokine array

A Multiplex assay (Merck, MCYTMAG-70K-PX32) was used to measure levels of multiple chemokines and cytokines in mouse plasma. The array was selected to measure a range of chemokines and cytokines, including ones which had been found to elevated in the literature or in previous work within the research group. This experiment examined differences between saline injected control mice, CIA-treated non-arthritic mice and CIA arthritic mice.

In line with the results from the ELISA, the array confirmed elevated IL6 in the arthritic mice. In all other cytokines and chemokines measured, either no significant differences in levels were found or levels in all samples were below the detection threshold (figure 3.7).

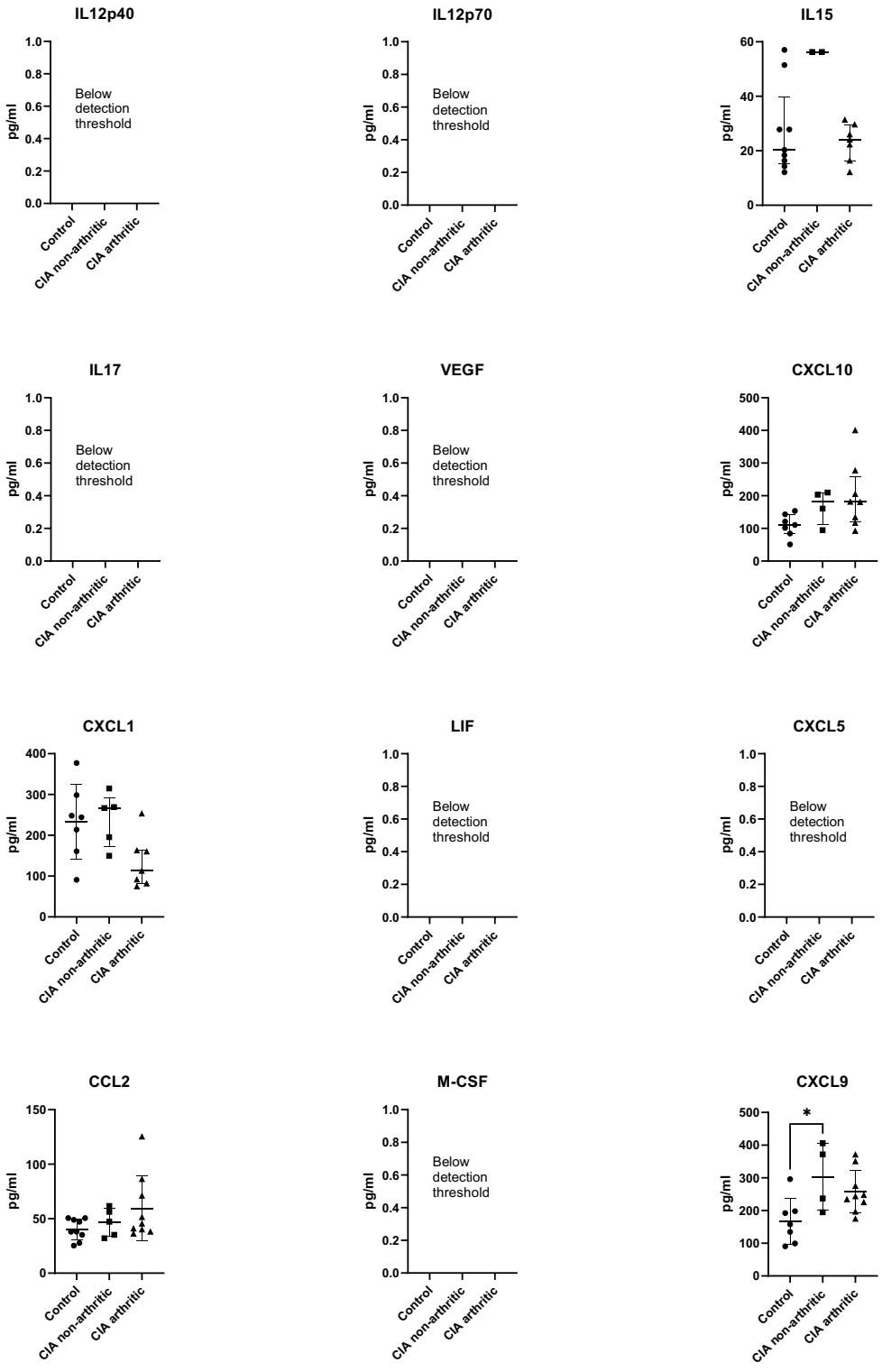

Figure 3.7 Plasma Multiplex assay of 31 inflammatory cytokines and chemokines
A total of 9 control mice and 15 CIA-protocol mice were used. Of the CIA-protocol mice a total of 9 developed arthritis with disease scores ranging from 5 to 12. Mice were culled at day 42 or when severity threshold breached. Blood was extracted via IVC and processed to obtain platelet-free plasma. Cytokine and chemokine levels were measured using pre-coated Multiplex kit. Quantification was determined by generating standard curve. Significance bar is only shown where p value <0.05

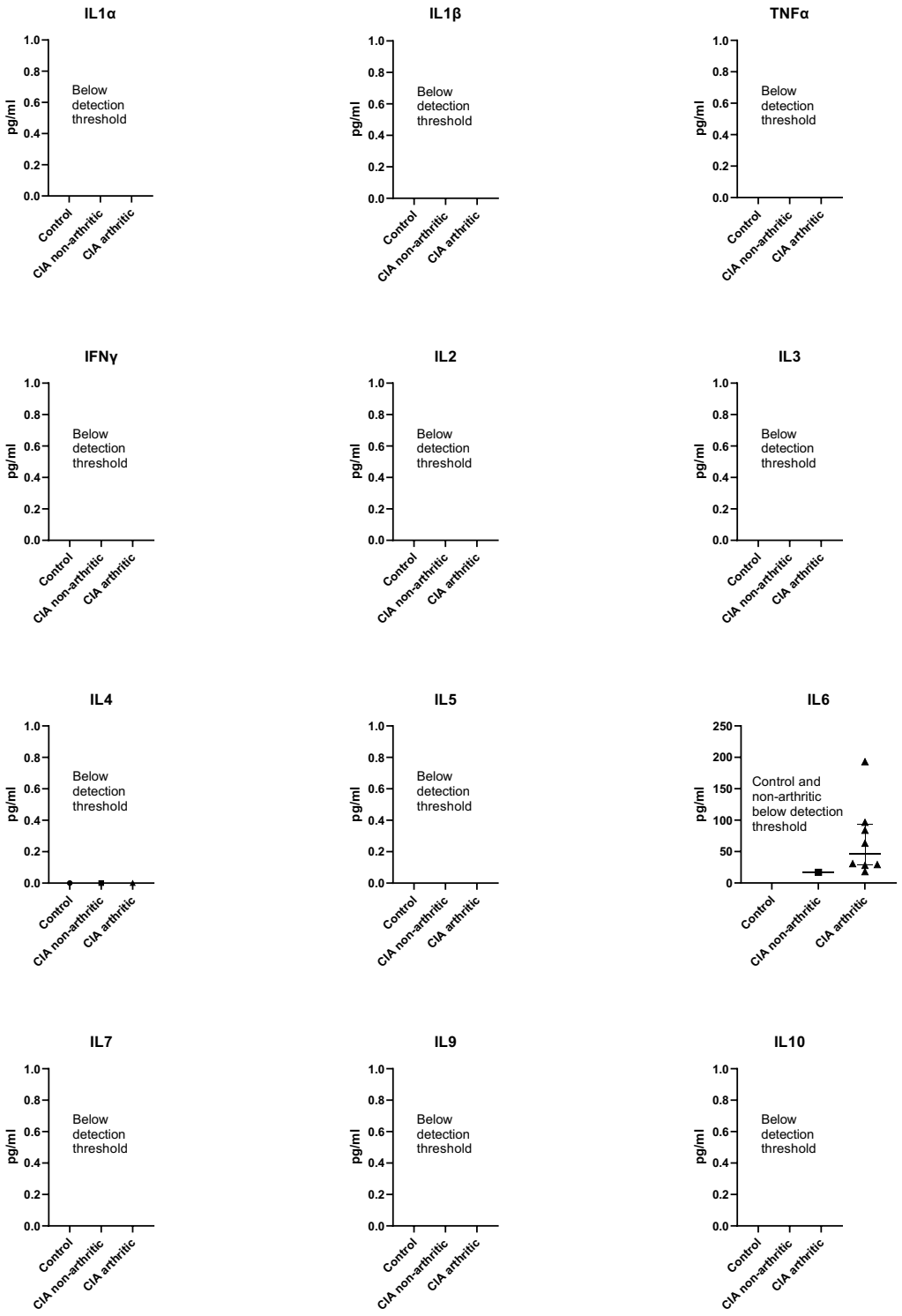

Figure 3.7 Plasma Multiplex assay of 31 inflammatory cytokines and chemokines
A total of 9 control mice and 15 CIA-protocol mice were used. Of the CIA-protocol mice a total of 9 developed arthritis with disease scores ranging from 5 to 12. Mice were culled at day 42 or when severity threshold breached. Blood was extracted via IVC and processed to obtain platelet-free plasma. Cytokine and chemokine levels were measured using pre-coated Multiplex kit. Quantification was determined by generating standard curve. Significance bar is only shown where p value <0.05

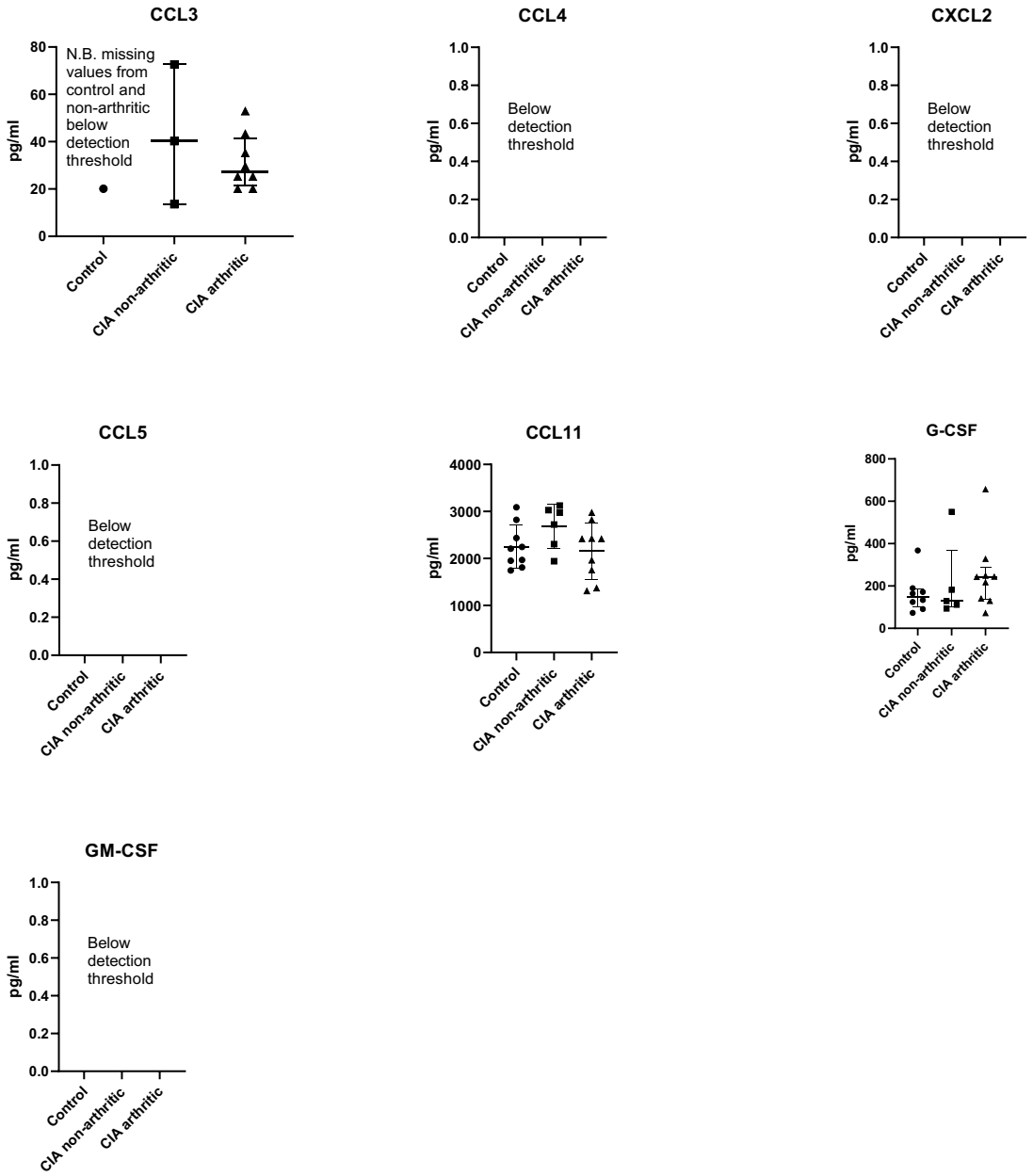

Figure 3.7 Plasma Multiplex assay of 31 inflammatory cytokines and chemokines
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3.2.6 PBL inflammatory transcriptional response in CIA

Various leukocytes including monocytes and T cells have an important role in the production of inflammatory cytokines and chemokines in the context of systemic inflammation in RA (Rana et al., 2018) (Roberts et al., 2015). The response of

peripheral blood leucocytes (PBLs) to CIA in my models was investigated as a measure of systemic inflammatory response. This was done by using qRT-PCR to measure the transcriptional change of a range of inflammatory cytokines and chemokines in RNA extracted from PBLs. The selection of cytokines and chemokines for testing was informed by previous work exploring the PBL transcriptional response to CIA (Sutthiwarotamakun, 2011) and to other peripheral inflammatory stimuli carried out within the research group (Nerurkar et al., 2017) (Thomson et al., 2014). Control mice injected with saline were compared with arthritic CIA mice (non-arthritic were not available for this experiment). The results are shown in figure 3.8.

The transcriptional response was surprisingly muted with only Cx3cl1 being upregulated in arthritic vs. control mice. In peripheral blood CX3CL1 is expressed by activated leukocytes (Ferretti et al., 2014) and augments T cell activity, facilitates monocyte recruitment and promotes inflammation (Thelen and Uguccioni, 2016).

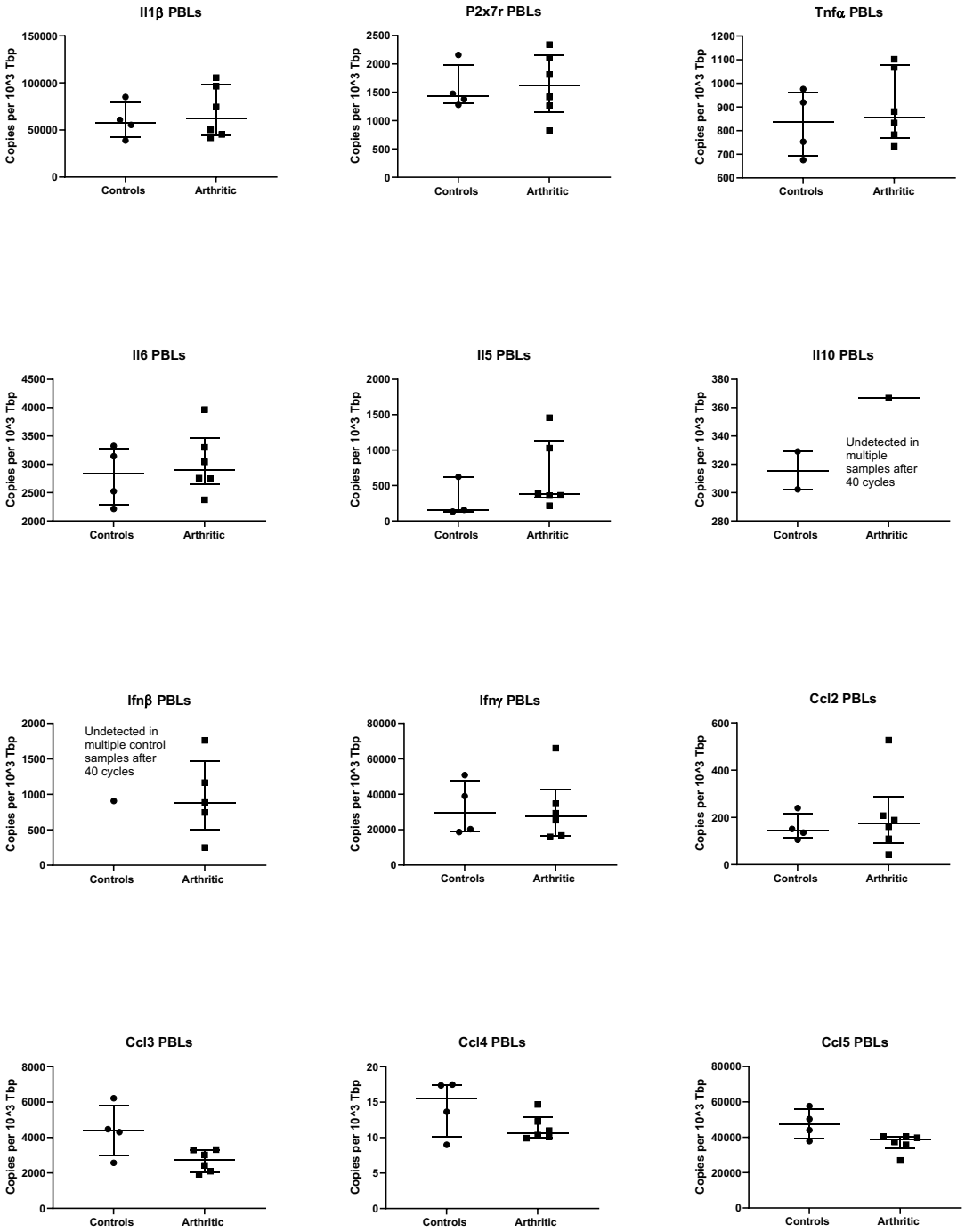
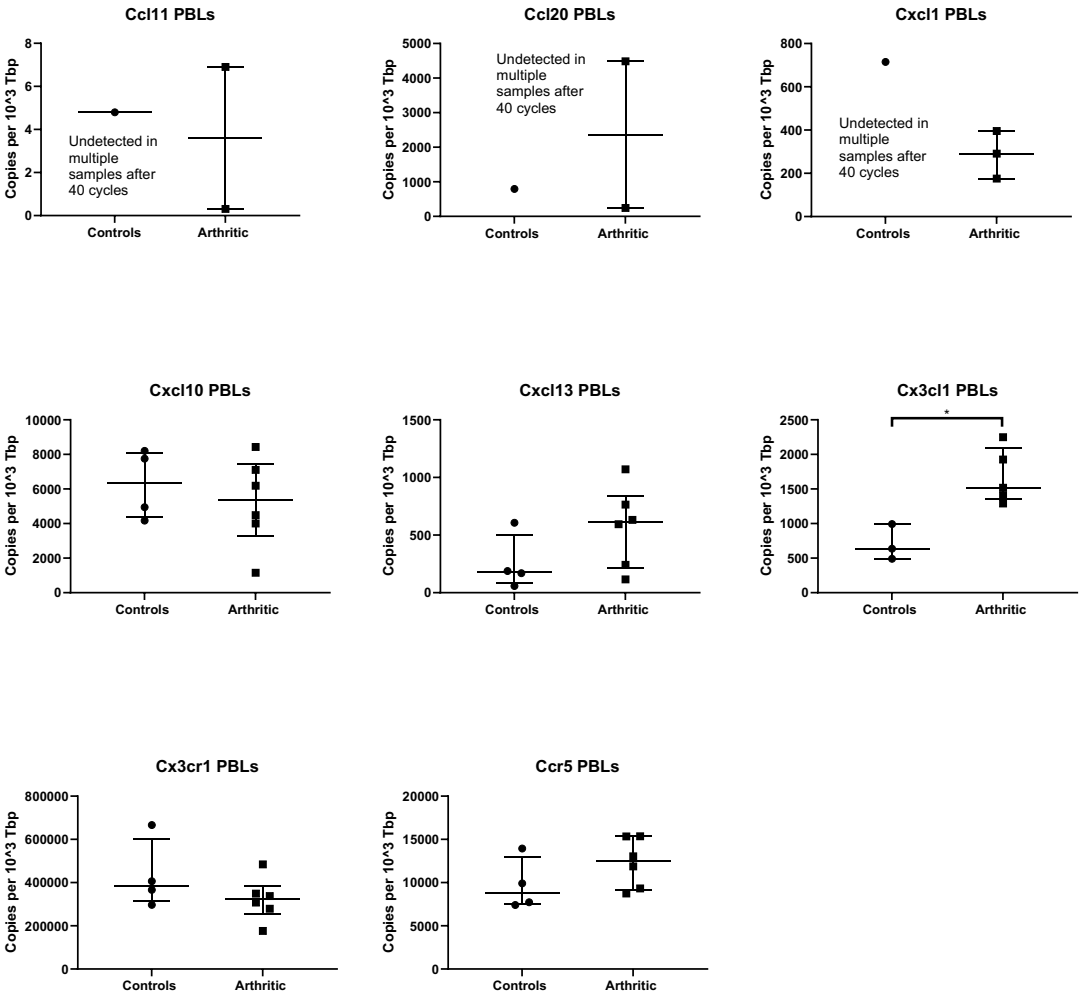


Figure 3.8 qRT-PCR analysis of PBL inflammatory transcriptional response in CIA
Arthritic mice were culled either at day 42 or when severity threshold breached, whichever occurred first. The arthritic mice all had arthritis scores ranging from 7 to 13 when culled with 3 or 4 paws affected. Clinically detectable arthritis had been present for between 10 and 14 days. Blood was extracted via the inferior vena cava. PBLs were separated from plasma and RNA was extracted and converted to cDNA for qPCR analysis. It was not possible to obtain blood from non-arthritic CIA stimulated mice. A range of inflammatory cytokines and chemokines were analysed. Statistical significance was determined using Mann-Whitney U test.



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3.2.7 Peripheral response: summary of key findings

The key findings are summarised here and discussed at greater length in discussion section at the end of this chapter. The key findings include:-

1. Arthritis in CIA is highly variable

The CIA models generated for these experiments showed a substantial degree of variation in terms of arthritic response between mice administered the CIA stimulus. Some mice did not develop any arthritis, whilst those that did develop

arthritis showed variation in timing of onset and severity. This variability is a well documented feature of CIA (Holmdahl et al., 2002).

2. CIA is associated with weight loss and splenomegaly

CIA is shown here to have systemic effects on body weight and also spleen weight, both of which can be indicators of systemic inflammation (Loukov et al., 2016). Body weight can be influenced by a multiplicity of factors, for example reduced feeding behaviours due to painful paws.

3. CIA is associated with elevated plasma IL6

IL6 was consistently elevated in arthritic mice vs. non-arthritic and control mice. IL6 is a key cytokine involved in the pathogenesis of arthritis in CIA (Alonzi et al., 1998). In human RA, IL6 is thought to influence development of arthritis by a variety of mechanisms, including activation of endothelial cell production of chemokines and recruitment of leukocytes to sites of inflammation (Romano et al., 1997), and by inducing synovial fibroblast proliferation (Mihara et al., 1995). No other differences in inflammatory cytokine or chemokines were detected in plasma.

4. The source of IL6 is unlikely to be from PBLs

No consistent inflammatory signature was seen in the PBL transcription of a range of inflammatory cytokines and chemokines, aside from elevated Cx3cl1 in arthritic mice vs. controls. Whilst no difference in Il6 mRNA levels was seen, it is likely that the source of IL6 is different e.g. other immunologically active cells/tissues, tissue macrophages, joint fibroblasts, lymphoid tissue etc.

3.3 Results: The brain response to CIA

Presented here are a series of experiments that were undertaken to evaluate the neuroinflammatory response in the context of CIA. As demonstrated in the preceding section, CIA is associated with a systemic inflammatory response characterised by weight loss, splenomegaly, elevated PBL transcription of Cx3cl1 and raised plasma IL6. In this context it was hypothesised that this systemic

inflammatory arthritic state would be associated with a neuroinflammatory response. A number of specific research questions arose from this hypothesis as detailed above in section 3.1.

3.3.1 The brain inflammatory transcriptional response in CIA

Altered transcription of cytokines and chemokines by cells and within organs is a key indicator of immune activation and inflammatory cell activity (Turner et al., 2014). Within the brain the source of these may be either resident cells, particularly glial cells, or recruited leucocytes (Becher et al., 2017).

The inflammatory transcriptional response of the brain in CIA was measured by testing a range of inflammatory cytokines and chemokines. The cytokines and chemokines were selected due to their attributes as inflammatory mediators (Cartier et al., 2005), and from previous work within the research group (Nerurkar et al., 2017) (Thomson et al., 2014) (Sutthiwarotamakun, 2011) and from published literature, showing their involvement in brain inflammatory responses. In addition, work by other groups using CIA and other RA models looking at brain response were reviewed for brain transcriptional responses to inform selection of candidate cytokines and chemokines of interest (Süß et al., 2020a) (del Rey et al., 2008) (Matsushita et al., 2021) – see section 1.7.1 for a brief review.

The brain inflammatory transcriptional response was measured using qRT-PCR with absolute quantification. The samples tested included saline-injected control mice, CIA-stimulus administered mice that did not develop arthritis and CIA arthritic mice. The arthritic mice showed a range of disease severity.

The results, presented in figure 3.9. show a clear relationship between arthritis and upregulation of brain Il1ß transcription. In relation to Il1ß, there was also a significant increase in P2x7r transcription, a key receptor involved in mediating the release of IL1ß (Di Virgilio et al., 2017). P2X7 receptor is expressed by almost all cells of the innate and adaptive immune system, including microglia and is involved in NLRP3 inflammasome activation (Di Virgilio et al., 2017).

The increased transcription of Il1ß was present only in the arthritic group, suggesting the response was a result of the ongoing arthritic process, rather than

the stimulatory events earlier in the model to which the non-arthritic CIA-protocol mice were also exposed.

There were no detectable differences in the other inflammatory cytokines tested. There was no clear association between arthritis and chemokine transcription aside from lower Ccl3 and Ccl4 transcription in the arthritic group vs. controls. The Ccl3 result was not replicated in the subsequent qRT-PCR analysis of hippocampus and rest of brain, presented in section 3.3.2, though higher Ccl4 transcription was observed in control hippocampus (figure 3.10). These findings are discussed in greater depth in section 3.5.3.

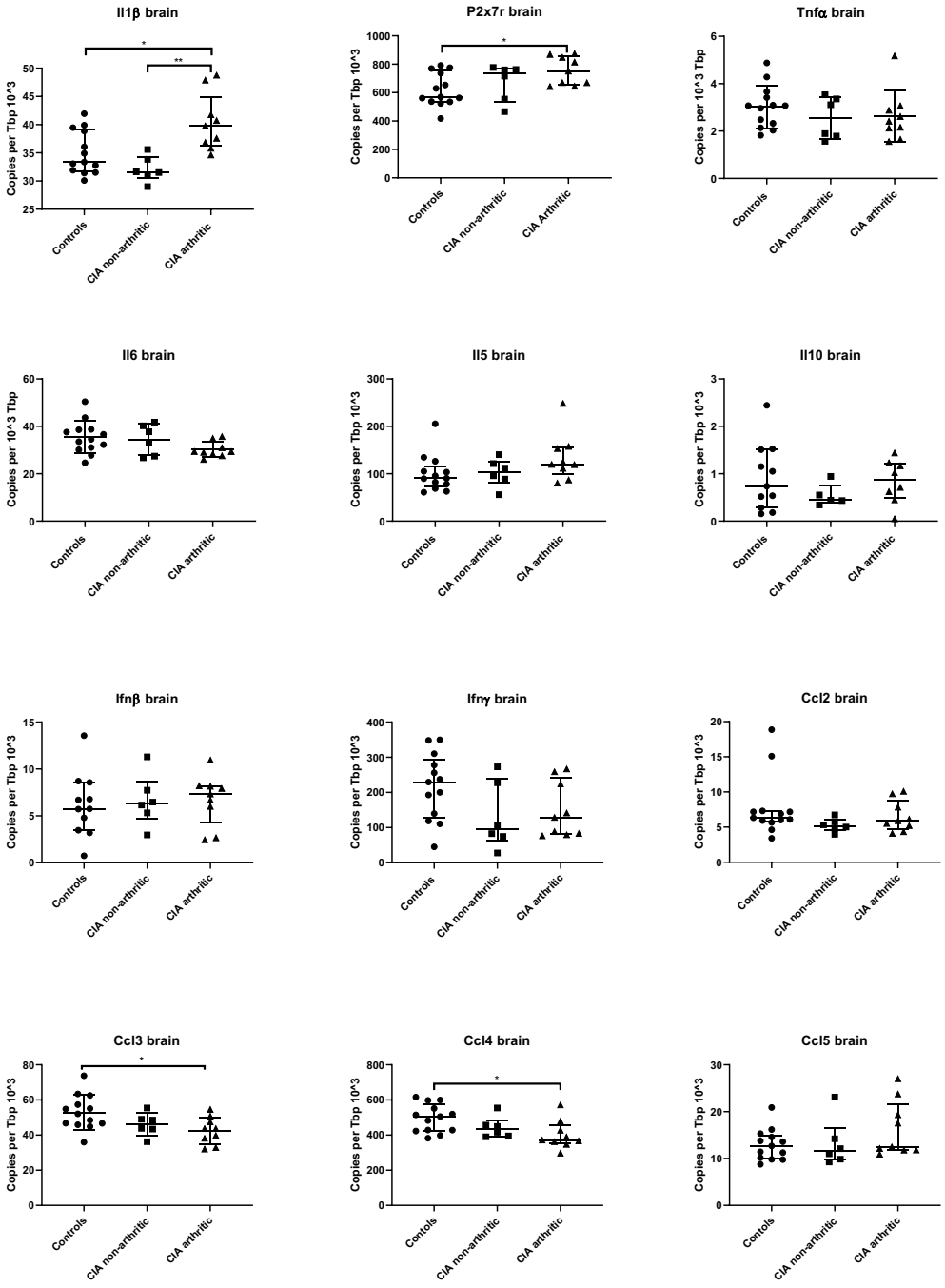
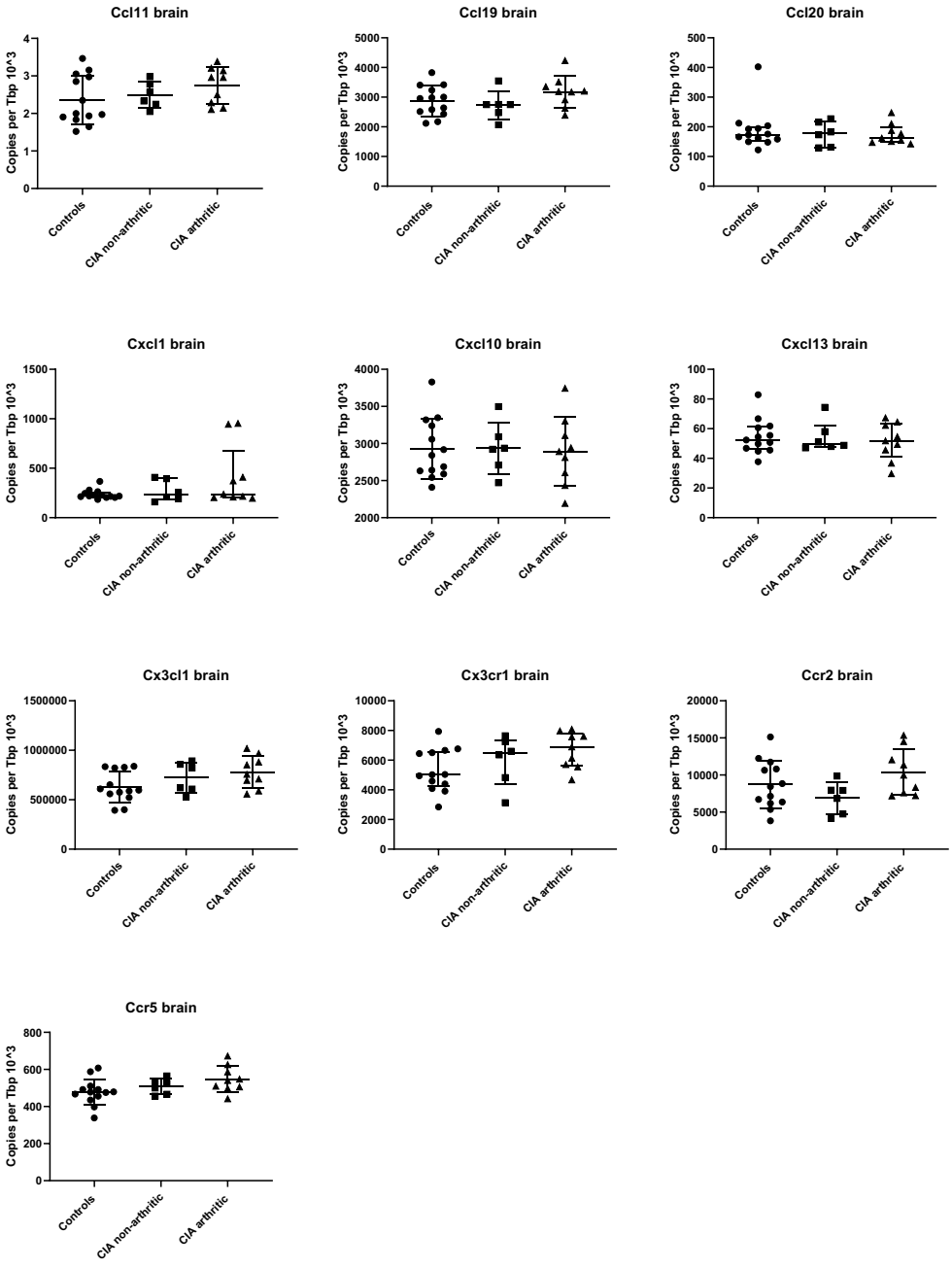


Figure 3.9 qRT-PCR analysis of brain inflammatory transcriptional response to CIA
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Arthritis severity scores ranged from 5 to 12. Following perfusion with ice cold PBS, brain was dissected and the right hemisphere retrieved. RNA was extracted and converted to cDNA for qRT-PCR analysis. A range of inflammatory cytokines and chemokines were tested in order to profile the brain inflammatory response. Statistical significance was determined using either ANOVA or Kruskal-Wallis depending on normality of data. Normality was determined using Shapiro-Wilk test and QQ plotting. Significance bar is only shown where p-value <0.05



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3.3.2 Inflammatory transcription in the hippocampus

The experiments to profile inflammatory cytokine and chemokine transcription were repeated and modified in order to determine if there were anatomical differences in the transcription patterns within hippocampus compared to the

rest of the brain. The experiment examined only arthritic CIA-stimulus mice and saline injected controls as the initial experiment showed no significant difference in brain inflammatory transcription between controls and non-arthritic CIA-stimulus administered mice. The same range of cytokines and chemokines were tested using qRT-PCR with absolute quantification.

The hippocampus was chosen as a region of interest as previously published evidence indicates altered hippocampal structure/function in response to inflammation (Chesnokova et al., 2016). Previous work in CIA has found elevated levels of Il1β mRNA along with other inflammatory changes, such as IBA1+ cell enrichment, within the hippocampus of arthritic mice (Andersson et al., 2018). The hippocampus is also known to be a key brain region involved in the pathogenesis of a range of neuropsychiatric disorders including mood disorders, psychosis and Alzheimer's dementia (Antoniades et al., 2018) (Chepenik et al., 2012) (Videbech and Ravnkilde, 2004) (Halliday, 2017).

The increased transcription of Il1β in the context of arthritis remained a consistent finding (figure 3.10). This was evident in both the hippocampus and in homogenate of the rest of the hemisphere. The increased P2x7r transcription was only evident in the rest of hemisphere excluding the hippocampus.

The higher rate of Ccl4 transcription in controls observed in the initial qRT-PCR experiment was reproduced in control hippocampus but not rest of hemisphere excluding hippocampus. The higher level of Ccl3 observed in the initial experiment (figure 3.9) was not reproduced. Transcription of Ccl11 appeared to be increased in the arthritic mouse brain hemisphere excluding hippocampus. Similarly, for Cxcl1. Il5 appeared to be higher in control hippocampus compared to arthritic. The significance of these results is considered later in the discussion section at the end of this chapter (section 3.5).

The results from these qRT-PCR experiments suggested further avenues for investigating the brain response to CIA. The elevated transcription of Il1β in concert with elevated levels of P2x7r and of select chemokines, point to an inflammatory brain response that could involve inflammasome activation, glial cell activation and leukocyte recruitment across the BBB. These areas are investigated in the following sections.

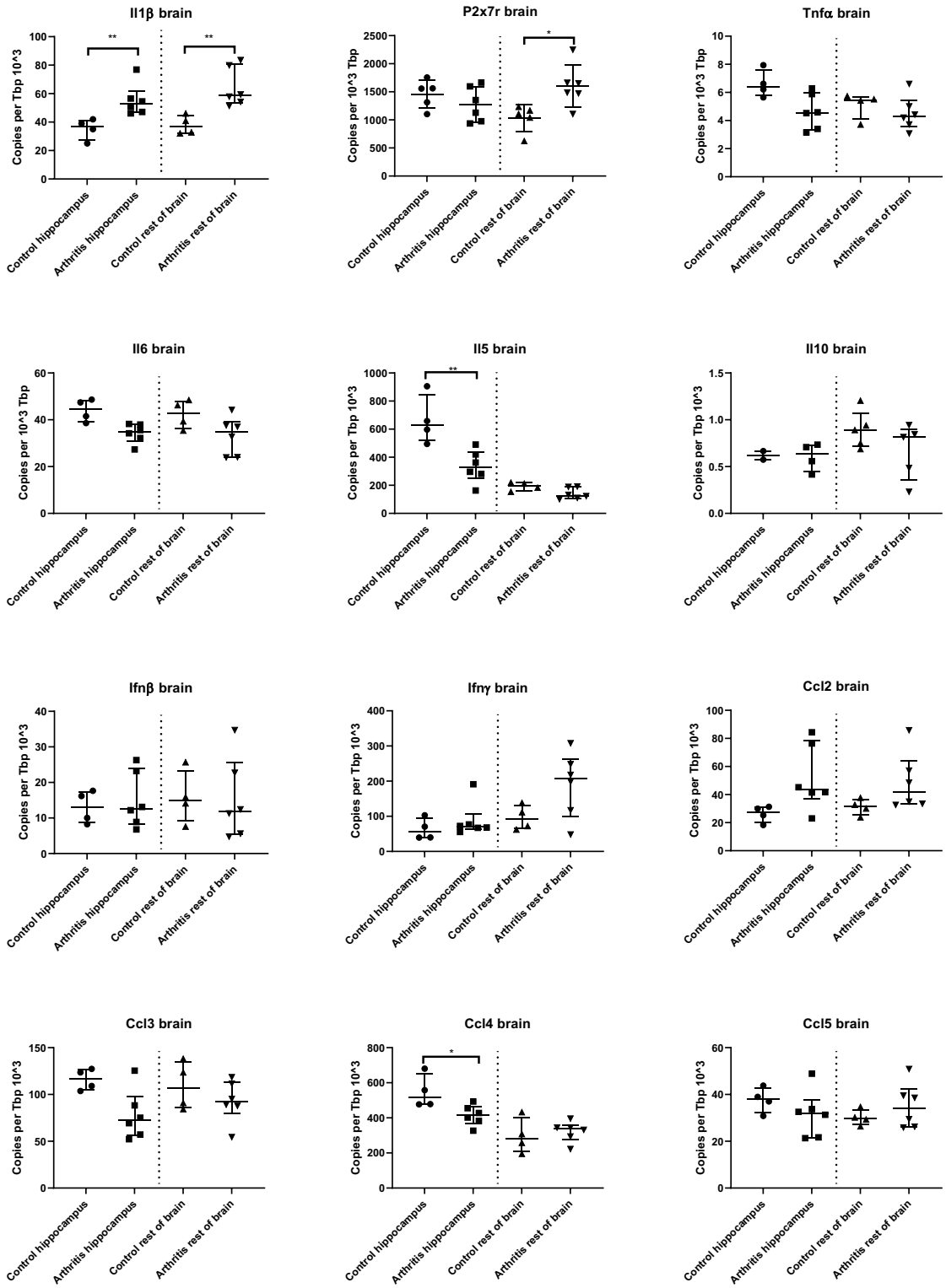
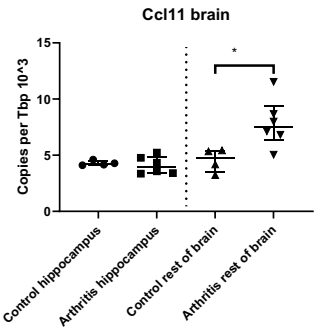
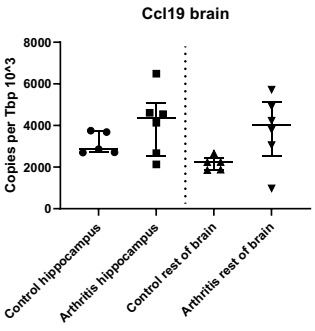
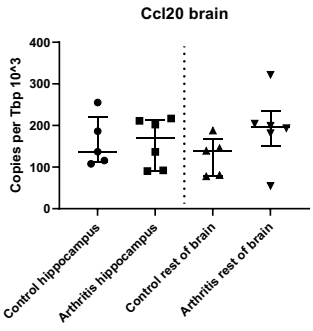


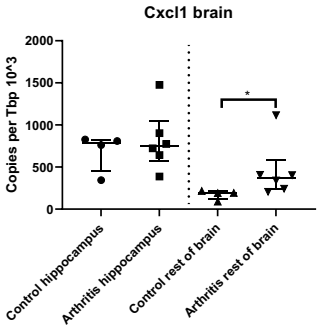
Figure 3.10 qRT-PCR analysis of hippocampus inflammatory transcriptional response to CIA

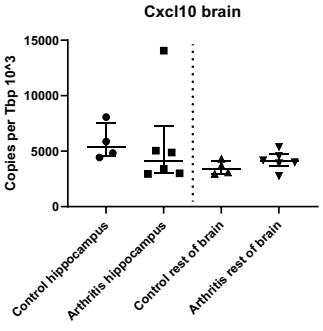
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular disease severity scores ranged from 2 to 9. Following perfusion with ice cold PBS, brain was dissected, and the right hemisphere retrieved. This hemisphere was then further dissected under magnification to separate hippocampus from the rest of the brain. RNA was subsequently extracted and converted to cDNA for qRT-PCR analysis. A range of inflammatory cytokines and chemokines were tested in order to profile the brain inflammatory response. Statistical significance was determined using Mann-Whitney U-test. Significance bar is only shown where p-value <0.05.

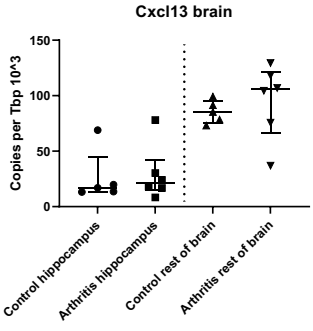

Ccl11 brain
Copies per Tbp 10^3
15
10
5
0
*
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain


Ccl19 brain
Copies per Tbp 10^3
8000
6000
4000
2000
0
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain


Ccl20 brain
Copies per Tbp 10^3
400
300
200
100
0
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain


Cxcl1 brain
Copies per Tbp 10^3
2000
1500
1000
500
0
*
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain


Cxcl10 brain
Copies per Tbp 10^3
15000
10000
5000
0
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain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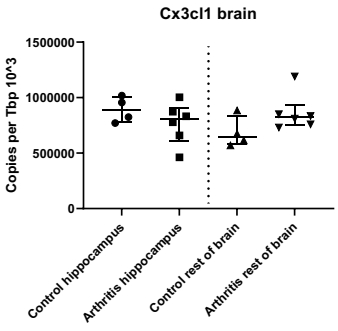
100
50
0
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain


Cx3cl1 brain
Copies per Tbp 10^3
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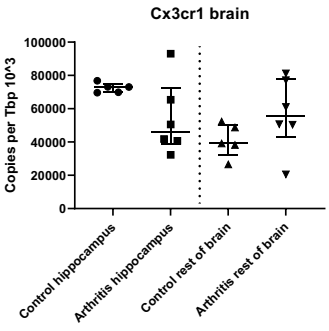
1000000
500000
0
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain


Cx3cr1 brain
Copies per Tbp 10^3
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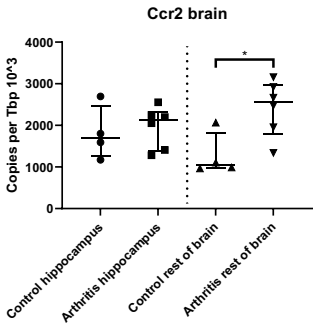
60000
40000
20000
0
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain


Ccr2 brain
Copies per Tbp 10^3
4000
3000
2000
1000
0
*
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain

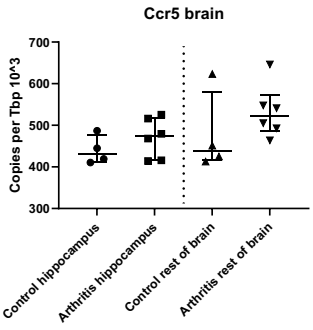

Ccr5 brain
Copies per Tbp 10^3
700
600
500
400
300
Control hippocampus
Arthritis hippocampus
Control rest of brain
Arthritis rest of brain

Figure 3.10 qRT-PCR analysis of hippocampus inflammatory transcriptional response to CIA
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular disease severity scores ranged from 2 to 9. Following perfusion with ice cold PBS, brain was dissected, and the right hemisphere retrieved. This hemisphere was then further dissected under magnification to separate hippocampus from the rest of the brain. RNA was subsequently extracted and converted to cDNA for qRT-PCR analysis. A range of inflammatory cytokines and chemokines were tested in order to profile the brain inflammatory response. Statistical significance was determined using Mann-Whitney U-test. Significance bar is only shown where p-value <0.05.

3.3.3 Brain Il1ß transcription and peripheral inflammatory burden

The experiments examining inflammatory transcription in the brain show that Il1ß transcription is consistently higher in CIA arthritic mice vs. controls and non-arthritic mice. This is evident in both the whole hemisphere and the hippocampus.

As discussed, CIA is a model in which there is substantial variation in timing and severity of arthritic response. Whilst presenting challenges, the variability can be leveraged to examine dose-effect type relationships between inflammatory phenomena in the brain and arthritis.

3.3.3.1 Brain Il1ß transcription and arthritis severity and chronicity

Regression analyses were performed, with Il1ß the dependent variable (figure 3.11). Regression analysis can inform both whether a relationship exists between variables and the extent to which this is the case. Linear regression analysis found that there is a significant relationship between arthritis severity score and brain Il1ß transcription with an r^2 of 0.5536 (p=*0.0215). Linear regression analysis did not show a significant relationship between brain Il1ß transcription and number of days of arthritis.

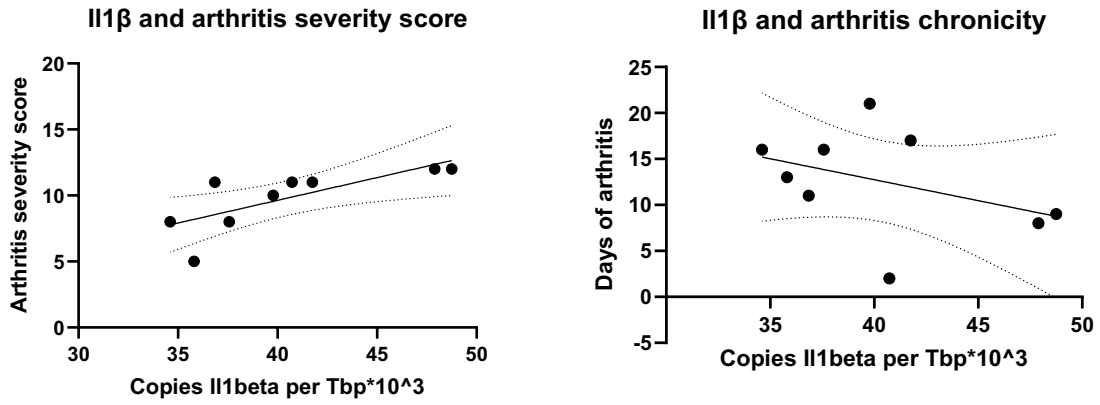


Figure 3.11 Linear regression analysis of brain Il1ß transcription and arthritis severity and arthritis chronicity
Mice underwent monitoring every 1-2 days to determine arthritis emergence and severity. Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Arthritis severity scores ranged from 5 to 12. Arthritis was present for between 2 and 21 days. Following perfusion with ice cold PBS, brain was dissected, and the right hemisphere retrieved. RNA was extracted and converted to cDNA for qRT-PCR analysis to determine Il1ß mRNA levels. Linear regression analysis demonstrates a significant relationship between brain Il1ß transcription and severity of arthritis (Y = 1.608*X + 24.69, r2= 0.5536, F= 8.682, p=*0.0215). The regression analysis for chronicity was not significant (p=0.2846).

To further explore the relationship and determine whether the chronicity of arthritis has a moderating effect on the relationship between arthritis severity and brain Il1ß transcription, a multivariable analysis was completed (figure 3.12). Multiple regression analysis is a predictive model which allows examination of the relationship between a continuous dependent variable (brain Il1ß transcription) and two or more independent/predictor variables (arthritis severity score and days of detectable arthritis). Multiple linear regression can determine the strength of the effect of the independent variables upon the dependent variable and can also predict the amount that the dependent variable will change in response to a change in the independent variables.

The ratio of number of dependent variable values per independent variables included was 3 and therefore the analysis was purely exploratory and must be interpreted as such. Although not well powered, multiple linear regression analysis did not indicate a statistically significant relationship between the variables of chronicity, severity, and brain Il1ß transcription.

Brain Il1β ~ Intercept + Arthritis severity score + arthritis chronicity

Intercept	27.41* (7.958)
Severity score	1.493 (0.6228)
Chronicity	-0.1263 (0.2538)
R^2	0.5713
Adjusted R^2	0.4284
No. observations	9

*Coefficients shown with standard error is shown in ( ) below. Statistical significance indicated by *(<0.05), **(<0.01), ***(<0.001) and ****(<0.0001).*

Figure 3.12 Multivariable analysis – Brain Il1ß and arthritis severity and arthritis chronicity
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Mice were monitored every 1-2 days for emergence of arthritis and measurement of articular severity score. Arthritis severity scores ranged from 5 to 12. Arthritis was present for between 2 and 21 days. Following perfusion with ice cold PBS, brain was dissected and the right hemisphere retrieved. RNA was extracted and converted to cDNA for qRT-PCR analysis to determine Il1ß mRNA levels. Multiple linear regression analysis was used to explore the relationship between brain Il1ß mRNA levels and articular severity score and plasma IL6 levels. The regression model was as follows:-

Il1ß ~ Intercept + arthritis severity score + arthritis chronicity

The overall regression was not significant (p=0.0788). Coefficients are shown, with statistical significance denoted with *, **, *** and ****. Standard error shown in ( ). R^2 and adjusted R^2 are also shown in the table along with number of observations. Arthritis articular severity score lost significance as a predictor variable when incorporated in a multivariable analysis with arthritis chronicity.

3.3.3.2 Brain Il1ß transcription and plasma IL6

A dose response relationship between articular severity score and Il1ß mRNA levels strengthens the suggestion of a causal relationship between disease activity and the inflammatory brain response. However, what factor of disease activity underlies this cannot easily be determined – for example, aside from joint inflammation, it could equally be pain, or some other factor, that associates with elevated brain Il1ß transcription. The clearest indicator of measurable peripheral systemic inflammation presented so far is the elevated levels of plasma IL6 protein. To further explore this question, linear regression

analysis was completed exploring whether IL6 could predict brain Il1ß (figure 3.13). The overall regression model did not reach statistical significance.

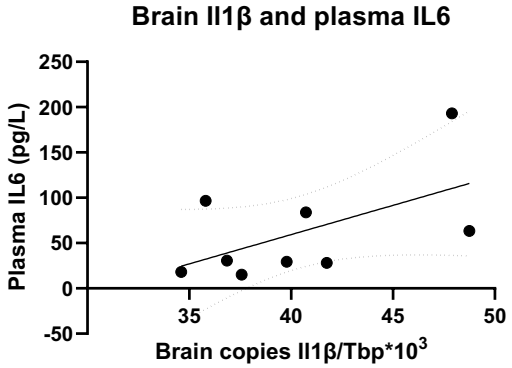


Figure 3.13 Linear regression analysis of brain Il1ß transcription and plasma
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Arthritis severity scores ranged from 5 to 12. Following perfusion with ice cold PBS, brain was dissected and the right hemisphere retrieved. RNA was extracted and converted to cDNA for qRT-PCR analysis to determine Il1ß mRNA levels. Blood was also retrieved at the time of culling and processed to extract platelet free plasma. IL6 levels were determined as reported earlier using multiplex assay as described in figure 3.6. Simple linear regression analysis of plasma IL6 as a predictor of brain Il1ß is not significant (p=0.1099).

3.3.3.3 Multivariable analysis

An exploratory analysis was undertaken to determine if a significant relationship emerged between plasma IL6, articular severity scores and brain Il1β transcription in a multivariable analysis. As before, the number of predictor variables per dependent variable is low and so results must be interpreted in this context.

Multiple linear regression analysis confirms the significant relationship between articular severity score and brain Il1β (p=*0.0242), but IL6 remains a non-sigificant predictor variable (figure 3.14).

Brain Il1β ~ Intercept + IL6 + Arthritis severity score

Intercept	24.30** (4.61)
Plasma IL6	0.03779 (0.01919)
Severity score	1.409* (0.4705)
R^2	0.7288
Adjusted R^2	0.6385
No. observations	9

*Coefficients shown with standard error is shown in ( ) below. Statistical significance indicated by *(<0.05), **(<0.01), ***(<0.001) and ****(<0.0001).*

3.14 Multivariable analysis – Brain Il1ß and arthritis severity and plasma IL6
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Mice were monitored every 1-2 days for emergence of arthritis and measurement of articular severity score. Arthritis severity scores ranged from 5 to 12. Arthritis was present for between 2 and 21 days. Following perfusion with ice cold PBS, brain was dissected and the right hemisphere retrieved. RNA was extracted and converted to cDNA for qPCR analysis to determine Il1ß mRNA levels. Blood was also retrieved at the time of culling and processed to extract platelet free plasma. IL6 levels were determined as reported earlier using multiplex assay. Multiple linear regression analysis was used to explore the relationship between brain Il1ß mRNA levels and articular severity score and plasma IL6 levels. The regression model was as follows:-

Il1ß ~ Intercept + IL6 + Arthritis severity score

The overall regression was significant (p=**0.0019). Coefficients are shown, with statistical significance denoted with *, **, *** and ****. Standard error shown in ( ). R^2 and adjusted R^2 are also shown in the table along with number of observations. Arthritis articular severity score was a significant predictor variable, whilst plasma IL6 did not emerge as a significant predictor variable, consistent with the simple linear regression analyses.

3.3.4 Leukocyte recruitment to the brain in CIA

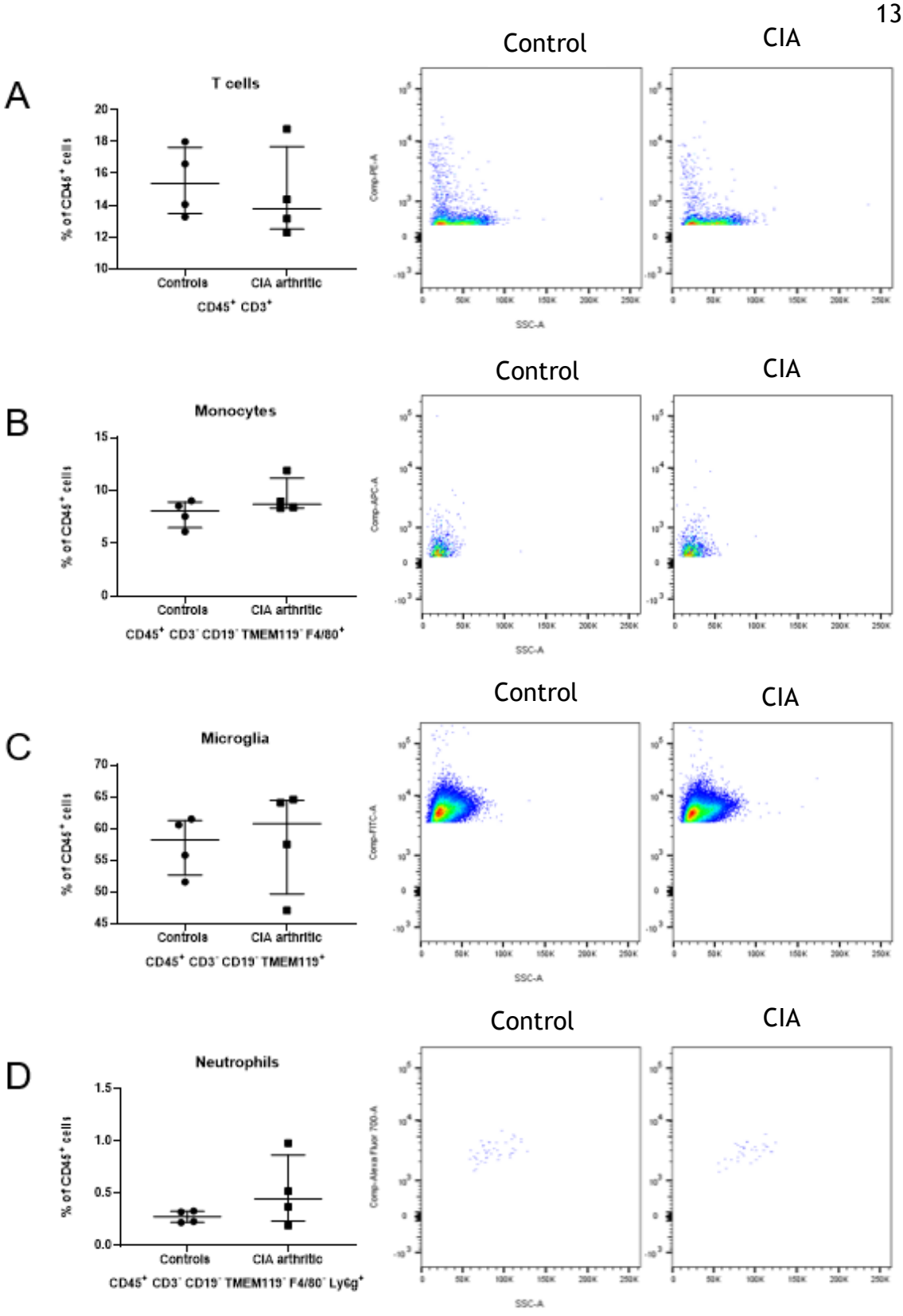
The results presented in this chapter so far show select differences observed in chemokine transcription in CIA mouse brains – significantly elevated brain transcription of Cxcl1, Ccl11 and Ccr2 in arthritic mice brains (homogenate of hemisphere when hippocampus removed).

To determine if leukocyte recruitment to the brain was a feature of CIA, a flow cytometry experiment was conducted on bloodless perfused brains from 4 control and 4 arthritic CIA mice with disease scores of 4, 8, 8 and 11. Arthritis had been detectable for 13, 15, 15 and 21 days. Flow markers included antibodies and fluorophores as shown in table 2.6 in methods chapter section 2.1.6. The following cell targets were identified in order to detect any differences in a broad range of immune cells:-

- CD45 – all immune cells
- CD3 – T cells
- TMEM119 – microglia
- F4/80 – monocytes
- Ly6g – neutrophils
- NK1.1 – NK cells
- CD19 – B cells

Gating strategy is shown in figure 2.1 in methods chapter, section 2.1.6.

The flow cytometry results indicated no difference in the proportion of microglia and of leukocytes stained for within the brain of control and CIA arthritic mice (figure 3.15). These results suggest that neither major leukocyte recruitment nor diffuse microglial proliferation are likely to be a feature of CIA.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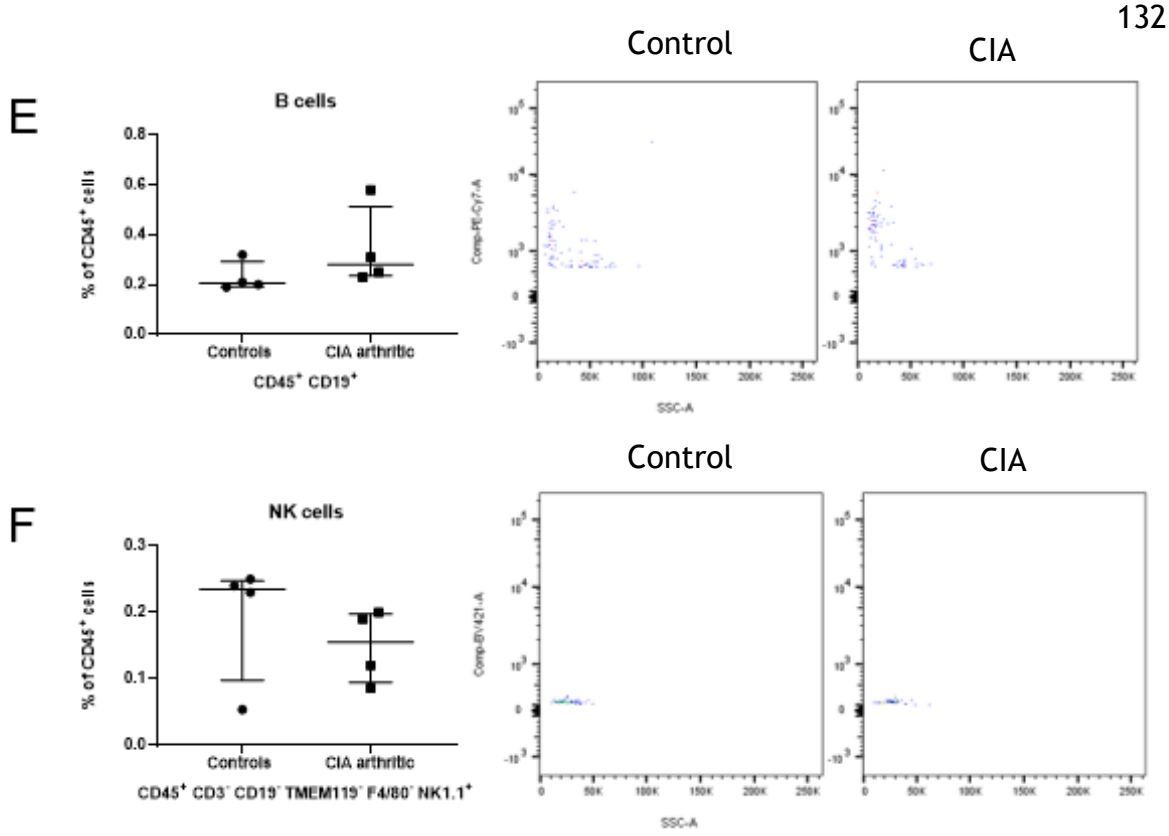


Figure 3.15 Flow cytometry analysis of brain leukocyte populations
Four arthritic mice and four control mice were culled and perfused with ice-cold PBS. The left hemisphere was then dissected and dropped into HBSS, processed into single cell suspension prior to undergoing myelin removal process and resuspension. Samples were plated and stained using fluorophore-tagged antibodies to mouse CD45, TMEM119, CD3, CD19, NK1.1, Ly6g and F4/80 (see table 2.1, methods section). A Live-Dead stain was also included. Samples were then run through a flow cytometer and fluorescence data extracted. Results are expressed as percentages of CD45+ cells in order to mitigate for technical variation in total cells retrieved from each sample. Using the gating strategy shown in figure x (methods section), it was possible to extrapolate proportions of T cells (A), monocytes (B), microglia (C), neutrophils (D), B cells (E), and NK-cells (F). Representative flow images are shown. Statistical significance was determined using Mann-Whitney test. There were no significant differences between control and arthritic mice in terms of T cells (p= 0.6857), monocytes (p=0.4857), microglia (p=0.6857), neutrophils (p=0.3429), B cells (p=0.2000) and NK cells (p=0.3429).

3.3.5 Glial cell response in CIA

The data presented so far regarding measurement of inflammatory cytokine and chemokine transcription indicates an association between CIA arthritis and brain transcription of Il1β and P2x7r. Within the CNS, P2x7r plays an important role in the activation and proliferation of microglia (Monif et al., 2009) promoting neuroinflammation with potentially neurotoxic consequences (Janks et al., 2018). Microglia form a key part of the innate immune system of the brain, acting as brain-resident macrophages, and are an important source of IL1β within the brain (Burm et al., 2015). Microglial IL1β secretion differs from

monocyte-derived macrophages in that its secretion tends to be more persistent and greatest in response to more prolonged stimuli (Burm et al., 2015). To further explore the potential sources of elevated brain Il1ß and P2x7r transcription, experiments were undertaken to examine glia response in the context of CIA. Whilst the flow experiment did not indicate a globally altered number of microglia in CIA, this did not rule out the possibility of anatomically localised differences in glial cell density, either through proliferation or intra-parenchymal migration. Such changes have been reported in a mouse model of arthritis previously (Süß et al., 2020a).

The original hypothesis was that peripheral inflammation would give rise to neuroinflammatory changes – including that there was neuroimmune communication between the peripheral environment and the brain across the BBB. The lack of major leukocyte recruitment across the BBB may suggest that this communication could be via the neural pathway and represent the transcriptional activity of resident cells, rather than originating from peripheral leucocytes that had migrated across the BBB.

To determine if CIA was associated with changes in regional microglial density, frozen brain sections were stained with anti-IBA1 antibodies. IBA1 is a marker for both microglia and peripheral myeloid cells (Ibanez et al., 2019).

To explore astrocyte response to CIA, IHC staining for glial fibrillary acidic protein (GFAP) positive cells was undertaken. GFAP is an intermediate filament exclusively expressed by protoplasmic and fibrous astrocytes and is an established marker used for IHC identification (Eng et al., 1971, Bignami et al., 1972). Astrocytes form the most abundant glial cell population within the brain with diverse functions including neuronal support, facilitating synaptic formation/plasticity and regulating innate and adaptive immune responses within the compromised brain (Colombo and Farina, 2016). Astrocytes are another potential source of Il1ß transcription (Didier et al.).

The experiments were designed to specifically explore different areas of brain with a view to drawing speculative inferences at a basic level regarding the potential functional meaning of any changes seen, and in an attempt to replicate similar experiments within the published literature that explored glia

response in the context of arthritis (Süß et al., 2020a). Images were obtained from four distinct brain regions – hippocampus, cortex, striatum and thalamus.

3.3.5.1 Hippocampal IBA1+ cell density

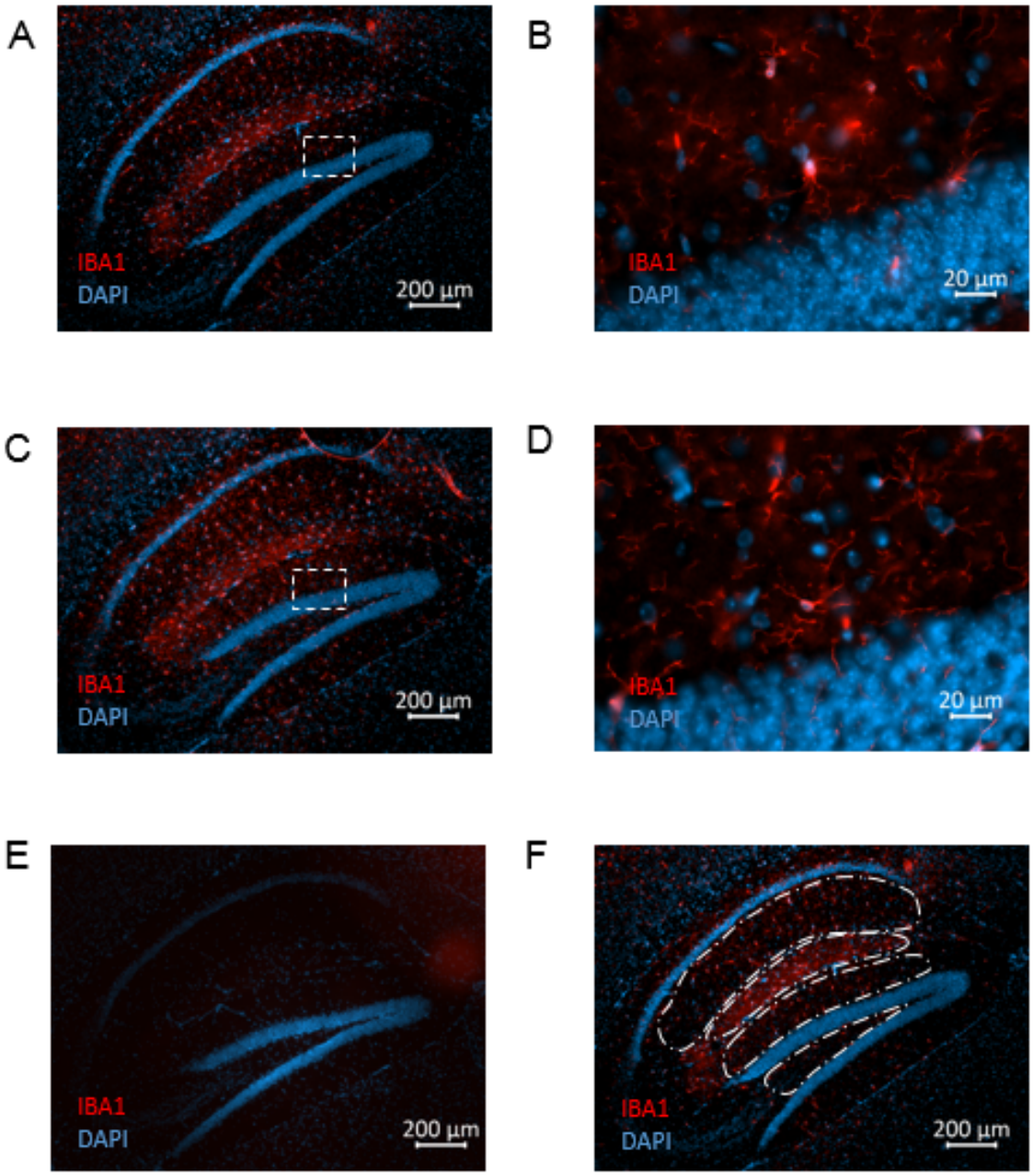
As previously discussed the hippocampus is a key brain region involved in neuropsychiatric disorders and affected by inflammatory stimuli (Chesnokova et al., 2016). The signalling receptor for IL1 exists in high density within the hippocampus (Cunningham et al., 1992), and therefore the hippocampus merits detailed examination. As noted in section 3.3.2, increased Il1β transcription was detected within the hippocampus.

Several anatomically distinct layers form the hippocampus. On sagittal sectioning, it is possible to reliably isolate the hippocampus regions on microscopy and perform cell counts within individual regions (see figure 3.16). These regions are:-

- Stratum radiatum
- Stratum lacunosum moleculare
- Stratum moleculare
- Dentate gyrus

Whilst the individual function of each region is unclear, they are believed to work in concert via integrated brain circuitry to perform key roles in cognition and memory (Maccaferri, 2011, Sloviter and Lømo, 2012).

Positively stained cells within the hippocampus were counted after strict blinding. Counts were completed and reported for each distinct hippocampal layer. These counts were also averaged across regions per mouse to report cell density within the hippocampus overall (figure 3.16). No significant differences were found between controls and arthritic mice in the hippocampus overall or within the 4 distinct hippocampal layers.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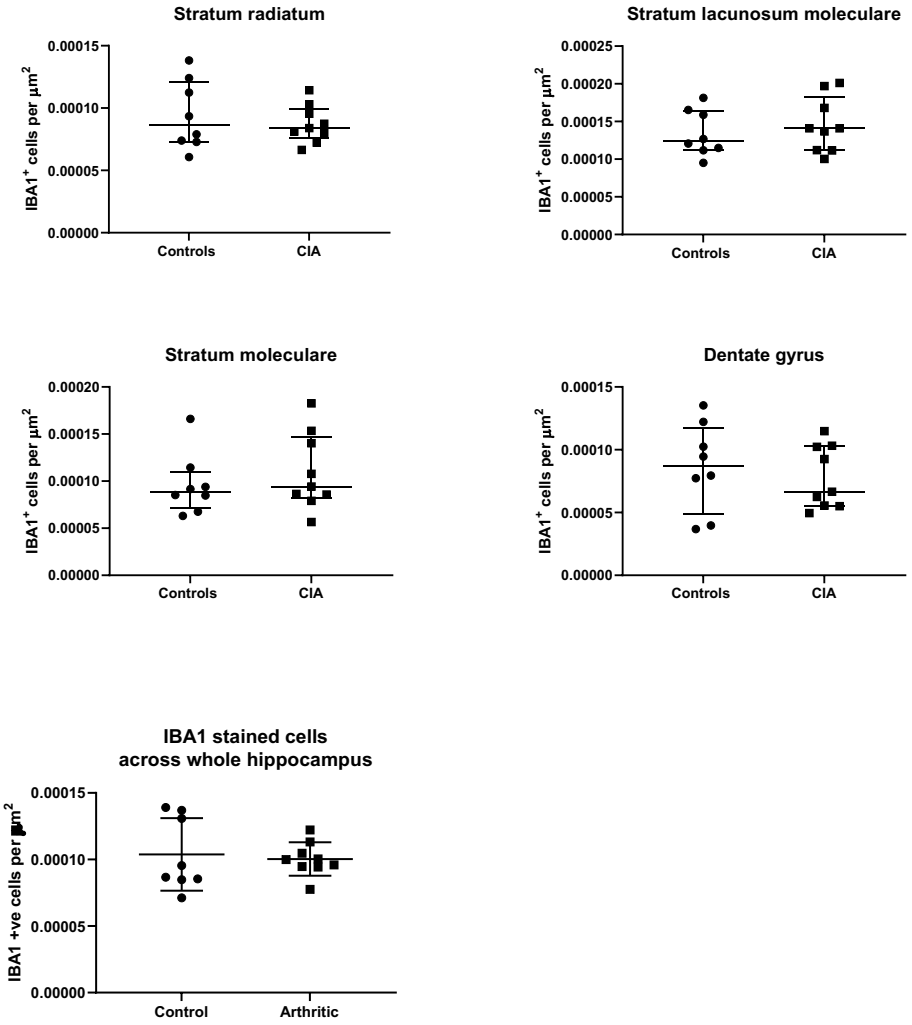
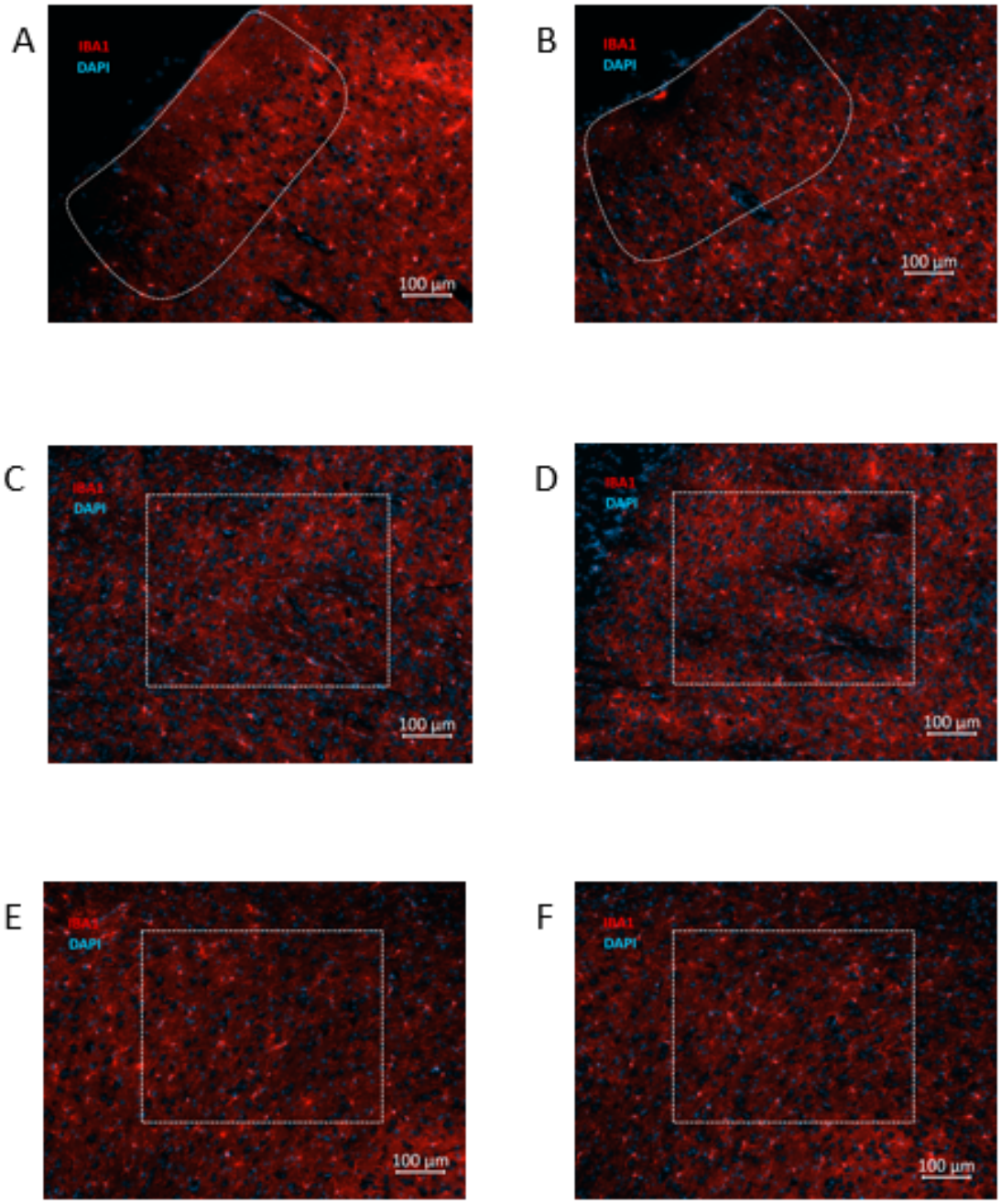


Figure 3.16 IBA1+ cell staining in the hippocampus
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Arthritis severity scores ranged from 5 to 12. Following perfusion with ice-cold PBS, brain left hemisphere was dropped into chilled 4% PFA for 24 hours prior to cryprotection by immersion in 30% sucrose for 24 hours before embedding in cutting media and freezing at -80^{o}C prior to cutting. Sagittal sections of frozen brains from a total of 8 control mice and 9 arthritic mice were cut at 10µm thickness. Six sections per mouse were stained with immunofluorescent staining with antibodies for IBA1 (red) with DAPI co-stain (blue). Sections were imaged at 5x magnification. Representative images show control hippocampus (A), with 40x zoomed image of IBA1+ cell (B) and arthritic hippocampus (C), with 40x zoomed image of IBA1 positive cell (D). Staining negative control image was obtained (E). Cells were counted within measured drawn areas as shown to ensure comparability between sections/mice. Images were blinded and counted for positively stained cells with the hippocampus divided into 4 distinct anatomical regions for the purposes of counting (F). Anatomical areas were identified with the aid of Paxinos Mouse Atlas. Counts were completed analysing individual layers of the hippocampus, including (from top to bottom) stratum radiatum, stratum lacunosum moleculare, stratum moleculare, and dentate gyrus. IBA1+ cell counts were compared for each region between control and arthritic CIA mice. Significance determined using Mann-Whitney U test or t-test with Welch's correction depending on normality of distribution. There were no significant differences between control and arthritic mice in cell density in stratum radiatum (p=0.8884), stratum lacunosum moleculare (p=0.6058), stratum moleculare (p=0.4807) and dentate gyrus (p=0.673). Once un-blinded, a mean number of IBA1+ cells across areas per hippocampal section and per mouse was calculated (p=0.7508) and Mann-Whitney. There were no significant differences between control and arthritic mice.

3.3.5.2 IBA1+ cell density in cortex, striatum and thalamus

Elevated transcription of Il1ß was detectable in the rest of the hemisphere, in addition to hippocampus, in arthritic mice. Therefore, imaging and analysis of IBA1+ cell density within select regions other than hippocampus was carried out, namely cortex, striatum, and thalamus. These regions were selected on the basis of evidence in the literature suggesting a glial cell response in these regions in the context of CIA (Süß et al., 2020a).

No significant differences were seen in IBA1+ cell staining between control and arthritic mice in the cortex or striatum. However, in the thalamus there were significantly more IBA1+ cells in CIA arthritic mice (figure 3.17). These findings are discussed in detail in section 3.5.3.4.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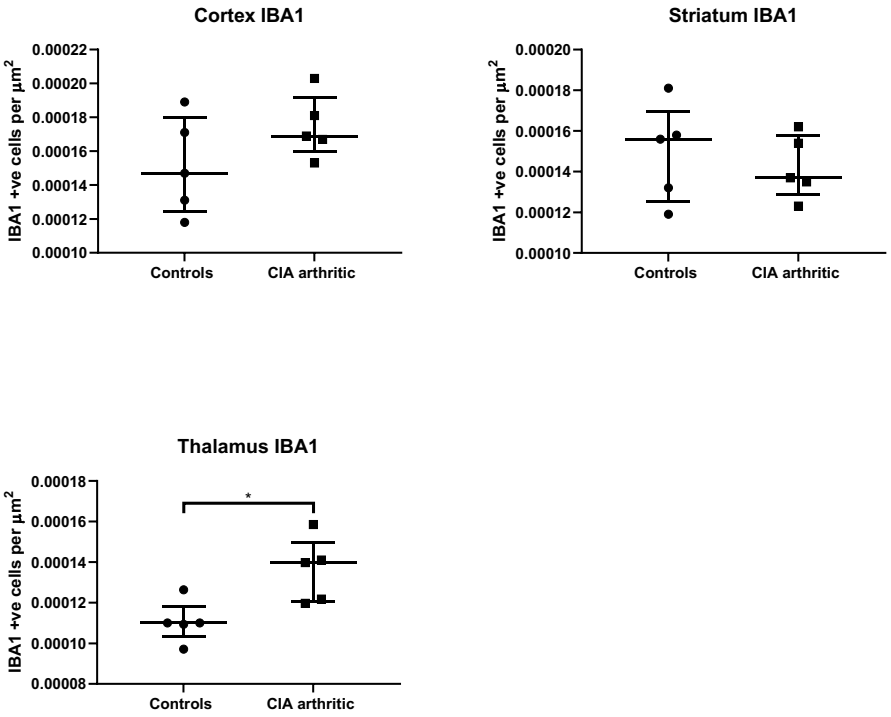
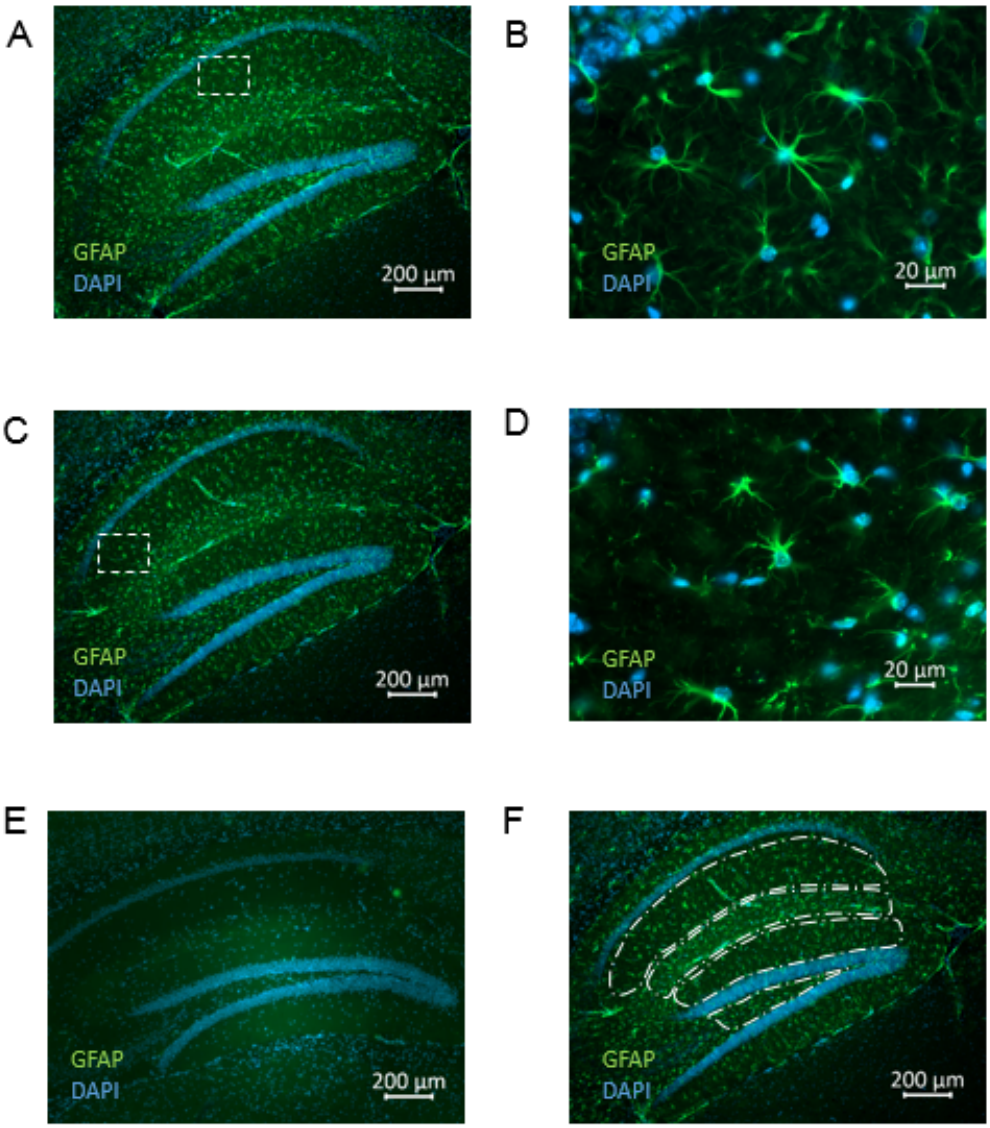


Figure 3.17 IBA1+ cell staining in cortex, striatum and thalamus
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular severity scores for CIA mice were 11 for 2 mice and 12 for 2 mice. Following perfusion with ice-cold PBS, brain left hemisphere was dropped into chilled 4% PFA for 24 hours prior to cryprotecting by immersion in 30% sucrose for 24 hours before embedding in cutting media and freezing at -80^{o}C. Sagittal sections of frozen brains were cut at 10µm thickness. Six sections per mouse were stained with immunofluorescent staining with antibodies for IBA1 (red) with DAPI co-stain (blue) from 4 control mice and 4 arthritic mice. Sections were imaged at 10x magnification. Cells were counted within measured drawn areas as shown in order to ensure comparability between sections/mice. Anatomical areas were identified with the aid of Paxinos Mouse Atlas. Representative images show control mouse cortex (A) and arthritic mouse cortex (B), control mouse striatum (C) and arthritic mouse striatum (D), and control mouse thalamus (E) and arthritic mouse thalamus (F). Images were blinded and processed to create measured cell counting areas within each image (areas within white dotted lines) and counted for positively stained cells. Once un-blinded, a mean number of IBA1+ cells per measured area and per mouse was calculated. Significance determined using Mann-Whitney. There were no significant differences between control and arthritic mice in IBA1-positive cells in cortex (p=0.3095) or striatum (p=0.8413). There were significantly more IBA1-positive cells in the thalamus of arthritic mice (p=*0.0317).

3.3.5.3 Hippocampal GFAP+ cell density

A similar staining and counting strategy as was used for IBA1 staining was employed to examine GFAP-positive cells within the hippocampus. No significant differences were found in cell count between normal saline injected controls and CIA arthritic mice (figure 3.18).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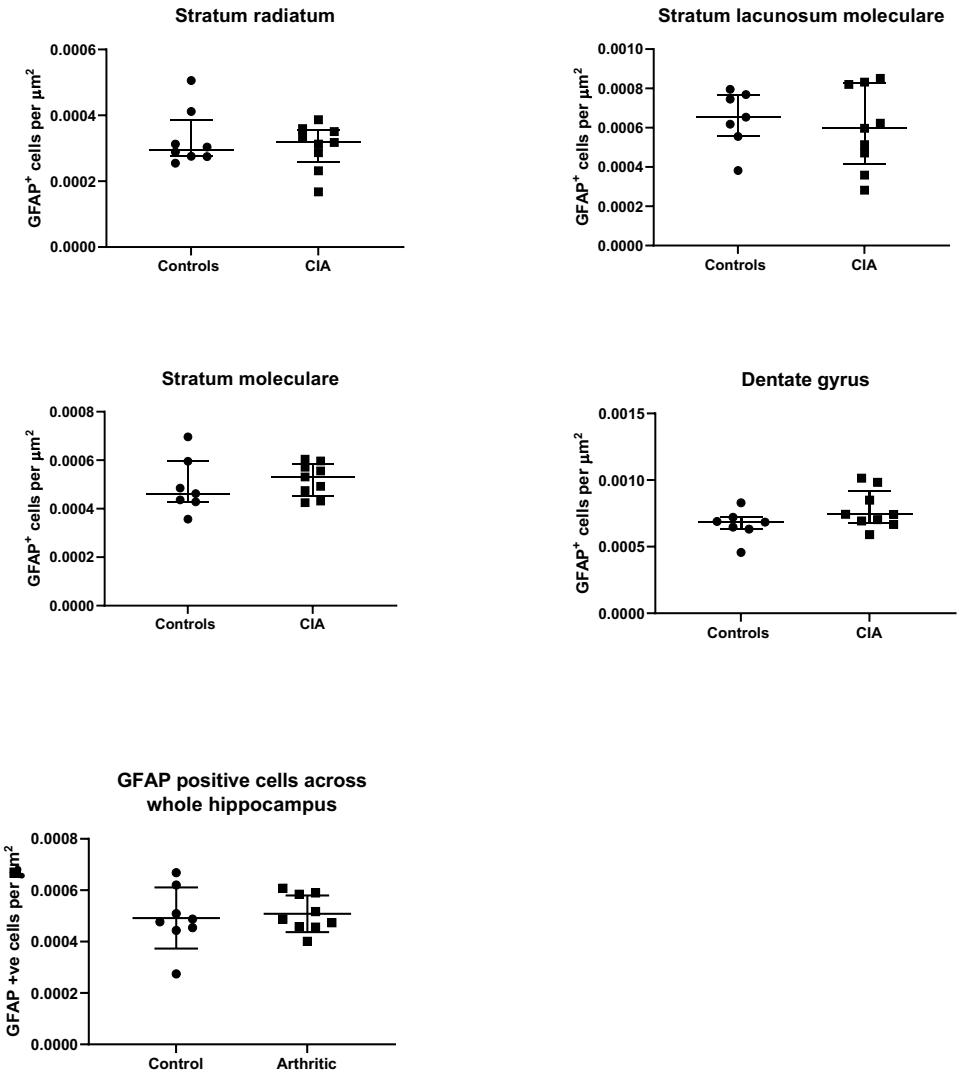
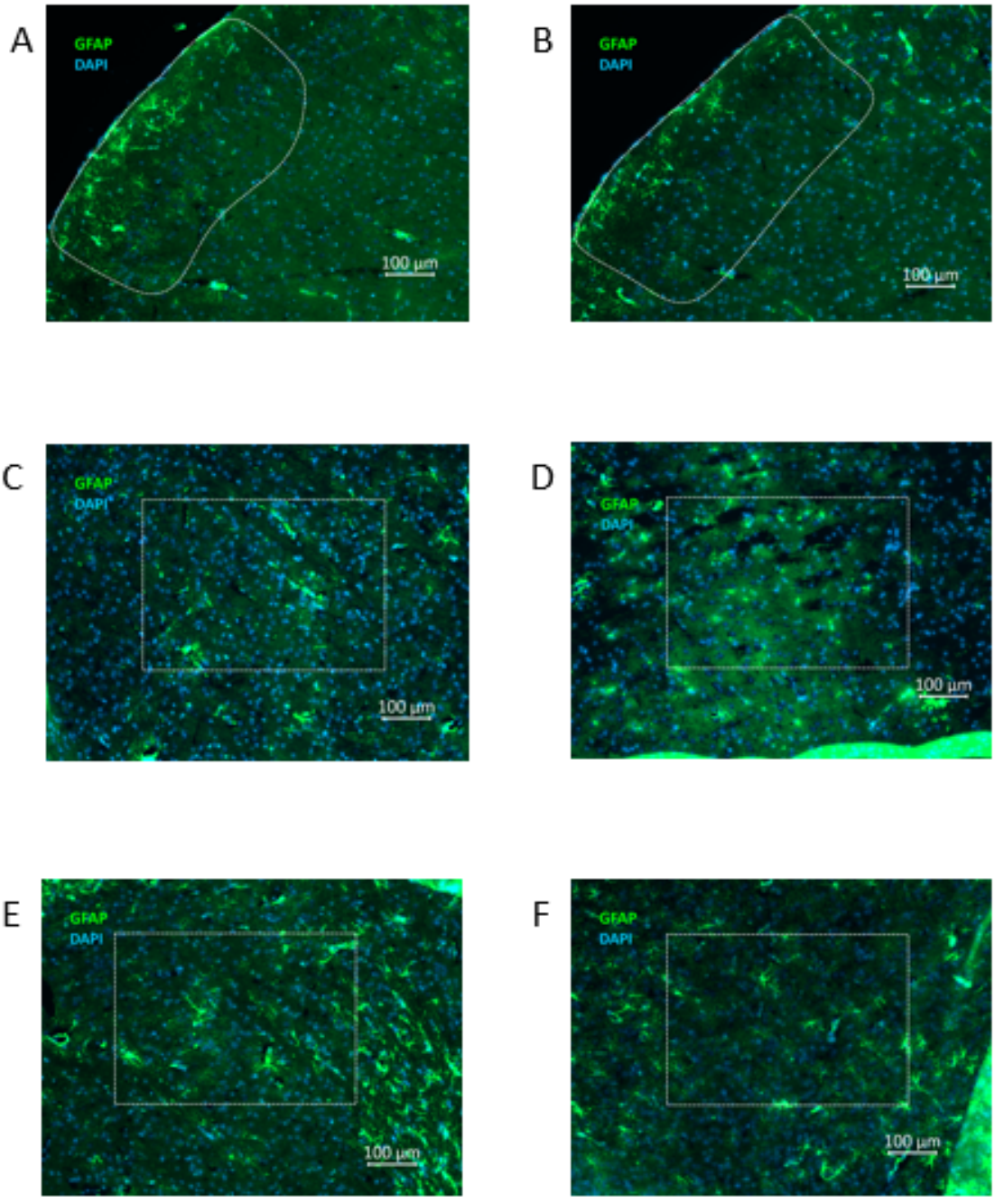


Figure 3.18 GFAP+ cell staining in the hippocampus
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Arthritis severity scores ranged from 5 to 12. Following perfusion with ice-cold PBS, brain left hemisphere was dropped into chilled 4% PFA for 24 hours prior to cryprotecting by immersion in 30% sucrose for 24 hours before embedding in cutting media and freezing at -80^{o}C prior to cutting. Sagittal sections of frozen brains from a total of 8 control mice and 9 arthritic mice were cut at 10µm thickness. Six sections per mouse were stained with immunofluorescent staining with antibodies for GFAP (green) with DAPI co-stain (blue). Sections were imaged at 5x magnification. Representative images show control hippocampus (A), with 40x zoomed image of GFAP positive cell (B) and arthritic hippocampus (C), with 40x zoomed image of GFAP positive cell (D). Staining negative control image was obtained (E). Images were blinded and counted for positively stained cells with the hippocampus divided into 4 distinct anatomical regions for the purposes of counting (F). Counts were completed analysing individual layers of the hippocampus, including (top to bottom) stratum radiatum, stratum lacunosum moleculare, stratum moleculare, and dentate gyrus. GFAP+ cell counts were compared for each region between control and arthritic CIA mice. Significance determined using Mann-Whitney U test or t-test with Welch's correction depending on normality of distribution. There were no significant differences between control and arthritic mice in cell density in stratum radiatum (p=0.5541), stratum lacunosum moleculare (p=0.5732), stratum moleculare (p=0.6064) and dentate gyrus (p=0.1059). Once un-blinded, a mean number of GFAP+ cells per hippocampal section and per mouse was calculated. Significance determined using t-test with Welch's correction (p=0.74). There were no significant differences between control and arthritic mice.

3.3.5.4 GFAP+ cell density in cortex, striatum and thalamus

GFAP staining in cortex, striatum and thalamus was investigated (figure 3.19). No significant differences were seen in GFAP staining between control and arthritic mice in the cortex, striatum or thalamus.



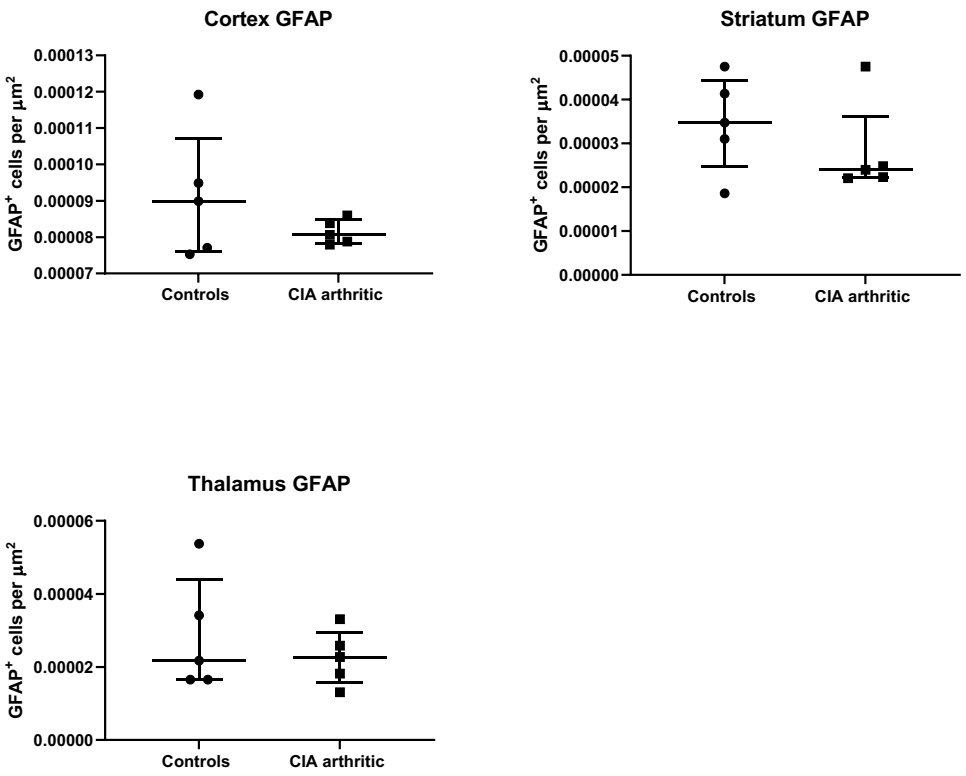


Figure 3.19 GFAP+ cell staining in cortex, striatum and thalamus
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular severity scores for CIA mice were 11 for 2 mice and 12 for 2 mice. Following perfusion with ice-cold PBS, brain left hemisphere was dropped into chilled 4% PFA for 24 hours prior to cryprotecting by immersion in 30% sucrose for 24 hours before embedding in cutting media and freezing at -80^{o}C prior to cutting. Six sections per mouse were stained with immunofluorescent staining with antibodies for GFAP (green) with DAPI co-stain (blue) from 4 control mice and 4 arthritic mice. Sections were imaged at 10x magnification. Cells were counted within measured drawn areas as shown in order to ensure comparability between sections/mice. Anatomical areas were identified with the aid of Paxinos Mouse Atlas. Representative images show control mouse cortex (A) and arthritic mouse cortex (B), control mouse striatum (C) and arthritic mouse striatum (D), and control mouse thalamus (E) and arthritic mouse thalamus (F). Images were blinded and processed to create measured cell counting areas within each image (areas within white dotted lines) and counted for positively stained cells. Once un-blinded, a mean number of GFAP positive cells per measured area and per mouse was calculated. Significance determined using Mann-Whitney. There were no significant differences between control and arthritic mice in GFAP positive cells in cortex ($p=0.6905$), striatum ($p=0.4603$) or thalamus ($p=0.7937$).

3.3.5.5 Glial cell transcriptional response in CIA

To detect changes in glial activation and proliferation at the transcriptional level in CIA, qRT-PCR was performed to measure transcription of Aif1 (gene name of IBA1), Tmem119 (unique marker expressed by microglia) and Gfap. Upregulation of these genes can accompany the CNS response to injury, infection and inflammation (Brenner, 1994) (Bennett et al., 2016) (Bodea et al., 2014).

The qRT-PCR experiments showed some differences in expression (figure 3.20). Aif1 was higher in control hippocampus compared to arthritic hippocampus. Additionally, Gfap appeared to be higher in the arthritic brain minus hippocampus compared to control brain minus hippocampus. The actual significance of these results is unclear. It is worth noting that these differences did not reproduce in qRT-PCR experiments looking at whole hemisphere (left hand graphs). Tmem119 showed no differences. Overall, such differences as were detected by qRT-PCR are difficult to account for.

It seems likely that there was not a substantial or diffuse glial cell response in response to arthritis, aside from the significant difference in IBA1+ cells seen in the thalamus.

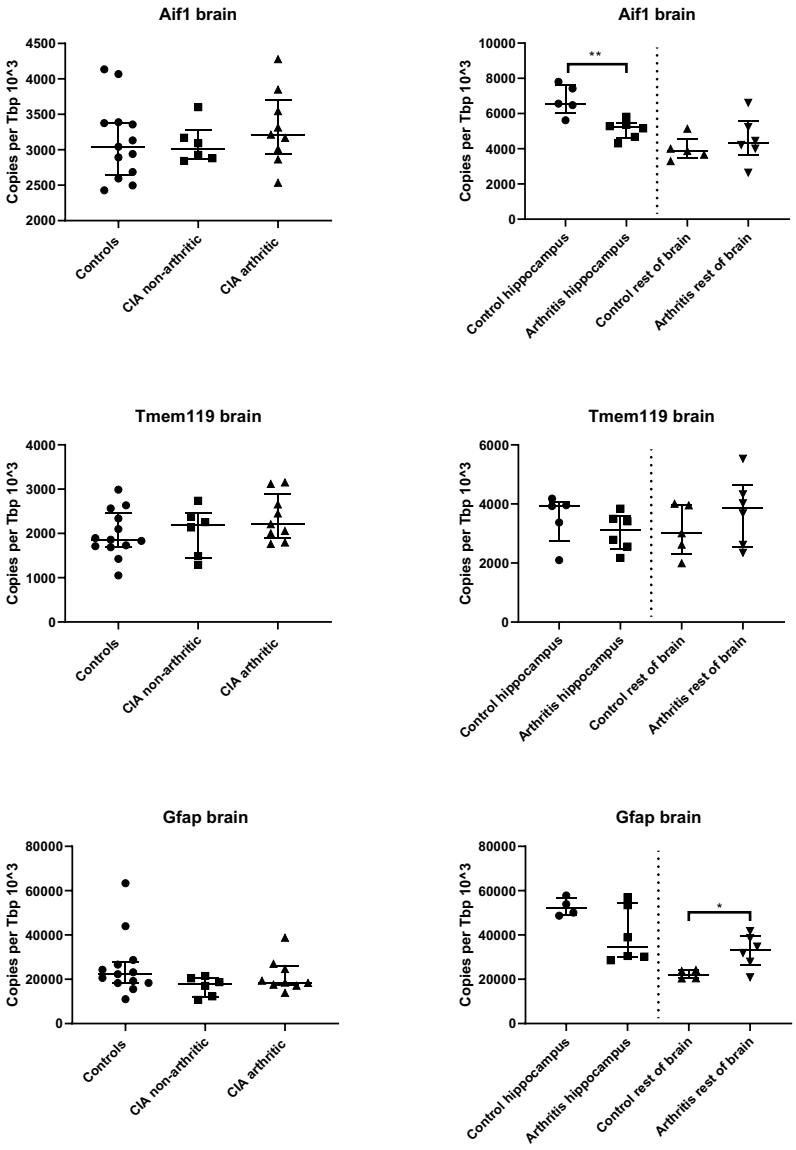


Figure 3.20 qRT-PCR analysis of brain glial cell transcription markers
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Arthritis severity scores ranged from 2 to 12. Following perfusion with ice cold PBS, brain was dissected, and the right hemisphere retrieved. RNA was extracted and converted to cDNA for qRT-PCR analysis. Graphs show mice retrieved from 2 separately run animal models. In a second model (right hand graphs) the experiment was repeated but on this occasion the right hemisphere was further dissected to separate hippocampus from rest of brain in order to determine differences in regional transcription. Three genes related to microglia and astrocyte activation and proliferation were tested. Statistical significance was determined using either ANOVA or Kruskal-Wallis depending on normality of data where >2 groups analysed or using Mann-Whitney where 2 group comparison.

3.3.6 Brain iNOS expression in CIA

The pro-inflammatory cytokine IL1β can be released by microglia and other immunologically active cells in response to a wide variety of brain insults,

producing diverse effects including inflammasome activation and oxidative stress, a feature of which includes increased activation of iNOS (Lucas et al., 2006).

Given the finding of elevated brain Il1ß transcription in association with CIA, it was decided to determine if this could be associated with increased iNOS protein expression. As before, the hippocampus was chosen because of the finding of elevated hippocampal Il1ß transcription. As noted earlier, increased IBA1+ cell staining was observed in the thalamus of arthritic mice vs. controls, and therefore thalamus was examined in addition to cortex and striatum.

To examine brain iNOS expression, immunohistochemical staining for iNOS in control and arthritic brain sections was undertaken. Images were taken to examine iNOS expression in the hippocampus (figure 3.21) and in the cortex, striatum, and thalamus (figure 3.22).

Positively stained cells were sparse in all regions in controls and arthritic mice. Overall, no significant differences were seen between control and arthritic mice in hippocampus, cortex, striatum, or thalamus. These results would suggest that major inflammasome activation is not a feature of the brain response in CIA in the areas studied.

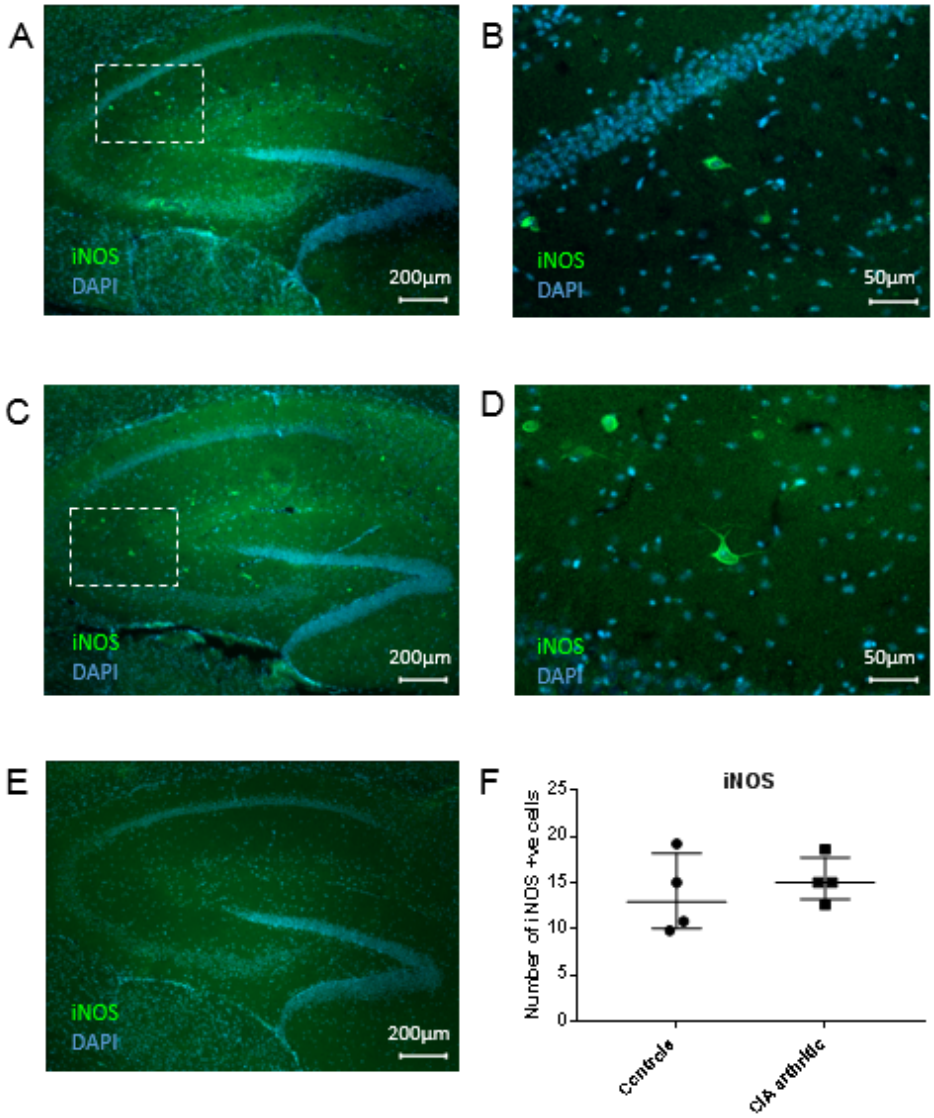
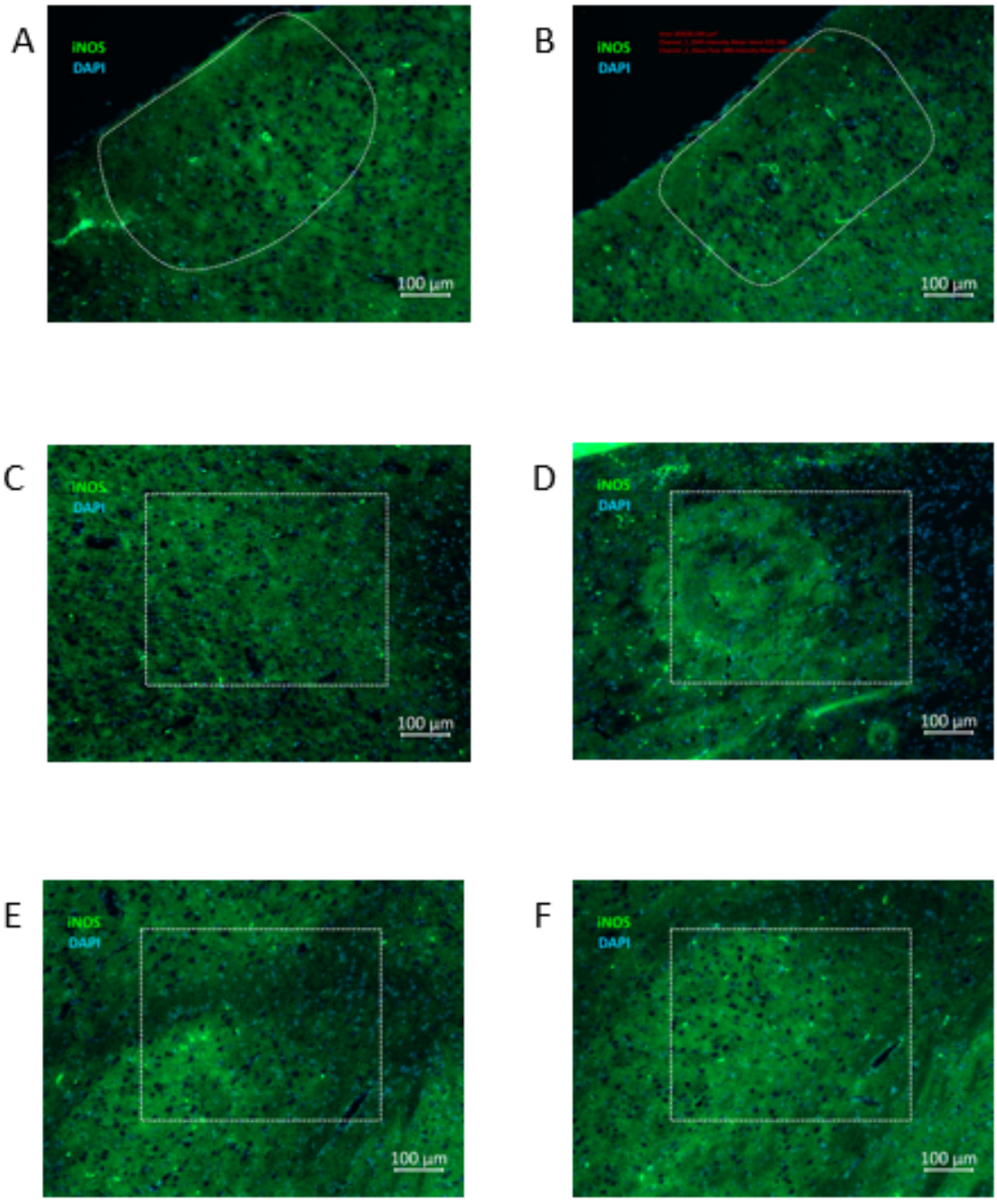


Figure 3.21 iNOS staining in the hippocampus
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular severity scores for CIA mice were 11 for 2 mice and 12 for 2 mice. Following perfusion with ice-cold PBS, brain left hemisphere was dropped into chilled 4% PFA for 24 hours prior to cryprotecting by immersion in 30% sucrose for 24 hours before embedding in cutting media and freezing at -80°C. Sagittal sections of frozen brains from 4 control mice and 4 arthritic mice were cut at 10µm thickness. Six sections per mouse were used for immunofluorescent staining for iNOS (green) and DAPI (blue) co-stain. Sections were imaged at 5x magnification. Representative images show control hippocampus (A), with 20x zoomed image of iNOS positive cell (B) and arthritic hippocampus (C), with 20x zoomed image (D). Negative control was included (E). Images were blinded and counted for all positively stained cells within the boundaries of the hippocampus. Once un-blinded, a mean number of iNOS-positive cells per 10µm hippocampal section was calculated (F) and significance determined using Mann-Whitney ($p=0.7$). There were no significant differences between control and arthritic mice.


A
iNOS
DAPI
100 µm
B
iNOS
DAPI
100 µm
C
iNOS
DAPI
100 µm
D
iNOS
DAPI
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E
iNOS
DAPI
100 µm
F
iNOS
DAPI
100 µm

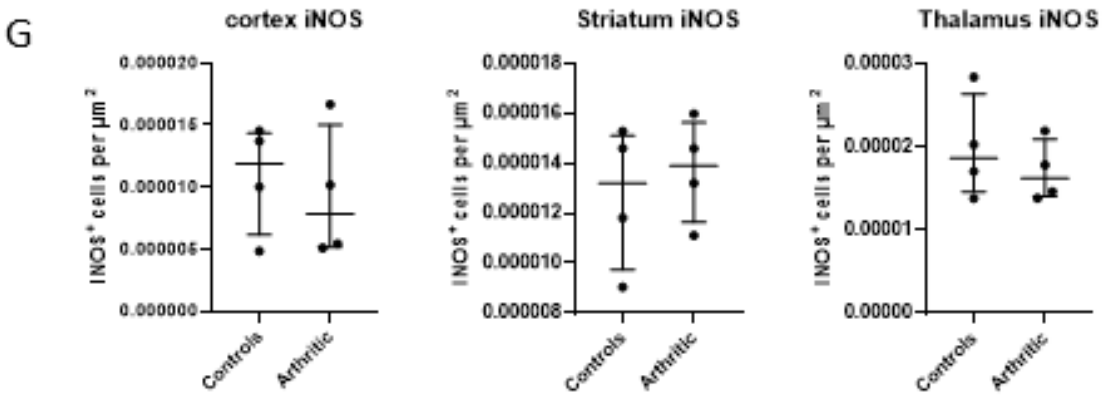

G
cortex iNOS
Striatum iNOS
Thalamus iNOS
iNOS+ cells per µm2
0.000020
0.000015
0.000010
0.000005
0.000000
0.000018
0.000016
0.000014
0.000012
0.000010
0.000008
0.00003
0.00002
0.00001
0.00000
Controls
Arthritic

Figure 3.22 iNOS staining in cortex, striatum and thalamus
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular severity scores for CIA mice were 11 for 2 mice and 12 for 2 mice. Following perfusion with ice-cold PBS, brain left hemisphere was dropped into chilled 4% PFA for 24 hours prior to cryprotection by immersion in 30% sucrose for 24 hours before embedding in cutting media and freezing at -80^{o}C prior to cutting. Sagittal sections of frozen brains from 4 control mice and 4 arthritic mice were cut at 10µm thickness. Six sections per mouse were used for immunofluorescent staining for iNOS (green) and DAPI (blue) co-stain. Sections were imaged at 10x magnification. Representative images show control mouse cortex (A) and arthritic mouse cortex (B), control mouse striatum (C) and arthritic mouse striatum (D), and control mouse thalamus (E) and arthritic mouse thalamus (F). Images were blinded and processed to create measured cell counting areas within each image (areas within white dotted lines) and counted for positively stained cells in order to count cells per square micron and allow comparison between samples. Anatomical areas were identified with the aid of Paxinos Mouse Brain Atlas. Once un-blinded, a mean number of iNOS positive cells per measured area and per mouse was calculated (G). Significance determined using Mann-Whitney. There were no significant differences between control and arthritic mice in iNOS positive cells in cortex (p=>0.999), striatum (p=0.8857) or thalamus (p=0.8857).

3.3.7 CIA and markers of brain plasticity

We hypothesised that the increased transcription of Il1ß, if translated at the protein level, could have a range of detectable effects on the mouse brain in the context of arthritis. The effects of IL1ß within the CNS can include activation of resident cells to express other cytokines and chemokines and promotion of a pro-inflammatory environment (Allan et al., 2005). The signalling receptor for IL1 exists in high density within the hippocampus (Cunningham et al., 1992), therefore one could expect this region to be particularly sensitive to the effects of elevated IL1ß. It was determined to investigate whether hippocampal neurogenesis and hippocampal neuronal density were affected in the context of CIA.

3.3.7.1 Hippocampal neurogenesis in CIA

Hippocampal inflammation has been shown to be detrimental for brain plasticity and neurogenesis (Yirmiya and Goshen, 2011). Neurogenesis is the formation of new-born neurons within the mature brain from precursor neural stem cells. It occurs exclusively within two distinct brain regions – the subgranular zone (SGZ) of the dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles (Ming and Song, 2005). These new-born neurons are believed to be highly plastic and undergo substantial synaptogenesis, supporting hippocampal

circuits important for cognition, and regulation of emotion, mood and anxiety (Schmidt-Hieber et al., 2004, Wang et al., 2000).

CIA has been found to associate with impaired hippocampal neurogenesis in work published by Andersson et al (Andersson et al., 2018).

Sagittal sections through the dentate gyrus were stained to detect doublecortin (DCX) which is a micro-tubule associated protein expressed by immature developing neurons and is a reliable marker for neurogenesis (Couillard-Despres et al., 2005).

The experiments failed to show any significant differences in DCX-positive cells between control and arthritic mice (figure 3.23). This suggests no detectable differences in the number of immature developing DG neurons, and therefore in these experiments it appears unlikely that hippocampal neurogenesis was significantly affected by CIA.

A further analysis was undertaken to detect if there was any association or trend between the number of days of arthritis or arthritis severity and DCX expression. Simple linear regression analysis was undertaken but found no significant relationship or trend (figure 3.24).

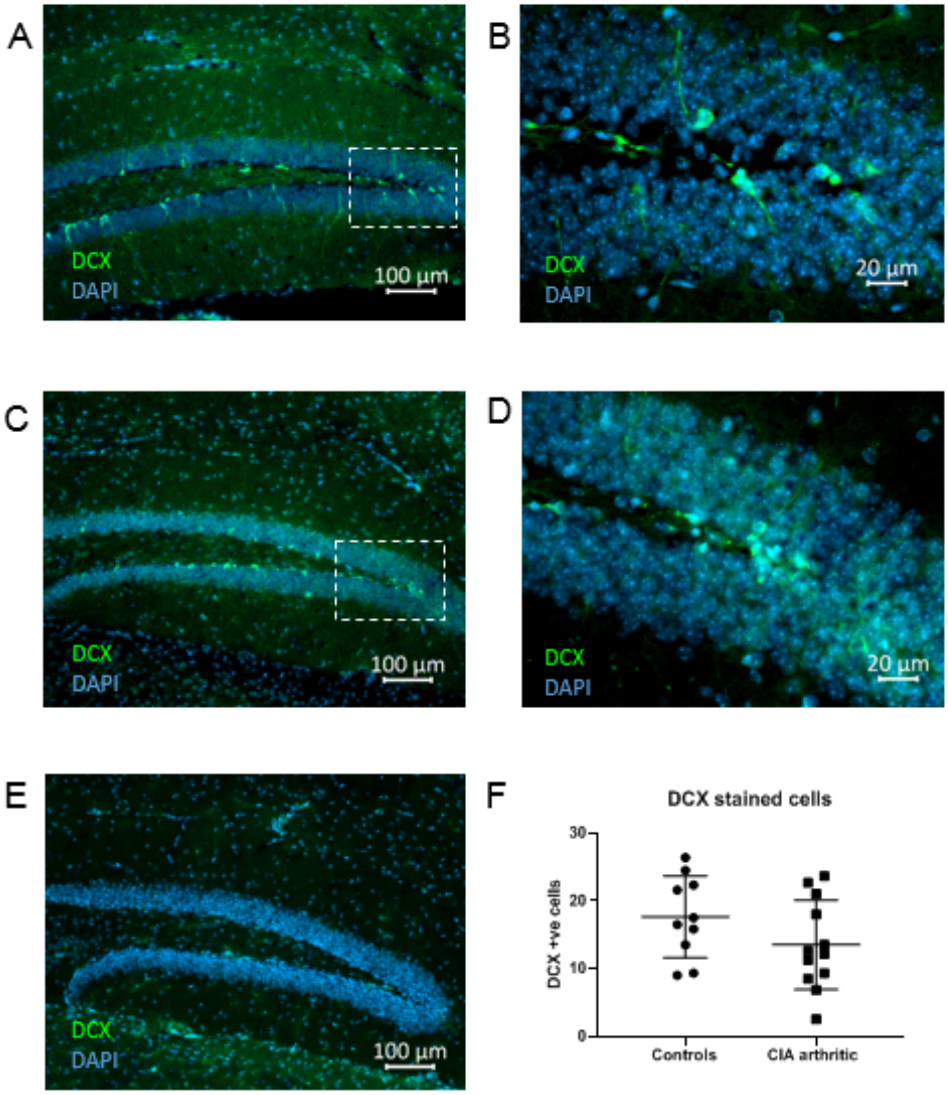


Figure 3.23 DCX staining in the dentate gyrus
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular severity scores for CIA mice ranged from 2 to 12. Following perfusion with ice-cold PBS, brain left hemisphere was dropped into chilled 4% PFA for 24 hours prior to cryprotecting by immersion in 30% sucrose for 24 hours before embedding in cutting media and freezing at -80°C prior to cutting. Sagittal sections of frozen brains from 10 control mice and 12 arthritic mice were cut at 10µm thickness. Six to twelve sections per mouse were stained for DCX (green) with DAPI (blue) co-stain. Sections were imaged at 10x magnification. Representative images show control dentate gyrus (A), with 20x zoomed image of DCX positive cell (B) and arthritic dentate gyrus (C), with 20x zoomed image (D). Negative control was included (E). Images were blinded and counted for positively stained cells across the dentate gyrus. Once un-blinded, a mean number of DCX-positive cells per section and per mouse was calculated (F). Significance was determined using t-test ($p=0.14$). There were no significant differences between control and arthritic mice.

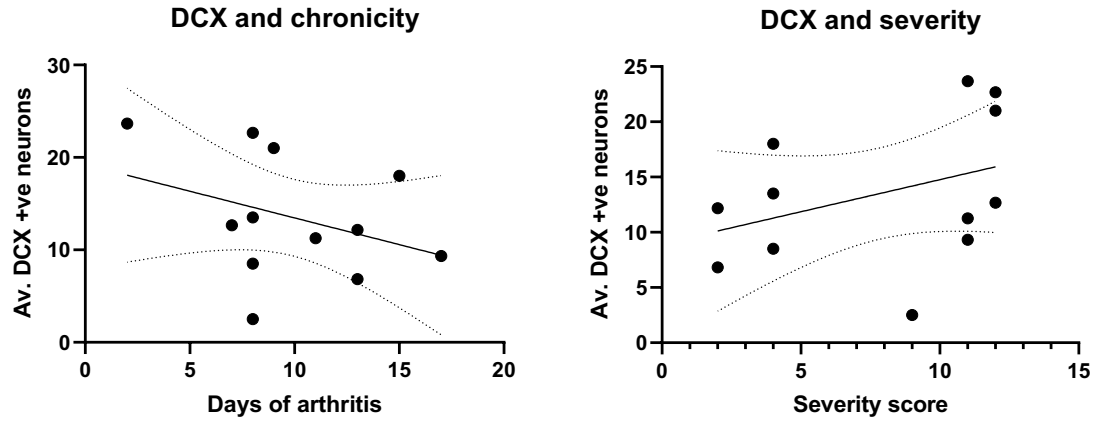


Figure 3.24 DCX cell count and chronicity and severity of arthritis
Sections were prepared and stained as described in figure 3.23 legend. To determine if there was a temporal relationship between DCX expression and onset of arthritis simple linear regression analysis was conducted. The overall regression model was not significant (p=0.2352). This was repeated for arthritis severity. The regression model was not significant (p=0.2552).

3.3.7.2 Transcriptional markers of neurogenesis and plasticity

As discussed above, Andersson et al (2018) found reduced DCX protein expression in the dentate gyrus of CIA mice. They also found reduced Dcx transcription (Andersson et al., 2018). Heterogeneity of disease response in the experiments presented in this thesis represent a major difference from the experiments reported by Andersson et al. (2018). To explore if a transcriptional difference, potentially preceding a protein expression change, could be detected, qRT-PCR analysis of brain Dcx mRNA levels was undertaken.

This was completed for brain tissue from the hippocampus and whole hemisphere. In addition, brain derived neurotrophic factor (Bdnf) transcription was measured. BDNF is a neurotrophin which may have a role in promoting DG neurogenesis (Chan et al., 2008), and more broadly supports neuronal survival and plasticity (Huang and Reichardt, 2001).

No significant differences were found in either Dcx or Bdnf transcription in controls vs. arthritic mice vs. CIA stimulus administered non-arthritic mice (figure 3.25). The lack of depleted Dcx transcription in association with CIA would tend to support the immunofuorescent staining results but does not entirely refute the possibility of a later/ more chronic effect on neurogenesis.

The lack of depleted Bdnf transcription, also tends to support the notion that CIA may not have major impacts upon brain plasticity.

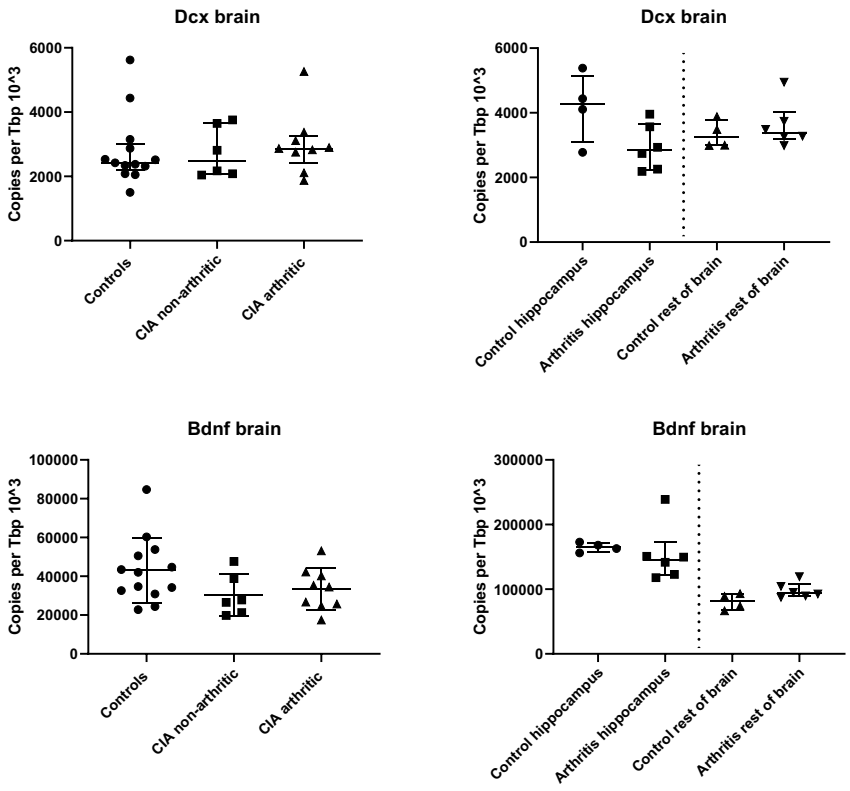


Figure 3.25 qRT-PCR analysis of brain markers of neurogenesis and plasticity
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular disease severity scores ranged from 2 to 12. Following perfusion with ice cold PBS, brain was dissected, and the right hemisphere retrieved. This hemisphere was then further dissected under magnification to separate hippocampus from the rest of the brain. RNA was subsequently extracted and converted to cDNA for qRT-PCR analysis. Statistical significance was determined using either ANOVA or Kruskal-Wallis depending on normality of data where >2 groups analysed or using Mann-Whitney for a 2-group comparison.

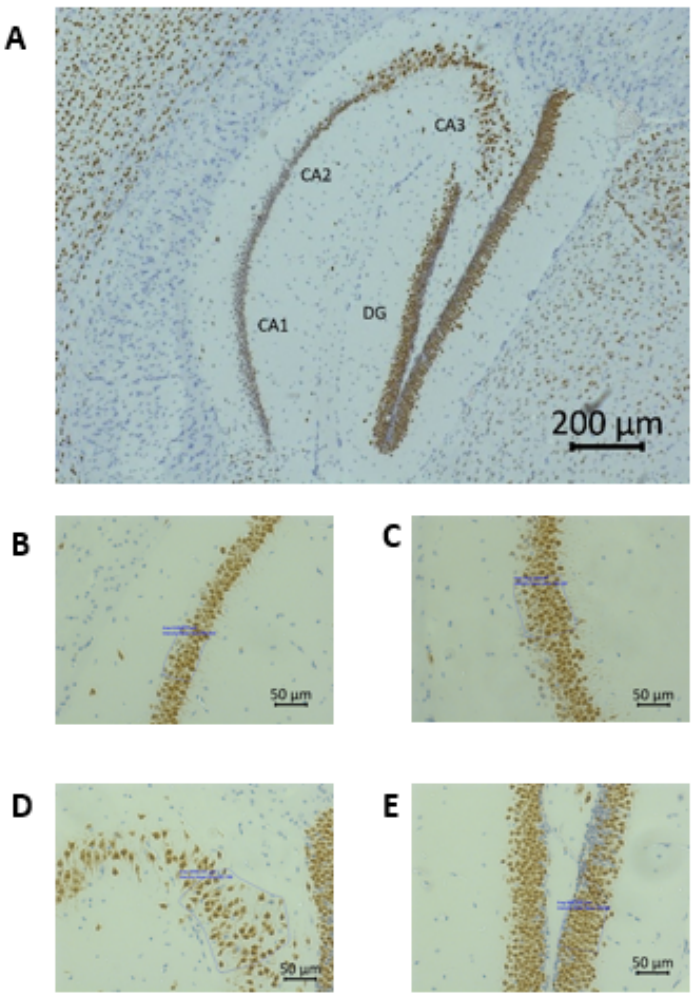
3.3.7.3 Hippocampal neuronal density in CIA

As noted in regard to the glia response in section 3.3.5 and neurogenesis in section 3.3.7.1, substantial signs of hippocampal inflammation have previously been reported by Andersson et al in the context of CIA (Andersson et al., 2018). Although they did not explore NeuN staining, they reported reduced hippocampal volume in CIA.

To determine if CIA influenced neuronal density in the hippocampus, a NeuN stain was used to identify mature neurons. In view of my findings of increased brain Il1ß transcription, I hypothesised that even in the absence of impaired

neurogenesis, it was possible that CIA may result in either more acutely impaired neurogenesis earlier in the course of the model, or alternatively result in neurotoxic sequelae leading to neuronal cell death or apoptosis.

No significant differences in neuronal cell density were observed in my experiments (figure 3.26). An overall difference in hippocampal volume cannot be excluded. Measurements of the overall thickness of neuronal layers within the hippocampal sections were not undertaken, as this was felt to be too susceptible to subtle differences in the precise spatial section of brain being examined despite care being taken to image slides from approximately the same depth between different mice.



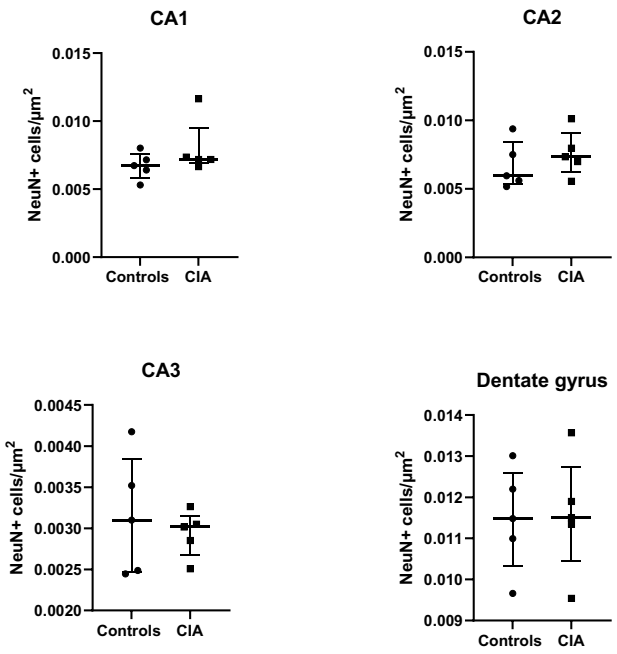


Figure 3.26 NEUN expression in hippocampus in CIA
Mice were culled either at day 42 or when severity threshold breached, whichever occurred first. Articular severity scores for CIA mice ranged from 7 to 13. Following perfusion with ice-cold PBS, brain left hemisphere was dropped into chilled formalin for 24 hours prior to being immersed in 70% ethanol prior to tissue processing and paraffin embedding. Six sections per mouse were chromogenically stained with antibodies for NEUN (brown) and counterstained with Gill's haematoxylin nuclear stain (blue) from 5 control mice and 5 arthritic mice. Sections were imaged at 10x magnification. Sagittal sections were cut at 7µm thickness. 6 sections per mouse were stained for NEUN using chromogenic staining and imaged at 20x magnification. Anatomical areas were identified with the aid of Paxinos Mouse Atlas. Representative images show whole hippocampus with labelled regions at 5x zoom (A), with 20x zoomed image of CA1(B) CA2 (C), CA3 (D) and DG (E). Cells were counted within measured drawn areas as shown in order to ensure comparability between sections/mice. The measured area in which cells were counted is shown. Images were blinded and cell counts were averaged and calculated per square micron and are presented unblinded in graphs. Images were blinded and counted for positively stained cells and significance determined using Mann-Whitney U test. There were no significant differences between control and arthritic mice(CA1 p=0.2222, CA2 p=0.5476, CA3 p=0.8413, DG p=>0.9999).

3.3.8 Brain response results: summary of key findings

The key findings are summarised here and discussed in detail in the main discussion in section 3.5.

1. CIA is associated with brain inflammatory transcription

In the context of CIA there is a consistent finding of elevated brain Il1β transcription. The related P2x7 receptor is also found to be transcribed in the

brain at elevated levels in CIA. No other differences in inflammatory cytokine transcription were detected.

The inflammatory chemokines Cxcl1, Ccl11 and chemokine receptor Ccr2 were also detected to be elevated in the brain, outside of the hippocampus.

2. Leukocyte recruitment across the BBB is not a feature of CIA.

Flow cytometry did not detect altered levels of a range of immune cells within the brains of CIA mice in comparison to controls. These included microglia, T cells, B cells, NK cells, neutrophils, and monocytes.

3. CIA is associated with increased density of IBA1+ cells within the thalamus.

IBA1 is a marker for myeloid cells, including microglia. However, there were no detectable differences in IBA1+ cell density within the hippocampus, cortex or striatum.

There were no detected differences in GFAP+ cells (astrocytes) in hippocampus, cortex, striatum, or thalamus.

4. Brain iNOS expression is not affected by CIA

There were no detectable differences in the expression of iNOS in the hippocampus, cortex, striatum or thalamus between CIA mice and controls. This suggests that substantial inflammasome activation and oxidative stress is not a major feature of the brain response in CIA.

5. Hippocampal neurogenesis and neuronal cell density is not affected by CIA

No detectable differences in DCX expression were seen in the dentate gyrus between CIA mice and controls. Neuronal cell density throughout the hippocampus was equal between CIA mice and controls. There was no difference in the transcription of the neurotrophin Bdnf.

3.4 Analysis of stage-dependent inflammation in the CIA model

3.4.1 Introduction

The data so far indicates a relatively circumscribed brain inflammatory response in CIA which appears to centre around increased brain transcription of Il1ß. This varies to some extent from the limited published literature exploring the brain response in CIA. One of the challenges encountered in the course of carrying out these animal models involved the substantial variability in disease response, in terms of severity and timing of arthritis. The CIA model is highly variable with varying severity of arthritis presenting at diverse timepoints. It is also a relatively long mouse model. These observations gave rise to the hypothesis that some changes may be detectable in the context of a more homogeneous group, and secondly, that more acute responses may be missed by experiments which examined the brain response at end-stage severity or day 42. It was also determined to seek to build up a clearer characterisation of how the inflammatory response in the periphery and the brain evolved throughout the model.

To determine if inflammatory changes occurred at earlier time points in the course of the model, a number of experiments were undertaken at pre-determined intervals throughout the course of the model (figure 3.27). Time-points were selected with a view to allowing analysis of the response to the CIA-challenge, in addition to the pre-/ early-arthritic stages. At the earliest time-points (24 hours and 7 days), the groups included a CFA-injected group in order to distinguish between the response to CFA and the response to CFA + T2C and the injection of sterile saline in control mice.

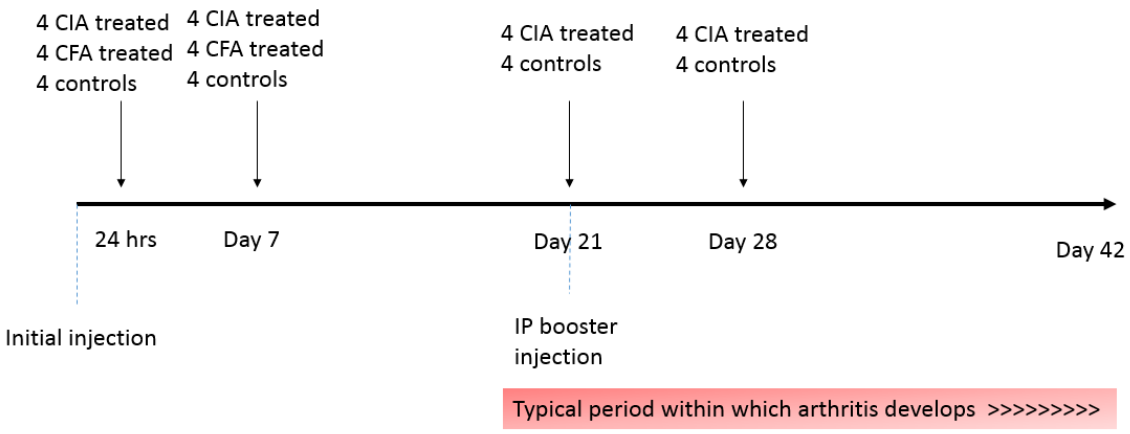


Figure 3.27 Timepoint sampling model design
Time points were selected throughout the model in order to determine the response to the CIA-challenge and the pre- /early-arthritic phase.

3.4.2 Arthritic response

Of the 4 mice administered the CIA-stimulus at baseline plus immunisation at day 21, only 1 mouse had clinically detectable arthritis by day 28, with onset on day 23 and by day 28 showing a severity score of 6 affecting 2 paws. It cannot be determined if the remaining 3 mice would have gone onto develop arthritis after day 28 if not culled.

3.4.3 Body weight over time

Body weight was measured at regular intervals throughout the time-points. Weight changes are summarised at the main time-points in figure 3.28. The control group appear to show a linear increase in weight over time, whilst the CIA stimulus and CFA-only injected mice show rather more irregular weight change. However, differences do not reach statistical significance.

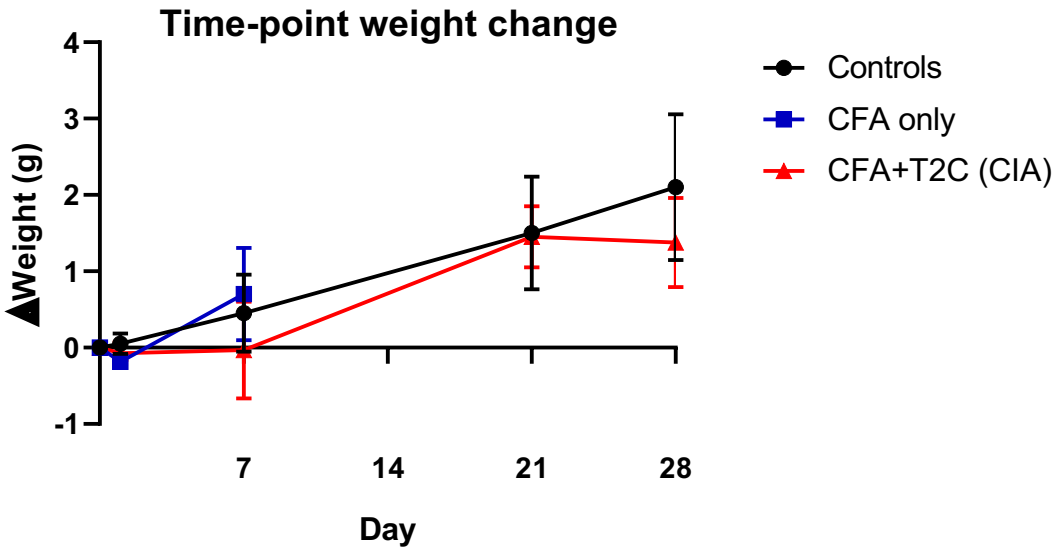


Figure 3.28 Body weight change in the course of the CIA model
Mice were administered either CFA+T2C (standard CIA stimulus), CFA only or sterile DPBS (controls). There were 16 controls at baseline, 12 by day 7, 8 by day 21 and 4 by day 28. There were 8 CFA only mice at baseline, 4 by day 7 and none thereafter. There were 16 CIA mice at baseline, 12 by day 7, 8 by day 21 and 4 by day 28. Mice were weighed regularly throughout the course of the model. Weight change from baseline is graphed with error bars indicating SD. Statistical significance determined using Mann-Whitney or Kruskal-Wallis. There was no significant difference in mean body weight change between groups.

3.4.4 Spleen weight over time

At each of the time-points sampled, spleen was dissected and weighed. Spleen weight relative to body weight was then calculated (figure 3.29). A trend of increased spleen weight appears to be apparent at all timepoints in CFA-administered and CIA-stimulus mice, reaching statistical significance at day 7 and day 28. As there are no significant differences in body weight between the groups (figure 3.28), this confirms that the increase in relative spleen weight observed is a result of spleen enlargement as opposed to being an artefact of body weight loss/cachexia. The red data-point indicates the arthritic mouse. At day 1 and day 7 this difference is indistinguishable from response to CFA, and indeed it would be biologically unlikely for a major adaptive immune inflammatory response to T2C to have become established at these stages. At the later time-points it is not possible to determine whether the difference represents either a past or lingering response to the initial CFA stimulus or an evolving inflammatory response to T2C, as there was no CFA-only group at day

21 or day 28 timepoints. Spleen hyperplasia has previously been reported in response to intradermal CFA injection (Bassi et al., 2012).

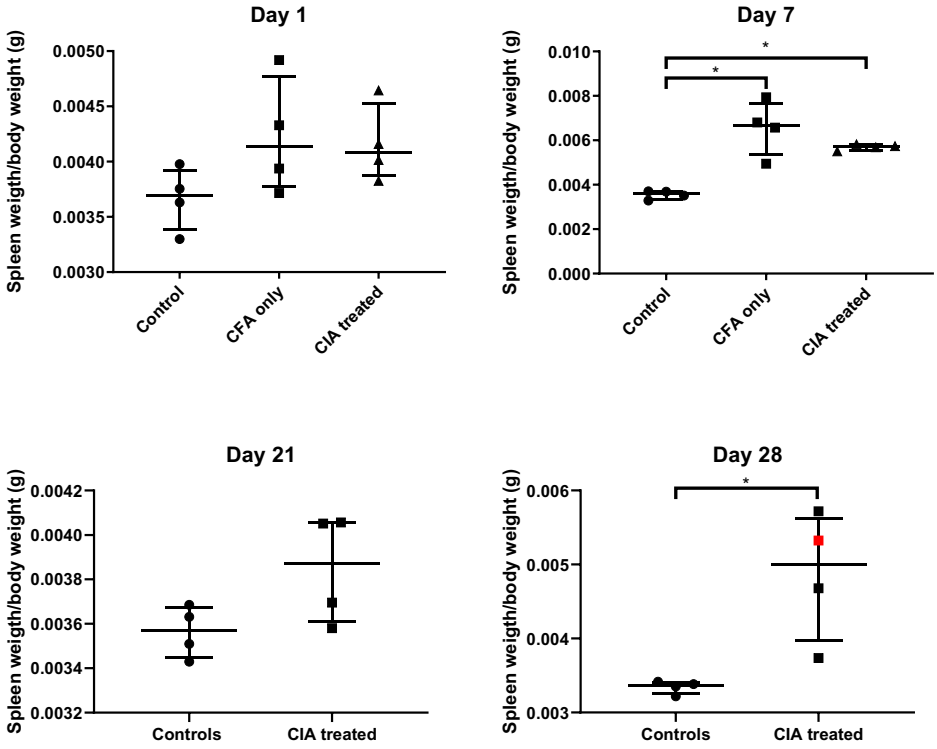


Figure 3.29 Spleen weight relative to body weight at timepoints throughout the CIA model
Spleens were dissected at the time of culling and weighed. Graphs show spleen weight normalised to body weight. Red dot represents mouse with clinically detectable arthritis. Statistical significance is determined using Kruskal-Wallis or Mann-Whitney.

3.4.5 PBL transcriptional response

PBL transcription of inflammatory cytokines and chemokines was measured using qRT-PCR with absolute quantification at the day 1, day 7, day 21 and day 28 time-points through the model (figure 3.30). As noted only one mouse developed clinically detectable arthritis – marked in red on the graphs in figure 3.30.

CFA injection was associated with increased PBL Il1β transcription at day 7 after injection. P2x7r transcription, which as discussed earlier is important in the production and release of IL1β was also elevated in PBLs at day 7 after CFA

injection. Elevated Il1ß transcription is also seen at day 28 in the context of CIA stimulus (1 arthritic, 3 non or pre-arthritic).

Tnfα PBL transcription is also noted to be elevated in the CFA- treated group at day 1 but not at other time points. No broader inflammatory transcriptional response in PBLs was observed at any time point in the cytokine and chemokine genes tested.

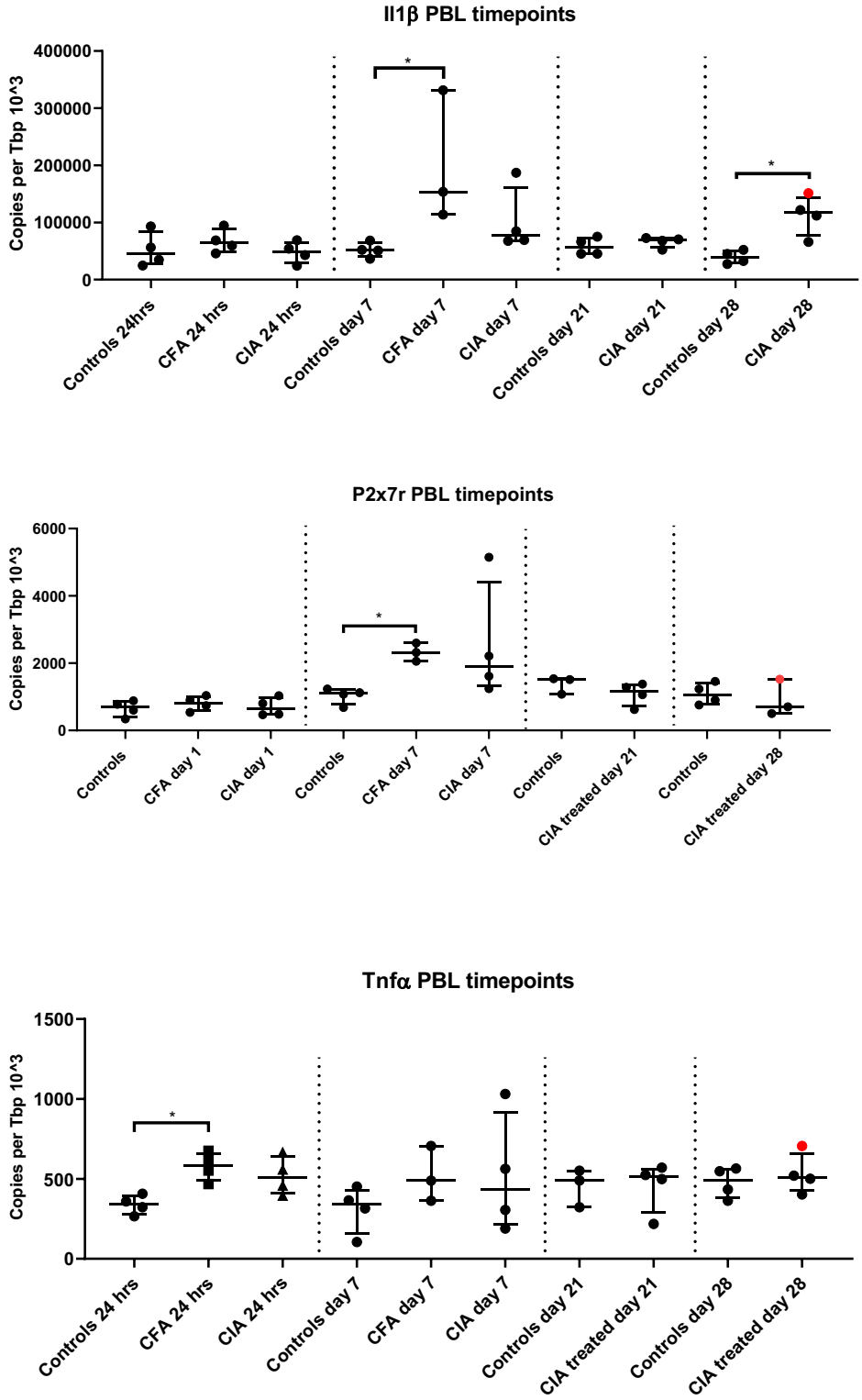


Figure 3.30 qRT-PCR analysis of PBL inflammatory transcriptional response at timepoints during the CIA model
Mice (n. 4 per group) were culled at day 1, day 7, day 21 and 28 post initiation of CFA/CIA stimulus. Blood was extracted prior to perfusion and processed to obtain PBLs. RNA was extracted and converted to cDNA for qRT-PCR analysis. A range of inflammatory cytokines and chemokines were tested. Only one mouse developed clinically detectable arthritis in the course of the experiment and is marked in red. Statistical significance was determined using Kruskal-Wallis where >2 groups analysed or using Mann-Whitney for 2 group comparison.

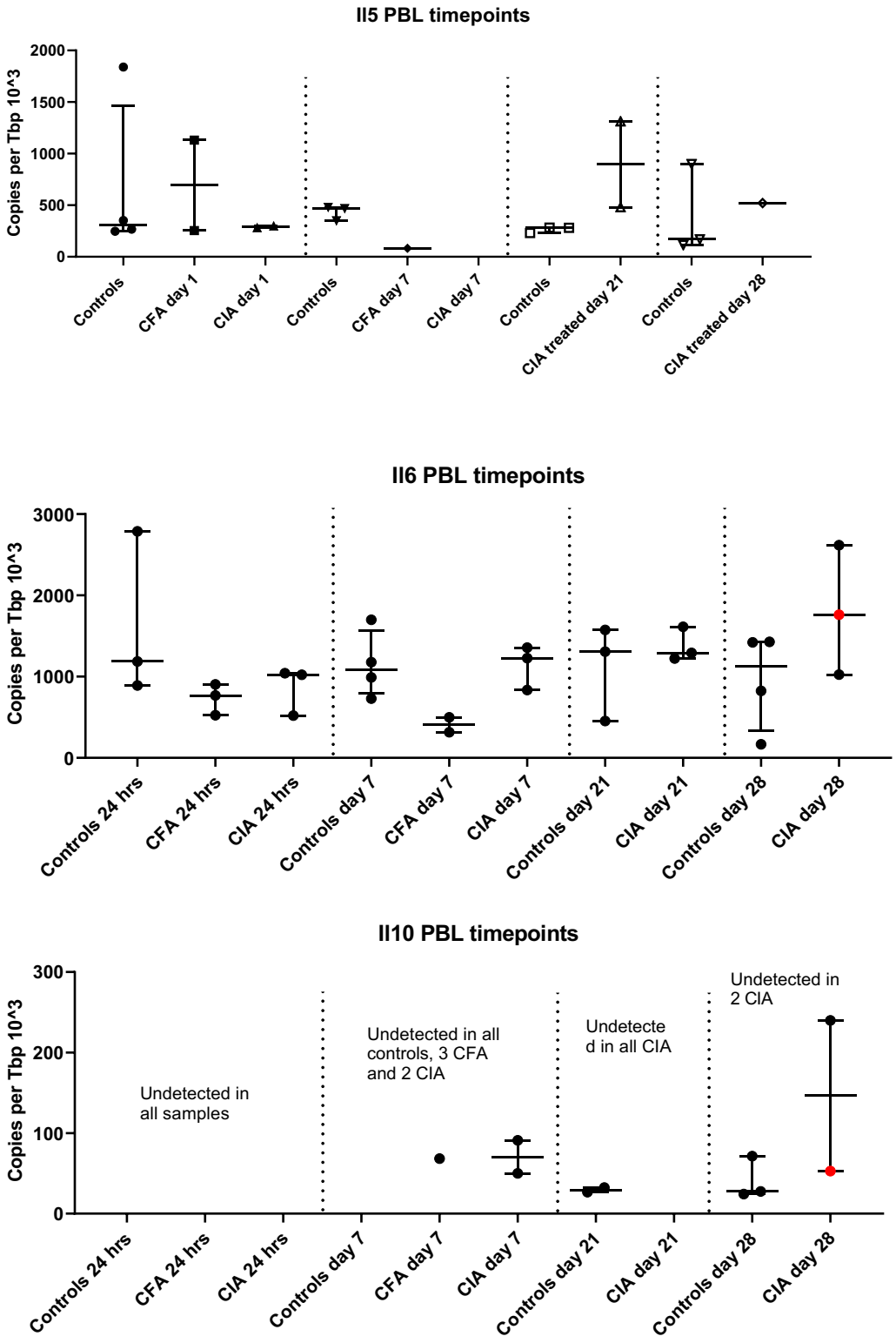


Figure 3.30 qRT-PCR analysis of PBL inflammatory transcriptional response at timepoints during the CIA model

Mice (n. 4 per group) were culled at day 1, day 7, day 21 and 28 post initiation of CFA/CIA stimulus. Blood was extracted prior to perfusion and processed to obtain PBLs. RNA was extracted and converted to cDNA for qRT-PCR analysis. A range of inflammatory cytokines and chemokines were tested. Only one mouse developed clinically detectable arthritis in the course of the experiment and is marked in red. Statistical significance was determined using Kruskal-Wallis where >2 groups analysed or using Mann-Whitney for 2 group comparison.

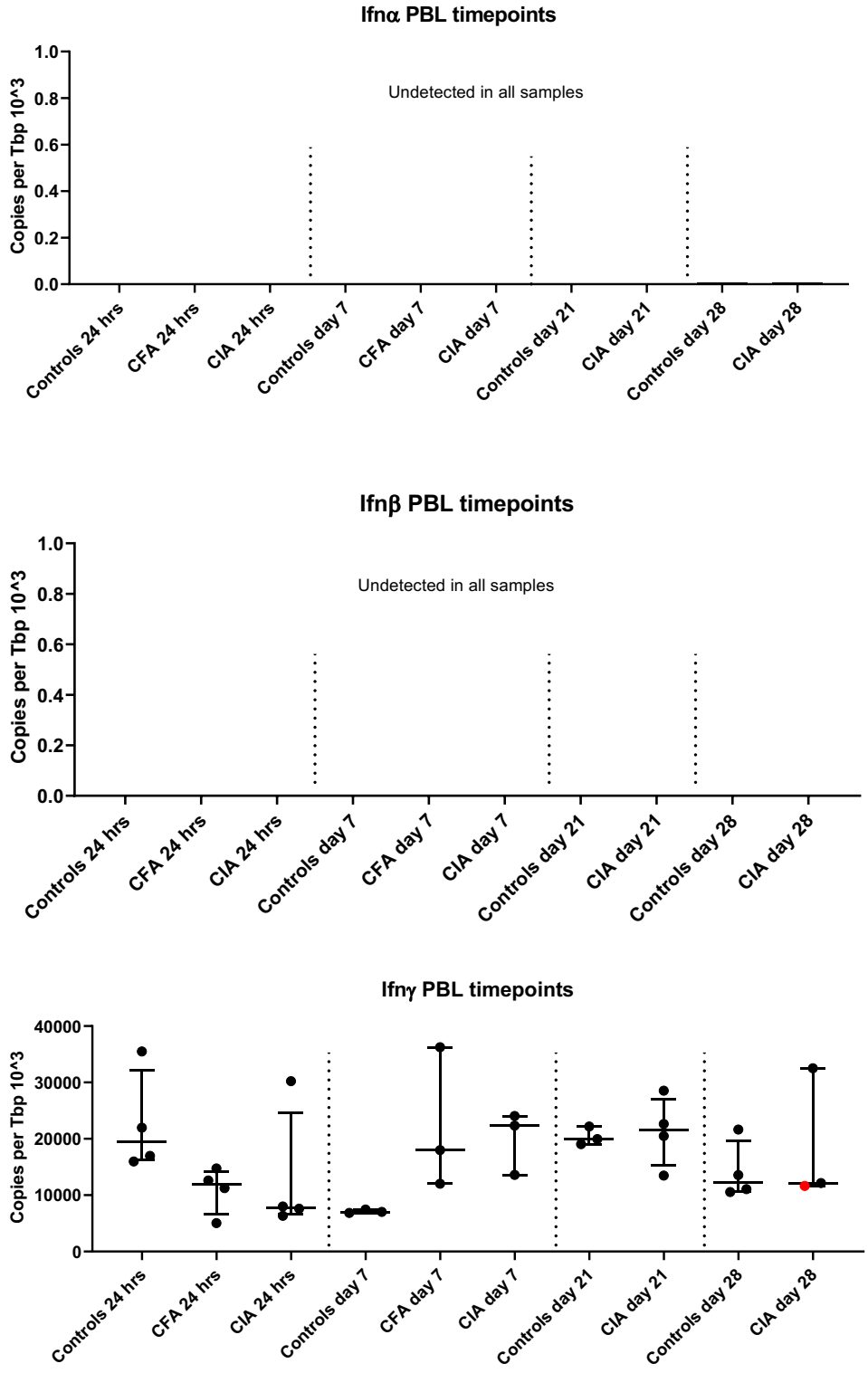


Figure 3.30 qRT-PCR analysis of PBL inflammatory transcriptional response at timepoints during the CIA model
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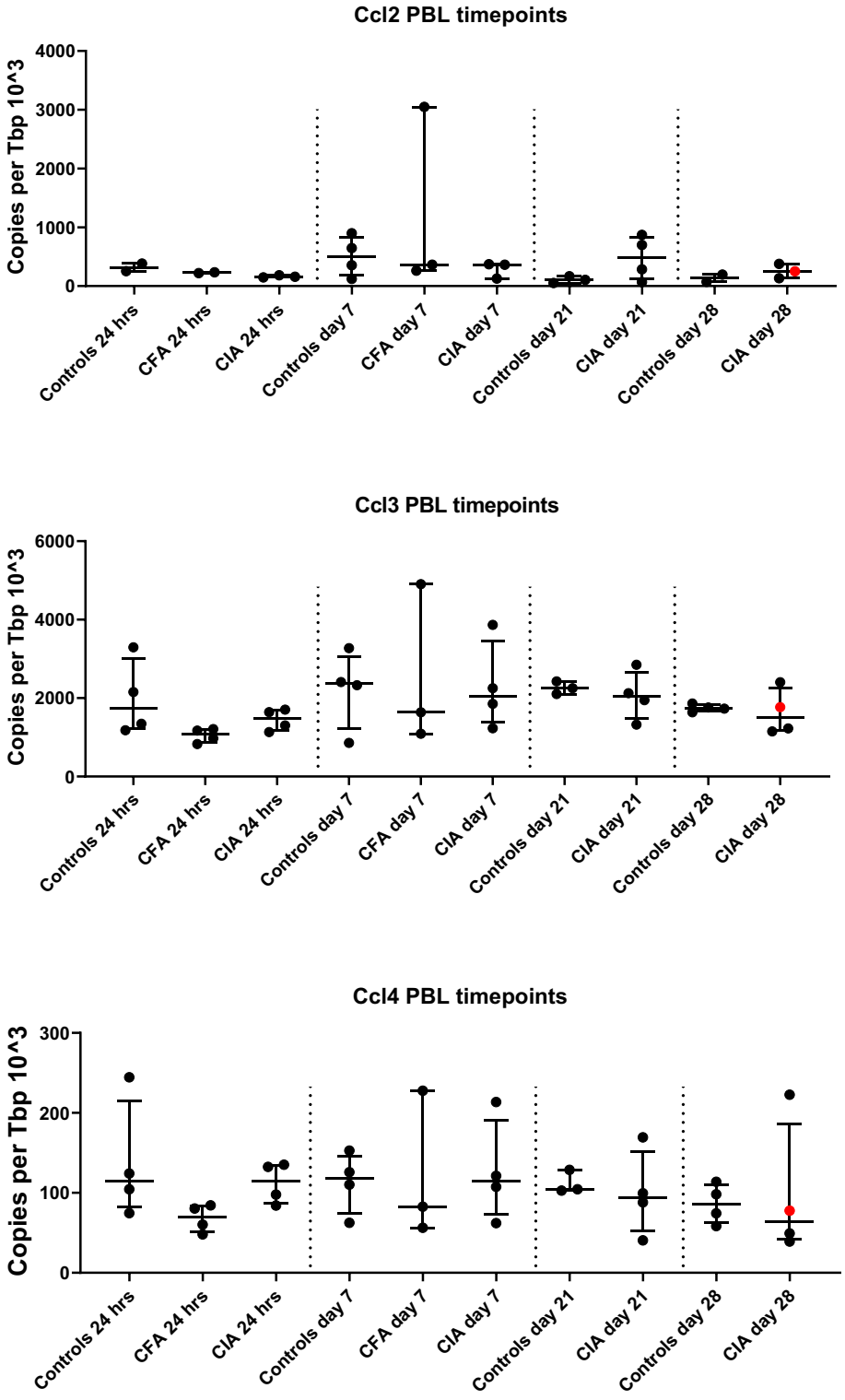
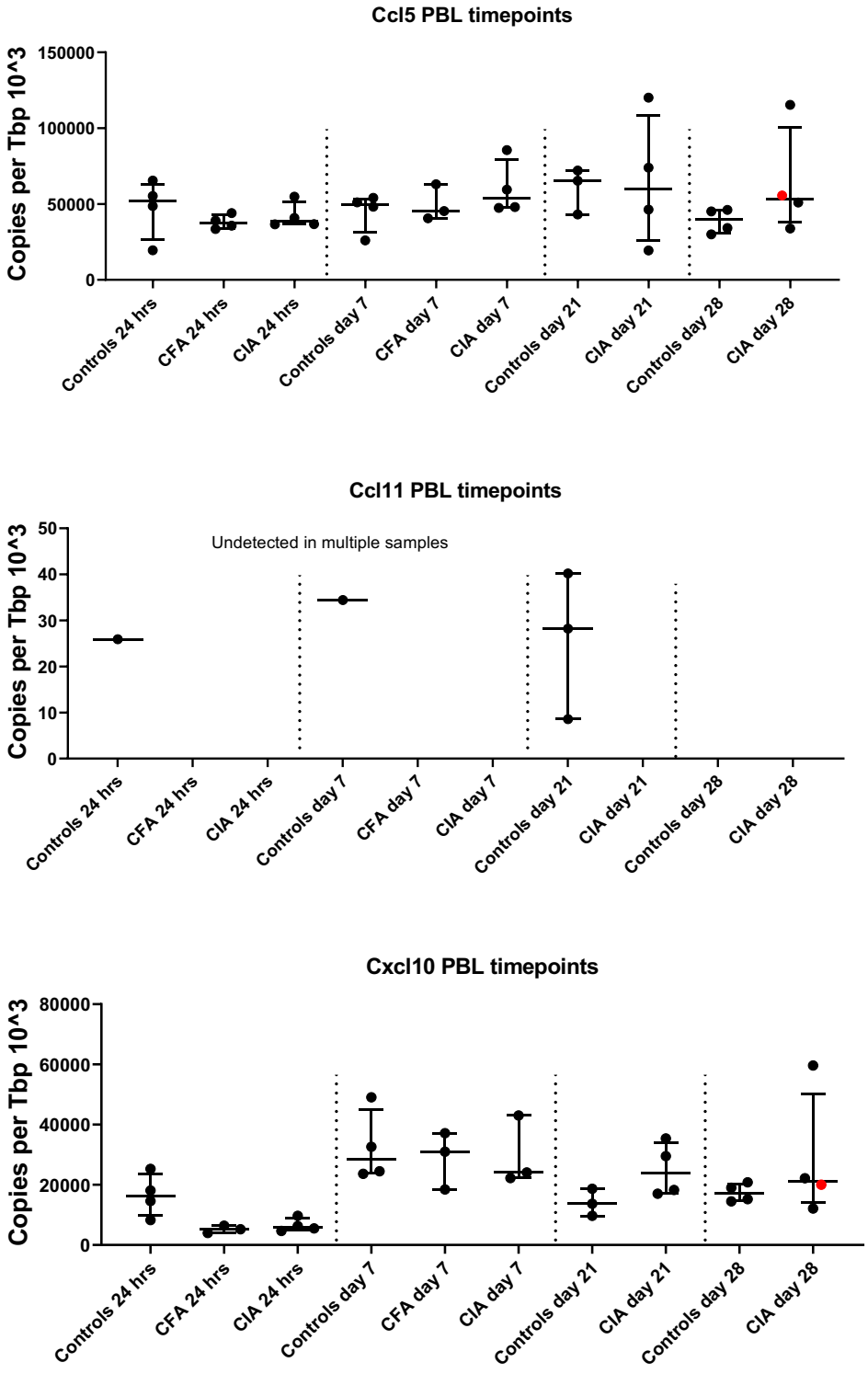


Figure 3.30 qRT-PCR analysis of PBL inflammatory transcriptional response at timepoints during the CIA model

Mice (n. 4 per group) were culled at day 1, day 7, day 21 and 28 post initiation of CFA/CIA stimulus. Blood was extracted prior to perfusion and processed to obtain PBLs. RNA was extracted and converted to cDNA for qRT-PCR analysis. A range of inflammatory cytokines and chemokines were tested. Only one mouse developed clinically detectable arthritis in the course of the experiment and is marked in red. Statistical significance was determined using Kruskal-Wallis where >2 groups analysed or using Mann-Whitney for 2 group comparison.



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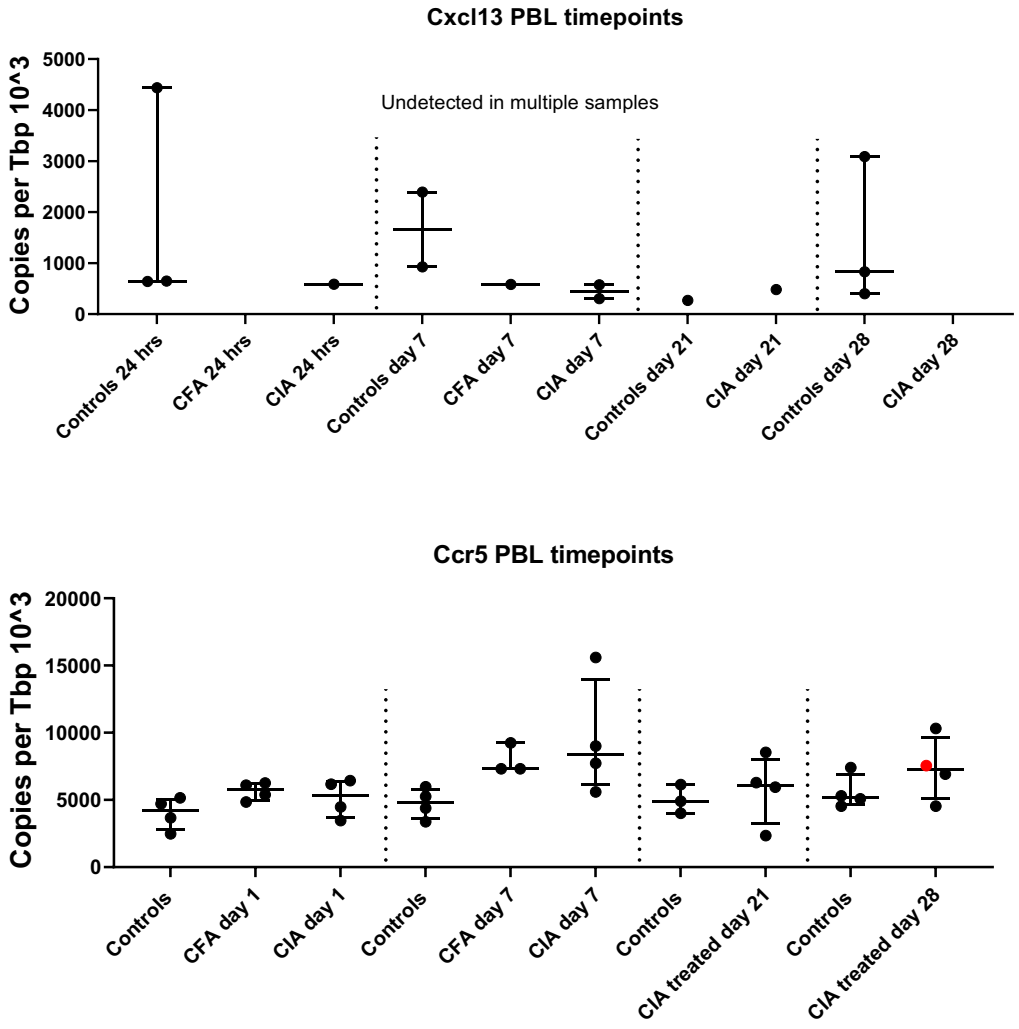

Figure 3.30 qRT-PCR analysis of PBL inflammatory transcriptional response at timepoints during the CIA model
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3.4.6 Brain transcriptional response

As in the original model described in section 3.3, brain transcription of a range of inflammatory cytokines and chemokines was measured at the predetermined time-points using qRT-PCR with absolute quantification. The results are presented in figure 3.31. A statistically significant finding of increased in brain Il1ß transcription at day 7 in response to CFA was noted, however, this was not

replicated in the CIA-stimulus group. By the later timepoints this had entirely attenuated.

Ccl2 transcription was elevated among the CFA treated group at day 1 after injection. The result was not statistically significant in the CIA-stimulus group but looking at the graph there is perhaps some indication of a distinct pattern in comparison to controls.

Cxcl1 was elevated at day 1 in the CFA treated group, but the CIA-stimulus treated group was almost identical to the controls, which somewhat undermines this finding. The result showing elevated Ccl19 at day 7 in CFA treated mice, is more convincing, as although not also statistically significant in the CIA-stimulus treated group, the grouping does look distinct from controls.

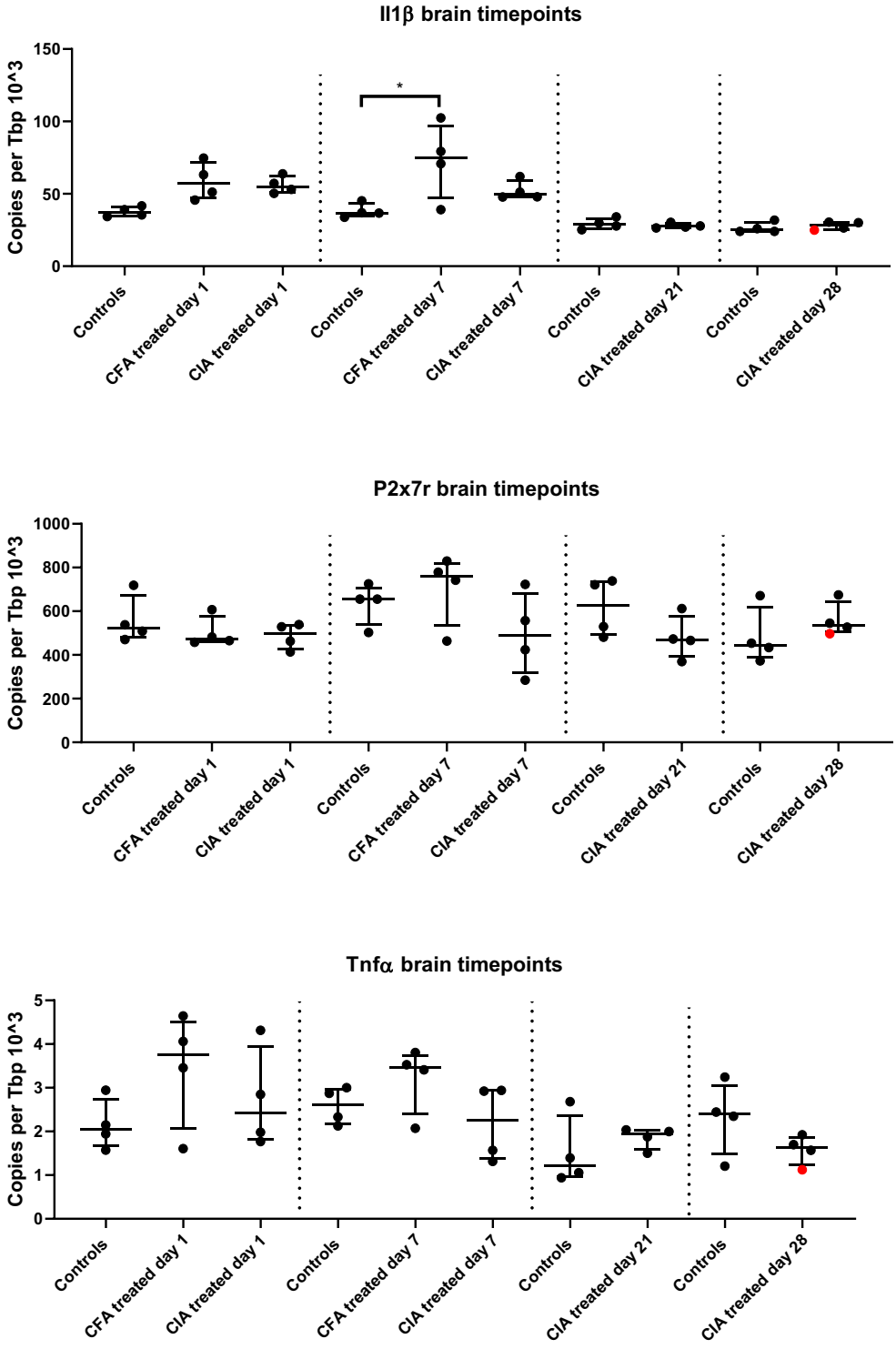

Figure 3.31 qRT-PCR analysis of brain inflammatory transcriptional response at timepoints during the CIA model

Mice were culled at day 1, day 7, day 21 and 28 post initiation of CIA stimulus. Only one mouse developed clinically detectable arthritis (marked in red). Following perfusion with ice cold PBS, brain was dissected, and the right hemisphere retrieved. RNA was extracted and converted to cDNA for qRT-PCR analysis. A range of inflammatory cytokines, chemokines and genes associated with plasticity and glial proliferation were analysed. Statistical significance was determined using Kruskal-Wallis where >2 groups analysed or using Mann-Whitney where 2 group comparison.

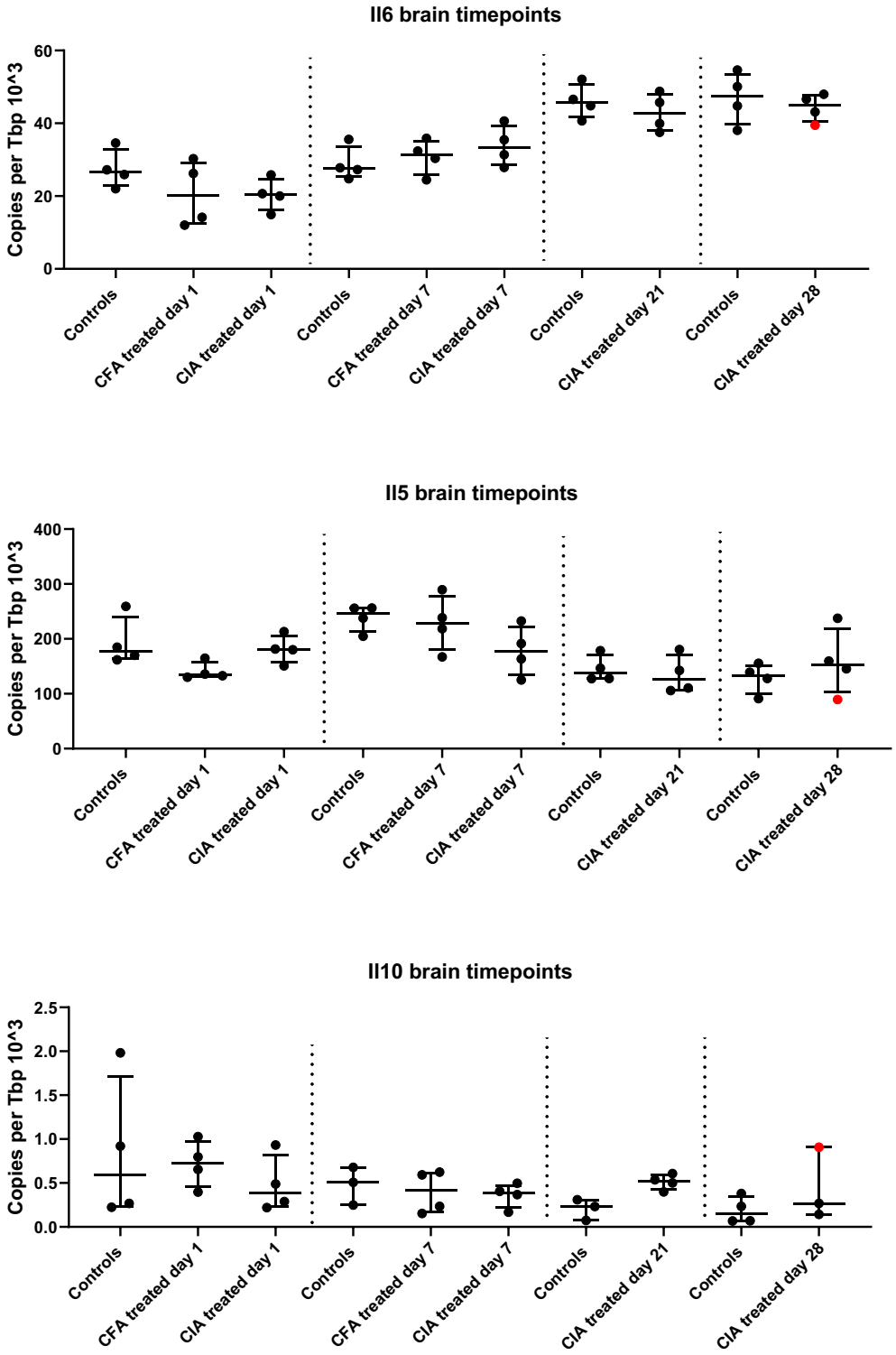

Figure 3.31 qRT-PCR analysis of brain inflammatory transcriptional response at timepoints during the CIA model
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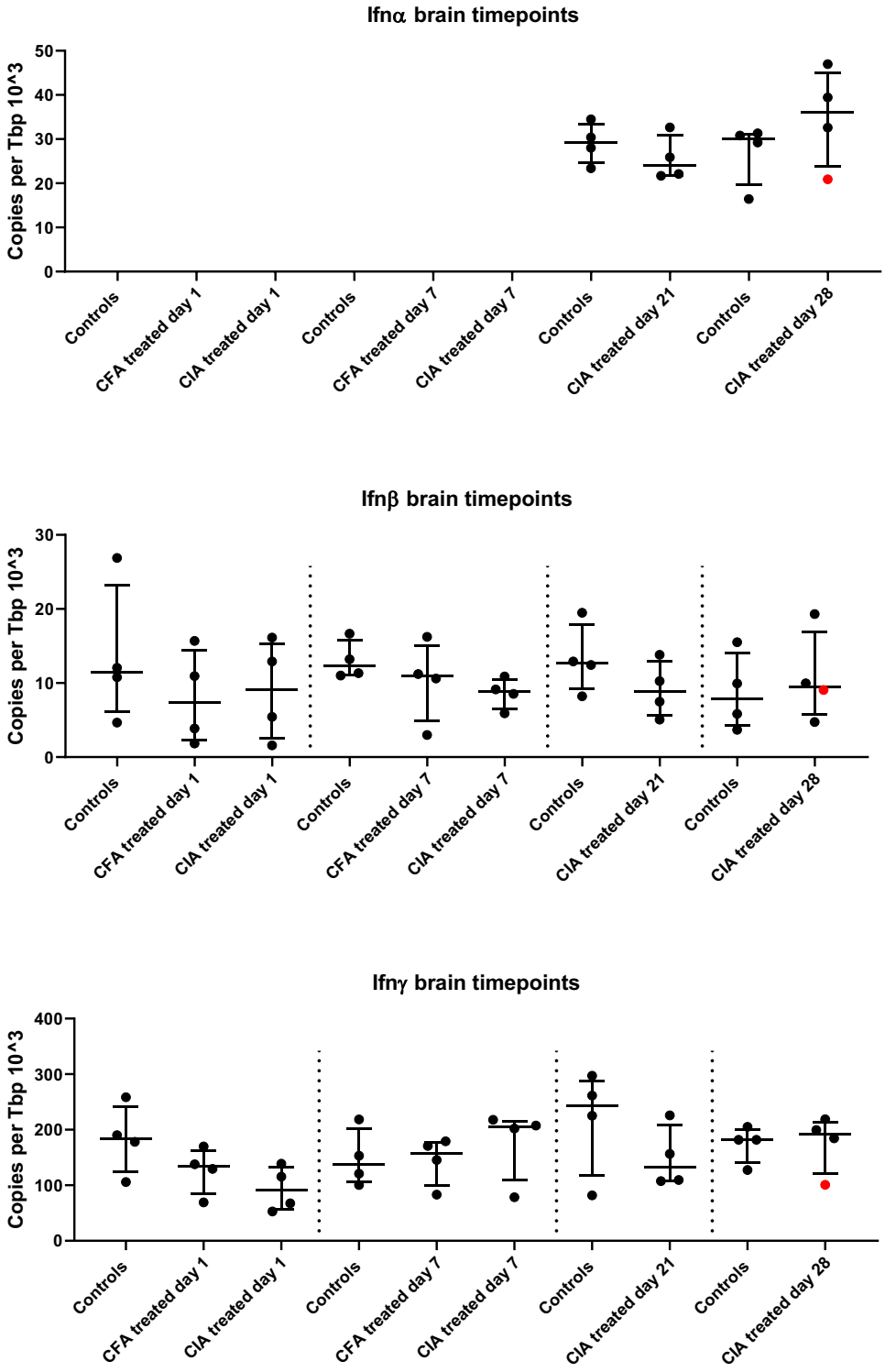

Figure 3.31 qRT-PCR analysis of brain inflammatory transcriptional response at timepoints during the CIA model
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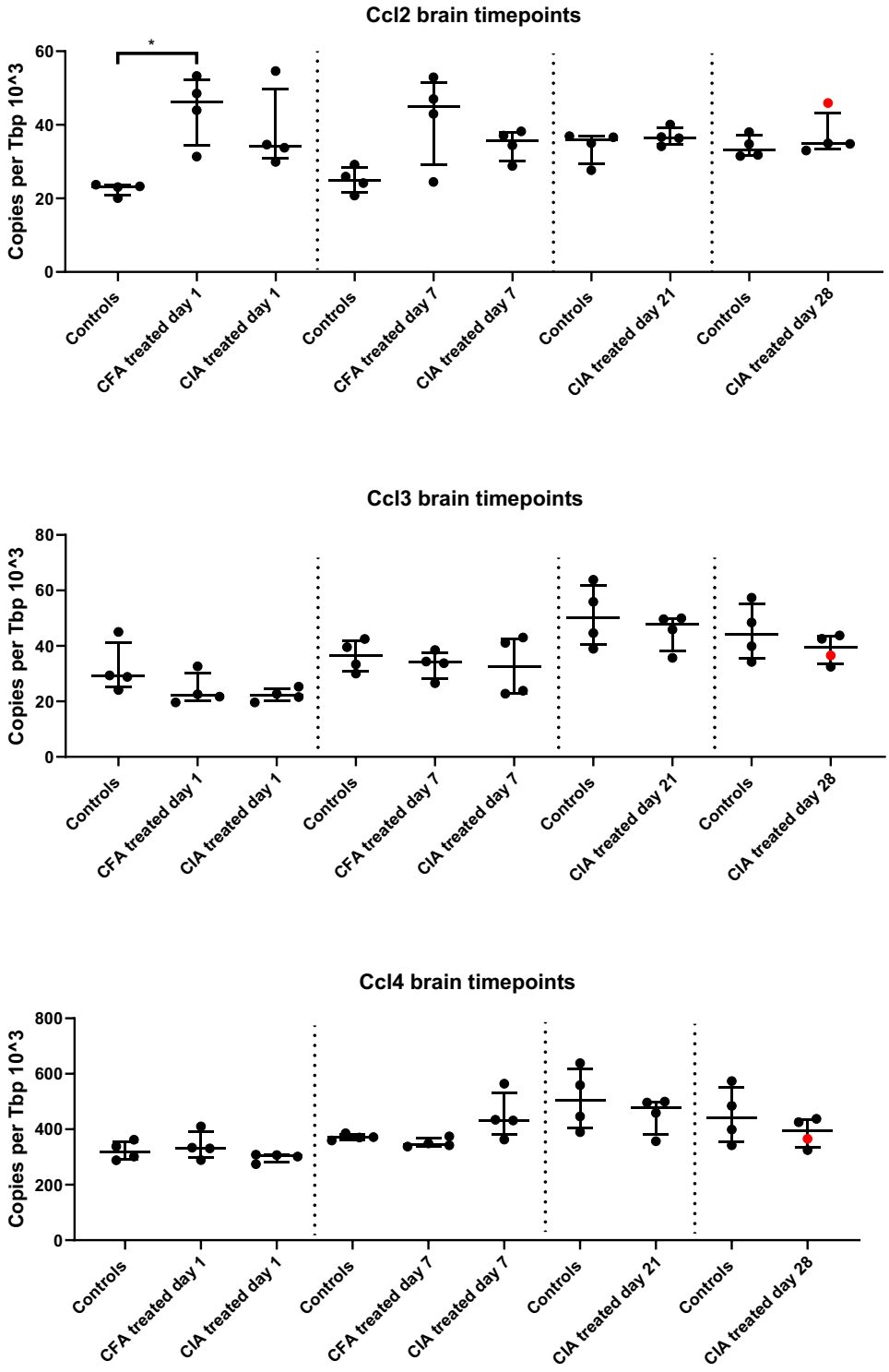

Figure 3.31 qRT-PCR analysis of brain inflammatory transcriptional response at timepoints during the CIA model
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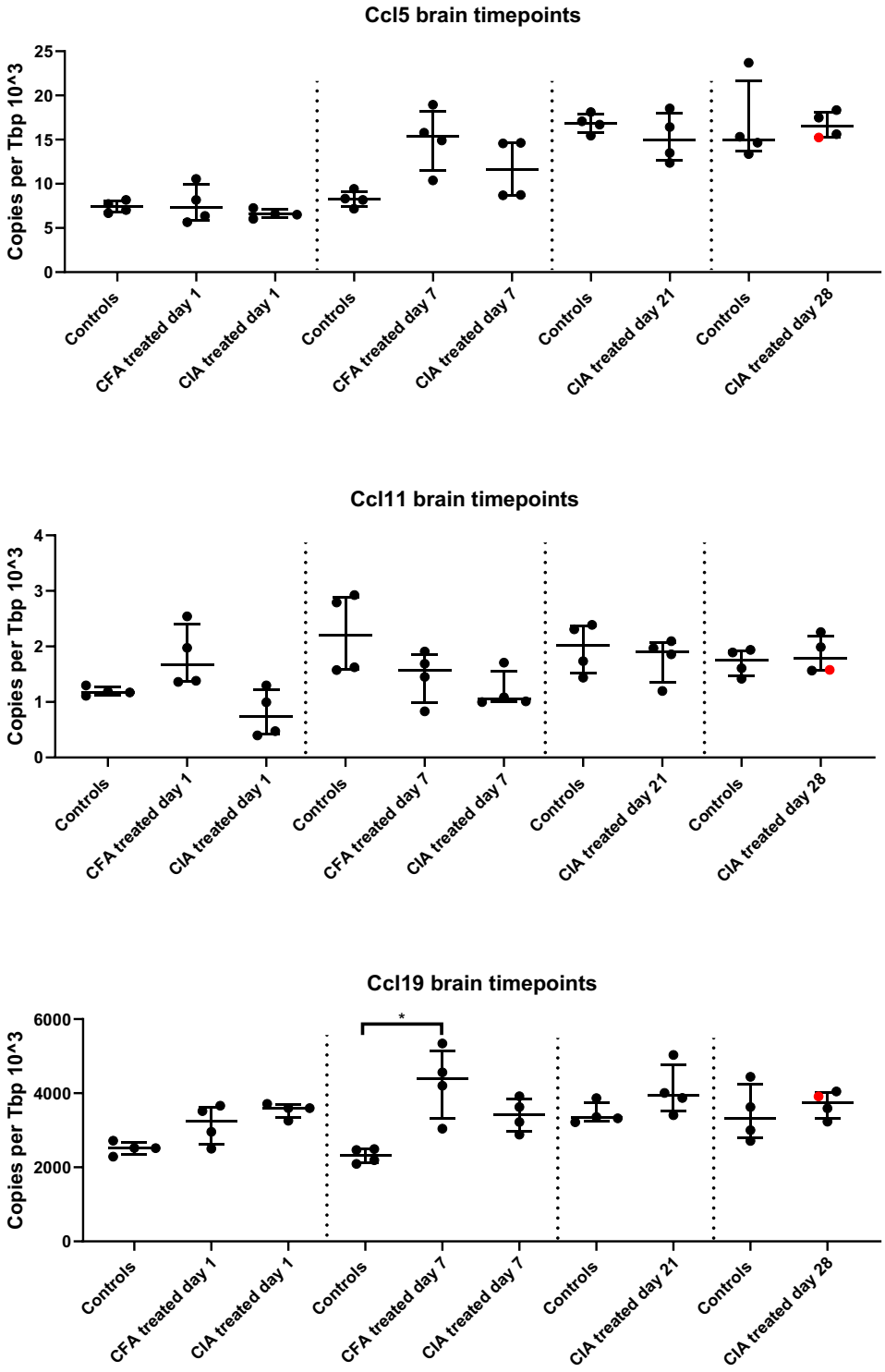

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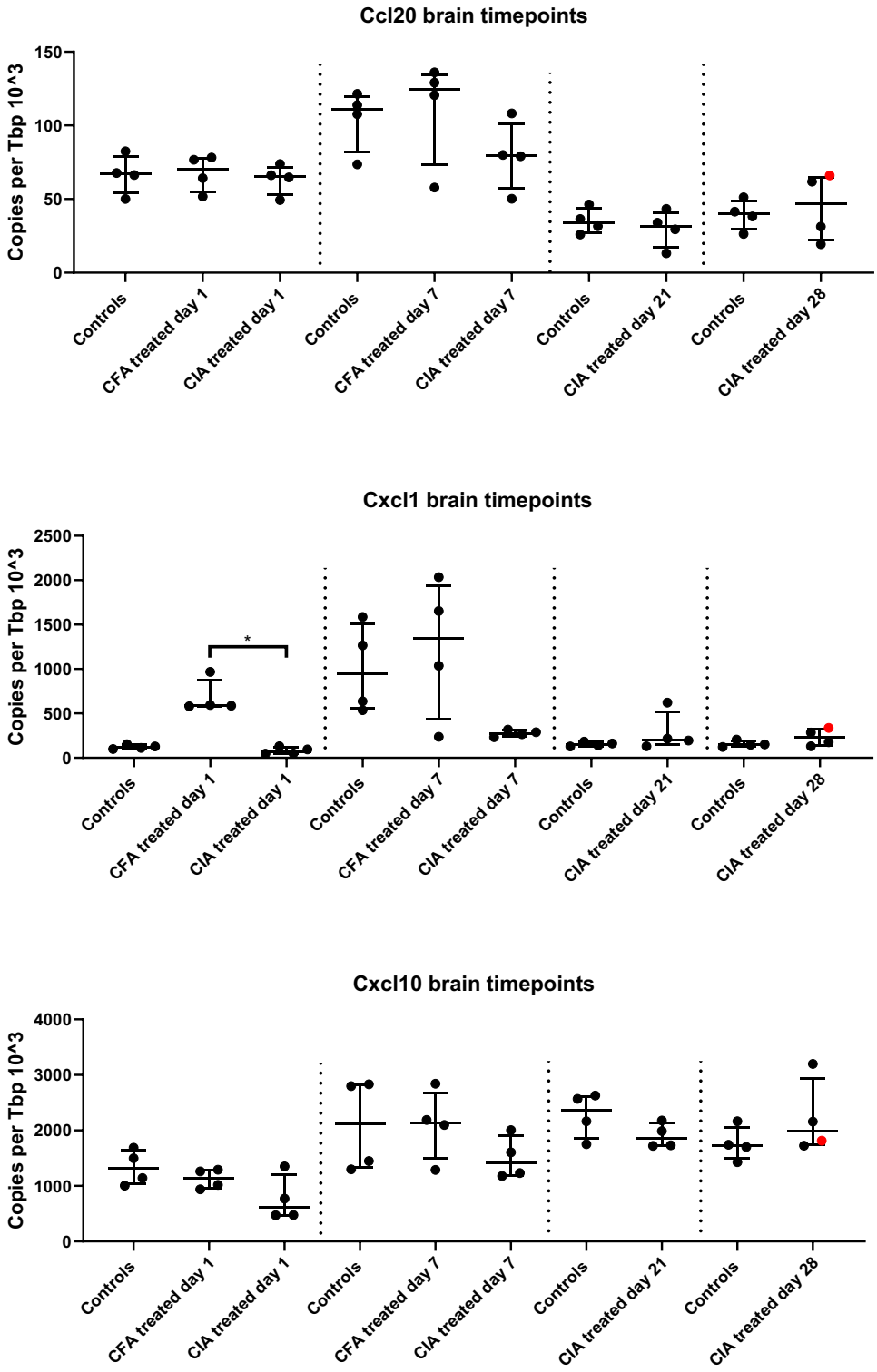

Figure 3.31 qRT-PCR analysis of brain inflammatory transcriptional response at timepoints during the CIA model

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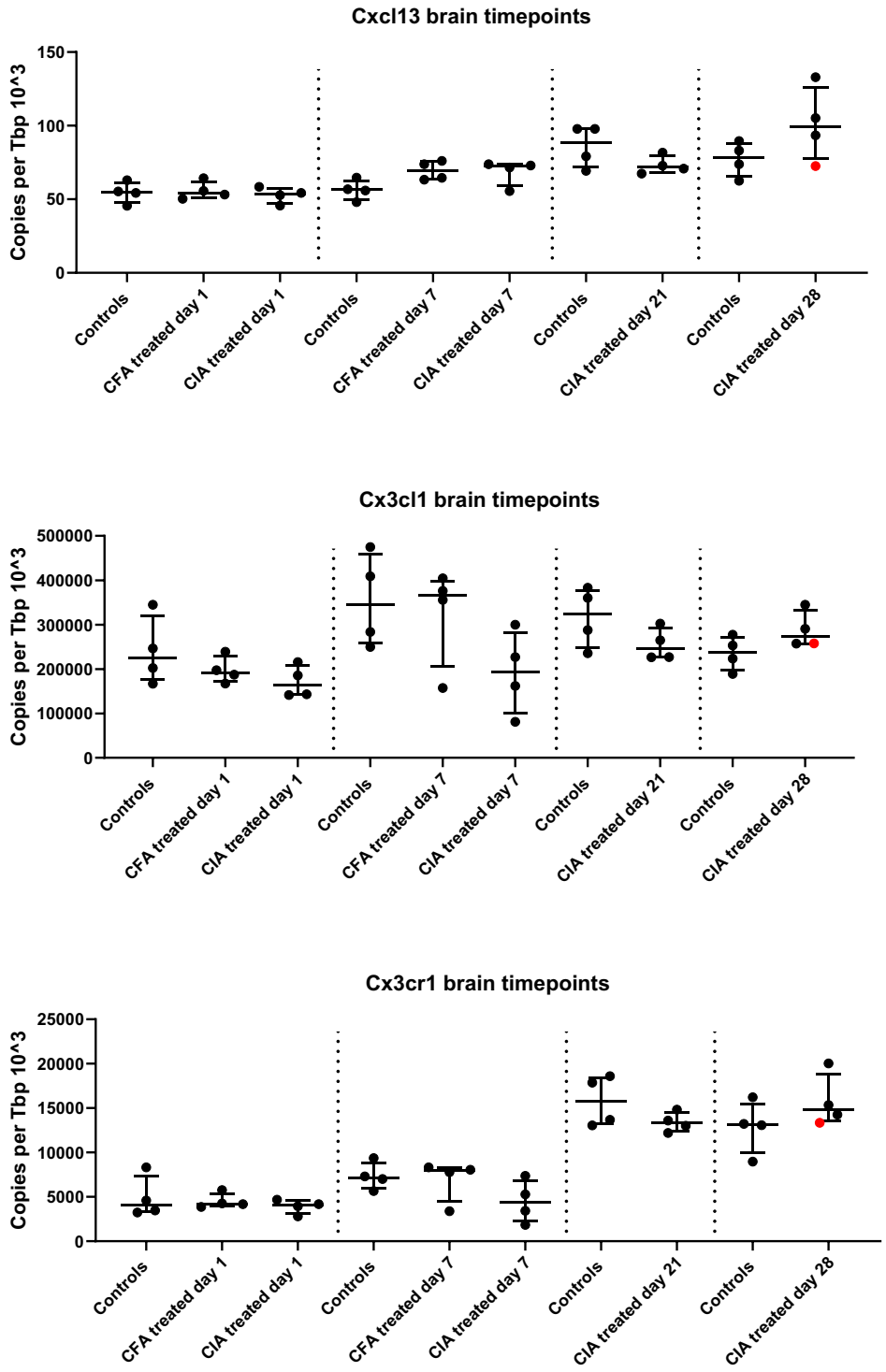
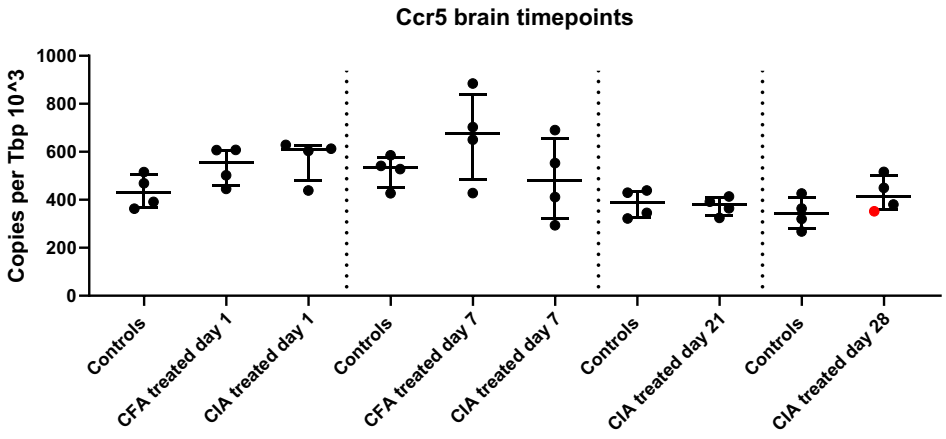


Figure 3.31 qRT-PCR analysis of brain inflammatory transcriptional response at timepoints during the CIA model

Mice were culled at day 1, day 7, day 21 and 28 post initiation of CIA stimulus. Only one mouse developed clinically detectable arthritis (marked in red). Following perfusion with ice cold PBS, brain was dissected, and the right hemisphere retrieved. RNA was extracted and converted to cDNA for qRT-PCR analysis. A range of inflammatory cytokines, chemokines and genes associated with plasticity and glial proliferation were analysed. Statistical significance was determined using Kruskal-Wallis where >2 groups analysed or using Mann-Whitney where 2 group comparison.



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3.5 Discussion

3.5.1 Introduction

The data presented in this chapter has sought to explore the original hypothesis and associated research questions set out in the introduction. The experiments presented sought to explore the hypothesis that CIA would produce a systemic inflammatory state, giving rise to a neuroinflammatory response. This hypothesis led to a series of sequential questions which were:-

1. Are there measurable changes in the brain transcription of a range of inflammatory cytokines and chemokines in response to an arthritic inflammatory stimulus?
2. Is there evidence of leucocyte recruitment across the BBB?
3. Does any inflammatory transcriptional response associate with glial cell changes?
4. Is there evidence of neurotoxic processes such as altered iNOS expression?

5. Are there changes related to neuroplasticity, such as hippocampal neurogenesis?

Following on from these initial questions, a further hypothesis and research question was generated exploring whether a distinct inflammatory transcriptional pattern occurred at earlier timepoints in the model was generated, leading to the time-points model.

The results of my experiments in response to these research questions is summarised and discussed below.

3.5.2 The peripheral inflammatory response in CIA

3.5.2.1 The arthritic response to the CIA stimulus

The animal models were successful overall in producing a characteristic CIA arthritic response. Penetrance of clinically detectable arthritis across the models was 71.4%, which is slightly below the usual range reported within the literature (>80%) (Brand et al., 2007) but is in line with disease penetrance locally reported by experienced colleagues working with this animal model. A feature of the arthritic response was its variability in terms of timing of onset and severity. This variability is a noted feature of CIA (Holmdahl et al., 2002).

Single IP injection of LPS between day 17 to 24 of the model has been reported in the literature to improve synchronicity of onset of arthritis (Caccese et al., 1992) (Hou et al., 2013). However, the problem of variation of severity remains. In the experiments reported in this thesis, it was decided not to follow this approach as we were interested in the effects of joint inflammation on the brain. To introduce an exogenous inflammatory stimulus such as LPS injection would change the nature of the experiments such that the relevance to joint inflammation would be undermined as it would not be possible to determine whether brain responses observed were related to arthritis or LPS.

3.5.2.2 Weight loss in CIA

Arthritic mice showed significant weight loss, likely consistent with a combination of systemic inflammation, pain, reduced feeding and disease-activity. The weight difference was only significant between controls and

arthritic mice, suggesting clinically detectable arthritis was an important factor in the emergence of weight loss.

3.5.2.2 Splenomegaly in CIA

Readily detectable spleen enlargement was found within CIA mice, in line with previously reported findings in CIA (Billiau and Matthys, 2011). The spleen is a secondary lymphoid organ with functions that include erythrocyte homeostasis, pathogen/antigen filtration and regulation of systemic immune responses (Bronte and Pittet, 2013). Systemic inflammation is known to represent a causal factor in mammalian splenomegaly (Loukov et al., 2016). There were distinct statistically significant differences in spleen weight between controls, CIA non-arthritic and CIA-arthritic mice. This would suggest that the effects of arthritis on spleen weight are distinct or additive to the effects of CFA. CFA by itself is understood to provoke marked excess spleen myelopoiesis and consequent spleen hyperplasia and structural disorganization (Billiau and Matthys, 2011). This is shown later in the chapter when CFA-only controls were used in the experiments examining stage-dependent inflammatory responses. Taken as a whole, these results reinforce that in addition to the effects of CFA, there is an additive effect of arthritis that exerts a significant effect upon the spleen over and above this, most likely due to immune stimulation and inflammation.

3.5.2.3 Blood measures of inflammation in CIA

The PBL transcriptional response was surprisingly muted with only Cx3cl1 being found to be upregulated in arthritic vs. control mice. In peripheral blood CX3CL1 is expressed by activated leukocytes (Ferretti et al., 2014) and augments T cell activity, facilitates monocyte recruitment, and promotes inflammation (Thelen and Uguccioni, 2016).

The lack of a broader transcriptional response in PBLs was perhaps surprising. Previous work has shown that CIA does cause elevated plasma protein levels of several inflammatory cytokines including TNFα, IL5 and IL6 (Teixeira et al., 2019). Indeed, elevated levels of IL6 have been shown to be a necessary condition for the development of arthritis in CIA (Alonzi et al., 1998). The experiments presented in this thesis found CIA to be associated with elevated levels of IL6 in the plasma of arthritic mice. This finding was in line with the

established literature. If the source of IL6 was from PBLs, then it is possible that at the time of culling, the transcriptional event for IL6 in PBLs had already concluded, though, in the context of an ongoing inflammatory process this seems unlikely. More likely, the source of plasma IL6 originates from an alternative cellular source, such as tissue macrophages, joint fibroblasts, lymphoid tissue, lymph nodes, synovium or other sites of immune activation or inflammation.

IL6 is a key cytokine involved in the pathogenesis of arthritis in CIA (Alonzi et al., 1998). In human RA, IL6 is thought to influence development of arthritis by a variety of mechanisms, including activation of endothelial cell production of chemokines and recruitment of leukocytes to sites of inflammation (Romano et al., 1997), and by inducing synovial fibroblast proliferation (Mihara et al., 1995). Chronically high systemic levels of IL6 in humans is associated with elevated risk of developing depression, fatigue and cognitive impairment (Felger and Lotrich, 2013).

TNFα, IL17, IFNγ and IL1β are important cytokines in both CIA and human RA (Notley et al., 2008) (Joosten et al., 2008) (McCann et al., 2014) but no differences were seen in the experiments presented here. Albeit, many of the measurements were below the detection threshold in the Multiplex array, raising the possibility that a more sensitive assay could have detected more subtle differences.

In searching for a systemic inflammatory response I investigated plasma. Alternative methods such as examining relevant tissues such as spleen or lymph nodes may have yielded more evidence of differences in inflammatory transcription or protein expression. Alternatively, it is possible that for some technical reason my CIA models exhibited a smaller than expected systemic inflammatory response.

Thesis data is available from a former group member who completed work on CIA and examined serum levels of inflammatory cytokines and chemokines (Sutthiwarotamakun, 2011). Elevated serum protein levels of IL1β, IL1α, CXCL1 and FGF2 were observed. Surprisingly IL6 was not elevated in those experiments, which as discussed earlier is thought to be important for arthritis emergence in CIA. Sutthiwarotamakun observed similar rates of arthritis (63%)

and variability in disease severity and timing of disease emergence. However, all mice were culled at day 42. It may be that severity thresholds and necessity to cull mice at an earlier stage were not an issue during these experiments – there is no mention within the animal welfare standards of severity thresholds and data within the thesis suggests disease scores for a number of mice exceeded the threshold that is currently used. A similar Luminex assay was used, but serum rather than platelet-free plasma was tested. The differences therefore could be attributable to variation in CIA disease or to technical factors such as tissue preparation or protein detection.

3.5.3 The brain inflammatory response in CIA

3.5.3.1 Brain inflammatory transcription in CIA

CIA was found to be consistently associated with upregulation of brain Il1β transcription. In relation to Il1β, there was also a significant increase in P2x7r transcription, a key receptor involved in mediating the release of Il1β (Di Virgilio et al., 2017). P2X7 receptor is expressed by almost all cells of the innate and adaptive immune system, including microglia (Di Virgilio et al., 2017). It activates innate immunity by promoting the NLRP3 inflammasome (Di Virgilio, 2007) and consequent production and non-canonical release of pro-inflammatory cytokines, particularly IL1β (Dubyak, 2012). Within the CNS the P2x7r is thought to play an important role in the activation and proliferation of microglia (Monif et al., 2009) promoting neuroinflammation with potentially neurotoxic consequences (Janks et al., 2018).

The increased brain transcription of Il1β was present only in the arthritic group, not the CIA-stimulus non-arthritic group, suggesting it was likely that the response was a result of the ongoing arthritic process, rather than the stimulatory events earlier in the model to which the non-arthritic CIA-protocol treated mice were also exposed. A significant relationship between arthritis severity and brain Il1β transcription level was demonstrated using simple linear regression. Severity appeared to be more important than chronicity, which did not significantly predict brain Il1β transcription. A further regression analysis exploring the relationship between brain Il1β transcription and plasma protein levels of IL6 did not find a significant relationship. These results were confirmed

with exploratory multiple linear regression analysis. To some extent this weakens the strength of the hypothesis that systemic inflammation is directly causative of elevated Il1ß transcriptional brain response, as one would expect a dose-effect relationship if there was a causal relationship. However, IL6 is only one very particular measure of systemic inflammation. The experiment may not have been powered adequately to detect a subtle relationship.

There were no detectable differences in the brain transcription of other inflammatory cytokines. There was no clear association between arthritis and chemokine transcription aside from modestly lower Ccl3 and Ccl4 transcription in the arthritic group vs. controls. The Ccl3 result was not replicated in the subsequent qRT-PCR analysis of hippocampus and rest of brain, presented in section 3.3.2, though higher Ccl4 transcription was observed in control hippocampus. The possibility of a type 1 error cannot be fully excluded, though this seems less likely given the replication in further qRT-PCR experiments. CCL4 interacts with receptor CCR5, along with CCL3 and CCL5, to produce a range of effects, including recruitment of leukocytes, and pro-tumourigenic actions (Mukaida et al., 2020). Ccl4 can be expressed by microglia and astrocytes, and elevated levels have been associated with neurodegenerative disorders (Sevenich, 2018). Although generally thought to be associated with deleterious brain effects (Cartier et al., 2005), there is some evidence that elevated CCL4 can be neuroprotective under certain circumstances (Kaul and Lipton, 1999). One could speculate that some process in the brain response in CIA could lead to reduced Ccl4 transcription in concert with brain other changes.

As with the work on the peripheral response in CIA, the brain response in CIA in these experiments also varies to some extent in comparison to earlier work. In keeping with the results presented here, Sutthiwarotamakun reported no difference in brain transcription of Il6 and Tnfα, despite finding elevated protein levels and hypothesised that the brain transcription event had already finished with resultant increased protein levels (Sutthiwarotamakun, 2011). Sutthiwarotamakun also reported elevated Ifnγ, Cxcl1 and Cxcl10 transcription but no difference in Il1ß. As with the differences in results from the peripheral tissues, the differences in the brain response compared to Sutthiwarotamakun's work may also be attributable to variation in CIA disease scores/chronicity. As

with all laboratory experiments, differences arising from to technical factors such as tissue preparation and/or gene detection, cannot be completely excluded. Reassuringly my finding of elevated brain Il1ß transcription has been reported in the published literature in a study by Andersson et al (2018) that examined the brain response in CIA (Andersson et al., 2018).

3.5.3.2 Hippocampal inflammatory transcription in CIA

Further experiments were undertaken to examine the brain response in the hippocampus compared to the rest of the hemisphere. The hippocampus was chosen as a region of interest due to its involvement in a range of neuropsychiatric disorders including mood disorders, psychosis, and Alzheimer's dementia (Antoniades et al., 2018) (Chepenik et al., 2012) (Videbech and Ravnkilde, 2004) (Halliday, 2017). Previously published evidence indicates altered hippocampal structure/function in response to inflammation (Chesnokova et al., 2016). Previous work in CIA has found elevated levels of Il1ß mRNA along with other inflammatory changes, such as IBA1+ cell enrichment, within the hippocampus of arthritic mice (Andersson et al., 2018).

The increased brain transcription of Il1ß in the context of arthritis remained a consistent finding. This was evident in both the hippocampus and in homogenate of the rest of the hemisphere. The increased P2x7r mRNA transcription was only evident in the rest of hemisphere, not the hippocampus. As discussed above, IL1ß is a key pro-inflammatory cytokine with diverse effects on immune and non-immune cells in the context of inflammation. Activity of the P2X7 receptor is particularly important in facilitating the release of IL-1ß (Monif et al., 2016). It may be therefore that whilst Il-1ß mRNA levels are elevated in hippocampus and the rest of the hemisphere, that areas where there is subsequently elevated IL1ß protein translation and release lie outwith the hippocampus.

As discussed earlier, the higher rate of Ccl4 transcription in controls observed in the initial qRT-PCR experiment was reproduced in control hippocampus but not rest of hemisphere. Transcription of Ccl11 appeared to be increased in the arthritic mouse brain hemisphere excluding hippocampus. Similarly, for Cxcl1. Il5 appeared to be higher in control hippocampus compared to arthritic.

CCL11 is involved in the pathophysiology of allergy and is most extensively characterised in relation to eosinophil recruitment, but is also involved in basophil and Th2 lymphocyte chemotaxis (Menzies-Gow et al., 2002). In the context of the CNS, CCL11 has been shown to have an inhibitory effect on neurogenesis when injected stereotactically to the hippocampus (Villeda et al., 2011).

CXCL1 can be expressed by glial cells and can also have stimulatory effects upon glial cells, and can be upregulated by IL1ß (Omari et al., 2006). This relationship has been shown to accelerate neurodegeneration in mouse prion disease (Hennessy et al., 2015). Elevated CXCL1 in the context of experimental autoimmune encephalomyelitis (EAE) has been shown to accelerate neurotoxicity (Grist et al., 2018) (Omari et al., 2009). CXCL1 is implicated in chemotaxis and recruitment of neutrophils to the CNS and in neurodegeneration (Murphy et al., 2000). Interestingly, CXCL1 is also implicated in mediating inflammatory pain modulation (Silva et al., 2017), which may be germane to the CIA model.

CCR2 is an inflammatory chemokine receptor, for which CCL2 is chemoattractant (Van Coillie et al., 1999). CCR2 is expressed by monocytes and microglia, and upregulation of expression of CCR2 by these cells can correlate with neuroinflammation and is observed with systemic administration of LPS (Cazareth et al., 2014). CCL2 and its receptor CCR2 have been shown to be upregulated in a range of neurodegenerative pathologies (Bose and Cho, 2013). As noted, Ccr2 was elevated in mouse brain excluding hippocampus in my experiments.

IL5 is a cytokine first characterised as a B cell growth factor expressed by T cells (Harada et al., 1985) and is also expressed by glial cells (Sawada et al., 1993). IL5 can have mitogenic effects upon microglia, stimulating proliferation with potentially protective effects in the context of injury or infection (Ringheim, 1995). It is not clear why a higher level of Il5 transcription was observed in control hippocampus.

In conclusion, the results from these qRT-PCR experiments suggested further avenues for investigating the brain response to CIA. The elevated transcription

of Il1ß mRNA levels in concert with elevated levels of P2x7r mRNA and of select chemokines, point to an inflammatory brain response that could involve inflammasome activation, glial cell activation and leukocyte recruitment across the BBB. These areas were subsequently investigated.

3.5.3.3 Leukocyte recruitment across the BBB in CIA

As discussed, select differences were observed in chemokine transcription in CIA mouse brains – modestly raised Cxcl1, Ccl11 and Ccr2. Flow cytometry was undertaken to determine if this transcriptional signature was associated with the presence of substantial differences in leukocyte populations within the brain. Populations of microglia, T cells, B cells, NK cells, neutrophils and monocytes were explored, but no significant differences were observed.

Brain leukocyte recruitment in the context of systemic inflammation is an area of ongoing research in the field. Systemic inflammation is believed to cause changes in the BBB that may facilitate leukocyte recruitment (Varatharaj and Galea, 2017). In the context of CIA, brain leukocyte recruitment has not been explored in the literature.

RA and other inflammatory rheumatic diseases are known to be associated with increased burden of cerebrovascular disease (Wiseman et al., 2016) (Kitas and Gabriel, 2011). This is thought to in part reflect a phenomenon of microvascular endothelial dysfunction in the context of a systemic proinflammatory state (Bordy et al., 2018). It is not known whether this phenomenon could result in increased permeability of the BBB. Increased BBB permeability is one of the mechanisms by which peripheral inflammation can be communicated to the brain (Chavan et al., 2017).

A 2010 study explored BBB in CIA in mice and found evidence of disruption of the BBB, particularly in CIA mice with very chronic persistent arthritis lasting 51-100 days since initiation of protocol, less so in the 21-50 day group (Nishioku et al., 2010). They did not look at leukocyte recruitment but instead focussed on levels of sodium-fluoroscein (Na-F) dye penetrating the brain and expression levels of TJ proteins. All mice in my experiments would fall into the less chronic group (21-50 days since inoculation). Although presence of Na-F within the brain can suggest altered BBB permeability, the size of a molecule of Na-F is many orders

of magnitude smaller than a leukocyte. Only active transport of large proteins and cells across BBB can occur under most circumstances.

The absence of any differences in leukocyte numbers between control and CIA mice in my experiments may still be in keeping with Nishioku et al's (2010) study showing differences in BBB integrity in CIA. BBB permeability may increase, without leukocyte recruitment. Alternatively, it may be that the BBB becomes more permeable at later time points than I measured and that leukocyte recruitment across the BBB, if it ever occurs, is a much later more chronic phenomenon - Nishioku et al reported the greatest degree of BBB permeability at 51-100 days.

The data presented in this thesis suggests that in the mid-stages of CIA (13-21 days of arthritis), leukocyte recruitment to the brain is not a major component of the brain response.

3.5.3.4 Glial cell response in CIA

In the context of the finding of upregulated brain Il1ß transcription in arthritic mice, a series of experiments were undertaken to determine if glial cell activation and proliferation was associated with this finding. There was no coincident evidence of hippocampal glia activation or proliferation, either in terms of IBA1+ and GFAP+ cell staining or at the transcript level. No significant differences were seen in IBA1+ or GFAP+ cell staining of the cortex or striatum. A statistically significant increased density of IBA1+ cells was observed in the thalamus of the CIA arthritic mice in comparison to controls. There were no significant differences in GFAP+ cell counts in thalamus.

My findings conflict with some published data which have suggested that CIA is associated with increased IBA1+ cell density within the hippocampus of arthritic mice, particularly within the DG (Andersson et al., 2018). There were some differences between the running of Andersson et al's animal experiments and my own. For example, it appears that they allowed mice to progress beyond the severity threshold of 10 on the UK Home Office Project Licence under which I worked (Andersson et al are not based within the UK). Additionally, they report less variation in severity and onset of arthritis, with all mice showing arthritis from day 21 and final severity scores ranging from 12 to 16. All mice were

culled at day 42. This uniformity and higher overall severity over a consistent period may have contributed to the difference seen in their experiments compared to my own. Reassuringly, Andersson et al found elevated Il1ß transcription within the hippocampus, echoing my experiment findings. They also reported markedly elevated plasma IL6, in accord with my findings.

Microglial proliferation and increased density of IBA1+ cells in the thalamus, as was found in my experiments, has been reported by Suß et al in the context of the hTNFtg transgenic mouse model of RA (Süß et al., 2020a). Their study also reported elevated IBA1+ cell populations in the cortex and striatum of arthritic mice, contrary to my findings in the CIA model. CIA is distinct from the hTNFtg model in many important aspects. The hTNFtg model involves ectopic over-expression of TNFα – a cytokine which was not found to be elevated in the plasma samples from the experiments reported in this thesis. Nonetheless it may suggest a common brain response between CIA and the hTNFtg RA model, perhaps in response to joint inflammation or an associated factor such as pain. Of note, Süß et al (2020a), found no indication of microglia response within the hippocampus, again similar to the findings reported here.

The thalamus is a key centre for processing sensory information, including the relay of nociception (Pinho-Ribeiro et al., 2017). A number of animal studies exploring pain have implicated glial cell activation and proliferation reactive to, and maintaining of, chronic pain (Ji et al., 2013). Microglial activation and proliferation in the thalamus is associated with induction of pain related behaviours (Zhao et al., 2007). There is some evidence that glial activation occurs in chronic pain in humans with studies showing enhanced glial marker signal in neuroimaging studies (Loggia et al., 2015).

Stress models in mice have reported microglial activation in the thalamus (Yirmiya et al., 2015). However, inflammatory communication may also underly this phenomenon and cannot be excluded - the thalamus is an important relay in the periphery-to-brain neural inflammatory reflex (Chavan et al., 2017).

In conclusion, increased levels of thalamic IBA1+ cells in the context of CIA could reflect either an inflammatory response to a peripheral inflammatory stimulus, or a pain-associated phenomenon or a combination of both.

3.5.3.5 Brain iNOS expression in CIA

Nitric oxide (NO) is an important molecule involved in immune effector and regulatory functions which is released by a range of cells including leukocytes, glial cells and endothelial cells (Bogdan, 2001). It is synthesised from L-arginine by three isoforms of nitric oxide synthase (NOS) enzyme: inducible NOS (iNOS), endothelial NOS and neuronal NOS, but the most potent producer of NO is iNOS (Bogdan, 2015). Pro-inflammatory cytokines such as IL1ß promote iNOS expression in macrophages and glial cells.

Within the CNS, NO has been dubbed 'Janus-faced' due to its dichotomous actions which can be neuroprotective in low concentrations but neurotoxic at higher concentrations (Calabrese et al., 2007). Glial cell iNOS-mediated release of NO has been suggested as a link between inflammation and neurodegenerative disorders and may be implicated in some of the morphological changes seen in the inflamed brain (Yuste et al., 2015).

Increased brain expression of iNOS has been observed in inflammatory models such as the LPS model of peripheral inflammation (Wong et al., 1996). Increased hippocampal iNOS expression has been observed in adjuvant-induced arthritis (AIA) in rats (Skurlova et al., 2011).

In the context of CIA, I found no indication of iNOS activation in comparison to controls. Staining was sparse throughout the hippocampus, cortex, striatum and thalamus. This would tend to support a conclusion of a quiescent brain inflammasome in CIA.

3.5.3.6 Hippocampal neurogenesis and neuronal density in CIA

Hippocampal neurogenesis is a process that is thought to be sensitive to inflammation, including chronic peripheral inflammation (Ekdahl et al., 2003) (Chesnokova et al., 2016). Hippocampal neurogenesis has been proposed as a mechanism by which inflammation may be transduced to mood, cognitive and behavioural change (Liu and Howard, 2018).

The finding of elevated Il1ß transcription in the brain and hippocampus of arthritic mice, if translated at the protein level, could be expected to have inhibitory effects on neurogenesis. Multiple animal studies have linked brain

IL1β to impaired neurogenesis (Ekdahl et al., 2003) (Goshen et al., 2008) (Spulber et al., 2008). It is hypothesised that the mechanism by which IL1β may inhibit neurogenesis may be via the kynurenine pathway, by upregulating enzymes such as IDO that are neurotoxic (Zunszain et al., 2012).

A recent study by Andersson et al. (2018) found impaired DG neurogenesis in the context of hippocampal inflammation in CIA (Andersson et al., 2018). However, in the experiments presented in this thesis no significant differences were found to suggest altered neurogenesis between CIA mice and controls.

In line with Andersson et al's study, DCX was chosen as a marker to detect changes in neurogenesis. Expression of DCX is associated with the appearance of neuroblasts, which peaks after 2 weeks and then declines as NeuN expression increases (Brown et al., 2003). It is possible that given the variable nature of the CIA model, differences in DCX expression could have been obscured. Whilst Andersson et al found a significant difference in DCX expression in CIA, their mouse models appeared to show much greater homogeneity in terms of timing of onset and overall severity in comparison to my own experiments. They do not report whether LPS was used to synchronise emergence of arthritis. They also allowed their mice to reach a higher severity of arthritis, with all mice showing severity of arthritis at time of culling well in excess of the maximum threshold in my experiments. Therefore, it may be that neurogenesis is affected in CIA after a more prolonged and severe inflammatory stimulus than was present in the animals used for the experiments presented here.

DCX is just one marker for neurogenesis and other markers exist, including bromodeoxyuridine / 5-bromo-2'-deoxyuridine (BrdU) uptake labelling and the proliferative marker Ki-67, which could be used as alternatives to confirm whether CIA has an impact on neurogenesis. BrdU uptake labelling is generally considered the definitive marker of neurogenesis due to its sensitivity in detecting cell proliferation. However, this must be administered in-vivo prior to culling and this was not an available procedure under the animal licence for the experiments presented here.

No differences were seen in Bdnf transcription between control and arthritic mice. BDNF is a neurotrophin which may have a role in promoting DG

neurogenesis (Chan et al., 2008), and more broadly supports neuronal survival and plasticity (Huang and Reichardt, 2001). Depleted hippocampal BDNF is associated with impaired neurogenesis in the context of neuroinflammation (Kubera et al., 2011). BDNF may be elevated in the context of acute neuroinflammation, forming part of an anti-inflammatory response, but tends to be depleted in the context of more chronic inflammation (Giacobbo et al., 2019).

Neuronal density within the hippocampus was examined by staining for the neuronal cell marker NeuN. Systemic inflammatory markers have been shown to associate with reductions in hippocampal volume (Zhang et al., 2016) (Satizabal et al., 2012). Reduced hippocampal volume can be observed in major depressive disorder (Belleau et al., 2019). However, no significant differences in neuronal density were detected in any of the hippocampal regions examined.

The data suggest that the neuroinflammatory response in CIA does not involve substantial hippocampal neurotoxicity or impaired neurogenesis.

3.5.4 The stage-dependent inflammatory response in CIA

To determine if a distinct inflammatory transcriptional pattern evolved over the course of the model, particularly at earlier, more acute time points, a time-point model was undertaken. This was in part driven by the marked variability of arthritic response in the initial end-point models; culling mice a set time-points relative to the initial stimulus was an attempt to rationalise the model to some extent. These experiments helped to characterise the pre-arthritic state in terms of PBL and brain inflammatory transcription.

3.5.4.1 The peripheral inflammatory response

The arthritic response was unfortunately more muted than in earlier models conducted, with only 1 of the 4 mice brought on to day 28 developing clinically detectable arthritis.

Significant weight loss outwith the context of clinically detectable arthritis was not seen in this model. Splenomegaly was seen at day 7 and at day 28, but not at day 1 or day 21, in mice injected CFA and injected CFA + T2C at baseline.

This confirms that CFA itself has an effect on spleen, in keeping with previously published literature (Billiau and Matthys, 2011). However, the fact that it had attenuated at day 21 before re-emerging at day 28 suggests that a pre/early-arthritic spleen response may have been emerging.

The analysis of PBL inflammatory transcription over time found that CFA injection was associated with increased PBL Il1ß transcription at day 7 after injection. P2x7r transcription, which as discussed earlier is important in the production and release of IL1ß was also elevated in PBLs at day 7 after CFA injection. Surprisingly, the statistical test (Kruskal-Wallis) did not indicate statistical significance in the CIA-stimulus group, even though they received equal dose of CFA. The grouping of data looks similar in terms of the median, but the variance is slightly higher, resulting in loss of significance.

Elevated Il1ß transcription is also seen at day 28 in the context of CIA stimulus (1 arthritic, 3 non/pre-arthritic). This diverges from the endpoint PBL qRT-PCR analysis reported in section 3.2.6 which found no difference in Il1ß, suggesting that there may be a PBL inflammatory transcriptional event in the very early stages of arthritis emergence in CIA.

Tnfα PBL transcription is also noted to be elevated in the CFA- treated group at day 1 but not at other time points. As with the Il1ß, the statistical test (Kruskal-Wallis) did not indicate statistical significance in the CIA-stimulus group, even though they received equal dose of CFA. Again, the grouping of data looks similar in the median, but the variance is higher, resulting in loss of significance.

In line with the findings at the endpoint experiments earlier reported in section 3.2.6, no broader inflammatory transcriptional response in PBLs was observed at any time point. The lack of a broader or more marked detectable PBL transcriptional response, even at very acute time-points in response to CFA injection is surprising. CFA is a non-specific stimulant of the innate immune system and has long been used experimentally to produce non-specific inflammation in mouse models (Rittner et al., 2005). CFA stimulates an adaptive immune response to its component mycobacterial antigens with prominent Th1 and Th17 lymphocyte activity that potentiates systemic inflammation. There is also an expansion in myeloid cell production and myelopoiesis (Billiau and

Matthys, 2011). It may be that a more marked inflammatory response would be detectable in other immunologically active cells and tissues.

3.5.4.1 The brain inflammatory response

A statistically significant finding of increased brain Il1ß transcription at day 7 in response to CFA was noted but by the later timepoints this had entirely attenuated. This is broadly in keeping with my findings of increased brain Il1ß occurring only in the context of arthritis.

Ccl2 transcription was elevated among the CFA treated group at day 1 after injection. Again, the result was not statistically significant in the CIA-stimulus group but looking at the graph there is perhaps some indication of a distinct pattern in comparison to controls. CCL2 is an important chemokine involved in the recruitment of monocytes and within the CNS, is involved in the modulation of microglial activation phenotype (Cazareth et al., 2014).

Cxcl1 was elevated at day 1 in the CFA treated group, but the CIA treated group were almost identical to the controls, which undermines this finding. Brain Ccl19 transcription was elevated at day 7 in CFA treated mice and although not also statistically significant in the CIA treated group, the CIA grouping does look distinct from controls. CCL19 is known to be involved in neuroinflammation in the context of experimental autoimmune encephalitis (Krumbholz et al., 2007) and may have a role in the maintenance of chronic inflammation in the brain (Columba-Cabezas et al., 2003)

Overall, these data tend to support the earlier findings of brain transcriptional changes in CIA being dependent upon clinically detectable arthritis, and that the response to CFA attenuates over time.

3.5.5 The utility of the CIA model in investigating brain responses

The CIA model has proven pedigree in the field of research exploring mechanisms of joint disease. It shares various immunological and pathological similarities with human RA (Brand et al., 2003) (Holmdahl et al., 2002). CIA has been a work-horse of RA research for 2 to 3 decades and is frequently used for the development and assessment of new anti-rheumatic therapeutics (Bevaart et

al., 2010). The strength of the model lies in its numerous immunopathological similarities with human RA; as a tissue-specific inflammatory model, it produces the complex inflammatory cascade that is of interest in studying the effects of peripheral inflammation on the brain.

A major limitation of the CIA model, encountered in the experiments set out in this thesis, is the marked degree of variability in timing of onset and severity of arthritis. This meant that it was impossible to construct experiments whereby the brain response could be mapped out over time in response to a reliable, replicable, and dose-equal stimulus. If the primary outcome of interest is within the primary disease site (i.e. the joints) this may be less challenging. However, where the outcome of interest lies within a remote organ such as the brain, with its own unique immune milieu, this becomes increasingly complicated to parse out. Severity-dependent changes or time-specific changes may become obscured by the wide variation in disease penetrance and disease-course.

3.5.6 The translational significance of findings

A broad swathe of evidence links mental disorder, particularly depression, to RA (Vallerand et al., 2019) (Matcham et al., 2013). Increasing evidence supports the more or less established hypothesis that a proportion of the variance in rates of mental disorder in RA is underpinned by inflammatory mechanisms (Nerurkar et al., 2019). Exactly what those mechanisms are and how they might be disrupted by therapeutics remains an ongoing area of intense research.

How RA affects the brain at the molecular and cellular level remains to be understood. What happens within the brain of patients with RA can only be studied either by proxy markers in vivo, for example neuroimaging or CSF sampling, or ex-vivo post-mortem. Despite recent advances in these methodologies, study in humans cannot yet reveal intricate mechanisms or be free from major distortion or confounding. These experiments sought to use a mouse model of inflammatory arthritis to explore the brain inflammatory response.

The most consistent finding in this series of experiments was of increased Il1β transcription within the brain in the context of arthritis and elevated plasma IL6. It is not possible to infer causality, though one of the mechanisms that could

explain this finding would include a brain response to peripheral inflammation via neuroimmune communication pathways. Alternative contributors such as pain response or stress cannot be excluded and indeed must also be actively considered.

Meta-analysis has provided evidence of elevated peripheral levels of IL6 associating with depressive disorders (Köhler et al., 2017). IL6 is also implicated in the burden of fatigue and pain in RA, both of which represent a major source of disability (Choy and Calabrese, 2018). It is extremely difficult to disentangle these related symptoms, which often co-exist and may share biological pathways (Penner and Paul, 2017). Fatigue and depression as constructs share a some overlap (Maes et al., 2012).

A prime cellular source for IL1ß within the brain is microglia, and increased cytokine production by microglia is associated with sickness behaviour and depressive phenotypes (Vollmer-Conna et al., 2004). Various strands of evidence support microglial activation as relevant to depression (Yirmiya et al., 2015) and neurodegenerative disorders (Hickman et al., 2018). However, although I observed elevated brain Il1ß transcription, I did not see evidence of major glial cell activation, aside from increased IBA1-positive cell density in the thalamus.

Indeed, in addition to not observing more general glial activation, I did not observe major inflammasome activation with iNOS expression, changes to neuroplasticity, hippocampal neuron loss or major leukocyte recruitment across the BBB. But this is not necessarily surprising – after all RA is not an encephalopathy. Chronic joint inflammation with modestly elevated brain IL1ß over long periods of time could very plausibly have deleterious consequences for brain health.

A major limitation of these experiments is a lack of any measures of behaviour. The significance of my findings in terms of how they could relate to sickness behaviour remains conjecture for the time being. Further experiments exploring behaviour changes in the context of CIA, for example measures of hedonic response, exploratory behaviour and cognitive tests could shed light on the functional associations of these findings. Very little work has been published characterising the behavioural phenotype of CIA mice. However, a study by

Brown et al (Brown et al., 2018), has explored this topic and demonstrated that CIA mice show anhedonia-type behaviours 5-6 weeks after initial immunisation, including reduced sucrose preference and reduced scoring for female urine sniffing test (FUST). They reported that administration of anti-TNFα treatment attenuated this phenotype and produced reduction of serotonin transporter (SERT) activity (Brown et al., 2018) – attractive evidence of the links between arthritic inflammation and serotonergically-mediated sickness behaviour in the context of RA.

In the course of these experiments, behavioural differences were noted between saline injected control mice and CIA mice. However, as formal behavioural experiments were not undertaken, this cannot be quantitatively examined. It was observed that CIA mice would show typical sickness-type behaviours including reduced activity, lethargy, hunching and reduced grooming. This tended to be most marked in the days following challenge and then emerge again around the development of arthritis and persist with disease. This is clearly relevant to the concept of sickness behaviour and fatigue in the context of human disease. IL6 is implicated in the burden of fatigue and pain in human RA (Choy and Calabrese, 2018), and meta-analysis has provided evidence of elevated peripheral levels of IL6 associating with depressive disorders (Köhler et al., 2017).

In conclusion, the data presented in this Chapter provide evidence of a distinct brain inflammatory response in a mouse model of RA. The next chapter sets out to explore the possible consequences of inflammatory arthritis in terms of mental disorder in a human cohort of RA cases, with a specific focus on fatigue.

Chapter 4

Fatigue and inflammatory variables in human RA

4 Fatigue and inflammatory variables in human RA

4.1 Introduction

The CIA model highlights that inflammatory arthritis is associated with systemic inflammation, as typified in this study by elevated levels of the inflammatory cytokine IL6 in plasma. The current study also found CIA to be associated with elevated gene expression of the inflammatory cytokine IL1ß within the brain.

The CIA model presents a number of challenges, not least variation in disease expression and penetrance. Nevertheless, it has been used extensively as an animal model to study RA because it shares important pathophysiological features with the human disease, both at the molecular/immune level and in terms of joint pathology.

RA is a chronic, often life-long condition. Animal models, by nature, tend to study the more short-term acute phase of disease. However, judicious translation across the species in the study of disease is valid and an important avenue for developing our greater understanding of disease mechanisms.

The role that inflammation and immune dysregulation plays in the association between RA and an increased risk of psychiatric disease remains poorly understood. As outlined in Chapter 3, the CIA mouse model of RA shows a brain inflammatory response in the context of joint-derived peripheral inflammation. This chapter seeks to explore using human data whether peripheral inflammatory variables in the context of human RA are associated with neuropsychiatric disorder.

The Scottish Early Rheumatoid Arthritis (SERA) Inception Cohort Study allows study from early stages of RA and includes measures relevant to psychiatric disease and sickness behaviour, including validated measures of mood, anxiety, and fatigue. In the following chapter I have sought to explore the factors associated with fatigue, a key dimension of sickness behaviour, in the context of early RA using the SERA cohort.

Recently published work has demonstrated a clear relationship between measures of disease activity and inflammation and elevated symptoms scores for

anxiety and depressed mood (Fragoulis et al., 2020). Fragoulis et al reported a strong correlation between DAS28 and anxiety and depression scores, and a correlation between CRP levels and depression but not anxiety scores.

Fatigue is another key sickness behaviour type symptom that is prevalent in RA, associated with significant disability, and consistently reported by patients to be among the most debilitating and problematic symptoms of RA (Wolfe et al., 1996) (Katz, 2017b). Fatigue can be persistent and difficult to treat (van Steenbergen et al., 2015) and thus has been recognised as one of the core outcome measures that should be included in RA clinical trials (Kirwan et al., 2005).

Based upon the work presented in Chapter 3, I proposed that the inflammatory brain signature associated with joint inflammation that was found in mice, could in human RA give rise to sickness behaviour. In particular, I proposed that measures of inflammation and associated disease measures would be predictors of fatigue. In this chapter, I have tested my hypothesis by exploring the following questions:-

1. To what extent are inflammatory variables associated with fatigue in RA?
2. To what extent are other variables including disease activity, pain and demographic characteristics apposite to inflammation associated with fatigue in RA?
3. Whether DMARD, biologic and antidepressant medications had an effect upon fatigue and anxiety/depression in RA?

The results of the analyses undertaken are reported in the following sections 4.2 to 4.6, with an in-depth discussion and synthesis of the results in section 4.7.

4.2 Results: SERA cohort patient characteristics

The data retrieved from the SERA cohort consisted of 1111 cases. Data regarding diagnosis was missing from 57 cases and so these were removed from the analysis leaving 1054 cases to ensure that the analysis was of inflammatory arthritis as far as was reliably possible. The database includes demographic and

clinical measures (as detailed in Chapter 2, section 2.2), with data available at baseline (first visit within 6 months of RA/UA diagnosis) and 6 and 12 months follow-up.

Fatigue rating was only undertaken on a proportion of the total cohort. Fatigue rating scales were undertaken in a total of 195 cases, with a mix of 145 cases at baseline, 123 cases at 6 months and 79 cases at 12 months. There was some inconsistency with the application of the fatigue rating in that some cases were scored at 6 months but not at baseline for example. The analysis presented in this chapter focusses primarily on this subset of the total cohort. A comparison of the basic diagnostic, demographic, and lifestyle variables of the total cohort vs. fatigue sub-cohort is shown in tables 4.1 and 4.2, and in figure 4.1. The fatigue sub-cohort appears largely comparable and representative of the cohort as a whole in terms of these variables.

4.2.1 Diagnosis

The total cohort consists of cases of newly diagnosed RA according to ACR/EULAR-2010 criteria (903 cases), UA (138), and yet to be classified (RF or CCP awaited) (13 cases). Within the fatigue sub-cohort there were newly diagnosed RA according to ACR/EULAR-2010 criteria in 162 cases, UA in 27 cases and RF/CCP awaited in 6 cases.

4.2.2 Basic demographics

Descriptive statistics of the SERA cohort are shown in Table 4.1. The total cohort consists of 681 female cases (64.6%) and 373 male cases (35.4%). This is a female : male ratio of approximately 2:1 which is slightly lower than the classically described ratio of 3:1 (Linos et al., 1980). However, it is broadly in keeping with the figures compiled by the Scottish Burden of Disease Study, 2015 – Rheumatoid arthritis technical overview (available from https://www.scotpho.org.uk/media/1448/sbod2015-arthritis.pdf – accessed on 13th April 2022) that reports a sex distribution of 68% female vs. 32% male.

The age range is not normally distributed. The mean age at baseline assessment for males is higher than females. This is in keeping with epidemiological data which has shown peak age of incidence in UK, USA, and Norway to be around 55-

64 in females and 65-75 in males. Very elderly onset (after 75) may be higher in men than in women (Symmons, 2002).

The age and sex distribution in the subset of the sub-cohort with fatigue rating scales is shown in table 4.2. The sex and age distribution does not vary substantially from the overall inception cohort. The female to male ratio is 1.64:1 and age standard deviation overlaps with the age in the whole cohort.

Table 4.1 Descriptive statistics of the whole SERA cohort: Age (yrs)

	All cases	Female	Male
Number of values	1054	681	373
Minimum Age (yrs)	18.00	18.00	19.00
25% Percentile	50.00	48.00	52.00
Median Age (yrs)	59.00	57.00	62.00
75% Percentile	68.00	67.00	69.00
Maximum Age (yrs)	88.00	88.00	86.00
Range	70.00	70.00	67.00
Mean Age (yrs)	57.97	56.68	60.34
Std. Deviation	13.72	14.14	12.59
Std. Error of Mean	0.4226	0.5419	0.6518

Table 4.2 Descriptive statistics of the SERA fatigue sub-cohort: Age (yrs)

	All cases	Female	Male
Number of values	195	121	74
Minimum Age (yrs)	24.00	24.00	24.00
25% Percentile	50.00	49.00	55.75
Median Age (yrs)	61.00	59.00	64.50
75% Percentile	70.00	68.50	70.25
Maximum Age (yrs)	85.00	85.00	84.00
Range	61.00	61.00	60.00
Mean Age (yrs)	59.97	58.39	62.57
Std. Deviation	13.23	13.45	12.52
Std. Error of Mean	0.9471	1.222	1.455

The SERA cohort is almost exclusively of 'white' ethnicity (1044). Other ethnicities listed were 'Indian Subcontinent' (3), 'South East Asian' (2) and 'Other' (5). The subset of the cohort with fatigue scores is defined as 'White' in 194 cases, with 1 case defined as 'South East Asian'.

Measures of socioeconomic status include SIMD quintile categories. The whole cohort distribution across SIMD categories is shown in figure 4.1, in addition to the fatigue sub-cohort. The spread is relatively even across demographic groups from 1 (most deprived) to 5 (least deprived) in the whole cohort, but less so in the fatigue sub-cohort where categories 3 and 4 are over-represented.

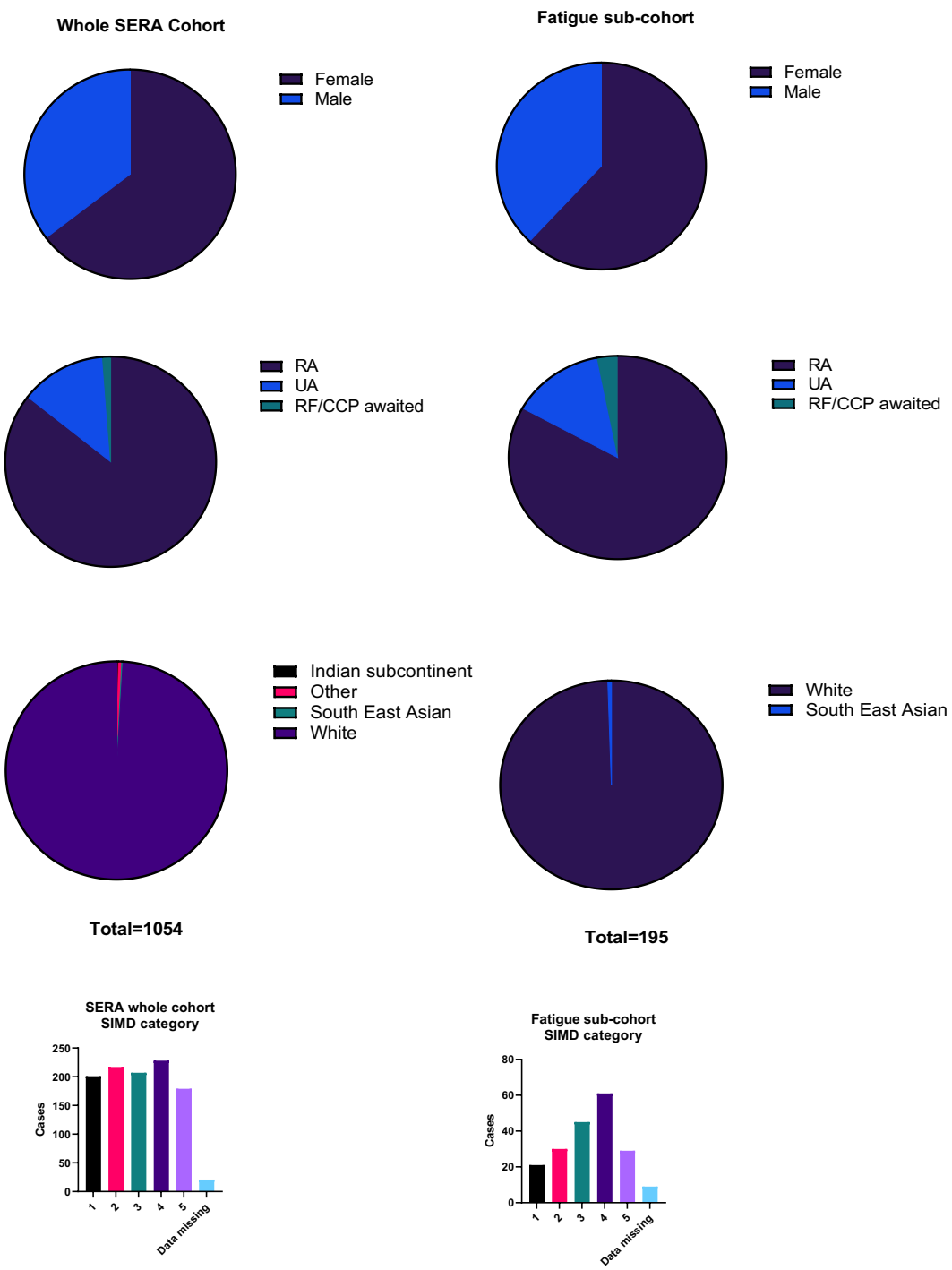


Figure 4.1 Basic demographic characteristics of cases within cohort of study
Graphs compare whole SERA inception cohort (n. 1054) with sub-cohort with fatigue data (n. 195). Various demographic and diagnostic variables are gathered as part of the SERA study. Variables as noted above are compared between the whole cohort and the sub-cohort in which fatigue data was available.

4.3 Results: Fatigue in the SERA cohort

Fatigue is measured in the SERA cohort using the Patient Reported Outcome Measures Information System (PROMIS) Item Bank v1.0 – Fatigue – Short Form 8a

questionnaire (see appendix 2.) (Bingham III et al., 2019). The fatigue rating scores are converted from raw scores to adjusted T-scores as per a previously validated conversion key (PROMIS Fatigue Scoring Manual, submitted to FDA with Initial Briefing Package on August 11, 2017 (available at - https://www.fda.gov/media/137977/download (accessed 13/04/2022)) (see methods section 2.2.1, table 2.1.). A T-score of 50 in PROMIS tools generally indicates the average in the US population – to which these rating scales are calibrated. A summary of the fatigue questionnaire is included in Appendix 2.

4.3.1 Fatigue change over time

Fatigue rating scales were completed as part of the SERA inception cohort project at baseline visit, 6 month visit and 12 month visit. Spread of T-scores is shown in figure 4.2.

The median fatigue T score in cases at baseline is 56.6. A trend of reducing fatigue scores in comparison to baseline measurement is observed at 6 months (median 51.5) and again at 12 months (median 48.1). Analysis of the difference in scores at these time points show a statistically significant reduction in overall fatigue scores at 6 months and 12 months relative to baseline (figure 4.3).

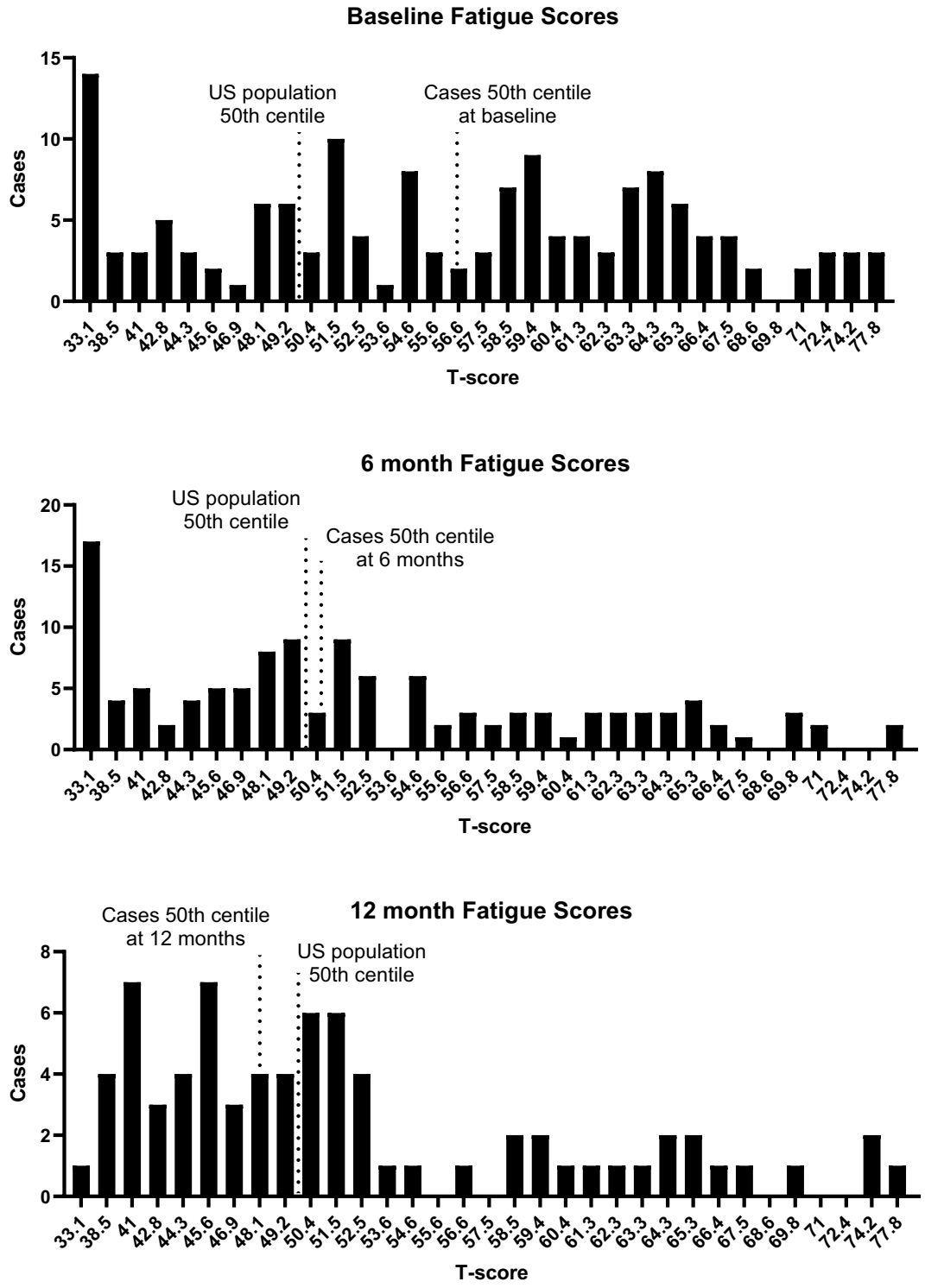


Figure 4.2 Spread of PROMIS Short Form 8A Fatigue Rating Scores at baseline visit (n. 145), 6 month visit (n. 123) and 12 month visit (n. 79)

Questionnaire raw scores converted to T scores (see table x.). T-score of 50 = 50th percentile of US population. 50th percentile in cases was calculated by obtaining median score. Median fatigue T-score in cases reduces at 6 months and again at 12 months.

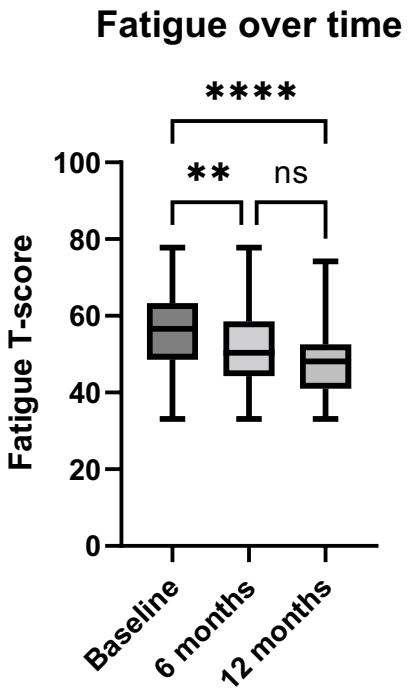


Figure 4.3 Fatigue scores over time
Fatigue T scores were compared over time at baseline (n. 145), 6 months (n. 123) and 12 months (n.79). Kruskal-Wallis test with multiple comparisons was used to determine significance. Fatigue scores differed significant from baseline at 6 months (p=0.0035) and 12 months (p=<0.0001). The difference between 6 months and 12 months was not statistically significant (p=0.285).

4.4 Results: Univariable analysis of fatigue in the SERA cohort

Statistical methods such as group comparisons and simple linear regression analysis were used to explore the relationship between fatigue and various predictor/explanatory variables, using the data collected within the SERA cohort. This analysis focussed particularly on exploring variables that were salient to inflammation, in line with the initial hypothesis, as well as characterising fatigue in terms of its demographic distribution.

4.4.1 Fatigue and diagnosis

Fatigue scores were compared according to diagnosis at baseline. As described above, cases were classified as either RA (ACR/EULAR-2010 criteria), UA or suspected RA awaiting RF/CCP to classify. Data on fatigue was available for RA (baseline n. 120, 6 months n. 106, 12 months n. 72), UA (baseline n. 25, 6 months n. 12, 12 months n. 5). There was no baseline data available on fatigue scores in cases yet to be classified at baseline. Differences in fatigue scores according to baseline diagnosis are shown in figure 4.4. No significant differences were observed.

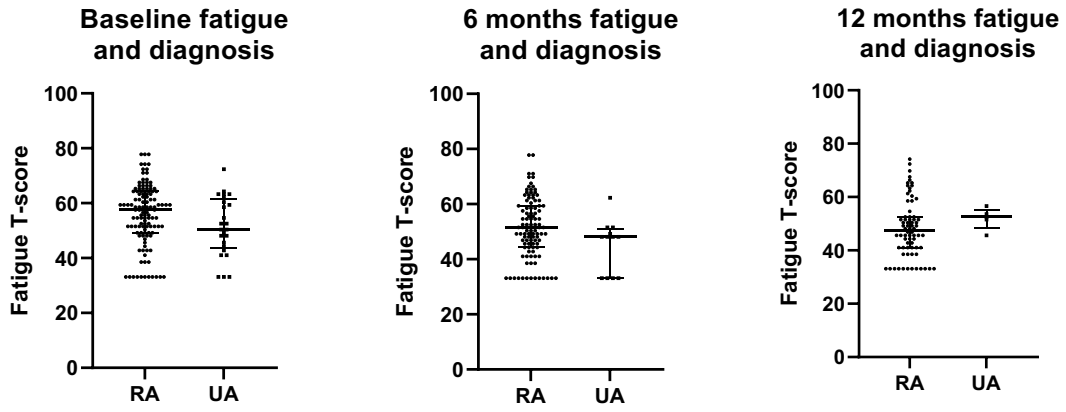


Figure 4.4 Fatigue scores and diagnosis
Fatigue scores between those with a baseline diagnosis of RA and UA were compared at baseline, 6 months and 12 months. Statistical significance was determined using Mann-Whitney U Test. The overall regression was not statistically significant at baseline (p=0.0523), 6 months (p=0.0501) or at 12 months (p=0.1893).

Within the SERA cohort, RA was diagnosed using the ACR/EULAR-2010 criteria. The ACR/EULAR-2010 scoring system was developed by a group of European and US experts as a scoring system for diagnosis and classification in early stage RA (Kay and Upchurch, 2012). The scoring system was subsequently validated and now forms an important part of assessment and monitoring of RA in clinical practice internationally. The ACR/EULAR-2010 classification criteria are shown in table 4.3. The scoring system contains a number of variables relevant to burden of joint and systemic inflammation and was therefore included for analysis in respect of fatigue scores.

Table 4.3 The ACR/EULAR-2010 scoring system

Domain	Category	Point score
A *Joint involvement*	1 large joint	0
	2–10 large joints	1
	1–3 small joints (large joints not counted)	2
	4–10 small joints (large joints not counted)	3
	>10 joints including at least one small joint	5
B *Serology*	Negative RFA and negative ACPA	0
	Low positive RF or low positive ACPA	2
	High positive RF or high positive ACPA	3
C *Acute-phase reactants*	Normal CRP and normal ESR	0
	Abnormal CRP or abnormal ESR	1
D Symptom duration	<6 weeks	0
	≥6 weeks	1

Adapted from source: (Kay and Upchurch, 2012)

Fatigue scores at baseline, 6 months and 12 months were analysed in respect of respective ACR/EULAR-2010 scores at these time-points using simple linear regression (figure 4.5). A significant relationship was not seen at baseline or 12 months. However, the regression model was statistically significant at 6 months, showing an association between higher ACR/EULAR scores and higher fatigue.

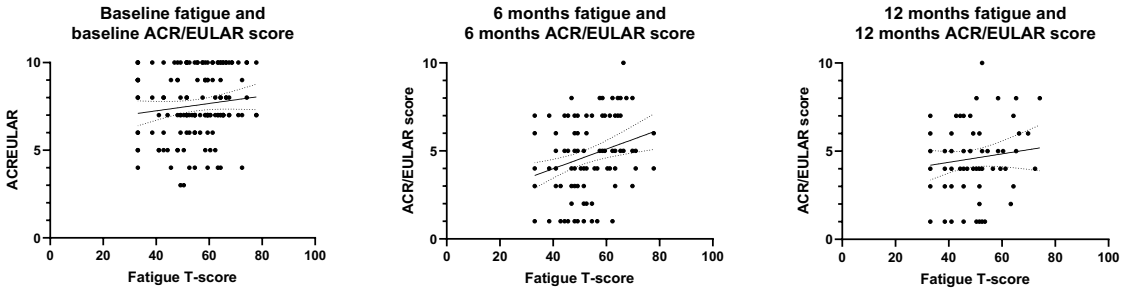


Figure 4.5 Fatigue scores and ACR/EULAR score
Simple linear regression was completed to test if ACR/EULAR scores at baseline, 6 months and 12 months predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression was not statistically significant at baseline (p=0.1521) or at 12 months (p=0.3058). The overall regression model was statistically significant at 6 months (fitted regression model Y = 0.05559*X + 1.763, r^2=0.07645, F=10.02, p=**0.002).

In addition to ACR/EULAR-2010 scoring, cases within the cohort also underwent assessment using the Visser Scoring system. The Visser score is derived from a standardised diagnostic evaluation of early arthritis which is useful in diagnosing RA and predicting severity and prognosis. Visser score can be used to predict whether inflammatory arthritis may be self-limiting, or persistent non-erosive in nature, or persistent erosive (Visser et al., 2002). A higher score indicates poor prognosis. The Visser score is reproduced in table 4.5. The scoring key is in table 4.6. In the SERA cohort, Visser score was measured as part of the baseline assessment and repeated at 6 months and 12 months. As with the ACR/EULAR-2010 score, the Visser score includes variables relevant to burden of joint and systemic inflammation and was therefore included for analysis.

Table 4.4 The Visser scoring system

Criterion	Persistent vs. Self-limiting scoring	Erosive vs. non-erosive scoring
≥6 weeks but <6 months duration	2	0
≥6 months	3	0
≥1 hour morning stiffness	1	1
Arthritis in ≥3 joint groups	1	1
Bilateral compression pain in MTPs	1	2
IgM-RF ≥5 IU	2	2
Erosion on hand or foot radiographs	2	infinite

Adapted from source: (Visser et al., 2002)

Table 4.5 Interpreting the Visser scoring system

Persistent vs. self-limiting		Erosive vs. non-erosive	
Total Visser Score	Probability of persistence	Total Visser Score	Probability of erosive arthritis
0	0.1	0	0.1
1	0.15	1	0.16
2	0.23	2	0.25
3	0.34	3	0.38
4	0.46	4	0.52
5	0.59	5	0.66
6	0.71	6	0.78
7	0.8	7	0.86
8	0.87	8	0.92
9	0.92	9	0.95
10	0.95	∞	1
11	0.97		
12	0.98		

Adapted from source: (Visser et al., 2002)

A regression analysis was completed to determine if Visser score predicted fatigue (see figure 4.6). Baseline Visser score was modelled with fatigue at baseline, 6 months and 12 months. This was done as baseline Visser score is used to predict the course of arthritis, and so comparing baseline Visser score with later time point fatigue scores was of interest – inferences could be drawn regarding the relationship between fatigue and course of arthritis. In addition, the 6 month Visser score was modelled with fatigue at 6 months and 12 month Visser score with fatigue at 12 months. The regression model was only significant when 6 month fatigue and 6 month Visser score were analysed.

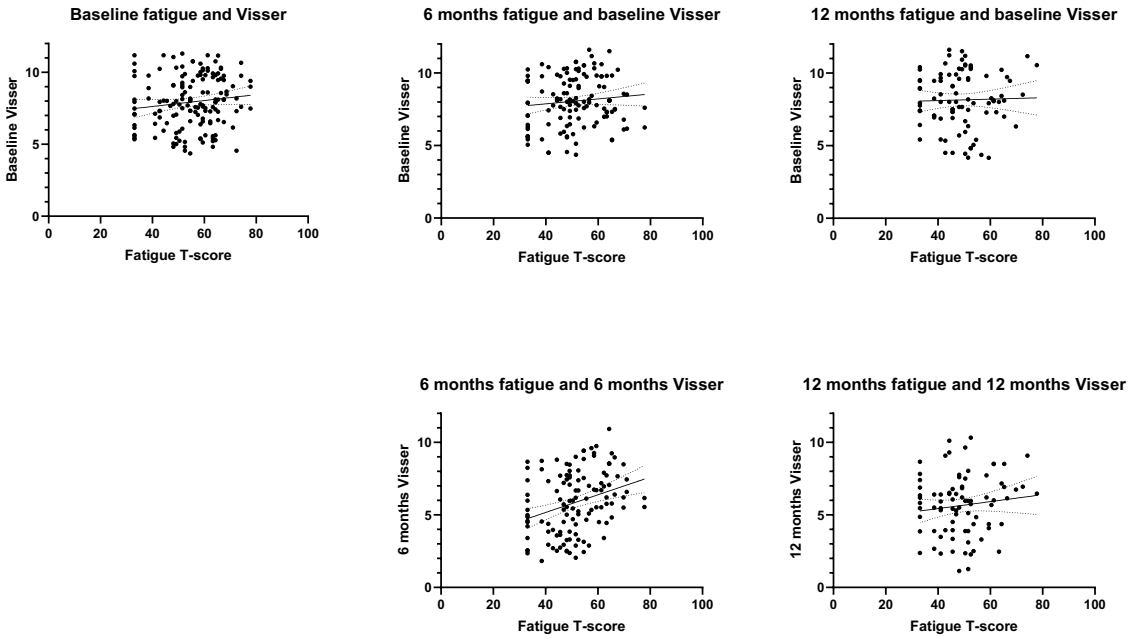


Figure 4.6 Fatigue scores and Visser score
Simple linear regression was completed to test if Visser scores at baseline and 6 months predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. When modelling baseline Visser scores the overall regression was not statistically significant at baseline (p=0.1101), 6 months (p=0.1236) or at 12 months (p=0.9777). When modelling 6-month Visser scores the overall regression model was statistically significant at predicting fatigue at 6 months (fitted regression model Y = 0.05996*X + 2.758, r^2=0.09377, F=12.52, p=***0.0006). The regression was not significant for 12 months Visser score and 12 months fatigue (p=0.3822).

4.4.2 Fatigue and demographic variables

Fatigue scores were compared between groups and analysed according to demographic variables, with an emphasis on variables relevant to inflammation.

Fatigue scores were higher in females than in males at baseline and 6 months, but this difference became non-significant by 12 months (figure 4.7). This could be due to loss of power as a result of lower n. value.

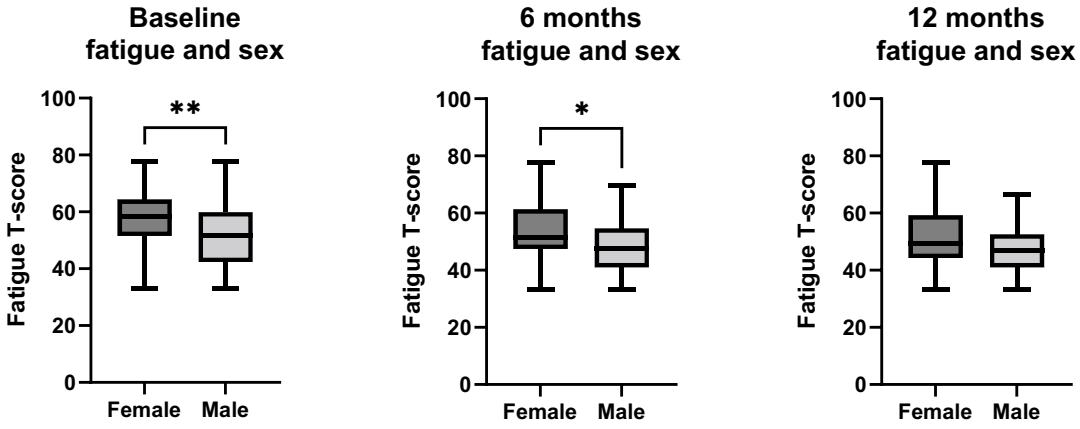


Figure 4.7 Fatigue scores and biological sex
Fatigue scores were compared according to sex. Data was not normally distributed and Mann-Whitney was used to determine significance. Female scores are significantly higher at baseline (p=**0.0015) and 6 months (p=*0.015) but not at 12 months (p=0.3035).

Aging is associated with a progressive inflammatory burden (Sarkar and Fisher, 2006). Immune tolerance of self-antigen can reduce with aging (Boren and Gershwin, 2004) and RA incidence peaks in later middle-age.

Simple linear regression analysis was completed to determine in age was significantly associated with differences in fatigue T-scores (figure 4.8). No significant relationship was found.

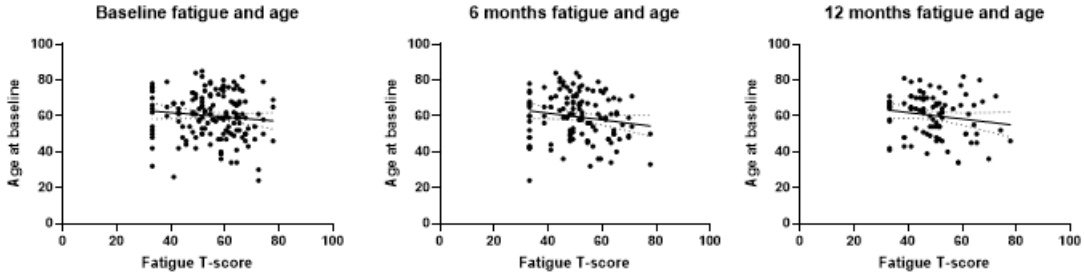


Figure 4.8 Fatigue scores and age
Simple linear regression was completed to test if age at baseline predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression was not statistically significant at baseline (p=0.1581), 6 months (p=0.0675) or 12 months (p=0.2177).

Socioeconomic status is a demographic variable with salience in respect of inflammatory disease and mental illness. Meta-analysis shows low socio economic status and unemployment is associated with elevated levels of

inflammatory markers such as CRP and IL6, and it is thought that increased propensity to systemic inflammation may be a key transducer of the association between deprivation and worsened health outcomes (Muscatell et al., 2020).

Comparisons of fatigue scores was made across baseline SIMD quintile groups at baseline, 6 months and 12 months (figure 4.9). The data was not uniformly normally distributed. Kruskal-Wallis with multiple comparisons was used to determine significance of any differences in scores between quintiles. There were no significant differences at any time-point. Simple linear regression was undertaken to determine if there was a relationship between SIMD-2012 score and fatigue. The regression was not statistically significant at baseline or 12 months, however there was a significant finding at 6 months where it appeared that a higher level of deprivation (lower SIMD-2012 score) appeared to predict lower levels of fatigue.

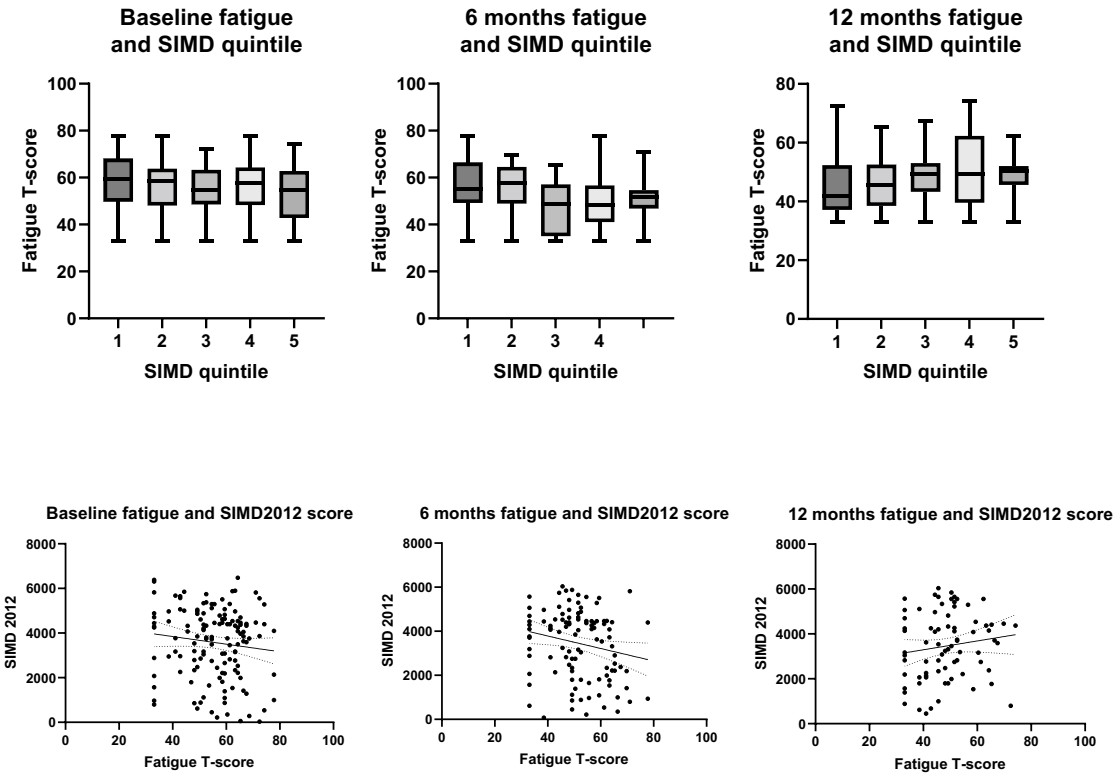


Figure 4.9 Fatigue scores and socioeconomic status
Fatigue scores and baseline SIMD quintile category were compared at baseline, 6 months and 12 months. Kruskal-Wallis and multiple comparisons test was used to determine significance of differences between quintiles. There were no significant differences at baseline (p=0.8553), 6 months (p=0.1284) or 12 months (p=0.5012). Simple linear regression was completed to test if baseline SIMD2012 score predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression was not statistically significant at baseline (p=0.1471) or 12 months (p=0.2231). The regression was significant at 6 months (fitted regression model Y = -28.39*X + 4921, r^2=0.03998, F=4.748, p=*0.0314).

Fatigue scores were also compared according to employment status at baseline (figure 4.10). Employment status was divided into multiple categories as determined by the original SERA data gathering. Fatigue scores were available from full-time employed (baseline n. 45, 6 months n. 31, 12 months n. 21), homemaker (baseline n. 5, 6 months n. 6, 12 months n. 1), part-time employed (baseline n. 18, 6 months n. 23, 12 months n. 16), retired (baseline n. 64, 6 months n. 51, 12 months n. 36), self-employed (baseline n. 7, 6 months n. 7, 12 months n. 4) and unemployed (baseline n. 6, 6 months n. 5, 12 months n. 1). There was no fatigue data for students in the cohort. Kruskal-Wallis test was used to determine significance of differences in scores between groups at baseline, 6 months and 12 months. Multiple comparisons tests were done only

on groups where the n. value was greater than 10. No significant differences were observed.

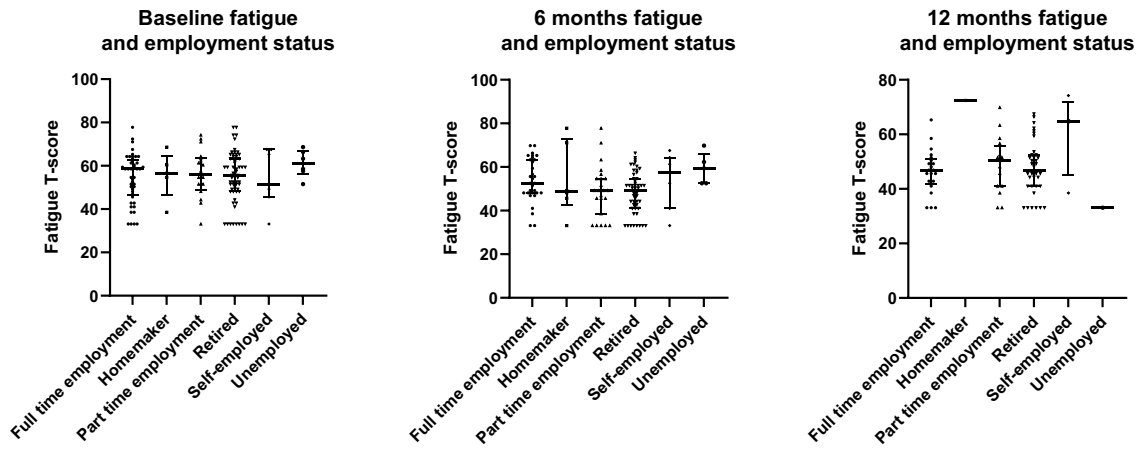


Figure 4.10 Fatigue scores and employment status
Fatigue scores and baseline employment status were compared at baseline, 6 months and 12 months. Kruskal-Wallis and multiple comparisons test was used to determine significance of differences between groups. There were no significant differences at baseline (p=0.8172), 6 months (p=0.0561) or 12 months (p=0.1243). Individual values are plotted in order to show the variation in n. between groups. Error bars indicate median and IQR.

4.4.3 Fatigue and lifestyle characteristics

Smoking is generally considered to have multiple pro-inflammatory sequelae (Sopori, 2002) (Gonçalves et al., 2011) and therefore is a relevant variable to be investigated in the context of understanding changes related to inflammatory burden in RA. Smoking has long been associated with increased risk of developing RA (Heliövaara et al., 1993) and with increased disease severity (Saag et al., 1997) (Manfredsdottir et al., 2006). Smoking appears to be associated with RF and ACPA auto-antibody production, the presence of which is associated with increased disease severity (van Wesemael et al., 2016).

Fatigue scores were compared between categories of smoking status (figure 4.11). Smoking status is divided into 'Current smokers' (baseline n. 36, 6 months n. 28, 12 months n. 16), 'Ex-smokers' (baseline n. 51, 6 months n. 45, 12 months n. 28) and 'Non-smokers' (baseline n. 66, 6 months n. 55, 12 months n. 47). No significant differences existed between smoking groups.

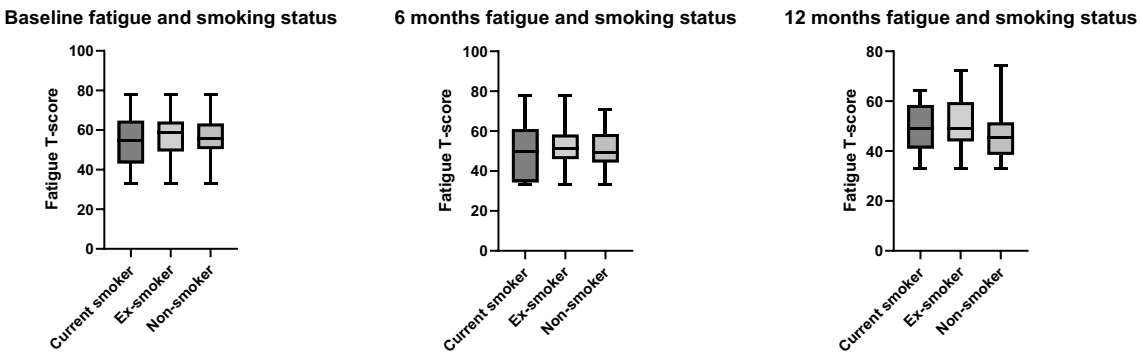


Figure 4.11 Fatigue scores and smoking status
Fatigue scores and baseline smoking status were compared at baseline, 6 months and 12 months. Kruskal-Wallis and multiple comparisons test was used to determine significance of differences between groups at baseline (not normally distributed), whilst ANOVA and multiple comparisons was used at 6 months and 12 months. There were no significant differences at baseline (p=0.8465), 6 months (p=0.2990) or 12 months (p=0.2085).

Alcohol can have complex interactions with inflammatory mechanisms within the body. In general, studies tend to suggest that alcohol consumption may be associated with reduced disease activity in RA (Turk et al., 2021) and may even be protective from developing RA (Scott et al., 2013). Weekly unitary intake of alcohol was analysed with respect to fatigue using simple linear regression (figure 4.12). The linear regression model was statistically significant for alcohol intake and fatigue scores at baseline – showing an inverse relationship, with higher intake being associated with lower levels of fatigue. The regression was not significant at 6 months and 12 months.

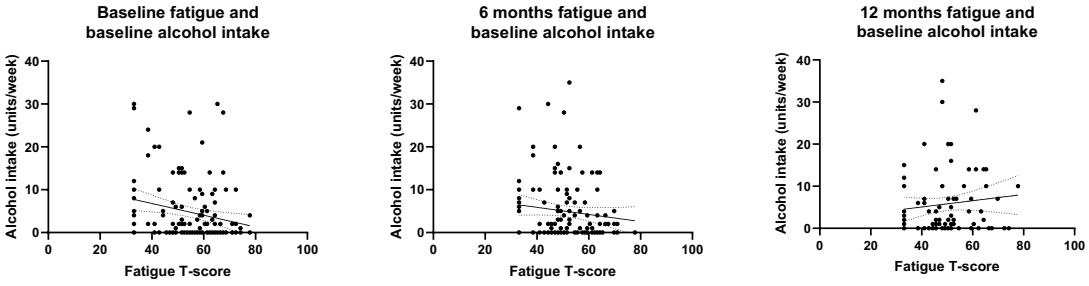


Figure 4.12 Fatigue scores and alcohol
Simple linear regression was completed to test if weekly alcohol intake at baseline predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression was statistically significant at baseline (fitted regression model Y= -0.1088*X + 10.71, r^2=0.02983, F=4.551, p=*0.0102). The regression was not statistically significant at 6 months (p=0.075) or 12 months (p=0.5774).

Excess body fat is known to be an important source of bodily inflammation and may enhance propensity to inflammatory disease (de Heredia et al., 2012). Baseline BMI is reported in the SERA dataset. The relationship between fatigue and baseline BMI was examined using simple linear regression (figure 4.13). No significant relationship between BMI and fatigue was observed using simple linear regression. The median baseline BMI among patients from whom we have fatigue data is 27.84kg/m^2.

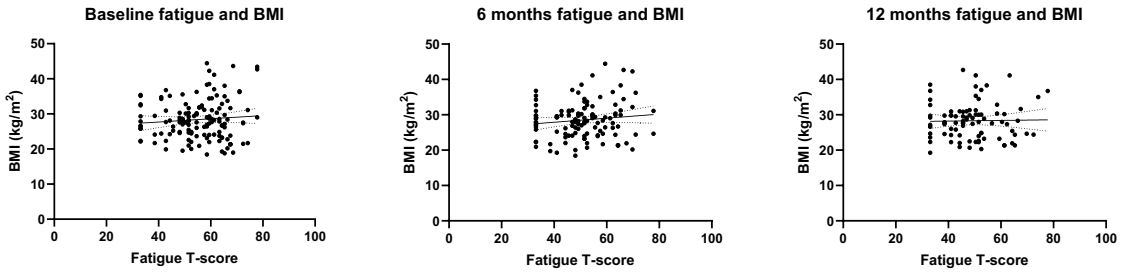


Figure 4.13 Fatigue scores and BMI
Simple linear regression was completed to test if BMI at baseline predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression was not statistically significant at baseline (p=0.1755), 6 months (p=0.0954) or 12 months (p=0.8564).

Data on serum cholesterol and high-density lipoprotein (HDL) is available within the SERA cohort. Total cholesterol/HDL ratio is superior in predicting risk for cardiovascular disease (Kinosian et al., 1994), an important complication of RA thought to be related to inflammatory burden. High total serum cholesterol (TC) levels and/or high TC/HDL ratio frequently associates with obesity and high dietary fat intake (Garrison et al., 1980) (Howell et al., 1997).

TC and TC/ HDL ratio was examined at baseline, 6 months and 12 months as predictor variables for fatigue at baseline, 6 months and 12 months in simple linear regression analysis (figure 4.14). Cholesterol data was limited at 6 and 12 months such that no useful analysis could be completed at these time-points. At baseline, no relationship was seen in the SERA cohort between fatigue and cholesterol or TC/HDL ratio.

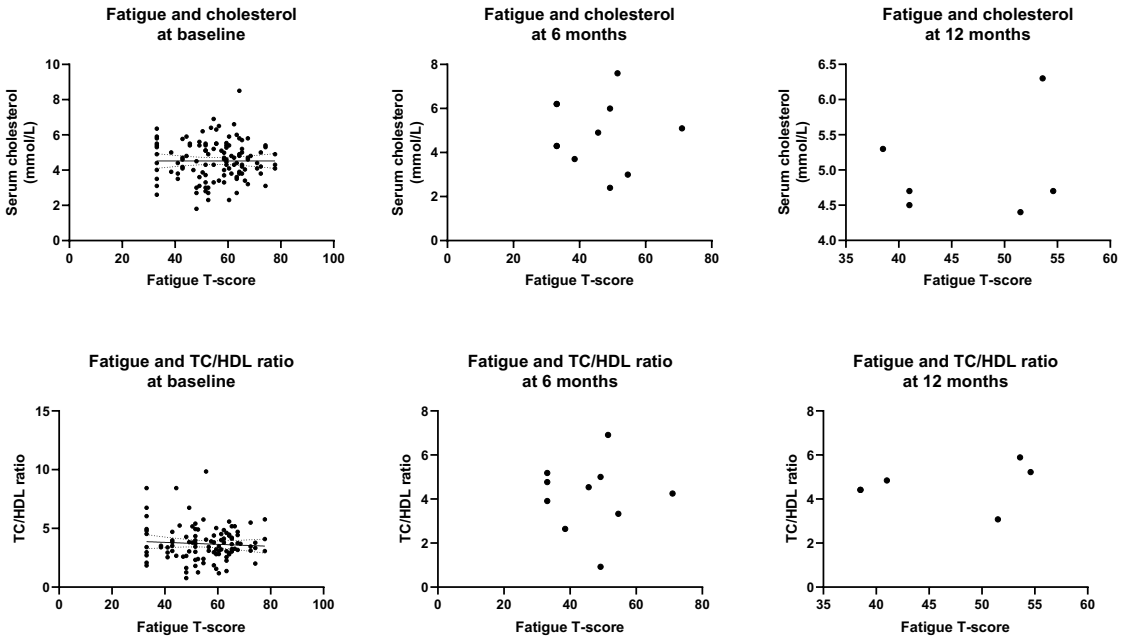


Figure 4.14 Fatigue and cholesterol
Simple linear regression was completed to test if total TC and TC/HDL ratio at baseline predicted fatigue T-scores at baseline. Data on cholesterol levels was too limited at 6 months and 12 months for meaningful analysis. Best fit line is shown with 95% confidence interval on either side. The overall regression model was not statistically significant either for cholesterol (p=0.8429) or TC/HDL ratio (p=0.7387).

4.4.4 Fatigue and disease activity

Various measures of disease severity and disease activity are reported within the SERA database, and are repeated at baseline, 6 months and 12 months. These are examined here both in univariable analysis and in a multivariable analysis of variables deconstructed from composite scores.

4.4.4.1 Fatigue and DAS28 scores

The DAS28 is among the most commonly used clinical assessment tools for measuring disease activity in RA. The DAS28 encompasses a scoring of clinical assessment of 28 specified joints (tenderness and swelling), a visual analogue scale of patient-reported global health (the PGA-VAS) and serum inflammatory markers - either ESR or CRP (Van der Heijde et al., 1993) (Wells et al., 2009). DAS28 score can be used to monitor treatment, with a value of ≤3.2 defined as the threshold for low disease activity and a score <2.6 being the threshold for remission (van Gestel et al., 1998).

DAS28 assessment was completed at baseline, 6 months and 12 months in the SERA cohort. Both DAS28-ESR and DAS28-CRP are reported. Fewer patients have ESR completed, particularly in the subset of patients in whom fatigue data is available. Both are explored here, however, in clinical practice CRP tends to be used more widely due to cost and ease of use. CRP may be more sensitive to more acute falls in systemic inflammation in comparison to ESR, and ESR tends to increase with age (Osei-Bimpong et al., 2007).

ESR was not universally tested in the SERA cohort, and therefore there is a more limited number of data points available in the patients with fatigue scoring (baseline n. 52, 6 months n. 34, and 12 months n. 13). More data was available for CRP, rendering the results more reliable (baseline n. 145, 6 months n. 112, 12 months n. 77).

Within the SERA cohort and among those patients with fatigue data available, the median DAS28-ESR scores are 4.209 at baseline (range 1.524 to 8.523), 2.946 at 6 months (range 0.485 to 5.029) and 3.073 at 12 months (range 1.124 to 5.82). The DAS28-CRP scores are 4.988 at baseline (range 1.664 to 7.825), 2.595 at 6 months (range 1.106 to 5.341) and 2.113 at 12 months (range 1.398 to 6.061). Sustained improvements in disease activity scores are seen in the cohort as would be expected in a cohort of actively treated patients.

Simple linear regression analysis of whether DAS28-ESR predicts fatigue was completed at baseline, 6 months and 12 months (figure 4.15). This was repeated for DAS28-CRP (figure 4.16). The regression analysis of DAS28-ESR and fatigue was significant only at 6 months, but not at baseline or 12 months. At 12 months the data was very limited (n. 13), thereby rendering results at that point more unreliable. However, there was a reasonable n at baseline (n. 52) and at 6 months (n. 34). The r^2 at 6 months is relatively high (0.4771) suggesting that a large proportion of the variance in fatigue at this point could be explained by high disease activity scores, but this is not in keeping with the higher n. value analysis at baseline or with the DAS28-CRP results (figure 4.16).

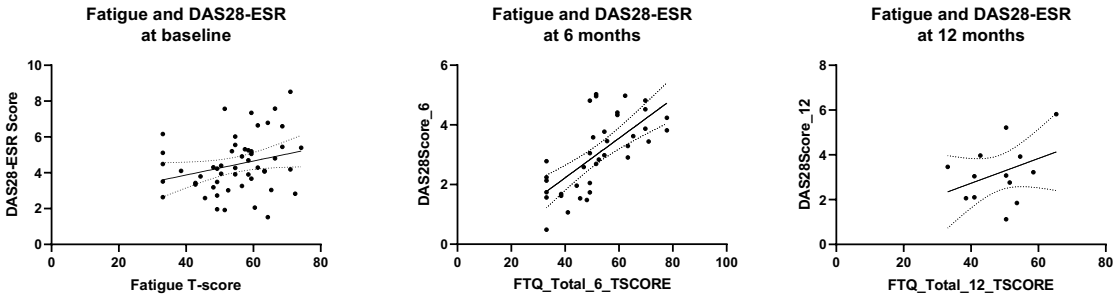


Figure 4.15 Fatigue and DAS28-ESR score
Simple linear regression was completed to test if DAS28-ESR scores predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at 6 months (fitted regression model Y = 0.06624*X - 0.4241, r^2=0.4771, F=31.93, p=****<0.0001). The regression was not significant at baseline (p=0.0528) or at 12 months (p=0.2109).

DAS28-CRP scores were similarly examined in regard to fatigue at baseline, 6 months and 12 months (figure 4.16). A very clear relationship is demonstrated by the regression analysis. The model is statistically significant at baseline, 6 months and 12 months. As before the r^2 increases at 6 months in comparison to baseline and is higher at 12 months also. This is consistent with the DAS28-ESR data, which is logical considering the scoring differs only in terms of the acute phase inflammatory marker included.

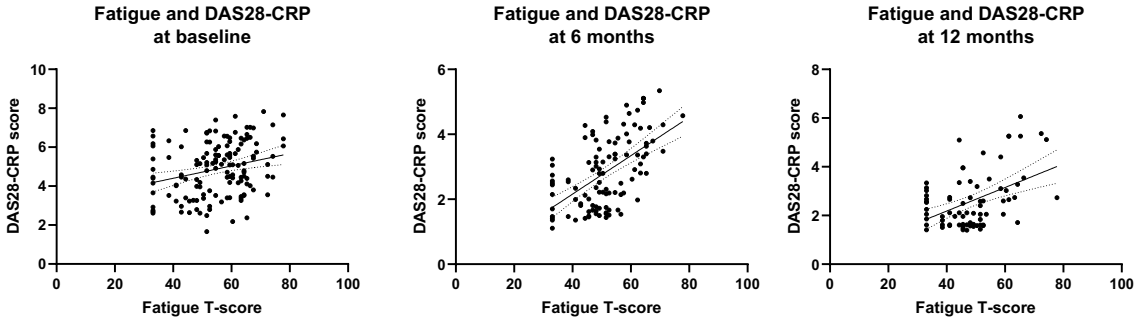


Figure 4.16 Fatigue and DAS28-CRP score
Simple linear regression was completed to test if DAS28-CRP scores predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at baseline (fitted regression model Y = 0.03310*X + 3.095, r^2= 0.07419, F= 10.58, p=**0.0015), at 6 months (fitted regression model Y = 0.05745*X - 0.1332, r^2=0.3209, F=51.98, p=****<0.0001), and at 12 months (fitted regression model Y = 0.04812*X + 0.2488, r^2=0.2063, F=16.37, p=***0.0001).

4.4.4.2 Fatigue and PGA-VAS and AGA-VAS

The PGA-VAS is a ubiquitously used patient-reported outcome measure in the assessment of early RA, and forms part of composite disease activity scores such as the DAS28. The PGA-VAS involves the patient rating their impression of current disease activity according using a visual analogue scale (Nikiphorou et al., 2016). Whilst subjective, the strength of the PGA-VAS is that it allows a patient-centred measure of disease experience. Its use is endorsed as a core component of data recommended for use in clinical trials related to RA (Felson et al., 1993).

The relationship between PGA-VAS and fatigue within the SERA cohort is explored using simple linear regression analysis (figure 4.17). The regression model was significant at baseline, 6 months and 12 months.

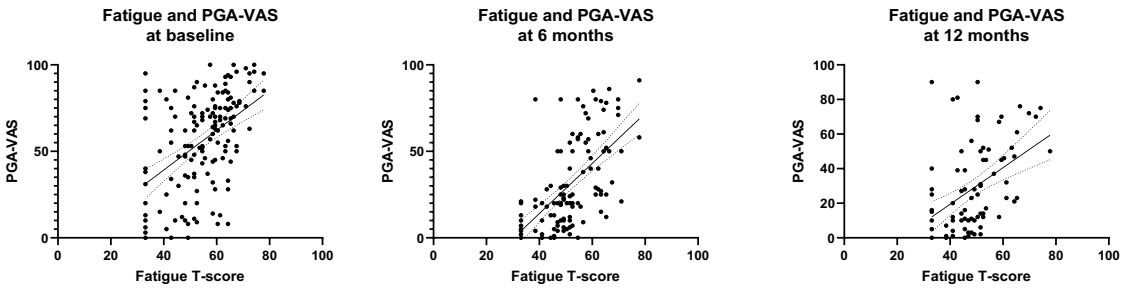


Figure 4.17 Fatigue and PGA-VAS
Simple linear regression was completed to test if PGA-VAS predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at baseline (fitted regression model Y = 1.140*X - 5.928, r^2= 0.2220, F= 40.80, p=****<0.0001), at 6 months (fitted regression model Y = 1.471*X - 44.60, r^2= 0.3992, F= 80.39, p=****<0.0001), and at 12 months (fitted regression model Y = 1.081*X - 23.43, r^2= 0.1989, F= 17.62, p=****<0.0001).

The AGA-VAS (also known as Physician Global Asssessment VAS) provides an important measure of disease activity (Felson et al., 1995). It is very similar to the PGA-VAS but is a clinician-reported measure of disease activity, rather than patient-reported. The AGA-VAS does not form part of the DAS28 scoring schedule.

The relationship between AGA-VAS and fatigue within the SERA cohort is explored using simple linear regression analysis (figure 4.18). AGA-VAS was found to be a significant predictor variable at baseline and 6 months but not at 12 months, with a lower r^2 than PGA-VAS.

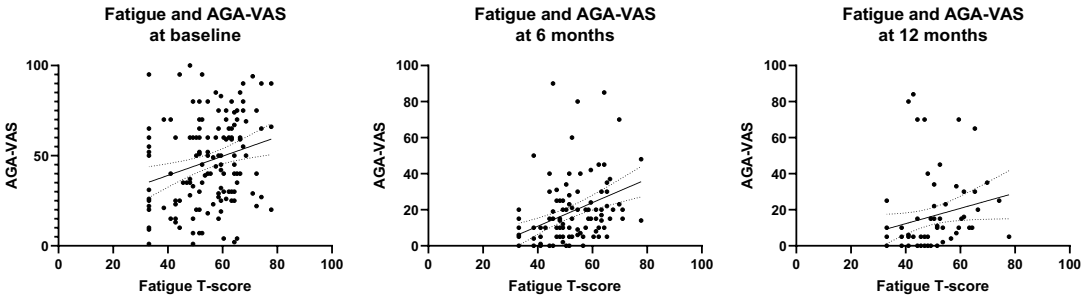


Figure 4.18 Fatigue and AGA-VAS
Simple linear regression was completed to test if AGA-VAS predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at baseline (fitted regression model Y = 0.4926*X + 19.48, r^2= 0.05355, F= 7.751, p=**0.0061), at 6 months (fitted regression model Y = 0.5385*X - 10.28, r^2= 0.1138, F=14.90, p=***0.0002), and at 12 months (fitted regression model Y = 0.4343*X - 7.111, r^2=0.06741, F=4.410, p=*0.0399).

Discordance between PGA-VAS and AGA-VAS is reported in the literature (Barton et al., 2010). Essentially, the two measures ought to measure the same phenomenon, albeit crucially, from different perspectives. Within the patients with fatigue measures in the SERA cohort there are also differences in equivalent scores (figure 4.19). The assessor reported scores are on average consistently lower than the patient reported scores across time points, with the differences becoming more pronounced at 6 and 12 months compared to baseline difference.

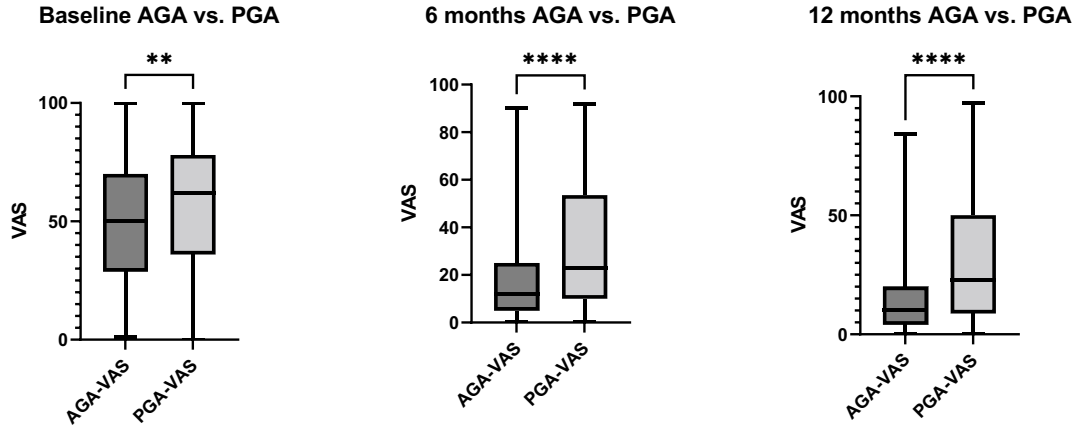


Figure 4.19 AGA-VAS vs. PGA-VAS
AGA-VAS and PGA-VAS were compared at baseline, 6 months and 12 months. Statistical significance of differences was determined using Mann-Whitney U test. PGA scores were significantly higher at baseline (p=**0.0032), 6 months (p=****<0.0001) and at 12 months (p=****<0.0001).

4.4.4.3 Fatigue and tender and swollen joints

An objective clinical assessment of 28 RA-prone joints forms a key component part of the DAS28. This includes a count of swollen joints and a count of tender joints. Within the SERA cohort, this test was completed at each follow-up visit.

Swollen joint count-28 (SJC-28) and tender joint count-28 (TJC-28) were explored in relation to fatigue levels using simple linear regression analysis (figure 4.20). Marked improvements in SJC-28 and TJC-28 are seen across the cohort in keeping with actively treated disease. SJC-28 emerges as a statistically significant predictor variable at 6 months and 12 months, whilst TJC-28 is a statistically significant predictor variable at all time points.

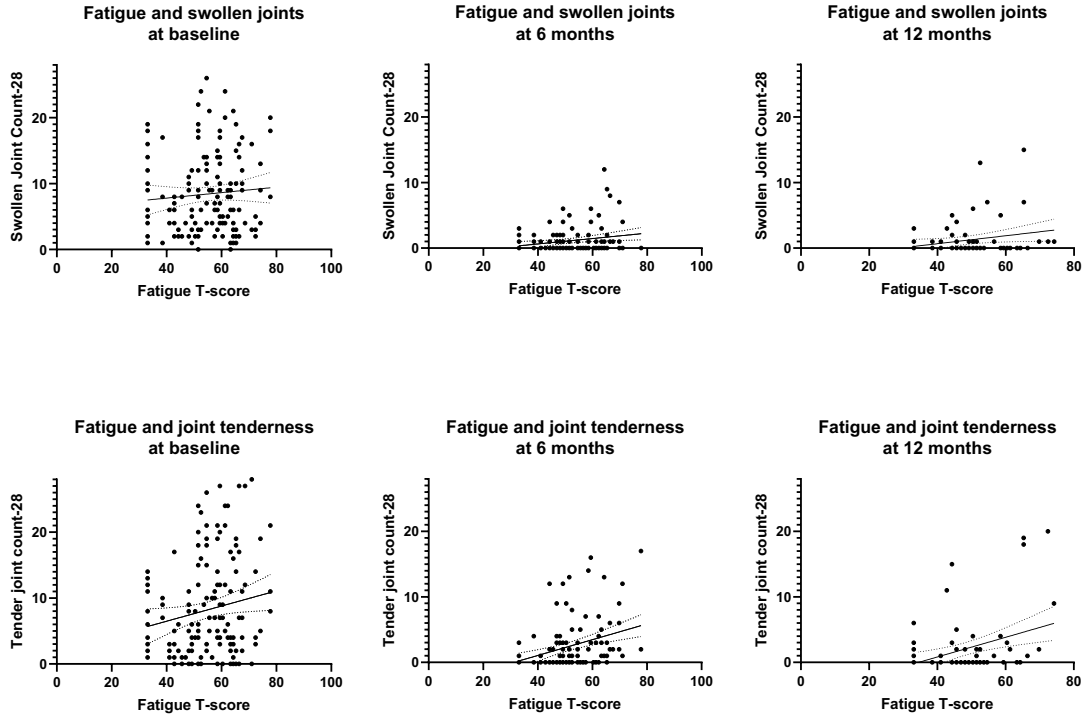


Figure 4.20 Fatigue and SJC-28 and TJC-28
Simple linear regression was completed to test if SJC-28 and TJC-28 predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. For SJC-28, the overall regression model was not statistically significant at baseline (p=0.3737). SJC-28 was a significant predictor variable at 6 months (fitted regression model Y = 0.04124*X - 1.015, r^2 = 0.05054, F=6.388, p=*0.0128) and at 12 months (fitted regression model Y = 0.06064*X - 1.753, r^2 = 0.05444, F= 4.088, p=*0.047). For TJC, the regression was statistically significant at baseline (fitted regression model Y = 0.1152*X + 1.876, r^2 = 0.03041, F= 4.485, p=*0.0359), 6 months (fitted regression model Y = 0.1205*X - 3.766, r^2 = 0.1259, F= 17.29, p=****<0.0001), and at 12 months (fitted regression model Y = 0.1501*X - 5.182, r^2 = 0.1283, F= 10.45, p=**0.0019).

4.4.4.4 Fatigue and ESR and CRP

ESR and CRP levels are general non-specific measures of inflammation that are measured in blood during routine clinical practice, and are characteristically elevated in active RA (Wolfe, 1997). Both ESR and CRP can form part of the DAS28 score and either can be used to generate DAS28 scores (DAS28-ESR and DAS28-CRP).

As mentioned above, the data on ESR within the SERA cohort is limited, particularly in the subset of patients with fatigue scores, as this test was not universally done for each SERA visit (baseline n. 52, 6 months n. 34, 12 months n. 13). ESR analyses are therefore likely to be underpowered. Data from CRP is more comprehensive with data for all patients with fatigue scores.

The relationship between fatigue and ESR at baseline, 6 months and 12 months was explored using simple linear regression analysis (figure 4.21). ESR was not found to predict fatigue in the regression analysis at baseline, 6 months or 12 months.

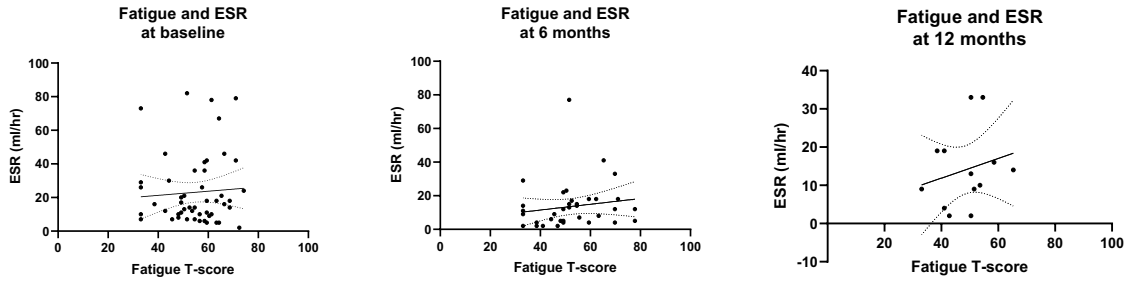


Figure 4.21 Fatigue and ESR
Simple linear regression was completed to test if ESR predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was not statistically significant at baseline (p=0.6543), 6 months (p=0.3469) or 12 months (p=0.4565).

Simple linear regression analysis for fatigue and CRP was explored at the same timepoints (figure 4.22). CRP was not found to significantly predict fatigue at baseline or 6 months. However, a statistically significant relationship was seen at 12 months, when CRP levels in the cohort were much lower.

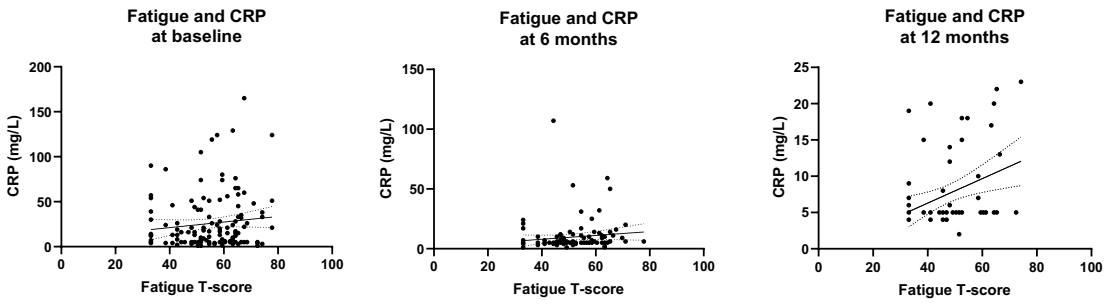


Figure 4.22 Fatigue and CRP
Simple linear regression was completed to test if CRP predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was not statistically significant at baseline (p=0.1885), 6 months (p=0.1808). The regression was statistically significant at 12 months (fitted regression model Y = 0.1687*X - 0.4532, r^2=0.1157, F=8.239, p=**0.0056).

4.4.4.5 Multivariable analysis of DAS28 variables

The results from univariable analysis presented above show that disease activity scales, such as the DAS28, are important predictors of fatigue. PGA-VAS is a consistently significant predictor of fatigue scores. There is a statistically significant relationship between DAS28-CRP at baseline, 6 months and 12 months. The PGA-VAS shows a consistent relationship as a predictor of fatigue at all time points with a relatively substantial r^2. Joint swelling was found to be a significant predictor of fatigue at 6 months and 12 months, and joint tenderness at all time points. However, CRP only appears to have a statistically significant relationship with fatigue at 12 months. ESR had no significant relationship with fatigue, although the n. was small.

To further explore the relationship between the factors described above, a multiple linear regression analysis was completed examining fatigue and the component measures from DAS28-CRP – PGA-VAS, SJC-28, TJC-28 and CRP at baseline, 6 months and 12 months (Model: Fatigue ~ Intercept + PGA-VAS + SJC-28 + TJC-28 + CRP) (figure 4.23). CRP was chosen instead of ESR due to the increased number of cases with CRP data compared to ESR.

The Multiple linear regression analysis found PGA-VAS to be a consistent predictor variable of fatigue across time. This was confirmatory of the univariable analysis.

The model found that SJC-28 was not a significant predictor variable at any time point. TJC-28 was a significant predictor variable at 6 months and 12 months only. These findings contradict the above univariable analysis of these variables.

CRP was not found to be a statistically significant predictor of fatigue. This contradicts the univariable analysis of CRP and fatigue at the 12 months point (see figure 4.22) but confirms the univariable analyses at baseline and 6 months.

Loss of significance of explanatory variables may indicate confirmation of the null hypothesis and that certain variables are not important once confounders are factored in. However, loss of significance of explanatory variables within multivariable analysis can also occur due to loss of degrees of freedom, whereby including more predictor variables in an analysis leads to lower precision and higher p-values. Over-fitting of data can be indicated by the emergence of non-Gaussian distribution of residuals. Statistical testing of distribution of residuals and QQ plotting indicated that the distribution was Gaussian at 6 months and 12 months, though deviated slightly at baseline (figure 4.24). This appears trivial on the QQ plot. Parameters that co-vary can compete for significance in a multiple linear regression analysis. There is some indication of SJC-28 and TJC-28 covariance at 12 months, but less so at baseline and 6 months (figure 4.24).

	Baseline	6 months	12 months
Intercept	41.78**** (2.245)	41.58**** (1.336)	39.84**** (2.113)
PGA-VAS	0.2263**** (0.03465)	0.2202**** (0.03553)	0.1328* (0.05467)
SJC-28	0.01798 (0.1544)	0.4198 (0.4450)	-0.1368 (0.5041)
TJC-28	0.02847 (0.1391)	0.5475* (0.2603)	0.6951* (0.3225)
CRP	-0.009333 (0.02950)	0.08715 (0.06101)	0.4126 (0.2349)
R^2	0.2683	0.4045	0.3175
Adjusted R^2	0.2469	0.3820	0.2720
No. observations	142	111	65

*Coefficients shown with standard error is shown in ( ) below. Statistical significance indicated by *(<0.05), **(<0.01), ***(<0.001) and ****(<0.0001).*

Figure 4.23 Multivariable analysis - Fatigue and PGA-VAS, SJC-28, TJC-28 and CRP
Multiple linear regression analysis at baseline, 6 months and 12 months. The regression models was as follows:-

Fatigue ~ Intercept + PGA-VAS + SJC-28 + TJC-28 + CRP

The overall regression was significant at baseline (p=<0.0001), 6 months (p=<0.0001) and 12 months (p=<0.0001). Coefficients are shown, with statistical significance denoted with *, **, *** and ****. Standard error shown in ( ). R^2 and adjusted R^2 are also shown in the table along with n. at each time-point. PGA-VAS was a significant predictor variable in at all time points. SJC-28 and CRP were not statistically significant predictor variables. TJC-28 was significant at 6 months and 12 months but not at baseline.

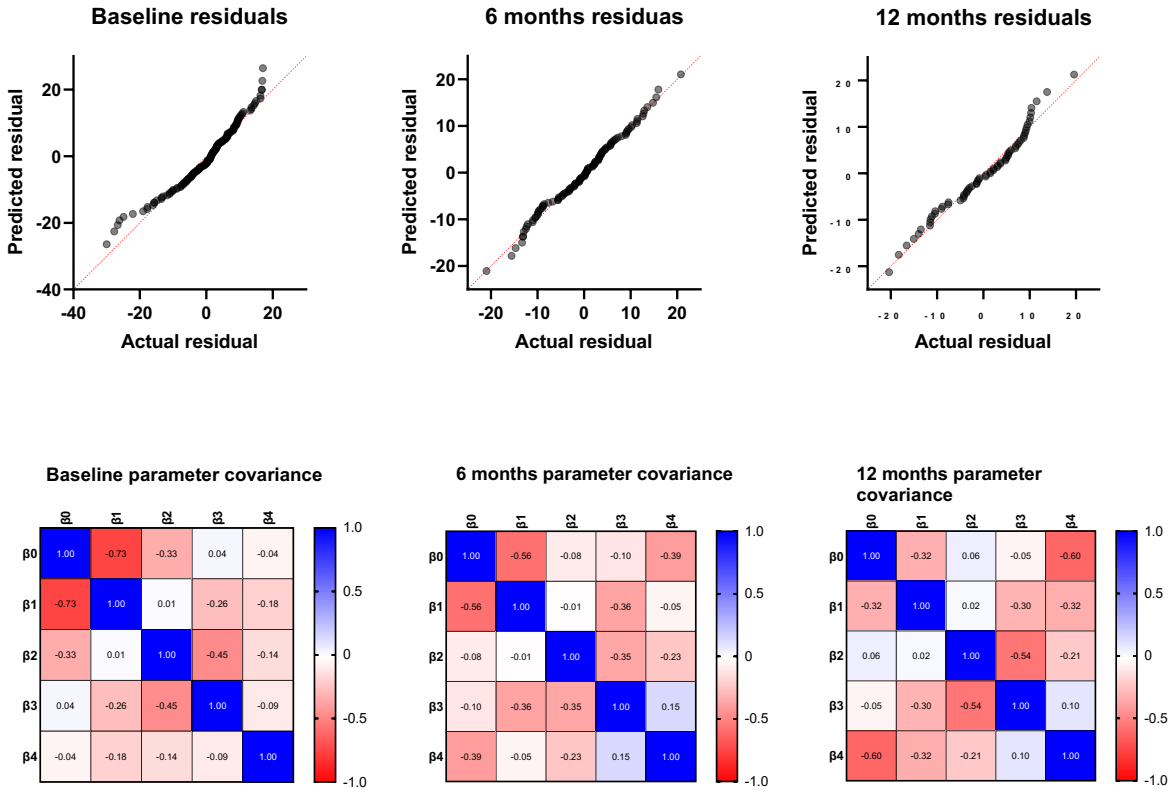


Figure 4.24 Multivariable analysis - Fatigue and PGA-VAS, SJC-28, TJC-28 and CRP - residuals and covariance
Multiple linear regression analysis at baseline, 6 months and 12 months. The regression models was as follows:-

Fatigue ~ Intercept + PGA-VAS + SJC-28 + TJC-28 + CRP

Distribution of residuals is presented in QQ plots. Statistical testing of normality of distribution was conducted using Anderson-Darling (A2*), D'Agostino-Pearson omnibus (K2), Shapiro-Wilk (W) and Kolmogorov-Smirnov (distance). Normality test was not passed in all tests at baseline, but was passed in all tests at 6 months and 12 months. Parameter covariance matrices are presented heat map form showing inverse covariance relationship in red and direct covariance in blue. Β0=intercept, Β1=PGA-VAS, Β2=SJC-28, Β3=TJC-28, Β4=CRP.

4.4.5 Fatigue and measures of health, functioning and pain

4.4.5.1 Fatigue and EuroQol (EQ)-VAS

The EQ-VAS is a patient-reported outcome measure (PROM) where participants are asked to mark on a visual analogue scale (VAS) of between 0 and 100 their assessment of the overall general health at the time of testing (Feng et al., 2014). A value is then obtained between 0 (worst imaginable health) and 100 (best imaginable health). EQ-VAS was completed in the SERA cohort at all time point visits.

Simple linear regression analysis was completed to determine if a relationship existed between patient-reported general health and fatigue, with EQ-VAS as the predictor variable, at baseline, 6 months and 12 months (figure 4.25). A clear relationship was observed, with high EQ-VAS (good patient reported general health) predicting lower fatigue T-score at baseline, 6 months and 12 months.

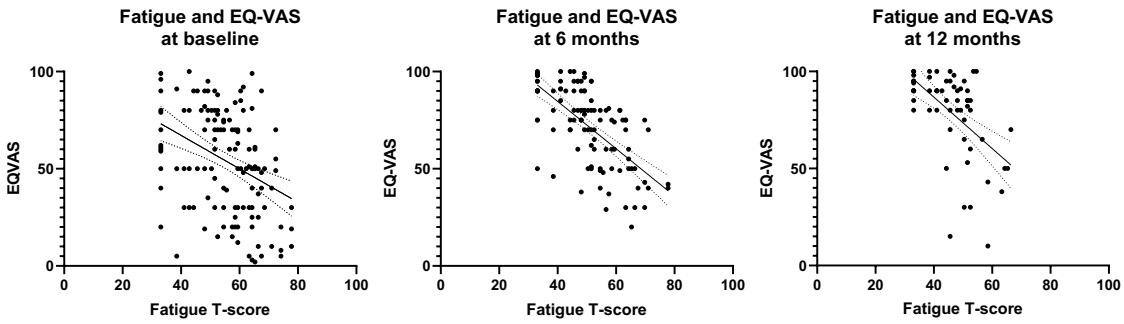


Figure 4.25 Fatigue and EQ-VAS
Simple linear regression was completed to test if EQ-VAS predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at baseline (fitted regression model Y = -0.8611*X + 101.6, r^2= 0.1385, F= 22.84, p=****<0.0001), at 6 months (fitted regression model Y = -1.216*X + 133.3, r^2=0.4191, F=78.64, p=****<0.0001), and at 12 months (fitted regression model Y = -1.326*X + 139.8, r^2=0.2970, F=24.50, p=****<0.0001).

4.4.5.2 Fatigue and HAQ

The HAQ (also known as HAQ-DI) is a validated measure of disability and functional status that was developed specifically for use in RA (Bruce and Fries, 2003) and is regarded as the 'Gold-Standard' measure of functional disability in rheumatological disorders (Maska et al., 2011). The questionnaire consists of 8 categories, encompassing 20 functions to assess ability to manage activities of daily living over the course of the week preceding assessment. The score ranges from 0 to 3, with score increments of 0.125. The content of the HAQ includes tasks such as attending to personal care and completing daily tasks, which are likely to be affected by major fatigue. HAQ scoring was completed in the SERA cohort at all time-point visits.

Simple linear regression analysis was completed to determine if a relationship existed between functional disability and reported fatigue, with HAQ as the predictor variable, at baseline, 6 months and 12 months (figure 4.26). The regression model was statistically significant at baseline, 6 months and 12

months. HAQ score was a consistent predictor of fatigue in the regression model with the r^2 around 0.3 to 0.36 at the separate time points.

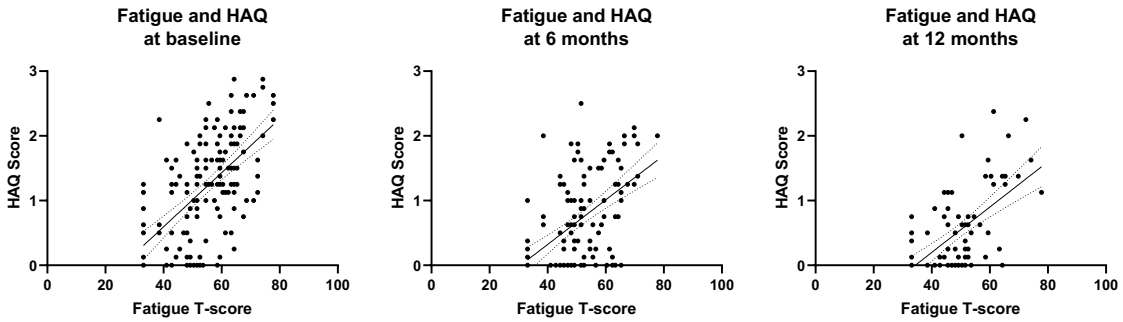


Figure 4.26 Fatigue and HAQ
Simple linear regression was completed to test if HAQ score predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at baseline (fitted regression model Y = 0.04115*X - 1.035, r^2 = 0.3511, F= 77.37, p=****<0.0001), at 6 months (fitted regression model Y = 0.03467*X - 1.066, r^2=0.3004, F=51.95, p=****<0.0001), and at 12 months (fitted regression model Y = 0.03532*X - 1.232, r^2=0.3665, F=41.08, p=****<0.0001).

4.4.5.3 Fatigue and Pain

The Pain-VAS is an established outcome measure validated in measuring patient experience of acute and chronic pain (Revill et al., 1976) (Jensen et al., 1986), including in the context of RA (Kalyoncu et al., 2009). Pain-VAS was completed in the SERA cohort at all study visits.

Simple linear regression analysis was completed to determine if a relationship existed between pain and reported fatigue, with Pain-VAS as the predictor variable, at baseline, 6 months and 12 months (figure 4.27). The regression model was statistically significant at baseline, 6 months and 12 months. A clear relationship was observed between higher pain and higher levels of fatigue.

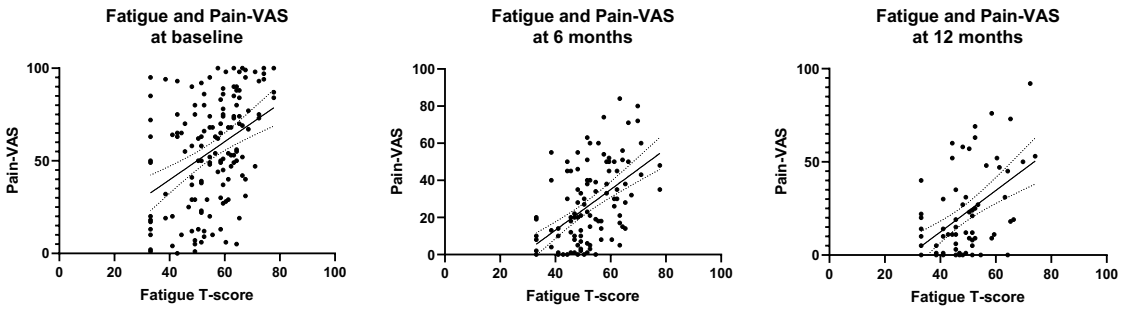


Figure 4.27 Fatigue and Pain-VAS
Simple linear regression was completed to test if Pain-VAS predicted fatigue T-scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at baseline (fitted regression model Y = 1.029*X - 1.377, r^2= 0.1600, F= 27.23, p=****<0.0001), at 6 months (fitted regression model Y = 1.095*X - 30.65, r^2=0.3108, F=53.66, p=****<0.0001), and at 12 months (fitted regression model Y = 1.112*X - 32.10, r^2=0.2674, F=25.55, p=****<0.0001).

4.4.6 Fatigue, depression and anxiety

Depression and anxiety symptoms are measured within the SERA cohort using the Hospital Anxiety and Depression Scale (HADS) (Zigmond and Snaith, 1983). The HADS is one of the most widely used clinical and research tools for the assessment of anxiety and depression since its development nearly 40 years ago. The HADS is a self-report questionnaire. Anxiety and depression symptoms are scored on separate subscales out of 21. An overall score out of 42 is also generated. The HADS has been validated across a number of settings, with a cut-off score of 8+ in each subscale having a diagnostic sensitivity and specificity in the range of 0.7 to 0.9 (Bjelland et al., 2002). The HADS scoring is summarised in table 4.7.

Table 4.7 Summary of HADS scoring interpretation guidance

Anxiety subscale score	Anxiety	Depression subscale score	Depression
0 to 7	Non-case	0 to 7	Non-case
8 to 10	Mild	8 to 10	Mild
11 to 14	Moderate	11 to 14	Moderate
15 to 21	Severe	15 to 21	Severe

Within the whole SERA cohort, HADS scoring was completed at baseline (n. 1041), 6 months (n. 862) and 12 months (n. 764). The data for the sub-cohort with fatigue scores was more limited (baseline n. 145, 6 months n. 129, 12

months n. 85). A summary of the distribution of scores within the cohort at these timepoints is summarised in table 4.8. HADS scores for cases who had also had fatigue scores recorded is summarised in table 4.9. HADS anxiety scores over time are shown in figure 4.28. A similar trend is seen whereby anxiety scores decrease over time. At baseline a substantial proportion of the cohort have clinically meaningful anxiety scores.

Table 4.8 Distribution of HADS anxiety scores in SERA cohort

Anxiety subscale score	% SERA cohort at baseline (n.1041)	% SERA cohort at 6 months (n.862)	% SERA cohort at 12 months (n.764)
0 to 7	63.1%	71.9%	73.7%
8 to 10	18.2%	13.7%	13.2%
11 to 14	13.7%	10.6%	9%
15 to 21	5%	3.8%	4.1%

Table 4.9 Distribution of HADS anxiety score in SERA fatigue sub-cohort

Anxiety subscale score	% SERA cohort at baseline *with fatigue scores* (n.145)	% SERA cohort at 6 months *with fatigue scores* (n.123)	% SERA cohort at 12 months *with fatigue scores* (n.73)
0 to 7	58.8%	68.2%	76%
8 to 10	24.2%	18.6%	18.1%
11 to 14	14.4%	11.6%	4.7%
15 to 21	2.6%	1.6%	1.2%

Distribution of HADS depression scores for the SERA cohort are shown in table 4.10, whilst scores for SERA cohort patients who also have fatigue scores is shown in table 4.11. The trend for reducing scores over time is repeated in the HADS depression data for both the whole cohort and the cohort with fatigue scores.

Table 4.10 Distribution of HADS depression score in SERA cohort

Depression subscale score	% SERA cohort at baseline (n.1041)	% SERA cohort at 6 months (n.862)	% SERA cohort at 12 months (n.764)
0 to 7	72.6%	79.6%	81.7%
8 to 10	15.2%	11.5%	11%
11 to 14	10.2%	6.9%	6%
15 to 21	2%	2%	1.3%

Table 4.11 Distribution of HADS depression scores in SERA fatigue sub-cohort

Depression subscale score	% SERA cohort at baseline *with fatigue scores* (n.145)	% SERA cohort at 6 months *with fatigue scores* (n.123)	% SERA cohort at 12 months *with fatigue scores* (n.73)
0 to 7	64.1%	76%	85.7%
8 to 10	24.1%	17%	8.3%
11 to 14	9.2%	4.6%	6%
15 to 21	2.6%	1.6%	0%

Differences in HADS scores over time are shown in figure 4.28. There is a clear reduction in scores for both anxiety and depression subscores by 6 months from enrolment in the study. There is not a significant difference between 6 months and 12 months.

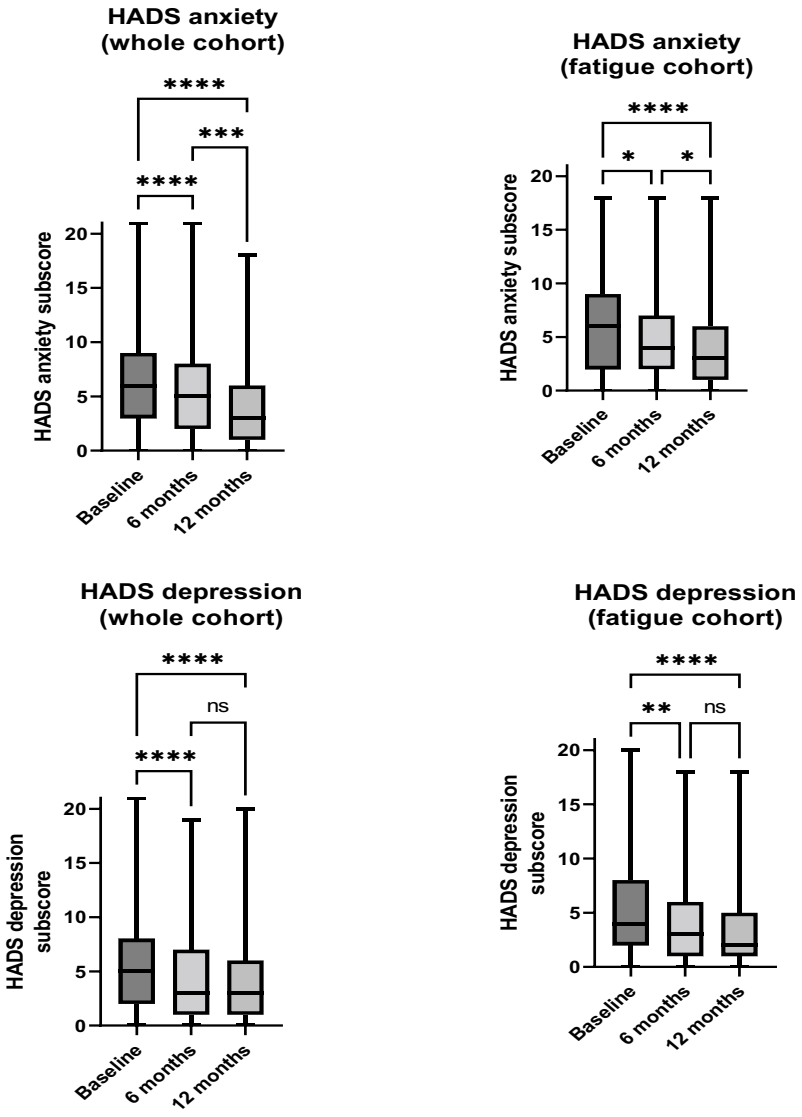


Figure 4.28 HADS scores over time in the SERA cohort
In the whole cohort, HADS scoring was completed at baseline (n. 1041), 6 months (n. 862) and 12 months (n. 764). The data for the part of the cohort with fatigue scores was more limited (baseline n. 145, 6 months n. 123, 12 months n. 73). Fatigue scores are compared over time using Kruskal-Wallis test with multiple comparisons. Significant reductions are seen over time in the HADS depression and anxiety scores. This is clearer in the whole cohort where there is more data. The largest changes in scores occur within 6 months for both anxiety and depression, with further significant differences in overall scores between 6 months and 12 months for anxiety but not for depression HADS subscores.

Simple linear regression analysis was completed to determine if a relationship existed between total HADS scores as the predictor variable, at baseline, 6 months and 12 months (figure 4.29). This was repeated for individual HADS

subscores of anxiety and depression. A very clear relationship is seen between fatigue and HADS score, including anxiety and depression subscores. HADS appears to account for a substantial proportion of the variance with an r^2 of around 0.4 at baseline, 6 months and 12 months. This is broadly replicated with the subscores of anxiety and depression.

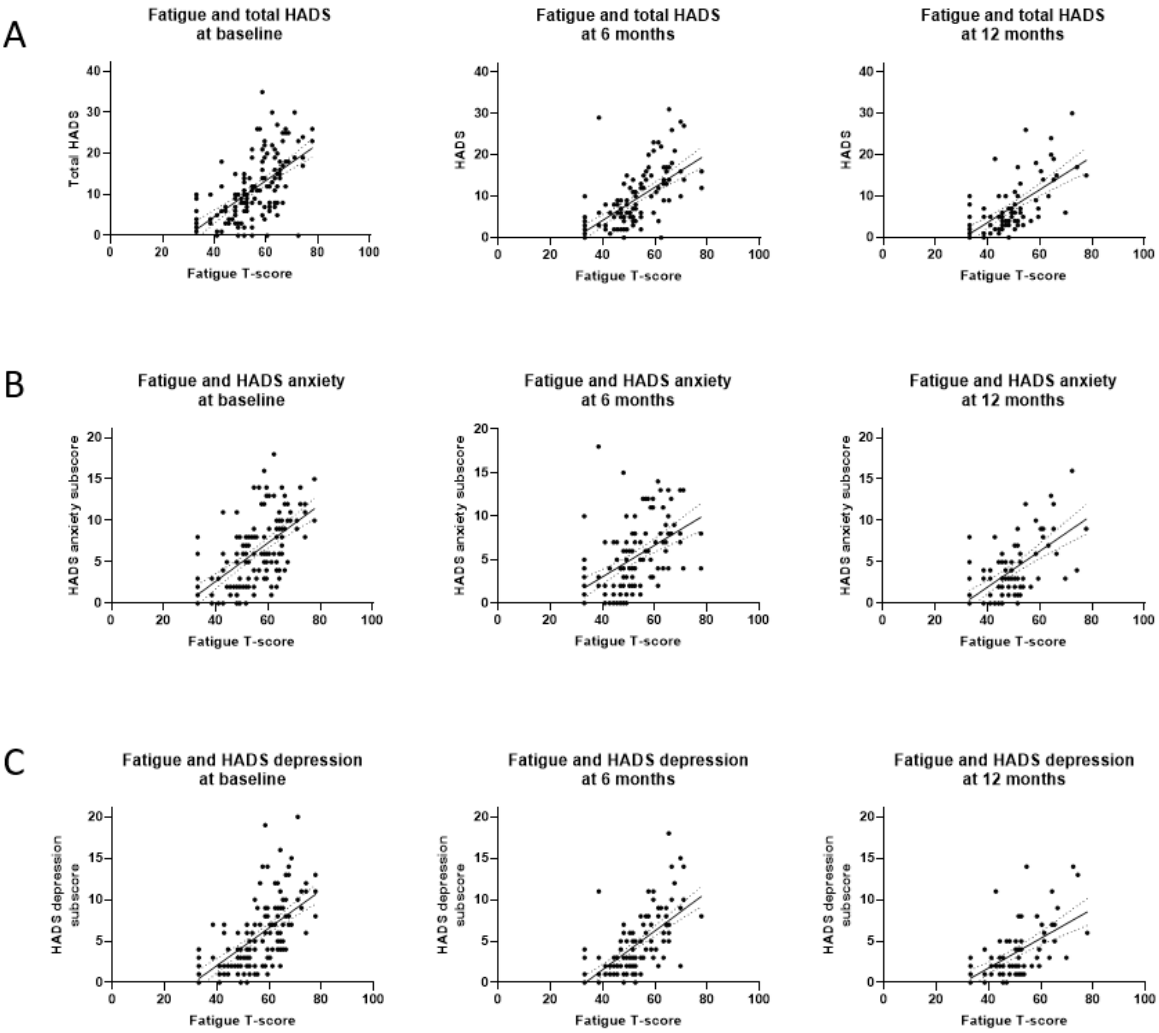


Figure 4.29 Fatigue and HADS
Simple linear regression was completed to test if total HADS at baseline, 6 months and 12 months predicted fatigue T-scores at baseline, 6 months and 12 months (A). This was repeated for HADS anxiety (B) and depression subscores (C). Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant (p=****<0.0001) at all time-points for all predictor variables tested:-

A – baseline: fitted regression model Y = 0.4500*X - 13.38, r^2= 0.4040, F= 96.92, p=****<0.0001

A – 6 months: fitted regression model Y = 0.4068*X - 12.04, r^2=0.3959, F=79.31, p=****<0.0001

A – 12 months: fitted regression model Y = 0.4105*X - 12.81, r^2=0.4296, F=53.48, p=****<0.0001

B – baseline: fitted regression model Y = 0.2382*X - 6.885, r^2= 0.3801, F= 85.24, p=****<0.0001

B – 6 months: fitted regression model Y = 0.1872*X - 4.447, r^2=0.2575, F=40.92, p=****<0.0001

B – 12 months: fitted regression model Y = Y = 0.2161*X - 6.697, r^2=0.3694, F=41.59, p=****<0.0001

C – baseline: fitted regression model Y = 0.2296*X - 7.086, r^2= 0.3870, F= 89.01, p=****<0.0001

C – 6 months: fitted regression model Y = 0.2369*X - 7.927, r^2=0.4856, F=112.3, p=****<0.0001

C – 12 months: fitted regression model Y = Y = 0.1945*X - 6.022, r^2=0.3746, F=41.93, p=****<0.0001

4.4.6.1 Fatigue and depression diagnosis

To determine if comorbid pre-existing depression was an important factor in fatigue in the SERA cohort, data on case medical histories was extracted. Out of 145 patients with fatigue scores at baseline, only 13 had a history of depression (8.97%), with one of the 13 also having a comorbid diagnosis of anxiety. There were no other cases with diagnoses of anxiety disorder or any other mental health disorder. Out of the 13 cases with a history of depression, this was noted as ongoing in 10 cases and in remission in 3 cases.

Fatigue scores were compared between patients with ongoing depression and no history of depression (figure 4.30). No significant differences were found.

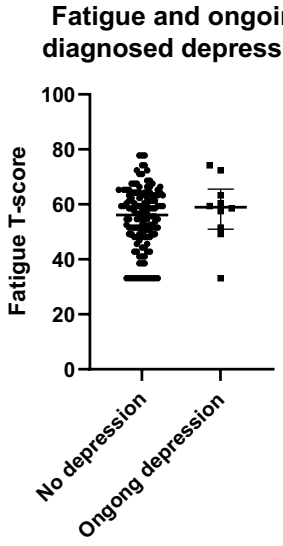


Figure 4.30 Fatigue and depression diagnosis at baseline Information on comorbid medical disorders was extracted from the SERA database. Presence or absence of documented history of ongoing depressive disorder at time of baseline SERA cohort assessment was noted. Baseline fatigue T scores were compared between cases with ongoing documented diagnosed depressive illness and cases without no history of depressive illness. Statistical significance was determined using Mann-Whitney U test. There was no significant difference in fatigue T-scores between the two groups (p=0.4918).

4.4.6.2 Fatigue and antidepressant medication

To further explore whether fatigue could be substantially related to depression, rates of antidepressant prescribing among the cohort was examined. Data was gathered to determine ongoing prescription of antidepressant medication at baseline assessment. Presence or absence of antidepressant medication at baseline was then used to compare fatigue scores (figure 4.31).

Out of 145 patients with fatigue scores at baseline, 18 were prescribed antidepressant medication. Of these 18 cases, 12 cases were prescribed an SSRI, whilst 6 were prescribed the tricyclic antidepressant (TCA) amitriptyline.

Medication doses are not known, and it must be borne in mind that in modern practice amitriptyline is more frequently prescribed to treat pain as opposed to depression.

No significant association was seen between prescription of antidepressant medication and fatigue scores at baseline.

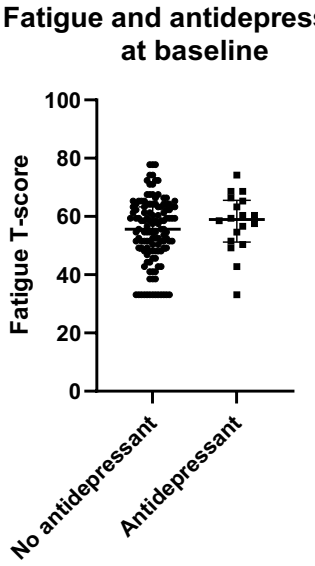


Figure 4.31 Fatigue and antidepressant prescribing at baseline
Information on antidepressant medication prescribing was extracted from the SERA database. Presence or absence of ongoing antidepressant prescription at time of baseline SERA cohort assessment was noted. Baseline fatigue T scores were compared between cases with ongoing documented diagnosed depressive illness and cases without no history of depressive illness. Statistical significance was determined using Mann-Whitney U test. There was no significant difference in fatigue T-scores between the two groups (p=0.2858).

4.4.7 Fatigue and laboratory blood measures

A range of blood tests relevant to inflammation and systemic illness were conducted in the SERA cohort with data available at baseline, 6 months and 12 months for renal function and full blood count.

4.4.7.1 Fatigue and renal function

Renal dysfunction in RA can be associated with high disease activity and inflammation in early stage RA (Koseki et al., 2001). Serum urea and creatinine data is available within the SERA cohort at baseline, 6 months and 12 months. Data on glomerular filtration rate is not. Urea and creatinine, when elevated to abnormal levels can indicate impaired renal function and can be a marker of systemic illness in the context of inflammatory disorders.

The relationship between urea and creatinine at baseline, 6 months and 12 months, with fatigue at these time-points was explored using simple linear regression analysis (figure 4.32). There was no significant relationship between urea and fatigue at any time point. Creatinine appeared to show an inverse association at baseline, but no significant relationship at 6 months or 12 months.

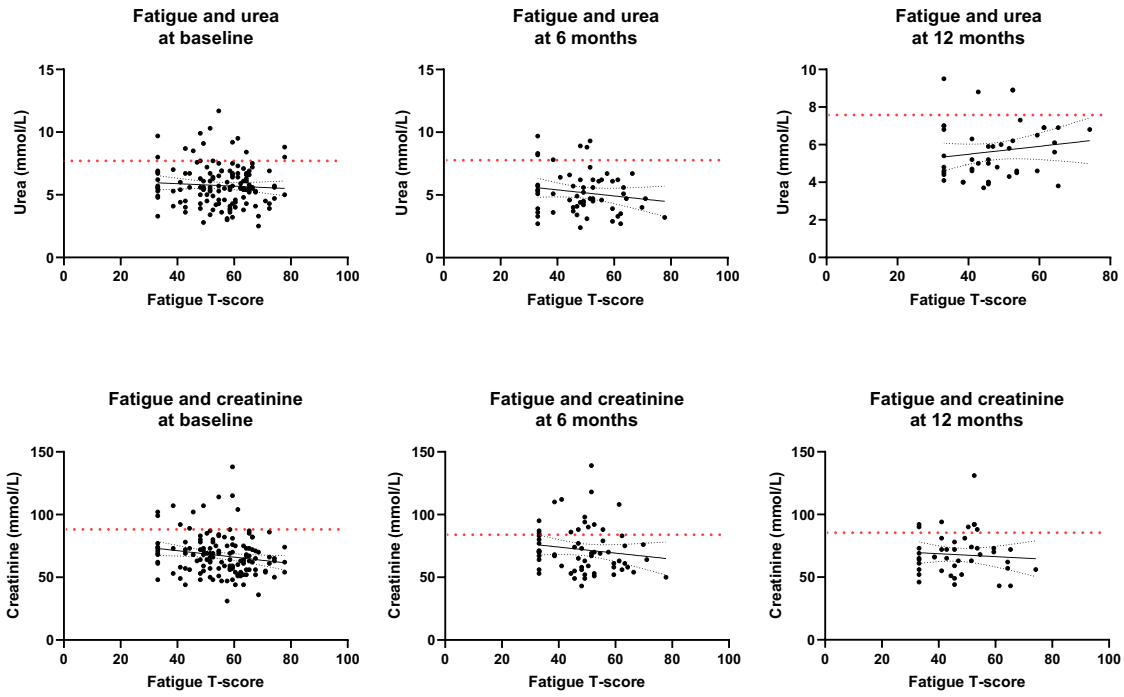


Figure 4.32 Fatigue and urea and creatinine
Simple linear regression was completed to test if serum urea and creatinine at baseline, 6 months and 12 months predicted fatigue T-scores at baseline, 6 months and 12 months. A red dotted line indicates the upper limit of the normal range (urea 7.8mmol/L, creatinine 84 mmol/L). Best fit line is shown with 95% confidence interval on either side. The overall regression model was not statistically significant for urea at any time-point (baseline p=0.6090, 6 months p=0.1563, 12 months p=0.7395). The overall regression model was not statistically significant for creatinine at any time-point (baseline p=0.0533, 6 months p=0.2452, 12 months p=0.9354).

4.4.7.2 Fatigue and anaemia

Data on haemoglobin levels in the SERA cohort can be obtained from FBC results at baseline, 6 months and 12 months. Anaemia can be a complication of inflammatory disorders as a consequence of organ dysfunction, inflammation and anaemia of chronic disease.

The relationship between Haemoglobin and fatigue at baseline, 6 months and 12 months within the SERA cohort was explored using simple linear regression analysis (figure 4.33). Few patients in the cohort were anaemic and the

proportion reduced over time, presumably in line with improvements in disease activity. No significant relationship was seen between haemoglobin and fatigue. In the minority of patients who were anaemic there is no trend observable in the graphs to suggest higher fatigue among anaemic cases.

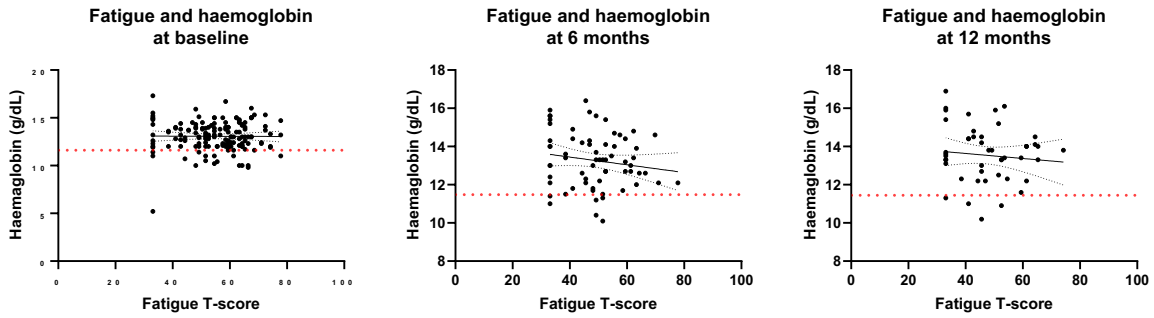


Figure 4.33 Fatigue and haemoglobin
Simple linear regression was completed to test if haemoglobin at baseline, 6 months and 12 months predicted fatigue T-scores at baseline, 6 months and 12 months. A red dotted line indicates the lower limit of the normal range (11.5 g/dL). Best fit line is shown with 95% confidence interval on either side. The overall regression model was not statistically significant at baseline (p=0.686), 6 months (p=0.1925) or 12 months (p=0.5241).

4.4.7.3 Fatigue and platelets

Reactive thrombocytosis is correlated with disease activity in RA (Ertenli et al., 2003). There is limited if any data available in the published literature specifically examining if there is a relationship between elevated platelet levels and fatigue.

The relationship between platelet levels and fatigue at baseline, 6 months and 12 months within the SERA cohort was explored using simple linear regression analysis (figure 4.34). A significant relationship was seen at baseline, but not at 6 months or 12 months.

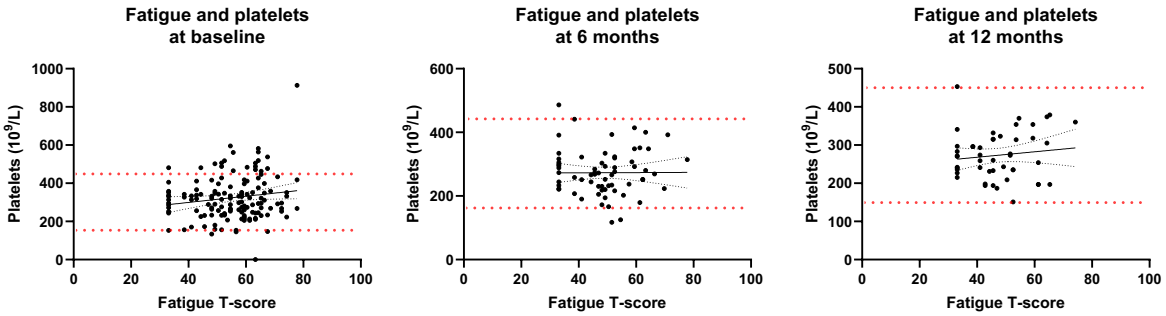


Figure 4.34 Fatigue and platelets
Simple linear regression was completed to test if platelets at baseline, 6 months and 12 months predicted fatigue T-scores at baseline, 6 months and 12 months. A red dotted line indicates the lower and upper limit of the normal range (15-450x10^9/L). Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at baseline (fitted regression model Y = 1.799*X + 221.2, r^2=0.03072, F=4.469, p=*0.0363), but not at 6 months (p=0.9792) or 12 months (p=0.2294).

4.4.7.3 Fatigue and peripheral blood leukocytes

Leukocytosis can occur in RA as an inflammatory manifestation or as a result of treatment with corticosteroids (Syed and Pinals, 1996).

Simple linear regression analysis was undertaken to determine if there was a relationship between total leukocytes and fatigue at baseline, 6 months and 12 months (figure 4.35). This was repeated for individual leukocyte populations of monocytes, lymphocytes, neutrophils and eosinophils (figure 4.36). Total WCC was elevated in a proportion of cases at baseline, but this was attenuated by 6 months. A similar pattern was seen in neutrophils. However, no significant relationship was observed between fatigue and either total WCC or differential blood count.

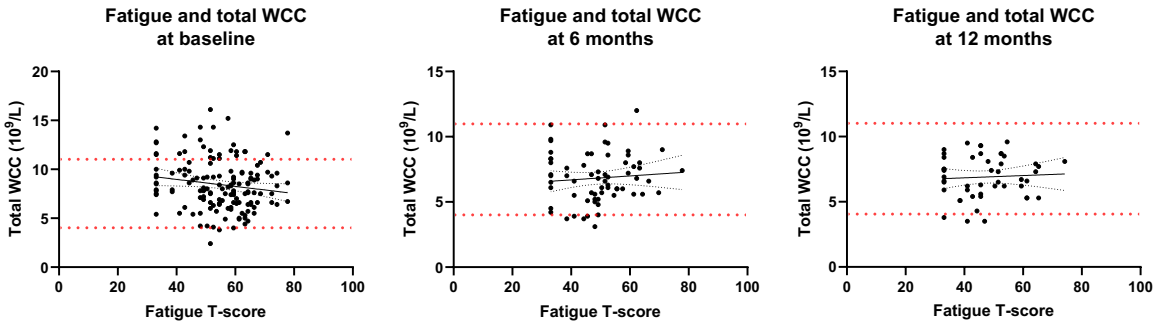


Figure 4.35 Fatigue and total WCC
Simple linear regression was completed to test if total WCC at baseline, 6 months and 12 months predicted fatigue T-scores at baseline, 6 months and 12 months. A red dotted line indicates the lower and upper limit of the normal range (4-11x10^9/L). Best fit line is shown with 95% confidence interval on either side. The overall regression model was not statistically significant at baseline (p=0.0626), 6 months (p=0.6289) or 12 months (p=0.4999).

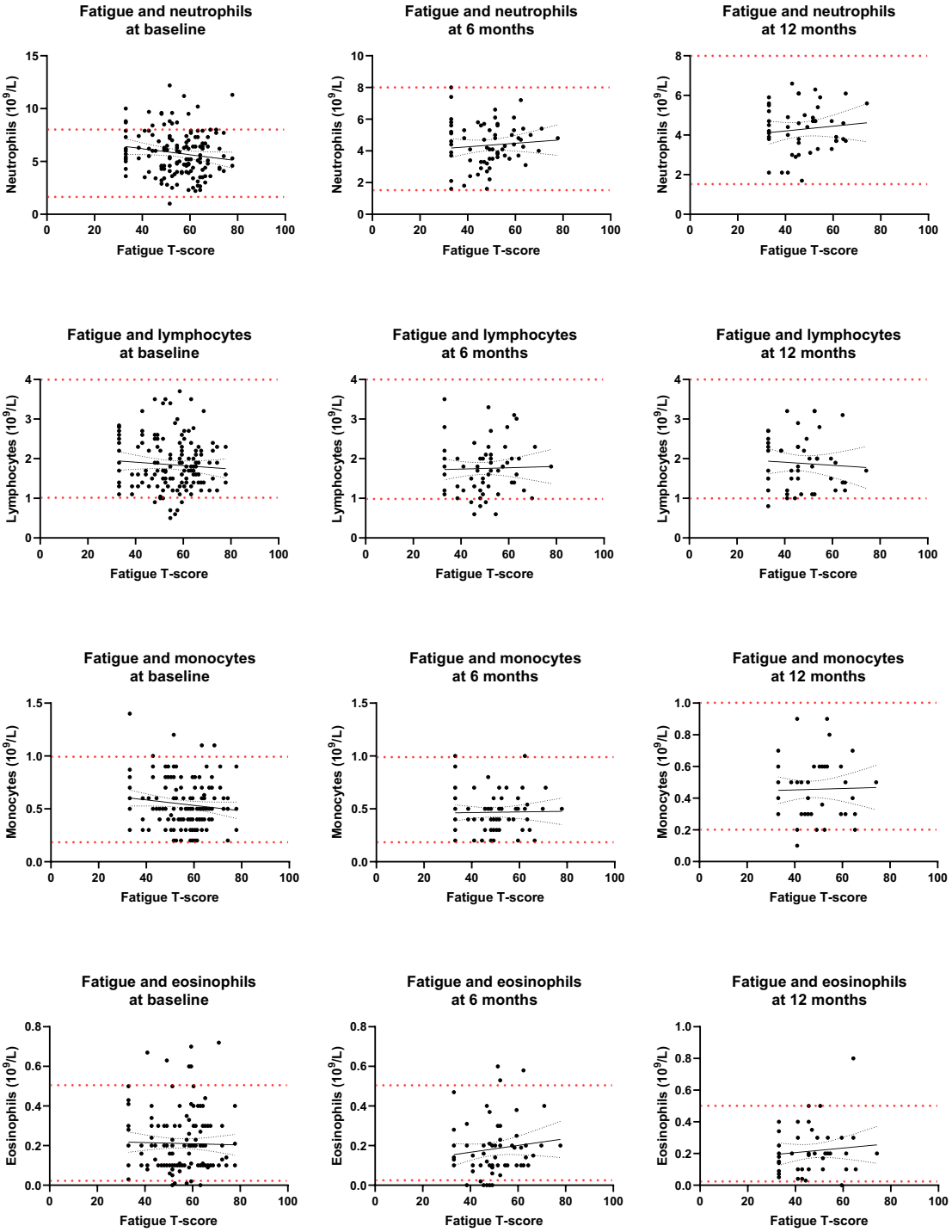


Figure 4.36 Fatigue and differential blood count
Simple linear regression was completed to test if neutrophils, lymphocytes, monocytes and eosinophils at baseline, 6 months and 12 months predicted fatigue T-scores at baseline, 6 months and 12 months. A red dotted line indicates the lower and upper limit of the normal ranges. Best fit line is shown with 95% confidence interval on either side. Neutrophils: the overall regression model was not statistically significant at baseline (p=0.0583), 6 months (p=0.6954) or 12 months (p=0.449). Lymphocytes: the overall regression model was not statistically significant at baseline (p=0.5967), 6 months (p=0.7554) or 12 months (p=0.9431). Monocytes: the overall regression model was not statistically significant at baseline (p=0.1571), 6 months (p=0.9915) or 12 months (p=0.558). Eosinophils: the overall regression model was not statistically significant at baseline (p=0.7366), 6 months (p=0.2308) or 12 months (p=0.2058).

4.4.8 Summary of key findings in univariable analyses

The data presented shows the results of univariable analysis of a range of demographic and lifestyle factors, and clinical and disease measures, and how they associate with fatigue in the context of inflammatory arthritis in the SERA cohort. A summary of the associations is shown in tables 4.12-4.17. P-values are noted if between 0.05 and 0.2 to inform selection of variables for multivariable analysis.

The univariable analyses tend to indicate that measures of disease activity are strong predictors of fatigue scores. However, basic inflammatory measures such as ESR and CRP tend to be less reliable than more symptom-based measures such as global assessment VAS, HAQ score, EQ-VAS and pain VAS. Another striking feature is the consistency with which HADS scores predict fatigue. These data raise the question as to whether fatigue is substantially a dimension of another variable, rather than a distinct phenomenological entity.

Limitations do exist in the data. Unfortunately, the number of cases within the cohort in which there is available data on fatigue, represents a relatively small proportion of the total cohort. However, the overall characteristics of the cohort in which there is fatigue data, does not vary significantly from the total cohort in terms of demographic or clinical characteristics. Univariable analysis cannot reliably determine the relative influence within the model of different predictor/independent variables. Nor can independent predictor parameter co-variance or multicollinearity be examined within a single regression model.

Table 4.11 Demographic variables and fatigue – summary of significant associations

	Timepoint		
Variable	**Baseline**	**6 months**	**12 months**
Sex	**	*	n.s. (p=0.1137)
Age	n.s.	n.s.	n.s.
SIMD2012 quintile	n.s.	n.s. (p=0.1284)	n.s.
SIMD2012 score	n.s.	*	n.s.
Employment status	n.s.	n.s. (p=0.0561)	n.s. (p=0.1243)

Table 4.12 Diagnostic variables and fatigue – summary of significant associations

Variable	Timepoint		
	Baseline	6 months	12 months
Diagnosis RA vs. UA	n.s. (p=0.0523)	n.s. (p=0.0501)	n.s. (p=0.1893)
ACR-EULAR score	n.s. (p=0.1521)	**	n.s.
Visser score	n.s. (p=0.1101)	***	n.s.

Table 4.13 Lifestyle variables and fatigue – summary of significant associations

Variable	Timepoint		
	Baseline	6 months	12 months
Smoking status	n.s.	n.s.	n.s.
Alcohol intake	*	n.s. (p=0.075)	n.s.
BMI	n.s. (p=0.1705)	n.s. (p=0.0954)	n.s.
Cholesterol ratio	n.s.	n.s.	n.s.

Table 4.14 Disease activity/health/disability/pain variables and fatigue – summary of significant findings

Variable	Timepoint		
	Baseline	6 months	12 months
DAS28-ESR	n.s. (p=0.0528)	****	n.s. (p=0.1713)
DAS28-CRP	**	****	***
PGA-VAS	****	****	****
AGA-VAS	**	***	*
SJC-28	n.s.	*	*
TJC-28	*	****	**
ESR	n.s.	n.s.	n.s.
CRP	n.s. (p=0.1855)	n.s. (p=0.1808)	**
EQ-VAS	****	****	****
HAQ	****	****	****
Pain-VAS	****	****	****

Table 4.15 HADS scores, depression, antidepressant medications and fatigue – summary of significant findings

Variable	Timepoint		
	Baseline	6 months	12 months
HADS anxiety score	****	****	****
HADS depression score	****	****	****
Total HADS score	****	****	****
Ongoing depression	n.s.	n/a	n/a
Ongoing antidepressant	n.s.	n/a	n/a

Table 4.16 Laboratory blood measures and fatigue – summary of significant findings

Variable	Timepoint		
	Baseline	6 months	12 months
Cholesterol ratio	n.s.	n.s.	n.s.
Urea	n.s.	n.s. (p=0.1563)	n.s.
Creatinine	n.s. (p=0.0533)	n.s.	n.s.
Haemoglobin	n.s.	n.s. (p=0.1925)	n.s.
Platelets	*	n.s.	n.s.
Total WCC	n.s. (p=0.0626)	n.s.	n.s.
Neutrophils	n.s. (p=0.0583)	n.s.	n.s.
Lymphocytes	n.s.	n.s.	n.s.
Monocytes	n.s. (p=0.1571)	n.s.	n.s.
Eosinophils	n.s.	n.s.	n.s.

4.5 Results: Multivariable analysis of fatigue in the SERA cohort

4.5.1 Introduction

To further explore the relationship between the various predictor variables and fatigue, a multivariable analysis was conducted, using multiple linear regression. Multiple linear regression allows generation of a multifactor model in the prediction of fatigue. It also allows comparison of the co-efficient of variance between predictor variables and variable multicollinearity within an inclusive model. Parameter covariance can also be determined in relation to the dependent variable.

Selection of candidate predictor variables for the multivariable analysis was guided by the original hypothesis, driven by the animal experiments conducted in CIA, that inflammatory variables would be strongly associated with sickness behaviours such as fatigue.

In line with generally accepted principles of multivariable analysis, a degree of variable rationalisation was required to ensure that the minimum ratio of the number of cases per predictor variable did not fall below 10. Additionally, including too many variables or irrelevant variables can produce misleading results due to 'noise' and over-fitting. But it is important to include appropriate variables that may emerge as important when confounding is controlled for. Therefore, variables relevant to inflammation and which had a p-value of less than 0.2 in univariable analysis at any time point were selected for inclusion in the multivariable analysis. For consistency, where possible the same variables were included for analysis at each consecutive timepoint. This approach was essentially a combination of using *a-priori* knowledge informed hypothesis with a rudimentary variant of the 'step-wise' approach to variable selection (Steyerberg, 2019).

4.5.2 Multiple linear regression of fatigue and inflammation-related variables

The only basic inflammation-specific variables measured within the SERA cohort are ESR and CRP. However, as these are relatively blunt measures, it was

decided to include variables that were also pertinent to disease activity. For example, although ACR/EULAR-2010 score and Visser score are essentially diagnostic and prognostic tools respectively, they include important measures within their composite scores such as RF titres and periarticular erosions, which are relevant to disease activity. ESR was not included due to the very low n. value at later time-points. Total WCC was included in preference to including neutrophils and monocytes separately, not only to rationalise the included variables but also because the p-value was lower for total WCC than either neutrophils or monocytes. DAS28-CRP was not included as its constituent parts were included individually. Platelets and total WCC were only included in the regression analysis at baseline as their limited measurement at 6 months and 12 months would have reduced the number of cases per predictor variable down to 3 at these time points, rendering the regression analysis uninterpretable.

The variables considered most relevant to inflammation and disease activity, with a p-value of <0.2 in univariable analyses, were:-

- ACR-EULAR score
- Visser score
- PGA-VAS
- AGA-VAS
- SJC-28
- TJC-28
- CRP
- Pain-VAS
- Platelets (baseline only due to low n.)
- Total WCC (baseline only due to low n.)

The multiple linear regression model was as follows:-

Fatigue ~ Intercept + ACR-EULAR score + Visser score + PGA-VAS + AGA-VAS + SJC-28 + TJC-28 + CRP + Platelets + Total WCC

The results of the multiple linear regression analysis are presented in figure 4.37.

	Baseline	6 months	12 months
Intercept	47.44**** (6.378)	39.97**** (2.541)	43.45**** (4.893)
ACR-EULAR score	0.6287 (0.7520)	-0.6811 (0.7983)	-0.5177 (1.258)
Visser score	-1.182 (0.9593)	0.8390 (0.7925)	-0.3784 (1.289)
PGA-VAS	0.1611** (0.05573)	0.1975*** (0.05227)	0.1175 (0.08199)
AGA-VAS	0.03417 (0.05180)	-0.002244 (0.06922)	-0.1401 (0.1363)
SJC-28	-0.01391 (0.2052)	0.4441 (0.5191)	0.4608 (0.6553)
TJC-28	0.003080 (0.1623)	0.4392 (0.3053)	0.4089 (0.4291)
CRP	-0.01780 (0.03743)	0.06560 (0.06864)	0.4791 (0.2720)
Pain-VAS	0.04505 (0.05007)	0.04273 (0.06400)	0.1210 (0.09937)
Platelets	0.01881* (0.009416)		
Total WCC	-0.7888 (0.4138)		
R^2	0.2856	0.4116	0.3081
Adjusted R^2	0.2235	0.3636	0.1904
No. observations	126	107	56

*Coefficients shown with standard error is shown in ( ) below. Statistical significance indicated by *(<0.05), **(<0.01), ***(<0.001) and ****(<0.0001).*

Figure 4.37 Multivariable analysis - Fatigue and disease activity/inflammatory variables
Multiple linear regression analysis was completed using variables at baseline, 6 months and 12 months. The regression models was as follows:-

Fatigue ~ Intercept + ACR-EULAR score + Visser score + PGA-VAS + AGA-VAS + SJC-28 + TJC-28 + CRP + Pain-VAS + Platelets + Total WCC

Platelet and neutrophil counts were excluded from multivariable analysis at 6 months and 12 months due to low number of data within cohort at these time points. The overall regression was significant at baseline (p=****<0.0001), 6 months (p=****<0.0001) and 12 months (p=**0.0036). Coefficients are shown, with statistical significance denoted with *, **, *** and ****. Standard error shown in ( ). R^2 and adjusted R^2 are also shown in the table along with n. at each time-point. PGA-VAS was a significant predictor variable in at baseline (P=**0.0046) and 6 months (P=***0.0003). Platelets were a significant predictor variable at baseline (p=*0.0481) with a low co-efficient (0.01881). No other predictor variables reached significance at any time point.

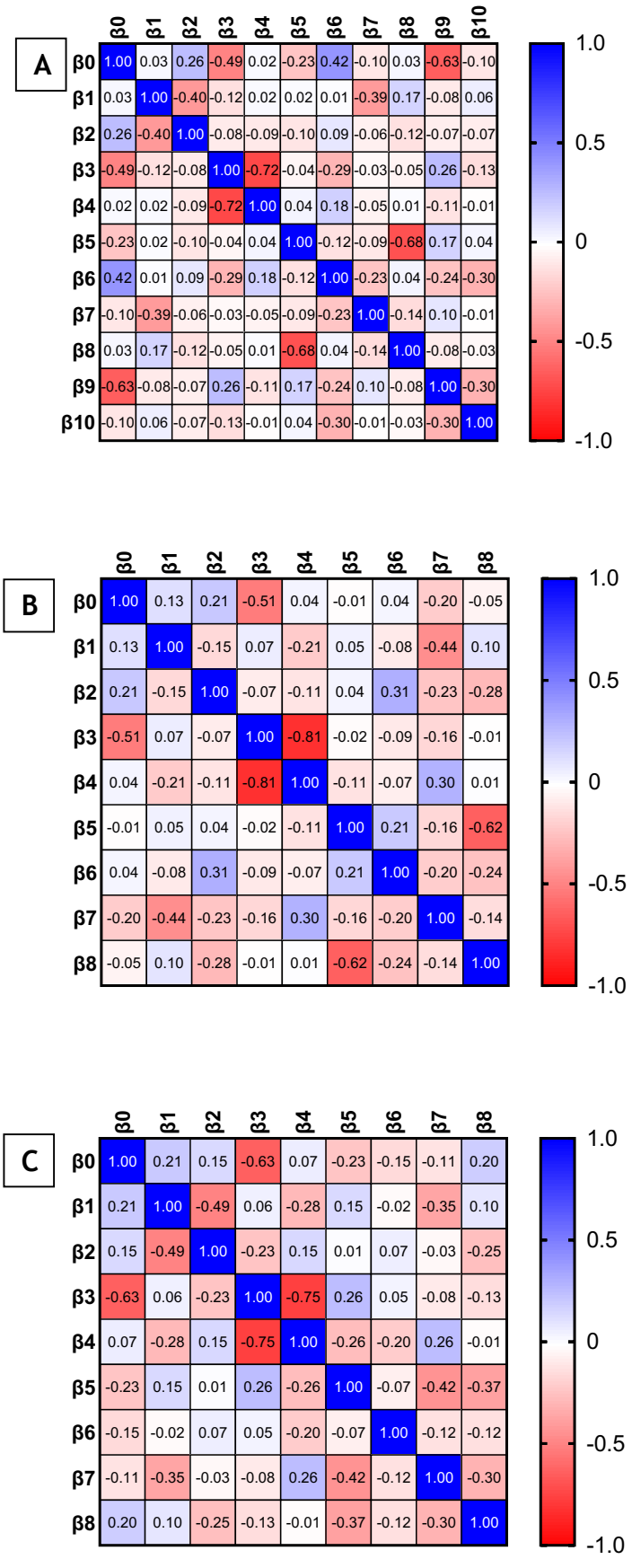


Figure 4.38 Parameter covariance matrix in multiple linear regression of fatigue at baseline (A), 6 months (B) and 12 months (C)

Β0 - Intercept
Β1 - SJC-28
Β2 - TJC-28
Β3 - Visser
Β4 - ACR-EULAR score
Β5 - PGA-VAS
Β6 - CRP
Β7 - AGA-VAS
Β8 - Pain-VAS
Β9 - Total WCC
Β10 - Platelets

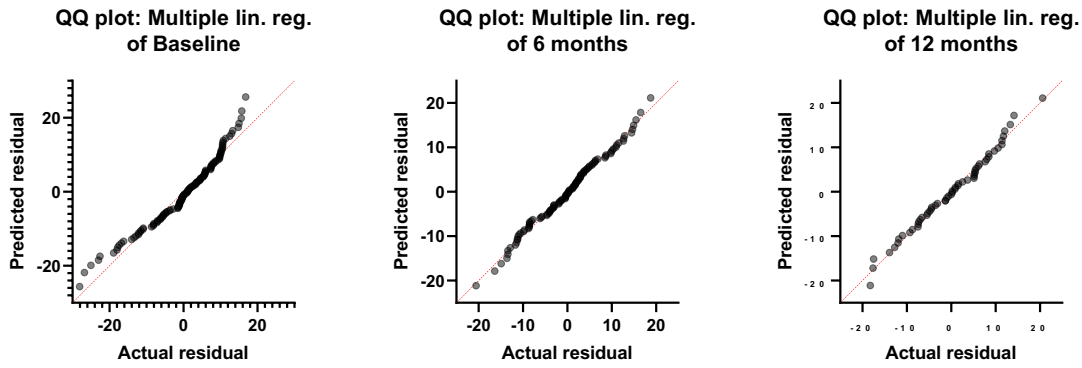


Figure 4.39 QQ-plot of residuals in multiple linear regression analysis of fatigue at baseline, 6 months and 12 months
Residuals are not normally distributed at baseline, but distribution is Guassian at 6 months and 12 months.

The results of the multiple linear regression analysis indicate that platelets (baseline) and PGA-VAS (baseline and 6 months) were significant predictor variables for fatigue. None of the predictor variables were significant at 12 months. However, the number of cases per predictor variable fell to 6.2 at 12 months due to missing data, limiting the interpretability of the regression at that time-point.

Overall, the results from the multiple linear regression analysis tend to support the null hypothesis and suggest that inflammatory and disease activity variables are not important predictors of fatigue in RA. That PGA-VAS was the clearest significant predictor variable could lead one to wonder whether fatigue is possibly a subjective epiphenomenon in inflammatory disease that is not well predicted by objective measures of disease activity or inflammation. Indeed, platelets were only marginally significant at baseline and their biological significance is not clear, given that the much more conventional inflammatory marker CRP was not found to be a significant predictor variable at any time-point. Moreover, in the univariable analyses, the strongest predictor relationships existed with PGA-VAS, depression and anxiety scores, self-reported pain scores and overall health and quality of life scores. Thus, it may be that sporadic significant predictor relationships between inflammatory variables and fatigue were purely confounders that happened to be co-incident with points during the evolution of the clinical picture when fatigue was elevated.

Alternative explanations for the loss of significance of the various predictor variables include loss of degrees of freedom – whereby including more predictor variables in an analysis leads to lower precision and higher p-values. This may well have been an issue in the regression analysis of the data from the 12 month time-point where the number of cases per predictor variables was low (6.2 cases per predictor variable). Over-fitting of data can be indicated by the emergence of non-Gaussian distribution of residuals. Normality of the residuals was determined by using the Anderson-Darling test, D'Agostino-Pearson omnibus, Shapiro-Wilk test and Kolmogorov-Smirnov test. In addition, the residuals were all plotted on a QQ plot (figure 4.39). However, normality testing of the residuals from the multiple linear regression indicates a non-normal distribution at baseline but Gaussian distribution at 6 months and 12 months. The appearance of the QQ plot at baseline suggests deviation from normal distribution is minimal.

Another possible contributor to the reduced number of significant predictor variables could include correlation of predictor variables. If variables are closely correlated, then one would expect to find strong parameter co-variance. Parameter covariance is shown in figure 4.38. in the form of a normalised covariance matrix which can be interpreted much the same as a correlation coefficient. There is clear parameter covariance between SJC-28 and TJC-28, and also between ACR/EULAR-2010 score and Visser score. Multicollinearity was also quite high for both Visser score and ACR/EULAR 2010 score with R^2 with other variables, ranging from 0.7045 to 0.7557 (Visser) and 0.6365 to 0.7641 (ACR/EULAR-2010) across the time-points.

4.5.3 Revised Multiple linear regression model

Based on the results of the initial multivariable analysis, a second rationalised model was designed with a view to reducing the risks of over-fitting and from loss of significance due to parameter covariance and multicollinearity. ACR/EULAR-2010 score was removed from the analysis as the Visser score includes within its composite score both RF level and presence/absence of articular erosions. Platelets and total WCC were removed to more consistently compare performance of the model across timepoints, whilst to minimise parameter competition between SJC-28 and TJC-28, the composite parts of the

DAS28-CRP were excluded and replaced by DAS28-CRP itself, with the exclusion of the PGA-VAS component. PGA-VAS was included despite the risk of parameter covariance with DAS28-CRP, as it was felt to be important to compare this more subjective disease measure with the other more objective measures, albeit within a composite score.

The revised multiple linear regression model was as follows:-

Fatigue ~ Intercept + Visser score + PGA-VAS + AGA-VAS + DAS28-CRP + Pain-VAS

The results of the multiple linear regression analysis are presented in figure 4.40.

	Baseline	6 months	12 months
Intercept	45.37**** (4.299)	37.36**** (2.637)	40.87**** (4.680)
Visser score	0.06399 (0.6283)	0.1979 (0.5093)	-0.5128 (0.9133)
PGA-VAS	0.2021*** (0.05768)	0.1526** (0.05403)	0.08064 (0.08418)
AGA-VAS	0.06387 (0.04937)	0.03353 (0.06023)	-0.07531 (0.1277)
DAS28-CRP	-1.628 (1.098)	2.121 (1.484)	2.313 (2.279)
Pain-VAS	0.04128 (0.04861)	0.04721 (0.06216)	0.1335 (0.1016)
R^2	0.2532	0.4034	0.2451
Adjusted R^2	0.2226	0.3742	0.1696
No. observations	128	108	56

*Coefficients shown with standard error is shown in ( ) below. Statistical significance indicated by *(<0.05), **(<0.01), ***(<0.001) and ****(<0.0001).*

Figure 4.40 Revised multivariable analysis - Fatigue and disease activity/inflammatory variables

Multiple linear regression analysis at baseline, 6 months and 12 months. The regression models was as follows:-

Fatigue ~ Intercept + Visser score + PGA-VAS + AGA-VAS + DAS28-CRP + Pain-VAS

Platelet and neutrophil counts were excluded from multivariable analysis at 6 months and 12 months due to low number of data within cohort at these time points. The overall regression was significant at baseline (p=****<0.0001), 6 months (p=****<0.0001) and 12 months (p=*0.0129). Coefficients are shown, with statistical significance denoted with *, **, *** and ****. Standard error shown in ( ). R^2 and adjusted R^2 are also shown in the table along with n. at each time-point. PGA-VAS was a significant predictor variable in at baseline (P=***0.0006) and 6 months (P=**0.0057) but not at 12 months (p=0.3427). No other predictor variables reached significance at any time point.

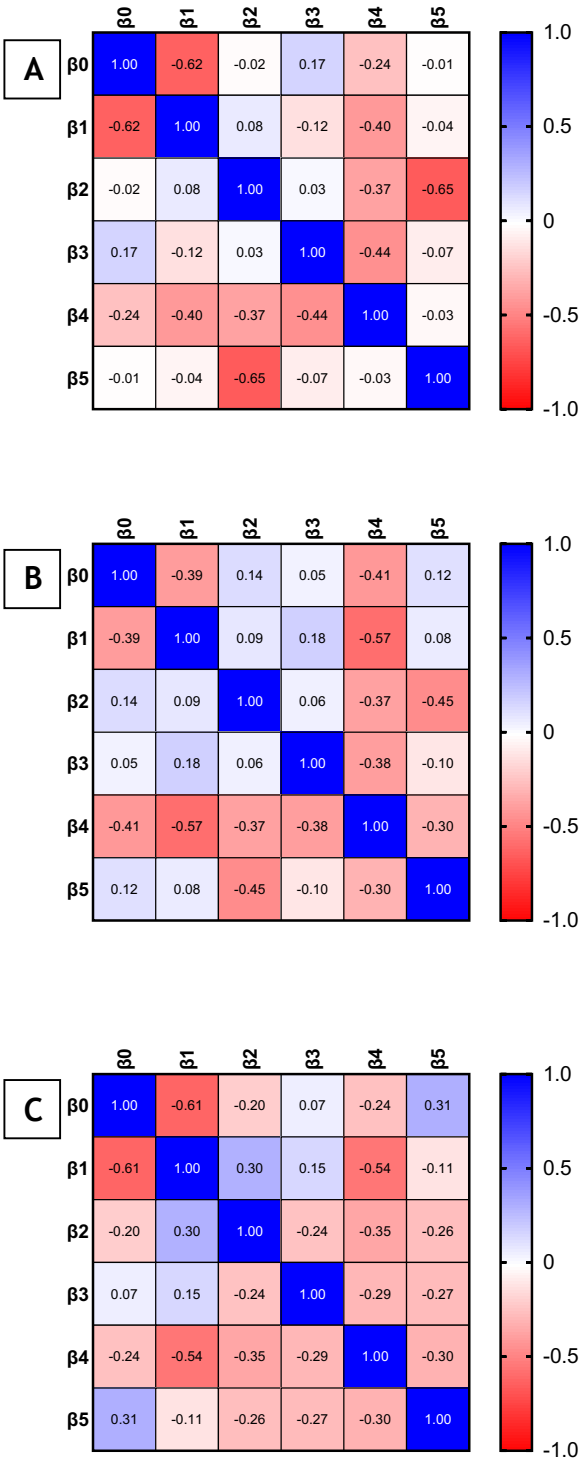


Figure 4.41 Parameter covariance matrix in revised multiple linear regression of fatigue at baseline (A), 6 months (B) and 12 months (C)

β0 - Intercept
β1 - Visser
β2 - PGA-VAS
β3 - AGA-VAS
β4 - DAS28-CRP
β5 - Pain-VAS

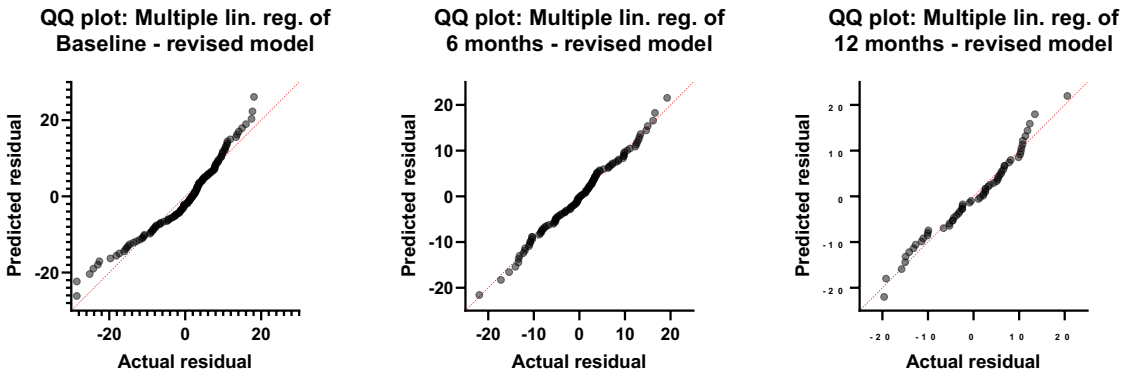


Figure 4.42 QQ-plot of residuals in multiple linear regression analysis of fatigue at baseline, 6 months and 12 months
Residuals are not normally distributed at baseline, but distribution is Gaussian at 6 months and 12 months.

The revised multiple linear regression model confirms the statistically significant relationship between fatigue and PGA-VAS at baseline and 6 months, but again this becomes non-significant at 12 months. The correlation co-efficients are similar to the initial model. As before the residuals are not normally distributed at baseline, but are Gaussian at 6 months and 12 months (figure 4.42). Multicollinearity is no longer apparent. Parameter co-variance is less, with only Pain-VAS and PGA-VAS unsurprisingly showing a degree of covariance, potentially resulting in competition for significance between the two parameters (figure 4.41). The number of cases within the regression analysis at 12 months in both multivariable regressions is low (n. 56), which could undermine the reliability of the results at this time-point due to reduced power.

4.6 Results: Effect of DMARD medication on fatigue, depression and anxiety

Recent prevailing practice in rheumatology in the UK and other developed countries has involved aggressive treat-to-target approaches to the management of RA and other rheumatological inflammatory disorders (Smolen et al., 2016) (Salomon-Escoto and Kay, 2019) (Lubrano and Perrotta, 2017). What effect this has upon fatigue and mood disorders in RA remains to be fully elucidated. However, we hypothesised that this robust treatment strategy, aiming at maximal clinical improvement and rapid attenuation of inflammation and

disease activity, may be contributory factor in the marked improvements seen in both fatigue and HADS scores over time within the SERA cohort. The improvements in fatigue scores over time, in particular, conflicts with more historical cohort findings that suggested that fatigue does not necessarily improve with treatment and improvements in disease activity (Druce et al., 2015c).

Another question of interest relates to fatigue in particular. The results presented so far appear to show that within the SERA cohort, inflammation as measured by CRP and ESR is not important in the prediction of fatigue. The multivariable analysis showed that even disease activity and pain measures were of lesser importance to subjective patient assessment of global disease activity using the PGA-VAS – the most reliable predictor of fatigue out of multiple measures of disease activity analysed.

Work completed by Fragoulis et al., (2020) has confirmed a relationship between inflammatory variables and depression/anxiety symptomatology within the SERA cohort (Fragoulis et al., 2020). However, inflammatory variables only contribute to a small fraction of the variance – when I repeated linear regression analysis on the whole cohort examining HADS and CRP at baseline, 6 months and 12 months, the r^2 was very low (0.01 at baseline. 0.02 at 6 months and 0.03 at 12 months (figure 4.43).

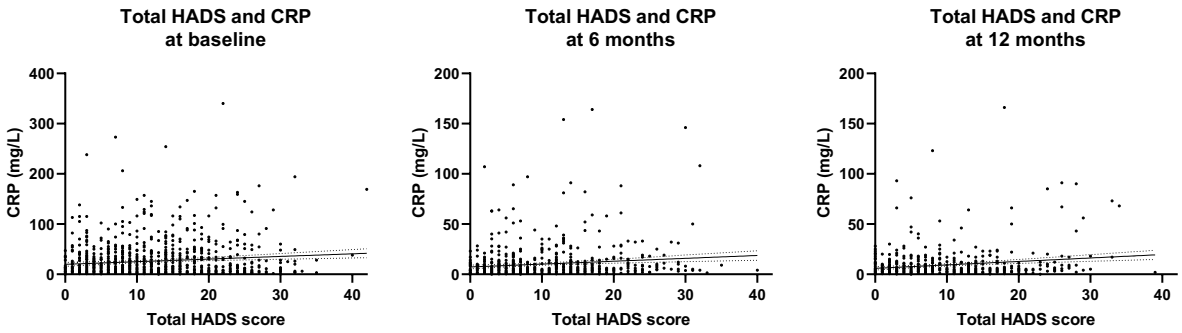


Figure 4.43 Total HADS score and CRP
HADS data from the whole SERA cohort was included in analysis. Simple linear regression was completed to test if CRP at baseline, 6 months and 12 months predicted total HADS scores at baseline, 6 months and 12 months. Best fit line is shown with 95% confidence interval on either side. The overall regression model was statistically significant at baseline (fitted regression model Y = 0.5242*X + 19.83, r^2=0.01327, F=13.15, p=***0.0003), 6 months (fitted regression model Y = 0.2862*X + 7.285, r^2=0.01785, F=13.76, p=***0.0002) and 12 months (fitted regression model Y = 0.3438*X + 5.912, r^2=0.03268, F=20.98, p=****<0.0001).

Fatigue is a subjective and potentially disparate phenomenon – there may be several different types of fatigue e.g. inflammatory fatigue, depressive fatigue, painful fatigue etc. Depression, likewise, is a phenomenon that in clinical and research practice is described by diagnostic systems that are based upon subjective experience and interpretation of symptoms. Both depression and fatigue are multifactorial in their causation.

Could it be that in a cohort treated early and comprehensively with immunosuppressive treatments, that sickness behaviours such as fatigue and depression driven substantively by inflammation become a limited source of morbidity, with other factors such as pain or disability accounting for a larger proportion of the remaining variance?

To explore these questions, analysis was completed examining the relationship between prescription of DMARDs and disease activity, inflammation, and sickness behaviour phenotypes (fatigue and HADS scores) at baseline within the SERA cohort.

4.6.1 Prescribing patterns

Data within the SERA cohort includes medication prescriptions, including broad categories such as DMARDS (classical DMARDS such as methotrexate, hydroxychloroquine, sulfasalazine, leflunomide) and biologics (such as etanercept, adalimumab, abatacept).

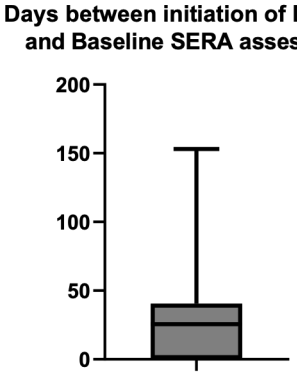
Data on medication prescribed at the time of baseline assessment in cases where there was a baseline fatigue rating was available in 145 cases. All but 27 cases were either already initiated, or initiated on the day of baseline assessment, on one or more DMARD medications at baseline.

No cases with fatigue scores were receiving ongoing corticosteroid medication at baseline. No cases were prescribed Biologic type medications at baseline. This is unsurprising, as these medications are generally reserved for cases in which there has been limited response to DMARD therapy.

4.6.2 DMARD prescribing

As noted, DMARDS were either initiated on the day of baseline assessment (34 cases) or were already prescribed (84 cases) by the time of baseline assessment in 118 of the cohort with fatigue data.

All cases within the cohort underwent baseline assessment within 6 months of initial diagnosis – therefore many cases had initiated therapy prior to undergoing baseline SERA cohort tests and assessments. This gap in some patients is purely an artefact of the practicalities of enrolling patients from standard care into cohort studies. The number of days between baseline assessment and initiation of DMARD therapy was calculated and is shown in figure 4.44.



Number of values	118
Minimum	0.000
25% Percentile	0.000
Median	25.50
75% Percentile	40.50
Maximum	153.0
Range	153.0
Mean	29.15
Std. Deviation	33.29
Std. Error of Mean	3.065

Figure 4.44 Descriptive statistics of days prescribed DMARD therapy at time of baseline SERA cohort assessment
118 out of 145 patients were either already commenced on DMARD therapy, or were commenced on therapy on the same day as SERA baseline assessment.

4.6.3 Length of DMARD exposure and inflammation and disease activity

Cases where DMARD had already been initiated or was initiated on the day of baseline SERA assessment, were included in an analysis to examine whether the number of days of exposure to DMARD had a significant explanatory relationship with variables relevant to inflammation and disease activity – including CRP, PGA-VAS, SJC-28 and TJC-28 (figure 4.45). No significant relationship was found

between length of exposure to DMARD therapy and any of the selected disease activity variables.

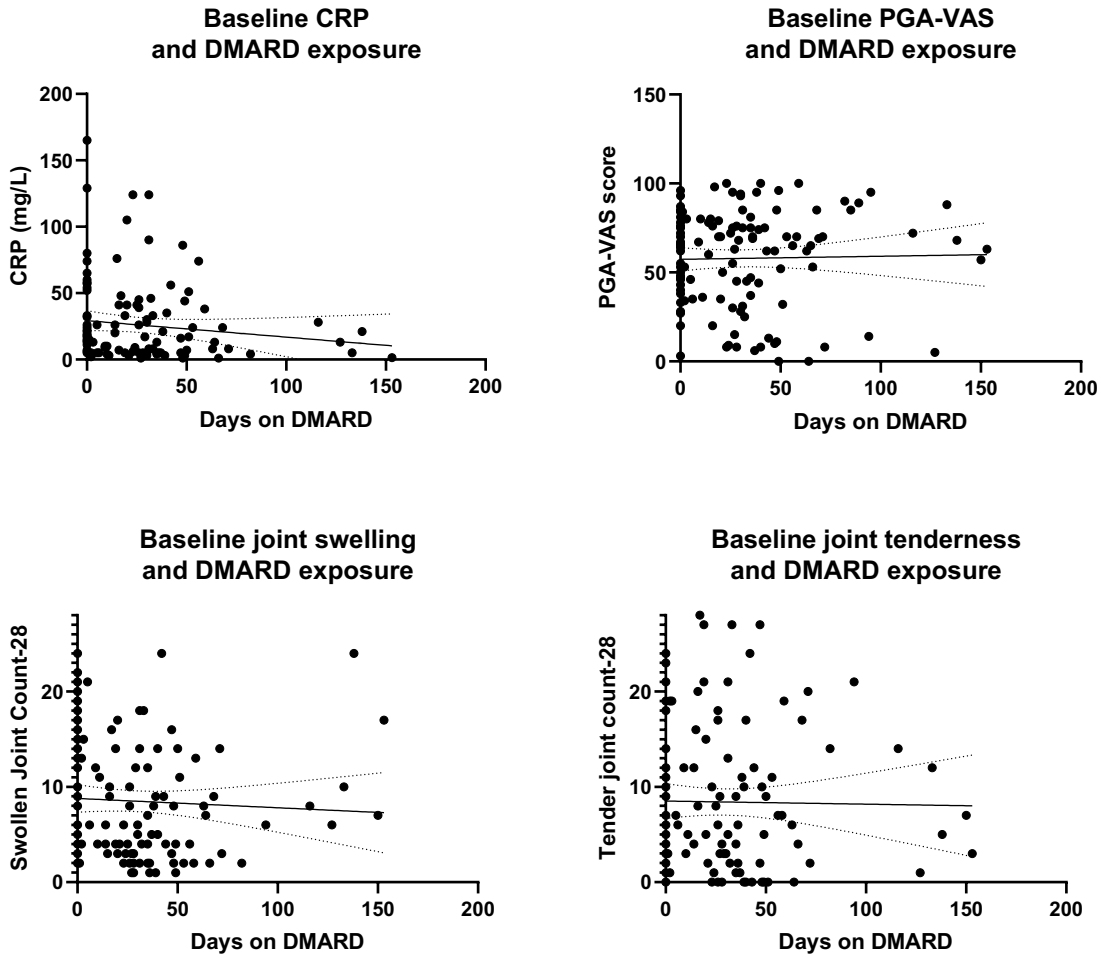


Figure 4.45 Exposure to DMARDs and CRP, PGA-VAS, SJC-28 and TJC-28
Simple linear regression was completed to test if the number of days of exposure to DMARD therapy predicted baseline scores of variables relevant to RA disease activity: CRP, PGA-VAS, SJC-28 and TJC-28. Best fit line is shown with 95% confidence interval on either side. Days of exposure to DMARD therapy did not significantly predict CRP (p=0.1863), PGA-VAS (p=0.8090), SJC-28 (p=0.5573) or TJC-28 (0.8771).

4.6.4 Length of DMARD exposure and fatigue

Analysis was conducted to determine if the length of exposure to DMARD therapy had any effect on the severity of fatigue. Simple linear regression analysis was conducted to determine if the number of days of DMARD therapy predicted fatigue (figure 4.46). There was no significant relationship between prior DMARD exposure and fatigue at baseline assessment.

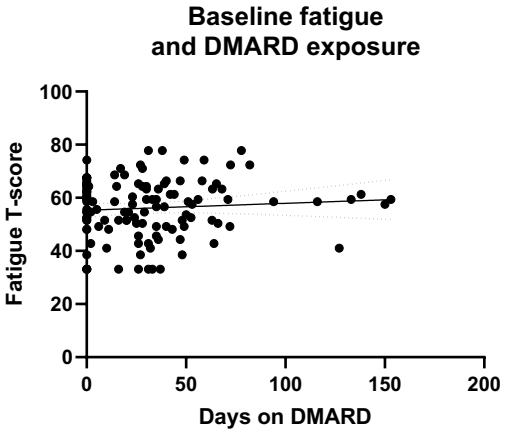


Figure 4.46 Fatigue and exposure to DMARDs
Simple linear regression was completed to test if the number of days of exposure to DMARD therapy predicted baseline fatigue T-score. Best fit line is shown with 95% confidence interval on either side. Days of exposure to DMARD therapy did not significantly predict fatigue T-score (p=0.3815).

4.6.5 Length of DMARD exposure and depression/anxiety

Analysis was conducted to determine if the length of exposure to DMARD therapy had any effect on the HADS anxiety scores and HADS depression scores. Simple linear regression analysis was conducted to determine if the number of days of DMARD therapy predicted HADS anxiety score and HADS depression score (figure 4.47). There was no significant relationship between DMARD exposure and HADS anxiety score or HADS depression score at baseline assessment.

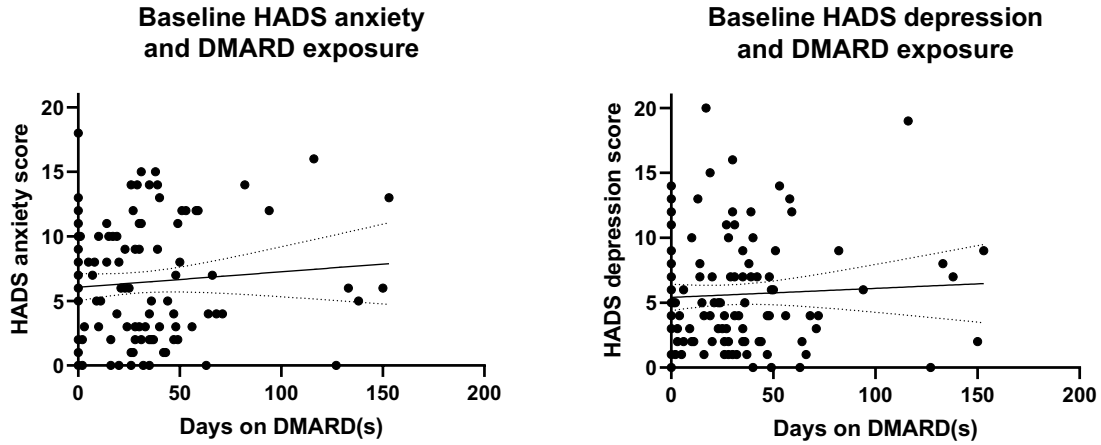


Figure 4.47 HADS and exposure to DMARDs
Simple linear regression was completed to test if the number of days of exposure to DMARD therapy predicted baseline HADS anxiety scores and HADS depression scores. Best fit line is shown with 95% confidence interval on either side. Days of exposure to DMARD therapy did not significantly predict HADS anxiety score (p=0.3366) or HADS depression score (p=0.5583).

4.7 Discussion

4.7.1 Introduction

The experiments presented in Chapter 3 sought to explore and characterise the brain inflammatory response in the context of inflammatory arthritis using the CIA mouse model. This chapter has sought to build upon these findings from the other end of the translational bridge by seeking to determine the functional consequences in terms of sickness behaviour – primarily fatigue – in the context of early-stage RA in a human cohort. This was based upon the hypothesis that peripheral inflammation in the context of RA would result in a brain response and subsequent expression of sickness behaviour including fatigue and altered mood. To test this hypothesis in the SERA cohort the following questions were explored:-

1. To what extent inflammatory variables are associated with fatigue in RA.
2. To what extent other variables including disease activity, pain and demographic characteristics apposite to inflammation were associated with fatigue and depression in RA.
3. Whether DMARD, biologic and antidepressant medications had an effect upon fatigue and anxiety/depression in RA.

4.7.2 Fatigue over time

Fatigue was found to be prevalent at baseline, with median scores well above the 50th centile of the general population. A marked improvement in fatigue scores was seen at 6 months and 12 months compared to baseline.

The majority of extant literature exploring fatigue in the context of RA tends to report that despite improvements in inflammation and disease activity, fatigue can remain a persistent symptom and cause of ongoing disability (Druce et al., 2015c) (Druce et al., 2015a). The results reported here from the SERA cohort contradict these findings.

It is unclear what mechanism underlies changes in fatigue in RA over time and with treatment – whether it may be an epiphenomenon of other symptom changes such as pain and disability (Pollard et al., 2006) or related to underlying

biological changes for example in inflammation (Almeida et al., 2016) (Chauffier et al., 2012).

The SERA cohort consists of a modern UK cohort of RA patients. Current practices within rheumatology promote early and comprehensive treat-to-target therapy whereby immune suppression is pursued with aggressive drug treatment with a view to effecting early and sustained disease remission (Smolen et al., 2016) (Smolen et al., 2020). Work completed on the SERA cohort by Fragoulis et al (2020) examining anxiety and depression, provided some basic information on treatment patterns within the SERA cohort. They reported that within the 1st year from entry to the cohort 85.3% were treated with methotrexate, 48.2% with sulfasalazine, 48.1% with hydroxychloroquine, 0.71% with leflunomide and 4.6% with biologic drugs (Fragoulis et al., 2020). They do not report the specific combinations or length of treatment or dosage, however, the figures suggest generally comprehensive treatment with a substantial proportion of patients receiving a combination of more than one DMARD early in the course of their illness.

The results presented here reinforce other recent findings from modern inception cohorts, including a recent study reported by Holten et al. (2022) which reported rapid and sustained improvements in Fatigue VAS scores over 24 months in aggressively treated RA (Holten et al., 2022). Similar findings have also been reported by Scott et al (Scott et al., 2020). Holdren et al. (2021) also report improvements in fatigue scores in a Canadian inception cohort (2007-2017), though suggest that fatigue improvements vary temporally from disease activity improvements by lagging behind (Holdren et al., 2021).

These results may suggest that improvements in fatigue, and potentially other sickness-behaviour symptoms such as depression and anxiety, may be achieved if patients are treated within a therapeutic window, during which their symptoms are not yet ‘hard-wired’ and are still remediable. Similar improvements in depression and anxiety scores have been reported by Fragoulis et al (2020) and are highlighted in this thesis in section (4.4.6). The findings presented here in regard to fatigue and psychiatric outcomes further support the importance of early remission-targeted therapy in RA.

4.7.3 Fatigue and diagnosis

Whilst the bulk of the SERA cohort consists of RA at baseline, a sizeable minority includes patients with UA. Scores between the groups were compared. A diagnosis of RA at baseline is not associated with higher fatigues scores at baseline, 6 months or 12 months. Diagnostic data is only available at baseline. UA may often manifest more clearly over time as RA or another inflammatory arthropathy. There is some evidence that UA at baseline is associated with a less severe clinical course, less functional impairment and higher chance of DMARD-free remission (Krabben et al., 2012). There is little published data directly comparing fatigue between RA and UA.

ACR/EULAR-2010 criteria were used to classify patients within the SERA cohort. As with other measures of disease manifestation in the cohort, ACR/EULAR-2010 scores reduce over time, presumably in response to effective treatment following diagnosis. No significant relationship was observed between fatigue and ACR/EULAR-2010 scores at baseline or 12 months, but there was a significant predictor relationship between ACR/EULAR-2010 at 6 months and fatigue score at 6 months. It could be that at 6 months a relationship emerges whereby patients with more resistant symptoms and persistently higher ACR/EULAR-2010 scores have ongoing higher levels of fatigue in comparison to those whose ACR/EULAR-2010 scores have improved. This does not appear to manifest at the 12 months timepoint.

Visser scoring was repeated on the cohort at each time-point during the cohort visits. The Visser score is generally used to produce an estimate of prognosis. It includes within its construct variables relevant to inflammation and disease severity such as presence of absence of peri-articular erosions. Baseline Visser scores do not appear to predict fatigue in the SERA cohort. However, the 6 month repeated Visser score does appear to show an association, with higher Visser score associating with higher fatigue, though this is not borne out at the 12 months timepoint. This may suggest some validity in terms of its relevance to disease severity, though this argument is not strong, given the lack of a relationship at 12 months. There is no data available among the published literature examining the relationship between Visser score and fatigue.

4.7.4 Fatigue and demographic variables

4.7.4.1 Sex

Female sex was found to be associated with a higher burden of fatigue at baseline and 6 months but not at 12 months. Female sex has previously been shown to correlate with higher reported fatigue levels in the context of RA (Nikolaus et al., 2013) (Davis et al., 2010). It is a recognised more general phenomenon that fatigue may be reported more frequently by females than males, though the complex reasons underlying this difference are not fully understood and may reflect external cultural/societal influences as much as internal psychological or biological factors (Bensing et al., 1999) (Watt et al., 2000). It is interesting to note that this difference appears to attenuate over time.

The presence of significant differences in the distribution of immune-mediated inflammatory disorders between the sexes is well established (Whitacre, 2001) (Quintero et al., 2012). This phenomenon is believed to be largely driven by genetic and hormonally driven differences in immune activity (Klein and Flanagan, 2016). There is also evidence that sex differences are important in understanding the emergence of sickness behaviour in the context of inflammation – such as fatigue, pain sensitivity and mood/anxiety changes (Lasselin et al., 2018) (Derry et al., 2015). There is some evidence that women are more susceptible to inflammation-induced mood changes than men, including from a recent study examining response to low dose endotoxin, in which despite similar IL6 and TNFα response levels, females reported greater levels of fatigue and depressed mood (Moieni et al., 2015).

4.7.4.2 Age

Aging is associated with a progressive inflammatory burden (Sarkar and Fisher, 2006). No significant relationship was found between age at baseline assessment and fatigue. Evidence on the association between age and fatigue in RA is inconsistent. Some previous research has indicated that younger age may be associated with higher fatigue levels in RA (Olsen et al., 2016), whilst other research shows no association with age (Feldthusen et al., 2016). Fatigue tends to increase with age in general (Avlund, 2010), however, current research tends

to suggest that age is not of major importance in the context of fatigue in RA (Uhlig and Provan, 2018).

4.7.4.3 Socioeconomic status

Socioeconomic status is increasingly recognised as an important demographic variable in respect of inflammatory disease (Muscatell et al., 2020). It is hypothesized that inflammation could represent an important pathway in translating some of the variance in mental health disparities associated with socioeconomic status, something that could be compounded in the context of inflammatory disease such as RA.

High quality cross-sectional data from Denmark found a strong correlation between fatigue and lower socioeconomic status in the context of RA, psoriatic arthritis and spondyloarthritis (Esbensen et al., 2020). Fragoulis et al (2020) reported that unemployment was consistently associated with high levels of both HADS anxiety and HADS depression scores within the SERA cohort.

Fatigue was examined in respect of SIMD-2012 quintile category and SIMD-2012 score. No significant relationship was found except between SIMD-2012 score and fatigue at 6 months – where an association between deprivation and lower levels of fatigue was observed. The actual significance of this is hard to determine given that it was not replicated at baseline or at 12 months.

RA can have major deleterious effects on socioeconomic status, particularly in the context of disease related physical limitations and disability (Albers et al., 1999) (Verstappen, 2017). It is a limitation of this analysis that socioeconomic status is only measured at baseline as one could hypothesise that high baseline and/or persistent fatigue levels could impact negatively upon subsequent socioeconomic status. We also do not know how levels of deprivation may impact upon an individual's propensity to self-report fatigue. One could speculatively hypothesise that lower socioeconomic status could be associated with greater deference towards medical professionals/appreciation of treatment, and/or lower expectations for general wellbeing, with resultant under-reporting of fatigue.

No association was seen between fatigue and employment status. Limitations included a low number of cases per category. Another limitation, similar to the analysis of socioeconomic status, is that we only have data regarding employment status at baseline and do not know how this status may have changed over time in the context of inflammatory arthritis. We have no way of knowing whether high fatigue scores at baseline could have impacted upon employment status over time. One could hypothesise that being employed could be a proxy for good functional status and therefore lower fatigue, but this is not discernible from this data.

4.7.5 Fatigue and lifestyle characteristics

4.7.5.1 Smoking

No differences in fatigue were seen between smokers, ex-smokers, and non-smokers. Smoking status is an important variable in the context of RA. Smoking has long been associated with increased risk of developing RA (Heliövaara et al., 1993) and with increased disease severity (Saag et al., 1997) (Manfredsdottir et al., 2006). Tobacco smoke has complex effects on the immune system including pro-inflammatory and dysregulating effects (Sopori, 2002, Gonçalves et al., 2011) . There is some evidence from the cohort studies literature that smoking may be associated with increased fatigue in RA (Katz et al., 2016) (Rat et al., 2012). In regard to HADS depression and anxiety scores in the SERA cohort, Fragoulis et al (2020) reported a correlation between smoking and higher HADS scores at 6 and 12 months but not at baseline. There are some limitations in the smoking data available in the SERA cohort, which only indicates smoking status at baseline and does not report cumulative dose of tobacco e.g. pack-years.

4.7.5.2 Alcohol

An inverse relationship was seen between weekly unitary alcohol intake and fatigue at baseline but not at 6 months or 12 months. Alcohol is known to have a generally suppressant effect on the immune system (Barr et al., 2016). A range of epidemiological case-control studies tend to suggest that alcohol has a protective effect in reducing risk of developing RA (Scott et al., 2013). Some research has shown a dose-dependent decreased risk (Källberg et al., 2009). A 2010 cohort study shows reports increasing frequency of alcohol is inversely

associated with RA disease severity including CRP, disease scores, pain and quality of life measures (Maxwell et al., 2010). Fatigue was not specifically examined in this study Interestingly, a study in CIA mice demonstrated reduced rates and severity of arthritis in mice administered ethanol (Jonsson et al., 2007).

The regression at baseline would tend to support the extant literature in terms of higher alcohol reducing disease severity, with fatigue either an epiphenomenon of this or otherwise. However, the relationship in this data appears weak and is not significant at later time points.

The nature of any association with fatigue is likely to be complex – for example, excessively fatigued individuals may refrain from alcohol consumption due to anxiety about worsening their health, or due to reduced socialising. Alcohol weekly unitary intake data is only available at baseline, not at 6 or 12 month time points, marking a limitation to the data. We can only say how baseline alcohol may affect fatigue, and therefore changes in alcohol consumption dynamic with fatigue score changes is unknown, potentially obscuring a relationship over time.

4.7.5.3 BMI and lipid profile

No significant relationship was seen between either BMI, blood cholesterol or TC/HDL ratio and fatigue.

Obesity has been shown to have dysregulating effects on immune function and increase the propensity to inflammatory states (de Heredia et al., 2012). Obesity has been found to be associated with higher levels of circulating inflammatory markers such as ESR and CRP in RA (George et al., 2017). Obesity has been described previously has having an association with fatigue symptoms in RA, though the causality and nature of the relationship is unclear (Katz et al., 2016). Inflammation has complex relationships with factors such as obesity and smoking and so indirect effects may be important (Katz, 2017b). High TC/HDL ratio has been found to associate with higher fatigue in multiple sclerosis in a relatively small cross-sectional study (Browne et al., 2019). High TC/HDL ratio has been reported in chronic fatigue syndrome (De Lorenzo et al., 1998), though the nature of the association with fatigue is entirely unclear i.e. cause or effect.

4.7.6 Fatigue and disease activity

The relationship between fatigue and the DAS28 scoring systems was analysed in addition to examining the relationship between fatigue and the component parts of the DAS28 in order to further understand what factors were important. AGA-VAS was also examined as it is also an important disease activity measure, though does not form part of the DAS28.

4.7.6.1 DAS28

The data from the DAS28-ESR was very limited due to a low number of patients being tested for ESR, rendering the results unreliable. DAS28-ESR was found to significantly predict fatigue in regression analysis at 6 months, but not at baseline or 12 months.

The data for DAS28-CRP was far more comprehensive and showed a clear relationship between disease activity and fatigue at all time-points. Interestingly the r^2 varies substantially from 0.07 at baseline, to 0.32 at 6 months and 0.21 at 12 months. These data may suggest that if a causal relationship exists between disease activity and fatigue, then at baseline, disease activity may be of lesser relative importance in determining fatigue compared to later time-points. Or, put differently, that other factors explain variance in fatigue more at baseline than later in time.

There is established evidence from systematic review linking fatigue to DAS28 scores (Madsen et al., 2016). This same systematic review found evidence for a link between fatigue and ESR, CRP and pain. Fatigue has previously been found to lag behind improvements in disease activity in early RA (Holdren et al., 2021).

It is logical that elevated disease activity scores such as DAS28-ESR/ DAS28-CRP, which encompass a broad range of factors such as joint inflammation, elevated inflammatory blood markers and global assessment of function should show an association with fatigue. The relative importance of each component variable was explored later using multivariable analysis.

4.7.6.1 PGA-VAS and AGA-VAS

A clear and consistent relationship was seen between PGA-VAS and fatigue in both univariable linear regression analyses and in later multiple linear regression analyses. PGA-VAS showed an r^2 of between 0.2 and 0.4 over the various time-points that it was measured. The r^2 at each timepoint is greater than for DAS28-CRP and the regression appears to show a clearer, more consistent association. This could be a reflection of the fact that fatigue is by nature a subjectively experienced symptom, that is well measured by subjective patient-reported scores such as the PGA-VAS. There is likely to be substantial overlap in fatigue and patient report of functional/general health or disease status.

The relationship between fatigue and the AGA-VAS is less clear than with the PGA-VAS, and although the overall regression is significant at baseline, 6 months and at 12 months. The r^2 at baseline, 6 months and 12 months is considerably lower for the AGA-VAS.

To better understand this disparity, PGA-VAS and AGA-VAS scores in the SERA cohort were compared. Essentially both are measures of the same construct, though from differing perspectives. PGA-VAS scores were found to be consistently and significantly higher on average than AGA-VAS scores, which replicates previous findings reported in the RA literature (Barton et al., 2010). This observation, in combination with the stronger association between PGA-VAS and fatigue scores, may again underline the subjective nature of fatigue as a symptom. The disparity could indicate that fatigue's persistence and its related effects on patient reported measures may to some extent be decoupled from more objective or disease-specific measures. It may be that the subset of patients with persistent fatigue symptoms, have fatigue that is less dependent upon disease-activity variables which may be of more particular salience to the clinician/assessor.

4.7.6.2 Swollen and tender joints

The relationship between fatigue and joint swelling and joint tenderness was examined by including the SJC-28 and TJC-28 joint counts that form part of the DAS28 in univariable linear regression analyses.

SJC-28 emerges as a statistically significant predictor variable at 6 months and 12 months, whilst TJC-28 is a statistically significant predictor variable at all time points. From a relatively modest r^2 of 0.03 at baseline, the r^2 increases at 6 months and 12 months to 0.13. The statistical significance of TJC-28 also increases at these time-points. If one assumes that tender joint scores are in part a measure of pain, then these results may indicate that joint pain becomes an increasingly important predictor of fatigue once other manifestations of disease activity have improved, leaving more of the variance to be accounted for by pain. Pain-VAS which was examined later showed a consistent relationship with fatigue in the SERA cohort.

4.7.6.3 ESR and CRP

Both ESR and CRP were examined in relation to fatigue for completeness and also because there is some indication that they measure distinct facets of inflammation. Whilst there is significant overlap, CRP may be a more useful test to measure disease activity in more acute situations as ESR is believed to be persistently sensitive to elevated levels of immunoglobulins and rheumatoid factor (RF) (Wolfe, 1997). Early changes in ESR and CRP predict treatment response (Heidari et al., 2007).

The ESR data was less reliable due to a low n. value at later time points, whilst CRP data was more comprehensive. ESR had no significant relationship with fatigue. CRP showed a significant relationship in regression analysis only at 12 months.

These findings of a rather limited relationship in terms of fatigue severity and primary markers of inflammation are to some extent borne out within the published literature which is mixed in terms of finding associations between fatigue and ESR/CRP. A major study by Druce et al (Druce et al., 2015a) examining fatigue in a large RA cohort of 2652 participants found similar results to the SERA cohort data, with no direct pathway from change in inflammation to altered fatigue. Effects were seen with DAS28, pain, disability, and mental health. Another earlier large cohort study (n. 2096) also shows similar findings to the SERA cohort data, reporting that the inflammatory components of DAS28 contribute minimally to fatigue (Bergman et al., 2009).

Some kind of relationship is thought to exist in the context of inflammatory conditions and even the general population with elevated levels of inflammatory markers, including ESR and CRP being linked to fatigue (Karshikoff et al., 2017). In the specific context of RA, a number of studies have found associations between fatigue and CRP/ESR or other inflammatory variables (van Steenbergen et al., 2015) (Lee et al., 2020a) but the evidence is inconsistent (Nikolaus et al., 2013).

That said, it should be remembered that ESR and CRP are very broad general measures of fatigue and are relatively blunt instrument measures. ESR and CRP represent epiphenomena of inflammation, as opposed more precise measures such as serum or tissue cytokine levels. Such data is rarely available in large cohort studies which predominantly rely upon routinely gathered blood measures.

4.7.6.4 DAS28 component multivariable analysis

To attempt to gain a deeper understanding of the disease activity factors important in predicting fatigue, a multiple linear regression was undertaken including the variables described above. The results of the univariable analysis of the component parts of the DAS28 suggest that broad/general measures of inflammation such as ESR and CRP may not have a direct explanatory relationship with fatigue. In addition, the relationship between swollen/ tender joints and fatigue lacked consistency over time.

The multivariable analysis of disease activity variables confirmed the importance of PGA-VAS as a predictor variable fatigue, with it being consistently significant across time. No other variable was significant except for TJC-28 at 6 and 12 months, similar to the univariable analysis. CRP and SJC-28 lost all significance.

Analysis diagnostics were performed in the form of multicollinearity testing, QQ plotting of residuals and parameter covariance analysis. The residuals were only slightly non-Gaussian at baseline and only modest parameter covariance between SJC-28 and TJC-28. Overall, the multivariable analysis of the component parts of the DAS28 would appear to support a conclusion that within the SERA cohort, the association between higher fatigue and higher disease activity scores is better explained by PGA-VAS, and to a lesser extent tender

joint counts, as opposed to swollen joint counts and inflammatory markers such as ESR and CRP. This may indicate that fatigue is best explained by pain and subjective measures.

4.7.7 Fatigue and Quality of life, disability and pain

General health, quality of life and disability are measured within the SERA cohort by the EQ-VAS and HAQ. Both EQ-VAS and HAQ showed strong and consistent relationships across time with fatigue in the univariable linear regression analyses. Both these measures have a significant overlap in terms of measuring subjective patient report of general health and disability/functioning. The strong association with fatigue is predictable. The HAQ measures ability to complete daily tasks, which could be impaired as much by fatigue, as by pain or reduced mobility. However, for both EQ-VAS and HAQ, causality cannot be satisfactorily implied or determined by a regression analysis. It is logical to deduce that whilst a high EQ-VAS or HAQ score is a predictor in the statistical sense, in the biological sense it is likely to represent an effect of high fatigue rather than a cause. Although an alternative consideration would include that a high EQ-VAS/HAQ score could suggest active or problematic disease, which in turn may associate with high fatigue.

Pain is measured within the SERA cohort by the Pain-VAS. Pain-VAS showed a strong and consistent predictor relationship with fatigue in univariable analysis across time. These results are much in keeping with previously published literature exploring this relationship (Garip et al., 2011) (Huyser et al., 1998) (Feldthusen et al., 2016) (Nikolaus et al., 2013). One cross-sectional cohort study explored the relationship between fatigue, pain and disease activity in addition to response of fatigue to DMARDs and etanercept (Pollard et al., 2006). Their analysis, using simple linear regression and multiple linear regression, supported the hypothesis that fatigue had a stronger relationship with pain and depression in RA, and that disease activity was a lesser factor. Previous research has indicated that variation in fatigue and pain scores occurs in temporal synchronisation suggesting an important dynamic relationship (Van Dartel et al., 2013a). Some evidence suggests that inflammatory variables and disease activity are less important in determining fatigue compared to pain scores (Druce et al., 2015a).

Why pain so closely associates with fatigue is unclear. It may be that individuals with high levels of pain are inhibited from activity and this then gives rise to lower energy and increased fatigue. Equally it could be that there is a common variable such as inflammation that could both cause changes to pain sensitivity and to fatigue perception via some centrally mediated pathway, that could equally be true for other sickness behaviour phenomena such as depression and anxiety. Hyperalgesia could be an adaptive response to avoid injury and conserve bodily resources in the context of physiological stress.

4.7.8 Fatigue, depression and anxiety

Mood and anxiety symptoms are measured within the SERA cohort by the HADS, which consists of depression and anxiety subscores.

Both depression and anxiety symptomatology are common at baseline in the cohort. Analysis confirmed that HADS scores improved over time within the part of the cohort that had fatigue scores, which is in keeping with previous analysis on the whole cohort by Fragoulis et al. (2020). Given that the cohort is made up of newly diagnosed patients involved in active treatment and follow-up, it appears that depression and anxiety symptoms are improving relatively quickly for a significant proportion of patients. Within the cohort the percentage of patients with clinically meaningful anxiety changes from 36.9% at baseline to 28.1% at 6 months and 26.3% at 12 months. The equivalent figures for depression are 27.4% at baseline, to 20.4% at 6 months and 18.3% at 12 months. Of course, the flip side is that a substantial proportion of patients have persistent meaningful anxiety and depression symptoms at 1 year from enrolment. Therefore, whilst active treatment of RA may be associated with reductions on average of depression and anxiety symptoms, these symptoms clearly remain problematic for a proportion of cases. This echoes past research that indicates that depression is a complex and sometimes difficult to treat phenomenon in the context of RA (Nerurkar et al., 2019).

A previous diagnosis of ongoing depression was noted at baseline assessment in 10 out of the 145 cases (6.9%), and one of these cases had a comorbid diagnosis of anxiety disorder. No other diagnoses of anxiety or other mental health disorder were noted among the cohort with fatigue data. Analysis of prescribing

of antidepressants was investigated and found only to be prescribed at baseline in a minority of patients (12.4%) which may suggest that depression and anxiety is under-recognised and undertreated at baseline. Of the patients on antidepressant at baseline, a third were prescribed tricyclic antidepressant medication – which is generally used for pain symptoms rather than mood/anxiety symptoms in current clinical practice. The data regarding diagnosis and medication prescribing prospectively in the cohort with fatigue scores shows that only 3 of the 145 patients were initiated on antidepressant medication in the 12 months from baseline assessment.

HADS scores showed a strong and consistent relationship with fatigue across time. These data are relevant to a core issue of discussion in research that explores fatigue in RA and fatigue more broadly. That is, whether fatigue is in itself a biologically meaningful construct or merely a concomitant manifestation of depression. Fatigue in RA is consistently found to associate with depressed mood, pain and disease activity but fatigue tends to respond poorly or incompletely to disease-modifying therapy (Katz, 2017a). Ultimately the answer is not a simple one. It is likely that in some cases fatigue is a distinct phenomenon from depression/anxiety, and in other cases it is heavily influenced by depression. The results above tend to support this, as a substantial proportion of the variance remains unaccounted for by the regression model.

The concept of sickness behaviour may also be relevant here. Fatigue and depressed mood are both features of this syndrome and therefore whilst they may not completely co-vary, similar underlying mechanisms may influence their phenotypic expression in certain circumstances, such as in the context of chronic inflammation.

Fatigue is a potentially multi-faceted and disparate phenomenon. It has been argued that a substantial proportion of the variance in fatigue in RA could be accounted for by comorbid depression (Fifield et al., 1998). Analysis of differences in fatigue between patients with ongoing diagnosed depression and without showed no significant difference, which would tend not to support the hypothesis that fatigue is purely a manifestation of depression. However, these results must be taken in context of limitations. The number of cases with depression is a very small sample and therefore there is a high probability of

bias. In addition, the accuracy of the diagnostic data is unknown and may underestimate the prevalence. Whilst incidence of ongoing diagnosed depressive illness is not unreasonable at 6.9%, the prevalence of any history of depressive illness in the cohort is quite low at 8.97%, considering this is a predominantly late middle-aged cohort. It is also striking that there is only a single diagnosis of anxiety disorder.

Analysis of fatigue in regard to the presence or absence of prescribed antidepressant medication showed no association with fatigue levels. The small n. value of cases, particularly in the antidepressant prescribed arm, limits the interpretability of these results. Nonetheless, they are in accord with the above findings relating to diagnosis of depression.

There is relatively limited high quality evidence exploring the efficacy of antidepressants in the particular context of RA (Silva Almodóvar et al., 2022), with the most recent Cochrane review including studies predominantly dating from the 1980s (Richards et al., 2012). A more recent meta-analysis found no clear evidence for improvements in depressive symptomatology with antidepressant treatment in the context of RA (Fiest et al., 2017). Fatigue was not reported as an outcome in any of the trials included within this more recent meta-analysis. Indeed, there is limited if any high quality data on the effects of antidepressant medication on fatigue in the context of RA. Fibromyalgia is a chronic pain disorder of unknown aetiology that is not related to RA. However, fatigue is a common and burdensome symptom in this condition, for which there is some evidence of reduced fatigue in response to antidepressant medication in the context of this condition (Üçeyler et al., 2008). The applicability to the context of RA though is limited.

In the context of inflammation, there is evidence that antidepressant medication can be anti-inflammatory and reduce fatigue symptomatology (Lee and Giuliani, 2019a). This relationship may complicate the interpretation of the results presented here exploring fatigue in response to antidepressant medication – whilst antidepressant prescription may indicate presence of a depressive illness which could worsen fatigue, antidepressants may have fatigue-reducing or anti-inflammatory effects themselves.

4.7.9 Fatigue and laboratory blood measures

4.7.9.1 Renal function

Major renal dysfunction and chronic renal failure is associated with clinically significant fatigue (Joshwa and Campbell, 2017). Renal involvement in disease is more common in other autoimmune disorders such as systemic lupus erythematosus (SLE), where kidney disease associates with higher fatigue levels (Dey et al., 2021).

There was no association between urea and fatigue in the SERA cohort data. Creatinine appeared to have an inverse association with fatigue at baseline, with higher levels of creatinine predicting lower fatigue. The creatinine finding at baseline is perhaps counter intuitive. However, the x-y plot shows only a very slight trend (figure 4.32). There is no sign of a non-linear relationship as could occur if for example individuals with very low muscle bulk, and consequently very low creatinine, had higher levels of fatigue – very few cases have a creatinine below the normal range. Equally very few cases have a creatinine level above the normal range, and even in these cases there is no clear relationship with fatigue. This is true for the urea data also. The regression analysis in creatinine at baseline only just reaches significance (p=*0.0262) and the r^2 is small (0.03) and the slope is shallow (figure 4.32). It appears unlikely that a meaningful inference can be drawn from this result, bearing in mind the above factors.

4.7.9.2 Haemoglobin, platelets and WCC

Anaemia and reduced oxygen carrying capacity in blood could reasonably be predicted to associate with increased fatigue. Anaemia is a relatively common finding in RA (Wilson et al., 2004). However, there is limited data to suggest that anaemia clearly associates with higher fatigue (Van Hoogmoed et al., 2010). However, no statistically significant relationship was observed in the analysis of the SERA cohort data.

Reactive thrombocytosis can be a feature of high disease activity in RA. Elevated platelets predicted higher fatigue at baseline in the SERA cohort, but not at 6 or 12 months. Few cases had elevated levels of platelets at baseline, and this dwindled significantly as a proportion of the total, presumably in

association with reducing disease activity among the cohort. A very modest trend towards higher fatigue at baseline and thrombocytosis may exist at baseline upon examining the graphed results. It is possible that the significant finding at baseline was an artefact of higher platelets occurring with more inflammation at a time when mean fatigue levels were high and therefore being purely a confounder.

No significant relationship was seen between either total WCC or any individual leukocyte type count and fatigue in the SERA cohort.

Leukocytosis is a common phenomenon in the context of a systemic inflammatory response. Monocytosis can be a feature of inflammatory disorders, including RA (Buchan et al., 1985). Leukopenia may also occur as a result of a rare and severe form of RA called Felty's syndrome (Balint and Balint, 2004). Leukopenia may also arise in response to DMARDs such as methotrexate or leflunomide (McKendry and Dale, 1993) (Lee et al., 2020b). Little or no research has been undertaken to examine the relationship between fatigue and leukocyte levels in RA.

4.7.10 Multivariable analysis

A multiple linear regression model was constructed to test the hypothesis that inflammatory disease activity variables would predict fatigue in early RA. As discussed earlier, multiple linear regression analysis allows comparison of predictor variables, accounting to some extent for confounding and allows comparison of variance coefficients and testing for multicollinearity and parameter covariance. Therefore, it has the potential to offer more insight into the factors important in determining fatigue and is potentially more robust than univariable analysis. The limitations of multiple linear regression including risk of over-fitting, are discussed in section 4.5, and examination of these limitations eventually led to a refined model as follows:

> *Fatigue ~ Intercept + Visser score + PGA-VAS + AGA-VAS + DAS28-CRP + Pain-VAS*

The model was devised by selecting variables thought to be relevant to either inflammation or disease activity based upon *a priori* assumptions. These were

then further rationalised by selecting those which had a p-value of <0.2 in univariable analysis, in a basic variant of the stepwise approach to variable selection.

PGA-VAS emerged as the only consistent predictor variable of fatigue in univariable and multivariable analysis. However, in the multivariable analysis, PGA-VAS lost significance as a predictor variable at 12 months, potentially weakening the association. The association is particularly weakened if we consider the SERA data to be purely cross-sectional in nature – meaning that the different time-points are used to confirm associations that are seen. However, this may not be entirely correct to assume, as the cohort of patients at 12 months will be clinically different than at baseline, and different factors may emerge as important in predicting fatigue in different clinical circumstances.

Overall, the revised multiple linear regression analysis would tend to more support the null-hypothesis i.e. that inflammatory disease activity variables are not reliable predictors of fatigue, with the exception of PGA-VAS. Within the SERA cohort, fatigue is not well predicted by objective measures of disease activity and is not predicted by measures of inflammation.

Although a measure of disease activity, PGA-VAS is the most subjective of the key measures, relying on a subjective estimation by the subject. What do these results suggest regarding fatigue and what it represents? One interpretation could be that fatigue is a truly subjective phenomenon that cannot be predicted by objective disease measures and may not have a strong relationship with blunt measures of inflammation. However, it is important to note that more precise and sensitive measures of inflammation such as cytokine levels are not available from this dataset. In addition, it is clear that as patients are treated in the cohort and disease activity levels improve, fatigue levels also improve in the majority of patients.

4.7.11 DMARD therapy and fatigue, depression and anxiety

There was a variation in the amount of time patients had been prescribed DMARD medication prior to baseline assessment. This allowed an analysis of whether the length of exposure to DMARD medication had any effect on fatigue scores at baseline. This was also repeated for HADS scores.

Firstly, days of exposure to DMARD therapy was examined in relation to baseline inflammation and disease activity. No significant relationship was seen with any of these variables tested. This is perhaps surprising as DMARD therapy represents the mainstay of RA treatment (Smolen et al., 2005) (Smolen and Steiner, 2003).

There was no significant relationship between DMARD exposure and fatigue at baseline assessment. There was also no significant relationship between days of DMARD exposure and HADS anxiety or HADS depression scores.

Whilst there is not a significant relationship between CRP or disease activity variables and length of DMARD exposure at baseline, no causal or explanatory inferences can be drawn from this analysis in regard to the significance of inflammation or disease activity in the context of fatigue, anxiety and depression. Several factors may underlie these findings. Firstly, DMARD medications, unlike for example corticosteroids, have a lag time to effect, typically taking up to 6 to 12 weeks before substantial clinically relevant improvements are seen. Only a minority of cases within the analysis had received therapy for between 6 to 12 weeks at the time of baseline SERA cohort assessment. We know that disease activity scores within the SERA cohort as a whole did improve over time with treatment at the sampling times of 6 months and 12 months in comparison to baseline, and DMARD therapy is the only plausible causative factor determining this.

Secondly there is considerable clinical variability in the cohort. For example, there is a diverse range of disease activity scores, even among the preponderance of patients started on DMARD therapy at or around the time of baseline SERA cohort assessment. This variability could obscure explanatory/predictor relationships.

Thirdly, the data regarding DMARD therapy is very ‘broad-brush’. The data presented her represents only in the broadest terms the presence of DMARD therapy. Data on the type, dose and combination of DMARD drugs was not included in the analysis presented here due to practical limitations. However, an in-depth and detailed analysis of this could reveal more subtle relationships.

4.7.12 What does this analysis tell us about fatigue in RA?

The research questions that were set out at the start of this chapter can now be answered. These were:

1. To what extent are inflammatory variables associated with fatigue in RA?
2. To what extent are other variables including disease activity, pain and demographic characteristics apposite to inflammation associated with fatigue in RA?
3. Whether DMARD, biologic and antidepressant medications had an effect upon fatigue and anxiety/depression in RA?

Firstly, inflammatory variables do not appear to be of major significance in predicting or explaining fatigue in early RA within the SERA cohort. Disease activity variables are somewhat more reliable than ESR and CRP, in predicting fatigue, though only PGA-VAS shows a reasonably consistent relationship and even then, loses importance at 12 months in multivariable analysis.

Other health and wellbeing related measures appear to have a more consistent relationship with fatigue, in particular anxiety and depression measures. Quality of life and disability measures, whilst consistently associated with fatigue levels, may indicate a consequence of fatigue rather than being explanatory variables. Pain shows a consistent relationship with fatigue in univariable analysis but not in multivariable analysis. Similar demographic relationships to fatigue inconsistently replicated with previously published literature. More subtle relationships may have emerged with more data.

Very early time-point exposure to DMARD medication doesn't appear to have an effect on fatigue or mood/anxiety scores. However, given that fatigue and HADS scores significantly improve over time in an actively treated cohort, it is plausible that the effects of DMARD medication do have an influence on fatigue, either directly or indirectly. Prescription of antidepressant medication doesn't appear to show a significant impact upon fatigue or HADS scores, but the data was limited.

The data presented here reinforce the complex and multi-dimensional nature of fatigue. A consensus definition of fatigue does not yet exist in the literature.

Fatigue is recognised in studies relating to RA as being of diverse aetiology in which psychological, biochemical and physiological mechanisms contribute to a multi-dimensional phenomenon (Repping-Wuts et al., 2009). Further research is required to fully understand the nature and causes of fatigue if we are to make advances in therapeutic approaches. Fatigue is among the most important symptoms commonly listed as problematic by RA patients and one in which there remains a high clinical need (Kirwan and Hewlett, 2007).

A tractable multidimensional model of fatigue in RA has previously been put forward by Hewlett et al. (2010) involving a dynamic interacting triad of key factors (Hewlett et al., 2011):-

1. RA factors e.g. inflammation, cortisol response, pain and disability, RA drugs, physiological changes.
2. Cognitive and behavioural factors e.g. over/under activity, illness beliefs, psychological stress, anxiety, depression.
3. Personal factors e.g. work/caring responsibilities, environment, general health, limited support.

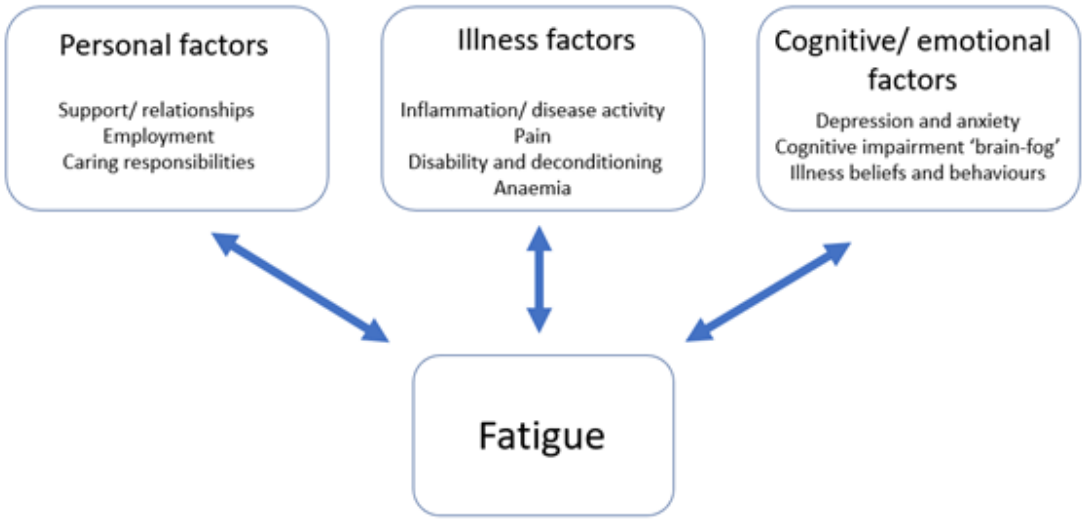


Figure 4.48 The multidimensional nature of fatigue
A dynamic interplay of factors is relevant to the emergence of fatigue in RA. Adapted from Hewlett, et al (2011).

The advantage of such a model is that it acknowledges the complexity of fatigue and yet also offers multiple targets for further research and ultimately for interventions aimed at reducing fatigue.

4.7.13 Clinical implications

Overall levels of fatigue (and depression/anxiety symptoms) improve within the SERA cohort over time. These results add to the evidence of the importance of early and robust disease modifying therapy for RA before sickness-behaviour type symptoms and deleterious brain changes can become more difficult to remediate. This contrasts with historical studies which tended to suggest that fatigue and mood changes could be more intractable in RA. The treat-to-target approach may have benefits for sickness behaviour and psychiatric sequelae, in addition to the proven benefits in joint disease prognosis.

The data comparing HADS scores with diagnoses of depression and anxiety and antidepressant medication suggests that depression and anxiety may be under-recognised and under-treated in RA. Given the strong association between HADS scores and fatigue, this could represent a relatively straightforward treatment strategy to improve both fatigue and depressive/anxiety symptomatology.

Given the multidimensional nature of fatigue, more fatigue specific therapies could be considered, including non-pharmacological approaches for those patients in whom fatigue is persistent. There is existing evidence for therapies such as graded physical activity (number needed to treat 7 for beneficial effect) (Cramp et al., 2013). However, there is limited evidence for other non-pharmacological approaches targeted at fatigue.

4.7.14 Strengths and limitations

A hypothesis-driven analysis has been undertaken using standard statistical analysis methods. This work substantially adds to the field of research of fatigue in early RA. This work explores the phenomenon of fatigue in RA from a variety of angles and helps to further construct a conceptual framework within which to better understand fatigue in inflammatory disease contexts.

The number of cases with fatigue data was somewhat limited. Stronger conclusions could have been drawn from the data had more patients completed fatigue assessment. Fatigue was introduced into the battery of SERA measures later in the recruitment and so only a minority of the total cohort had completed this assessment. However, when examined, there were no major

differences in terms of clinical aspects of demographics between the sub-cohort with fatigue ratings and the whole SERA cohort.

Fatigue was measured within the SERA cohort using the (PROMIS) Item Bank v1.0 – Fatigue – Short Form 8a questionnaire (see Appendix 2.) (Bingham III et al., 2019). Whilst this is a clinically validated tool, it has not been extensively used in the RA literature up to this point, and although validated for use in RA, it is not RA-specific. The results from this study cannot be entirely comparable with other studies in which different fatigue ratings are used. Other commonly used instruments include the Fatigue Checklist Individual Strength (CIS-fatigue), the fatigue-VAS and the RA-specific Multidimensional Assessment of Fatigue Scale (Hewlett et al., 2007). Fatigue is recognised as a challenging concept to measure and there is no recognised "gold standard" within the field (Santos et al., 2019). The PROMIS v1.0-Fatigue-SF-8a is to some extent a multidimensional instrument. The questions focus on fatigue severity, physical effort and motivation and do not cover concentration – a concept which may have some overlap with "brain fog" or cognitive problems and is arguably an important facet of fatigue. Other fatigue rating instruments such as the CIS-fatigue do include dimensions that cover concentration (Worm-Smeitink et al., 2017).

An important confounder that was not measured in the SERA cohort was sleep. Sleep quantity and quality is thought to have a meaningful relationship with fatigue in the context of RA (Katz et al., 2016). Evidence from cohort studies that have included measures of sleep in RA have suggested that sleep problems exacerbate fatigue (Irwin et al., 2012) (Austad et al., 2017). Factors which have previously been shown to affect fatigue such as disease activity, pain and anxiety/depression symptoms, are also factors which may impair sleep. Sleep may be an important mediator, however, to date there exists a paucity of literature that has included a comprehensive mediation analysis of the range of factors hypothesised to be related to the burden of fatigue in RA.

Chapter 5

Discussion

5 Discussion

5.1 Introduction

Inflammatory disorders, in particular RA, are associated with brain dysfunction in the form of sickness behaviour and elevated risk of psychiatric diseases, including depression, and an enhanced longer term risk of dementia (Matcham et al., 2013) (Hewlett et al., 2011) (Ungprasert et al., 2016). The mechanisms underlying these associations remains to be elucidated, though in recent times there has been an increased emphasis on the importance of immune-to-brain communication as a putative aetiological pathway (Nerurkar et al., 2019). Strong evidence from multiple strands of study confirms that the brain will respond to peripheral inflammatory stimuli and the routes of inflammatory communication between the periphery and brain have begun to be relatively well delineated (Chavan et al., 2017) (Capuron and Miller, 2011). Crucially however, and despite much study, the actual importance of this brain response to inflammation in the emergence of psychiatric disease and certain sickness-behaviour phenotypes, such as persistent fatigue, remains uncertain.

Much of our current understanding of the brain response to peripheral inflammation is based upon animal models that employ potent systemic inflammatory stimulators such as LPS endotoxin or poly(I:C). A criticism of many of these models is that they essentially amount to studying responses in the context of very acute illness, bacterial sepsis, or major viral infection. Whilst their utility lies in the expedient study of cytokine stimulation, their relevance to more chronic inflammatory conditions, in which the associations with psychiatric disorder are well noted, is uncertain. Animal models by their nature tend to be relatively acute and modelling of human disease is inherently challenging. However, the study of more disease-relevant tissue-specific inflammatory models such as CIA may yield more translatable findings; translation is of fundamental importance in moving the field forward in our understanding of disease and in the identification of new therapeutic targets. The literature exploring the brain response in animal models of arthritis (as summarised in Chapter 1) is limited and there is much scope for further investigation.

This thesis set out to explore the brain inflammatory response to inflammatory arthritis using methods that span animal models to human observational study, in an attempt to provide a translational approach.

5.2 Discussion of key findings

The experiments presented in Chapter 3 found that inflammatory arthritis in the CIA model was associated with elevated brain transcription of the pro-inflammatory cytokine Il1ß. This finding was replicated in homogenate of both the hippocampus and the rest of the hemisphere and remained a consistent finding despite significant heterogeneity of arthritic response. In association with this, elevated brain P2x7r transcription was also detected. P2X7 receptor function is well characterised in regard to its role in microglia activation and IL1ß release as part of the brain inflammasome (Gustin et al., 2015) (Campagno and Mitchell, 2021). These findings tend to point to a microglial source for the elevated Il1ß transcription, though no diffuse changes were seen in terms of IBA1+ cell density, aside from a statistically significant increase within the thalamus. There was also no indication of elevated brain inducible nitric oxide synthase to suggest major inflammasome activity. Transcription of Il1ß appears to relate to arthritis severity, though whether this inflammatory signature in the brain arises in response to peripheral inflammation or pain – or indeed some other factor – cannot be determined. This phenomenon did not associate with changes indicative of altered plasticity, such as impaired neurogenesis or reduced hippocampal neuronal density. Despite elevated transcription of some chemokines, no increased leukocyte recruitment to the brain was detected.

These results are intriguing. The relatively low-grade but consistent brain inflammatory signal of Il1ß transcription in association with arthritis in the CIA model may provide some interesting insights into human disease. What might the effects of a subtly pro-inflammatory brain environment be over longer periods of time, as in the context of a chronic human inflammatory disorder?

An increasing body of literature indicates that neuroinflammatory states are major factors in the development of sickness behaviour in the immediate/short

term, mental disorders such as depression in the medium term, and predispose to neurodegenerative conditions in the long term (Dantzer et al., 2008) (Khansari and Sperlagh, 2012). As discussed, RA has been linked with an increased risk of developing such problems, including a major association with fatigue, cognitive difficulties, depression and an elevated risk of developing dementia (Pollard et al., 2006) (Matcham et al., 2013) (Ungprasert et al., 2016). Given what we know about the cascade effects of cytokines within the brain – such as interference with neuronal functioning, neurotoxicity, dysregulated neurotransmission, altered plasticity – it seems reasonable to suppose that at least some of the variance in the association between RA and neuropsychiatric disease may be explained be neuroinflammation. Although evidence of a major inflammatory brain state was not seen in the CIA experiments presented in Chapter 3, this is perhaps not surprising. After all, brain dysfunction in RA is not an encephalitic syndrome, but more in keeping with a concert of more modest inflammatory brain changes with insidious effects, occurring in the context of peripheral inflammation. This would be in keeping with a low-grade inflammatory brain milieu, such as was seen in the experiments described.

The experiments in Chapter 4 were based upon the hypothesis that in human RA, peripheral inflammation would communicate with the brain, resulting in a brain response with behavioural manifestations including elevated fatigue. Data from the SERA cohort study was interrogated to answer research questions related to this hypothesis. Fatigue and HADS scores were found to reduce significantly over time, particularly between baseline and 6 months, but also between 6 and 12 months. However, univariable and multivariable analysis failed to show any convincing relationship between fatigue and variables associated with inflammation and disease activity. Basic inflammatory measures such as ESR and CRP showed no explanatory relationship with fatigue. Rather, fatigue was best predicted by subjective measures, in particular the PGA-VAS – a subjective patient reported measure of disease. Significant associations were also seen between fatigue and predictor variables such as pain, quality of life and HADS scores. These results tend to support the null hypothesis. Previous work has been conducted with data from the SERA cohort using correlation analyses has shown that depression scores do have a significant association with disease activity and inflammation (Fragoulis et al., 2020). When I examined this using

simple linear regression analysis, I was able to confirm this association. But the biological importance of this association may be limited – the r^2 was very low (~0.01 to 0.03), suggesting that inflammation as measured by CRP may only explain a very small fraction of the variance in depression scores.

These results present a more nuanced picture than may be hypothesised based upon the data from experimental studies reported in the literature, as detailed in Chapter 1, examining inflammatory stimuli and cytokine-induced sickness behaviour. These studies include various cytokine-stimulus mouse models that are clearly shown to demonstrate phenotypes indicative of various fatigue-type sickness behaviours (Dantzer et al., 2006), and also human experimental literature in which mild inflammatory stimuli are shown to cause both elevated fatigue symptomatology and brain activity indicative of fatigue signalling (Stefanov et al., 2020) (Harrison et al., 2009). However, there is some evidence from the clinical fatigue literature that would chime with the results reported in this thesis, whereby fatigue in RA has been found to be a persistent symptom with limited association with inflammatory variables (Druce et al., 2016) (Druce et al., 2015c) (van Steenbergen et al., 2015) (Pollard et al., 2006).

To contextualise the results presented in Chapter 4 it is important to consider exactly what, and in whom, has been measured. The SERA cohort consists of early-stage/newly diagnosed patients undergoing aggressive, modern, treat-to-target therapy. Overall inflammation and disease activity levels within the cohort drop precipitously over time in the context of robust immunosuppressive treatments tailored to quickly control arthritis. The work from the animal models reported in this thesis is really most applicable in a translational context to an untreated acute RA patient. The SERA cohort differs from earlier studies in that it is a modern treat-to-target cohort, as opposed to older cohort studies drawing on patient data prior to the advent of treat-to-target in around 2010. This may explain why improvements in fatigue and HADS scores are seen in the SERA cohort, in comparison to older cohorts that found fatigue and rates of depression to be more persistent problems. The findings of improving fatigue in the context of modern treatment are echoed in a recently published study of fatigue in a treat-to-target cohort (Holten et al., 2022).

The fact that fatigue scores improve significantly in the context of active treatment of RA following diagnosis, and yet there appears to be no association between fatigue and inflammatory variables leads to two possible conclusions. Firstly, it may be the case that in early RA, inflammation and disease activity variables are not important factors in the causation of fatigue and that other factors that co-vary with treatment and improvement in symptoms, explains fatigue, for example pain or some other biological, social, or psychological factor. This is not implausible in the context of fatigue, which is after all a multidimensional and complex phenomenon. We may also wonder if the same might be said for depression and anxiety-type symptoms in early RA given that the variance explained by inflammatory variables is so minimal.

The alternative conclusion is that inflammation is involved in the causation of fatigue and mood changes via a subtle or nuanced mechanism that is not well quantified by the inflammatory variables available for analysis within the SERA cohort. It would seem simplistic to remove inflammatory mechanisms from consideration altogether, particularly given that inflammation is such a key part of the RA disease process and the key therapeutic target of anti-rheumatic therapies, treatment with which appears to coincide with marked improvements in fatigue symptoms. ESR and CRP, the inflammatory variables measured in the SERA study, are general inflammatory markers and may lack the sensitivity to reflect more sensitive inflammatory signalling that could be of mechanistic importance in the emergence of sickness behaviour, fatigue, and depressive symptoms. This is even more true for proxy measures of inflammation such as WCC, platelets or clinical disease activity measures such as DAS28. There remains the possibility that more subtle signals such as elevated systemic levels of particular inflammatory cytokines could yet be of importance in RA in association with sickness behaviour or psychiatric disease. For example, peripheral IL6 has been shown to associate with in experimental studies of sickness behaviour and fatigue both in mice and humans (Stefanov et al., 2020) (Bluthé et al., 2000). Firm evidence of a specific role of IL6 in RA is less certain, though it is thought that it may play an indirect role in fatigue symptoms perhaps acting via HPA axis activation to give rise to fatigue and mood symptoms (Choy and Calabrese, 2018). There is stronger evidence for IL6 in regard to depression symptoms in RA (Hodes et al., 2016) and the anti-IL6 biologic

Sirukumab has shown some efficacy in improving depressive symptoms in RA (Smolen et al., 2014).

Overall, it seems likely that the peripheral immune response in RA, and other chronic inflammatory conditions, forms at least part of the process whereby the brain mounts some sort of response that results in behavioural changes and enhanced risk of neuropsychiatric disease. A direct relationship between the periphery and the brain may be unlikely in view of the unique nature of the CNS immune-specialised environment. To add to the complexity of the relationship, it may be a dynamic that evolves over time in the context of a range of environmental, biological, and indeed psychological variations.

5.3 Strengths and limitations

Strengths and limitations have been discussed in each results chapter, but the key points bear repeating. An important strength of the results reported in CIA is that they represent key findings relating to the brain inflammatory response in a tissue-specific model of inflammatory arthritis. These findings add to what is as yet a relatively naïve field of research. This is an important area of study as there is a need to move towards more disease faithful models involving tissue-specific inflammatory stimuli such as CIA. The bulk of experimental data investigating brain responses to peripheral inflammation does not utilise such models. Existing knowledge is largely based upon experiments that involve either administration of potent cytokine stimulators such as LPS, which are more translatable to acute severe inflammation, or involve administration of particular cytokines such as IL6 or IL1ß. Whilst these experiments are helpful in dissecting the role of individual proteins, they are not well adapted to help us describe the concert effect of multiple inflammatory signals generated by tissue inflammation, which is arguably more faithful to human inflammatory disease.

However, the strength and reliability of the findings relating to the neuroinflammatory response in CIA is to some extent limited by the high degree of variability inherent in the model. This means that there is the potential for significant findings to be obscured. Despite this variability, a consistent

significant finding of elevated brain Il1ß transcription emerged and the variability in arthritis severity was leveraged to demonstrate a predictor relationship between arthritis severity and brain Il1ß using linear regression analysis.

Objective measures of sickness behaviour derived from the animal experiments would have strengthened the translational potential of the results. Clear signs of sickness behaviour such as hunching, reduced grooming, reduced feeding and reduced motor activity were observed during the running of the experiments but formal behavioural experiments, for example tests of cognition, hedonic responses such as sucrose preference, burrowing, and exploratory activity were not undertaken. A characterisation of the behavioural phenotype associated with CIA would be an informative addition to the literature and represents an important future direction.

The SERA fatigue data has been analysed using a combination of hypothesis-driven univariable and multivariable analysis. This study of fatigue adds to the literature in the field as it is based upon a modern treat-to-target early RA cohort, measures of which are relatively rare in the current literature. However, the data on fatigue was limited by a low n. value, particularly at later time points such as at 12 months and in certain predictor variables. This may have led to the analysis being underpowered for some variables at these time points and increased the risk of type 2 error. Another limitation in terms of exploring the effects of peripheral inflammation on fatigue was inherent to the dataset, in that the measures of inflammation were relatively basic and non-specific. Blood measures of cytokine and other inflammatory mediators was not available, though this is in keeping with the nature of most large cohort studies.

This thesis attempts to present this body of work in the form of a translational approach, attempting to approach the question of the role of peripheral inflammation in the generation of a brain response, from either side of the translational bridge. A translational approach may be considered a strengthening quality in a piece of research, in that it can enhance the validity and salience of the findings. However, attempting a translational approach from the extremes of the bridge, i.e. from the experimental animal model to the clinical cohort study, is challenging and the success of this approach may fairly

be argued over. The brain is an inherently difficult organ to study in humans due to its complexity and relative inaccessibility; the challenges faced in translation in this study are not unique. Translational studies however will remain an important endeavour in the field of biological and clinical research and are key to efforts aimed at understanding and better treating human disease.

5.4 Future directions

An important step in strengthening the translational bridge would be to study the behavioural phenotype of mouse models of inflammatory arthritis. This would also be essential in determining whether brain inflammatory changes observed have functional consequences relevant to sickness behaviour and human psychiatric disease. There has been some study of behavioural phenotypes in hTNFtg mice (described in section 1.7.1). Though these mice do not appear to manifest major sickness behaviours relevant to depression such as loss of hedonic responses, locomotion is reduced – linked either to arthritis or fatigue (or both) (Süß et al., 2015). Limited if any behavioural experimentation is reported in the literature relating to other arthritis models such as CIA. A single study was identified by literature searching that reported reduced hedonic responses in CIA (Brown et al., 2018). Greater behavioural phenotyping in arthritis models is required.

Further studies of the brain response to tissue-specific inflammatory stimuli are needed. Whether CIA is the correct model to undertake such experiments is uncertain. The disease variability observed in the experiments presented in this thesis limited the analysis of the neuroinflammatory response and would present major challenges to any behavioural study design. Whilst the hTNFtg model of arthritis is consistent and chronic in its disease presentation, it may be limited by the nature of its primary pathogenic mechanism involving a single cytokine driver – TNFα. This marks a major diversion from the human context. CAIA (see section 1.7) shares many similarities to CIA but shows greater consistency in response and may be a better suited model for examining the brain inflammatory response and behavioural phenotype in inflammatory arthritis.

However, in comparison to CIA, CAIA bypasses important immuno-pathogenic stages that may be important in human RA, such as autoantibody production, and requires additional stimulation with LPS to manifest arthritis. By their nature, no animal model will be without limitations.

Experimental methods in determining cellular responses to inflammatory stimuli have advanced in recent years, to the extent that gaining mechanistic understanding of pathogenic processes is becoming more realisable. The field of RNA sequencing and single-cell RNA sequencing has matured to the extent that it can be harnessed as a reliable and effective method to understand differential gene expression from tissues and the activity of distinct cell populations, for example quiescent vs. activated microglia (Borst and Prinz, 2020). Building on this, spatially resolved transcriptomics offer a method of delineating the transcriptional profile of cells in situ (Stark et al., 2019). Such an approach is particularly important when studying neuroinflammatory responses given the diverse functional implications of regional responses in the brain, and could be leveraged to explore translational implications relating to human neuroimaging data. Future exploration of the brain response to tissue-specific inflammatory models would be greatly enhanced by utilising such approaches.

At the other end of the translational spectrum, clinical studies that incorporate more specific measures of peripheral inflammation would enhance a mechanistic understanding of disease phenomena. The SERA cohort could be further interrogated in terms of the importance of peripheral inflammation in the expression of fatigue, depression and anxiety scores using such an approach. The SERA study encompasses biobanked serum samples which could be analysed to determine whether peripheral cytokine levels were associated with fatigue or HADS scores in early RA.

A challenge in human clinical and experimental studies is the relative inaccessibility of the brain. In view of the immune-specialism and protective compartmentalisation of the brain, it is in most circumstances incorrect to assume that whatever is measured in the periphery, is directly reflected in the CNS, regardless of the extent of immune-to-brain communication. Post-mortem studies may have a place in the study of brain responses, but are so beset by confounding (e.g. cause of death) in many circumstances as to render them

uninterpretable. Future studies could give consideration to CSF sampling as practicable and incorporating neuroimaging for at least a subset of patients in cohort studies. This would go some way to enhancing our appreciation of CNS responses in association with inflammatory disease and their behavioural and neuropsychiatric manifestations.

5.5 Conclusions

The work presented in this thesis supports the existence of a complex relationship between peripheral inflammation in inflammatory arthritis and the brain and behavioural response. The experiments presented show that a brain neuroinflammatory response can be detected in a tissue-specific mouse model of peripheral inflammation. What the consequences of this neuroinflammatory response could be if translated to human RA is yet to be determined. The existing literature leads us to suspect that over time a neuroinflammatory environment will predispose to the emergence of troublesome symptoms such as fatigue, cognitive problems and an enhanced risk of neuropsychiatric disease, including mood disorders and dementias. Fatigue is a problematic sickness behaviour type symptom commonly seen in RA. The experiments conducted using human RA data in this thesis suggest that in early RA, although fatigue improves markedly coincident with active RA treatment, peripheral inflammatory variables such as CRP and DAS28 scores are not clear predictors of fatigue. Depression scores are only marginally predicted by CRP. It appears that whilst a meaningful brain response to inflammatory arthritis is likely to exist, it may involve a nuanced and complex interplay. This is a field that clearly warrants further investigation with an emphasis on translational approaches in order to enhance our understanding and treatment of mental disorders in the context of inflammation.

Appendices

Appendix 1: Companies referenced in methods

Abcam
Abcam Plc.
Discovery Drive
Cambridge Biomedical Campus
Cambridge CB2 0AX
UK

Agilent
5301 Stevens Creek Boulevard
Santa Clara
CA 95051
USA

BD Biosciences
BD Becton, Dickinson and Company
1 Becton Drive
Franklin Lakes
NJ 07417-1880
USA

Biolegend
BioLegend
8999 BioLegend way
San Diego
CA 92121
USA

Bio-Rad
Bio-Rad Laboratories Ltd.
Station Road
Watford WD17 1ET
UK

Bright Instruments Ltd.
Unit 1 Sovereign Park
Laporte Way
Luton LU4 8EL
UK

CellPath
CellPath Ltd.
Mochdre Industrial Estate
Newtown
SY16 4LE
UK

Denovix
DeNovix Inc.
3411 Silverside Road
Wilmington
DE 19810
USA

Envigo
Envigo UK Ltd.
Huntingdon PE28 4HS
UK

Gibco
Gibco UK
See ThermoFisher Scientific

GraphPad Software
GraphPad Softward
2365 Northside Drive
San Diego
CA 92108
USA

Greiner
Greiner Bio-One Ltd.
Brunel Way
Stonehouse
UK

Integrated DNA Tehcnologies
Integrated DNA Technologies Inc.
1710 Commercial Park
Coralville
IA 52241
USA

MD Biosciences
MD Biosciences Inc.
3510 Hopkins Pl
Oakdale
MN 55128
USA

Menarini
Menarini Diagnostics Ltd.
405 Wharfedale Road

Winnerish RG41 5RA
UK

Merck
Merck Life Science UK Ltd.
Croxley Green Business Park
Watford WD18 8YH
UK

Miltenyi Biotech
Miltenyi Biotech Ltd.
Almac House
Church Lane
Bisley GU24 9DR
UK

National Diagnostics
National Diagnostics
305 Patton Drive
Atlanta
GA 30336
USA

New England Biolabs
New England BioLabs UK Ltd.
75-77 Knowl Piece
Hitchin SG4 0TY
UK

Novus
Novus Biologicals LLC
10730 East Briarwood Avenus
Centennial
CO 80112
USA

Qiagen Ltd.
Skelton House
Manchester Science Park
Manchester M15 6SH
UK

SantaCruz
Santa Cruz Biotechnology Inc.
2145 Delaware Avenue
Santa Cruz
CA 95060
USA

Sigma
Sigma-Aldrich Company Ltd.
The Old Brickyard
New Road
Gillingham
Dorset SP8 4XT
UK

Starlab
STARLAB UK, Ltd.
5 Tanners Drive
Milton Keynes MK14 5BU
UK

ThermoFisher
ThermoFisher Scientific
168 Third Avenue
Waltham
MA 02451
USA

TissueTek
Sakura Finetek USA Inc.
1750 West 214th Street
Torrance
CA 90501
USA

Vectorlabs
Vector Laboratories – 2B Scientific
77 Heyford Park
Upper Heford OX25 5HD
UK

VWR
VWR International Ltd.
Hunter Boulevard
Magna ark
Lutterworth LE17 4XN
UK

Westburg Life Sciences
Westburg BV
Arnhemseweg 87
3832 GK Leusden
Netherlands

Zeiss
Car Zeiss Microscopy
Carl-Zeiss-Strasse 22
Oberkochen 73447
Germany

Appendix 2: The PROMIS Fatigue 8a - Adult v1.0 Short Form

- Have you had problems with fatigue for more than three months? (Y/N)
- During the past 7 days:-
    - I feel fatigue –
      Not at all(score 1)/ A little bit(score 2)/ Somewhat(score 3)/ Quite a bit(score 4)/ Very much(score 5)

    - I have trouble starting things because I am tired –
      Not at all(score 1) / A little bit(score 2) / Somewhat(score 3)/ Quite a bit(score 4)/ Very much(score 5)

- In the past 7 days:-
    - How run-down did you feel on average?
      Not at all(score 1) / A little bit(score 2) / Somewhat(score 3) / Quite a bit(score 4) / Very much(score 5)

    - How fatigued were you on average?
      Not at all(score 1) / A little bit(score 2) / Somewhat(score 3) / Quite a bit(score 4) / Very much(score 5)

    - How much were you bothered by your fatigue on average?
      Not at all(score 1) / A little bit(score 2) / Somewhat(score 3) / Quite a bit(score 4) / Very much(score 5)

    - To what degree did you fatigue interfere with your physical functioning?
      Not at all(score 1) / A little bit(score 2) / Somewhat(score 3) / Quite a bit(score 4) / Very much(score 5)

- In the past 7 days:-
    - How often did you have to push yourself to get things done because of your fatigue?
      Not at all(score 1) / A little bit(score 2) / Somewhat(score 3) / Quite a bit(score 4) / Very much(score 5)

    - How often did you have trouble finishing things because of your fatigue?
      Not at all(score 1) / A little bit(score 2) / Somewhat(score 3) / Quite a bit(score 4) / Very much(score 5)

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