The roles of interferon biomarkers in monitoring patients with systemic lupus erythematosus and other connective tissue diseases

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I would also like to thank Ms Sabina Khan, Dr Khaled Mahmoud, Ms Huma Cassamoali, Ms Zoe Wigston, Dr Antonios Psarras, Dr Yasser El-Sherbiny, Dr Marta Aguilar Zamora, Dr Shouvik Dass, Ms Heather Owston, Ms Elizabeth Hensor and Dr Sabih-Ul Hassan for their contributions and support throughout this project. AstraZeneca provided fellowship funding for this project and I acknowledge their major financial contribution. I thank my family, friends and partner for their support during this time of great personal and professional growth. Finally, but most importantly, I acknowledge the love and life of my grandmother Teresa Kavur. Her strength of character, wisdom and grace has been my greatest inspiration and it is to her that I dedicate this thesis.

Candidate’s role in this project

The initial concept and design for the studies in this thesis was established by myself, Dr Md Yusof and Dr Vital. Clinics in Leeds were attended under the supervision of Dr Vital, and all 279 and 43 participants were consented and recruited by myself into the studies described in Chapter 4 and Chapter 6, respectively. In the study described in Chapter 5, Dr Md Yusof recruited and consented all 166 participants in clinics in Leeds under the supervision of Dr Vital. I extracted all data from the clinical records of these participants. For Chapter 4 and Chapter 6 studies, I collected all of the clinical data. The research bloods were processed and stored by Dr Antonios Psarras, Mrs Diane Corscadden, Mrs Katie Mbara and Ms Zoe Wigston. The IFN scores were originally derived by Dr Hensor (statistician) and Dr Vital. Research databases were constructed by myself, however assistance was received from Ms Heather Owston in Chapter 5. In terms of statistical analyses and the writing of the manuscript, I carried out the initial work, and supervisors checked and revised my work.
List of publications arising from this thesis

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**Oral presentations (first author):**


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- The Annual Combined Northern and Yorkshire Rheumatology Meeting in September 2018

**Poster presentations (first author):**


Abstract

Background: Type I interferon (IFN-I) is thought to have a central role in the pathogenesis and activity of autoimmune connective tissue disease (CTD). As a heterogeneous condition, CTD is often a challenge to manage, which is further made difficult by the lack and imprecision in diagnostic tools. IFN has shown promise as biomarkers in correlation studies on disease activity in CTD. Real world challenges in CTD management could be addressed with a validated IFN biomarker.

Objectives: (i) to examine the role of IFN biomarkers in the prediction of flares and glucocorticoid requirements in SLE; (ii) to examine the use IFN assays in distinguishing patients who meet definite CTD classification criteria from a cohort of patients labelled as UCTD; and (iii) to examine the relationship between IFN biomarkers and patient-reported outcomes in patients At-Risk, with UCTD and with established CTD.

Methods: A prospective study was conducted in a (i) SLE cohort and a (ii) UCTD cohort attending routine clinics. Comprehensive clinical assessment focusing on (i) disease activity and glucocorticoid requirements, and (ii) classification criteria for SLE, SS, IM and SSc, was conducted in conjunction with IFN biomarker sampling. (iii) A cross-sectional study of patient-reported outcomes was administered together with IFN biomarker sampling in At-Risk, UCTD and established CTD patients attending routine clinic.

Results: (i) High IFN Score A, IFN Score B and Memory B cell tetherin were associated with flares and increased glucocorticoid requirements in a cohort of SLE patients; (ii) IFN Score A was higher in those who were re-classified into CTD than those remained undifferentiated, thus could be used to distinguish between these two groups, and patient’s initially labelled as UCTD; (iii) correlation between IFN Scores and PROMs varied widely among diagnoses of CTDs, with the strongest correlation found in patients with UCTD.

Conclusion: In this thesis, I have demonstrated several potential uses of IFN assays in the monitoring of patients with CTD with respect to prediction of flares and glucocorticoid requirements in SLE; the distinction of classifiable CTD from UCTD; and understanding the relationships between IFN and patient-reported outcomes. These findings need validation in a longitudinal cohort to inform their applicability in clinical practice.
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Abbreviations

ACR  American College of Rheumatology
ANA  Antinuclear antibodies
BAFF  B cell activating factor
CTD  Connective tissue disease
CVD  Cardiovascular disease
DEFINITION  Defining the role of interferon in autoimmune conditions
DM  Dermatomyositis
DMARDs  Disease-modifying anti-rheumatic drugs
EBV  Epstein-Barr virus
EDTA  Ethylenediaminetetraacetic acid
EQ-5D-5L  5-level EQ-5D version
ESSDAI  EULAR Sjögren’s Syndrome Disease Activity Index
EULAR  European League Against Rheumatism
FA  Factor analysis
FACIT-Fatigue  Functional Assessment of Chronic Illness Therapy
HMGCR  3-hydroxy-3-methylglutaryl coenzyme A reductase
HSCT  Haematopoietic stem cell transplantation
IBM  Inclusion body myositis
ICECAP-A  ICEpop CAPability measure for Adults
IFN  Interferon
IFN-I  Type I interferon
IFN-II  Type II interferon
IL  Interleukin
IM  Inflammatory myositis
IMNM  Immune-mediated necrotising myopathy
ISGs  Interferon-stimulated genes
LN  Lupus nephritis
MCTD  Mixed connective tissue disease
NSAIDs  Non-steroidal anti-inflammatory drugs
OCTD  Overlap connective tissue disease
PBMCs  Peripheral blood mononuclear cells
pDCs  Plasma cytoid dendritic cells
PPIA  Peptidyl prolyl isomerase A
PM  Polymyositis
PROMs  Patient-reported outcomes measured
pSS  Primary Sjögren’s syndrome
RA  Rheumatoid arthritis
RNA  Ribonucleic acid
RNP  Ribonucleoprotein
SF-36  RAND® 36-Item Health Survey version 1.0
SLE  Systemic lupus erythematosus
SLICC  Systemic Lupus International Collaborating Clinics
SNP  Single-nucleotide polymorphisms
SR14  SLE Responder Index 4
SRP  Signal recognition particle
SS  Sjögren’s syndrome
SSc  Systemic sclerosis
sSS  Secondary Sjögren’s syndrome
Th17  T helper 17
TLRs  Toll-like receptors
UCTD  Undifferentiated connective tissue disease
Chapter 1 Introduction

The autoimmune connective tissue diseases (CTDs) are a group of chronic systemic rheumatic diseases that cause significant morbidity and mortality\cite{1, 2}. Systemic lupus erythematosus (SLE) is the prototypical CTD, however Systemic sclerosis (SSc), Sjögren’s syndrome (SS) and inflammatory myositis (IM) and variants of these disorders are included in the concept\cite{2}. While classification criteria have been developed to define definite subsets of CTD, no diagnostic criteria have been generated for any of the entities within the CTD spectrum\cite{2}. This contributes to delays and inaccuracies in the management of the disease. The mean interval between onset of symptoms and the diagnosis of SLE has been estimated at 22 months\cite{3}. Inaccurate diagnosis of SS (e.g. as RA or SLE) is described in up to 34% of SS cohorts, and diagnostic delay is frequently reported among patients with SSc and IM\cite{4-6}.

As classification criteria have not been developed, reports of the prevalence and incidence of CTD are limited\cite{7}. North America estimates a prevalence of CTD of 0.27% and describe the incidence as “very low” \cite{8}. Despite this, CTDs represent a substantial proportion of hospital admissions and demand higher than average levels of healthcare utilisation \cite{8}. The all-cause annual healthcare cost has been estimated at £15,000 per person among all individuals with CTD based on US figures\cite{8}. Moreover, significant impairments in quality of life, life expectancy and work productivity are evident for all subsets of CTD \cite{9, 10}. The estimated ten year survival rate for SSc is reported at 60 – 73%, >90% for SLE, 90% for SS, and 73% for the antisythetase syndrome (a subset of IM) \cite{11-13}. High unemployment and absenteeism rates of up to 70% accounted to CTD-related illness have been reported in CTD cohorts \cite{14}.

Current methods for the diagnosis and monitoring of CTD is mainly indirect and relies on a comprehensive assessment aimed matching findings to known clinical syndromes \cite{15, 16}. Many patients are incorrectly given a diagnosis of a CTD and exposed to toxic medications\cite{17}. Of the limited biomarkers that are currently available in the clinical setting, none have universal application within the CTD population \cite{18, 19}. There is therefore a need to identify biomarkers that can discriminate CTD from mimickers to prevent misdiagnosis and delays in treatment.

Type I interferon (IFN-I) plays a fundamental role in the pathogenesis of SLE, as indicated by genetic susceptibility data and lupus-like disease in monogenic interferonopathies\cite{20}. It has also been implicated in SSc, SS and IM\cite{21-23}. Since IFN-I activity varies between individuals and is associated with disease activity in SLE,
it is of interest as a biomarker across the CTD spectrum[20]. Previous work in our group showed that IFN assays could predict progression in an At-Risk cohort and correlate with lupus flares and features[24, 25]. Therefore investigating the roles of interferon in SLE and other CTDs is the theme of this thesis.

1.1 Thesis hypothesis
The unifying hypothesis of this thesis is:

The use of interferon biomarkers will aid the prognosis and management of autoimmune connective tissue diseases

1.2 Overview of planned investigations
Planned investigations to test the unifying and hypotheses for each chapter were divided into three Work-Packages (WPs). These are summarised in Figure 1.

Figure 1 Planned work of this thesis

Focus areas on the role of IFN biomarkers in SLE and other CTDs

SLE
Examine the role of IFN biomarkers in glucocorticoid and disease flare prediction (WP1)

Other CTDs

UCTD
The use of IFN assays in distinguishing patients who meet definitive CTD classification from a cohort of patients labelled as UCTD (WP2)

At risk and established CTDs including UCTD
Examine the association between IFN assays & patient-reported outcomes in at risk, UCTD and established CTD (WP3)

Figure 1. Definition of three work packages (WPs)
Chapter 2 Literature Review

2.1 Nomenclature, classification and diagnostic criteria

2.1.1 The spectrum of CTD

Defined CTD entities exist within a spectrum of more heterogeneous entities that lack defining clinical features, laboratory, imaging, or other diagnostic tests (Figure 2). In other words, defined CTDs, such as SLE, SS, SSc, IM, can be conceived as part of a spectrum including mixed CTD (MCTD), overlap CTD (OCTD), undifferentiated CTD (UCTD). In terms of the variant forms of CTD, OCTD represents the full expression of two or more defined CTDs [26]. MCTD has mixed clinical features and antibodies against ribonuclear proteins. UCTD represents antinuclear antibodies (ANA) and clinical features of CTDs that do not fulfil criteria for any one disease. UCTDs may evolve into a defined syndrome, remain undifferentiated or regress into remission.

Approximately 50% of patients with an apparent autoimmune rheumatic disease cannot be given a specific diagnosis in the first year of observation[27] and nearly 25% of patients exhibit features of two or more diseases[28]. This concept facilitates the definitive diagnosis of CTD while permitting the option of evolving, transient or variant forms of disease. However, prior research has usually enrolled homogeneous patient populations.

2.1.2 CTD classification and diagnostic criteria

Classification criteria are important for research and epidemiological purposes. They are standardised definitions that are primarily intended to enable uniform cohorts in clinical studies[29]. Classification criteria prioritise the capture of patients who share key features of the specific disease, but are not intended to capture all patients affected by the condition. They serve the useful purpose of defining (relatively) homogeneous cohorts that can be compared across studies and geographic regions, and provide precision in scientific and medical communication.
Clinical spectrum of the CTDs. Traditional CTDs (i.e. SLE, SS, SSc and IM) and composite, transient or undefined variants (i.e. MCTD, UCTD and OCTD).
In contrast, diagnostic criteria are broad and must reflect all the possible different features and severity of a disease (heterogeneity)[29]. Both the specificity and sensitivity of diagnostic criteria need to be high[29]. Consequently, in the case of the CTDs, classification criteria will exclude a greater number of true cases than diagnostic criteria (greater false negatives), as diagnostic criteria will tend to risk the inclusion of patients with “mimicker” conditions so include all actual disease cases (i.e. greater false positives)[29]. The strength of diagnostic criteria and classification criteria is impacted by contextual factors such as the prevalence and homogeneity of the disease in question and the frequency of disease mimickers[29]. Single universal diagnostic criteria are usually not possible due to disease variance across different geographic areas, race and ethnicities[29]. If adequate internal and external validity for diagnosis is demonstrated in a given population, classification criteria could in theory be diagnostic. For example, when classification criteria have absolute (100%) sensitivity and specificity, classification and diagnostic criteria are synonymous and have the capacity to identify every affected individual[30].

The utility of classification criteria in routine clinical practice is limited. There is a propensity within the rheumatology community to defer to classification criteria when the diagnosis is uncertain, however this will be to the detriment of some patients who will consequently be denied corrective treatment[29]. Given the complexity of the phenotype model of CTD, diagnostic criteria will remain out of reach for CTD until a more discerning framework is established. Until such a time, the diagnostic process will depend on a subjective combination of clinical signs and symptoms, results of available tests, the immunological and autoantibody profile, and the knowledge of epidemiology of the geographical region[28].

2.2 The autoimmune connective tissue diseases

2.2.1 Systemic lupus erythematosus

Systemic lupus erythematosus is often referred to as the “prototypical CTD.” It is a chronic autoimmune disease characterised by an aberrant autoimmune response to nuclear antigens that can affect almost any organ or tissue[1]. It is a heterogeneous disease with a diverse range of clinical manifestations that has a typical relapsing and remitting course over the lifetime. Lupus predominantly affects women of child-bearing age (female: male ratio of 9.1) and has a peak incidence of onset between the ages of 16 and 55 years[2]. The worldwide prevalence of SLE currently ranges from 20 to 150 cases per 100,000 population, with the highest prevalence reported in North America and Puerto Rico, and the highest incidence reported in people of African, Caribbean, Hispanic or Asian racial ancestry compared with white Europeans[31]. Increased genetic risk burden in these populations and the associated increased tendency for autoantibody reactivity may explain the global ethnic and racial variations. Overall,
lupus appears to be increasing in worldwide prevalence as the disease gains recognition and survival rates improve, alongside chronicity of the condition[32]. The current 10-year survival rate of lupus is approximately 97%[12].

2.2.1.1 SLE diagnostic and classification criteria

Diagnostic criteria for lupus are yet to be established. Classification criteria for lupus have been developed as a means to identify uniform and comparable groups of subjects for research purposes. The most widely used criteria for SLE was developed by the American College of Rheumatology (ACR) in 1982 and later revised in 1997 (ACR-97)[33]. The ACR-97 criteria are a composite of clinical and immunological criteria with classification achievable in the presence of 4 of 11 criteria[34, 35]. The criteria have undergone various attempts at refinement including a weighing system in the Cleveland Clinic version, and new supplemental criteria such as antiphospholipid antibodies in the Boston criteria[36], however these changes have failed to generalise into the mainstream[37]. The major criticisms of the ACR-97 were that they (1) over-represented cutaneous lupus with four cutaneous criteria; (2) omitted hypocomplementaemia, one of the most important characteristics for SLE; (3) lacked validation in an independent cohort and within multiple ethnic groups; and (4) failed to receive development contributions from non-rheumatology stakeholders, such as from dermatologists or nephrologists[33].

The Systemic Lupus International Collaborating Clinics (SLICC) is an international group that presented a new set of classification criteria in 2012 (SLICC-12). The criteria required four of 17 criteria, including one of 11 clinical criteria and one of six immunologic criteria, or biopsy-proven SLE nephritis in the presence of ANA or anti-dsDNA antibodies (Table 1). SLICC-12 performed with fewer misclassifications (62 vs. 74, p=0.24) and had a greater sensitivity (97% vs. 83*; p<0.0001) but less specificity (84% vs. 96%, p<0.0001) compared to ACR-97 within validation cohorts[38]. A 2018 systematic review concluded that the performance of SLICC-12 was best for adult SLE whilst ACR-97 was best for juvenile SLE[39]. In adult SLE (nine studies: 5236 patients, 1313 controls) SLICC-12 had a high sensitivity (94.6% vs. 89.6%) and similar specificity (95.5% vs. 98.1%) compared to ACR-97. For juvenile SLE (four studies: 568 patients, 339 controls), SLICC-12 demonstrated higher sensitivity (99.9% vs 84.3%) compared to ACR-97, but much lower specificity (82.0% vs. 94.1%) and therefore presented a higher risk of false positives (Table 1).

2.2.1.2 SLE pathogenesis

The current model of lupus pathogenesis is based on the sequential breakdown of self-tolerance in genetically pre-disposed individuals due to environmental influences, leading to activation of the innate and adaptive immune systems, and critically, the
production of antibodies to nuclear material [1]. In rare cases SLE may be associated with the deficiency of a single gene (i.e. the C1q and C4 complement components) but in most cases the disease is the result of combined effects of variants in a large number of loci[32].

Table 1 The Classification of SLE: ACR-97 and SLICC-12 criteria

<table>
<thead>
<tr>
<th>ACR-97</th>
<th>SLICC-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Four of the 11 criteria are needed for the classification of SLE</td>
<td>Four of 17 criteria, including one of 11 clinical criteria and one of 6 immunologic criteria OR biopsy-proven SLE nephritis with ANA</td>
</tr>
<tr>
<td><strong>Clinical criteria</strong></td>
<td><strong>Clinical criteria</strong></td>
</tr>
<tr>
<td>1. Malar rash</td>
<td>1. Acute cutaneous lupus OR subacute cutaneous lupus</td>
</tr>
<tr>
<td>2. Discoid rash</td>
<td>2. Chronic cutaneous lupus</td>
</tr>
<tr>
<td>3. Photosensitivity</td>
<td>3. Oral ulcers</td>
</tr>
<tr>
<td>5. Non-erosive Arthritis</td>
<td>5. Synovitis OR tenderness in &gt;1 joint and &gt;30 min of morning stiffness</td>
</tr>
<tr>
<td>6. Pleuritis or pericarditis</td>
<td>6. Serositis</td>
</tr>
<tr>
<td>7. Renal Disorder</td>
<td>7. Renal</td>
</tr>
<tr>
<td>8. Neurologic Disorder</td>
<td>8. Neurologic</td>
</tr>
<tr>
<td></td>
<td>10. Leukopaenia OR lymphopenia</td>
</tr>
<tr>
<td></td>
<td>11. Thrombocytopenia</td>
</tr>
<tr>
<td><strong>Immunologic criteria</strong></td>
<td><strong>Immunologic criteria</strong></td>
</tr>
<tr>
<td>10. Positive ANA</td>
<td>2. Anti-dsDNA antibody</td>
</tr>
<tr>
<td></td>
<td>3. Anti-Sm</td>
</tr>
<tr>
<td></td>
<td>4. Antiphospholipid antibody</td>
</tr>
<tr>
<td></td>
<td>5. Low complement</td>
</tr>
<tr>
<td></td>
<td>6. Direct Coombs' test</td>
</tr>
</tbody>
</table>

Table 1. SLE classification criteria.

2.2.1.3 SLE genetic factors and immune pathophysiology

Most single-nucleotide polymorphisms (SNPs) linked to SLE are components of non-coding DNA regions of immune response-related genes[40, 41]. Some genes are specific to SLE whilst others have been linked with several other autoimmune diseases (i.e. PTPN22 with diabetes and Graves thyroiditis)[42]. Certain SNPs associated with SLE have been identified to contribute to aberrant dendritic cell function and IFN signalling (TREX1 and STAT4); whilst others are linked to dysfunction of immune-complex processing and innate immunity such as impaired interleukin (IL) expression (C1QA, ITGAM); T cell function and signalling (STAT4 and PTPN22); cell cycle, apoptosis and cellular metabolism (CASP10); and transcriptional regulation (MECP2) in SLE[32]. Although the literature is growing, the loci identified so far can only account for about 15% of the heritability of SLE[43]. An altered copy number of certain genes, such as FCGR3B[44], TLR7[45], and C4[46] which influences gene dosage has also been linked to SLE disease expression.
Environmental factors are known to initiate SLE disease and activity. Epigenetic changes through DNA hypomethylation due to exposure to certain medications (i.e. tetracyclines) can induce the onset of SLE, which is sometimes defined as drug-induced SLE[47]. Smoking and ultraviolet light increase the risk of SLE through unknown mechanisms[48]. Viral infection, such as with the Epstein-Barr virus (EBV), have been implicated in epidemiological studies in the initiation and exacerbation of SLE through the potentiation of aberrant innate immune pathways[49]. Female hormones contribute to the increased prevalence of SLE in women[50]. Pregnancy and exogenous oestrogen or progesterone administration increases the severity of SLE, however the mechanism of how hormones influence SLE is unknown. The X chromosome may be an independent SLE risk factor based on castrated X chromosome knockout murine models. Mouse models manipulated to express XX or XXY combinations, compared to XO or XY combinations, were found to have increased severity in SLE[51].

DNA methylation and histone modification contribute to DNA accessibility and thus gene expression; contributing to the development of lupus in healthy persons. Hydralazine and procainamide inhibit DNA methylation and are linked with a lupus variant called drug-induced lupus. Hypomethylation of the regulatory region of genes involved in disease pathogenesis (ITGAL, CD40LG, CD70, and PPP2CA) have been reported in SLE[32]. Experimental treatment in lupus-prone mice with an inhibitor of histone deacetylase, Trichostatin A, has been shown to improve disease through the blockade of histone deacetylase-mediated recruitment and suppression of the IL-2 promoter[52].

Innate and adaptive immune responses are pathogenic in patients with SLE, where early signalling events are amplified or primed[53]. Pathways implicated include T cells, lipid rafts, IL17, adhesion molecule CD44, CXCR4 receptors, plasma cells, autoreactive B cells; normal and plasmacytoid dendritic cells (pDCs), IFN, and loss of inhibitory immune pathways for IL-2. The resultant impact is the development of damaging immune complexes composed of that antinuclear antibody bound to nuclear tissue deposit into tissues and organs. These complexes arise in large amounts in blood and tissues and are slow to clear due to numerical and functional depletion in Fc and complement receptors. In the renal glomeruli, sequential deposition of immune complexes into the subendothelial and mesangial areas first, followed by deposition in the basement membrane and subepithelial areas is seen[32]. Immune deposition has also been shown in the tissue beds of skin and the central nervous system. Immune complexes may bind to receptors expressed by tissue-specific cells and alter their function, however more prominently, the complexes activate the influx of immune cells.
and the complement cascade, generating localised inflammation. The mix of migrant peripheral T cells, polymorphonuclear cells and B cells contribute towards the local auto-inflammation and feedback to the overall autoimmune process.

Non-immune cells contribute to disease expression through facilitating antigenic presentation with an increase in secretion of pro-inflammatory cytokines. This has been observed in renal glomerular cells when exposed to IFN-γ, and keratinocytes following exposure to ultraviolet light for which the latter become apoptotic and release nuclear material[32]. The expression of additional organ-specific molecules is influential in determining the pattern of organs or tissue involvement in lupus. Expression of tumour necrosis factor (TNF) receptor 1 is linked with lupus skin disease, however is protective against lupus nephritis (LN) [54].

2.2.1.4 SLE and the antinuclear autoantibody spectrum

Antibodies against nuclear antigens are a pre-requisite diagnostic feature of SLE and form one of the immunological criteria of both the ACR-97 and SLICC-12 SLE classification criteria (Table 1). There are a wide spectrum of autoantibodies specific to lupus and some autoantibody specificities have well-recognised clinical associations, such as ribosomal P antibody and lupus cerebritis[32]. Antibodies against phospholipids and β2-glycoprotein 1 are sometimes present in patients with lupus however are indicative of the antiphospholipid syndrome (APLS) which features recurrent thrombosis and pregnancy morbidity. An in-depth discussion of ANAs is detailed in Chapter 2.3.6.

Within the lupus autoantibody specificities only a few have site-specific effects linked with manifestations of lupus disease. Anti-T-cell antibodies are associated with the suppression of IL-2 production. Anti-blood-cell antibodies are linked with cytopaenia. Lupus nephritis is associated with anti-DNA antibodies, antibodies against the collagen-like region of C1q, anti-nucleosome antibodies and the presence of anti-chromatin antibodies. Anti-Ro antibodies have a predilection for myocytes and the cardiac conduction system and are associated with congenital foetal heart block. Some anti-DNA antibodies cross-react with cerebral cell-specific antigens (i.e. N-methyl-d-aspartate receptors), and increase the risk of lupus cerebritis and neurocognitive defects.

2.2.1.5 SLE treatment pathways

Patients with SLE are treated with a range of anti-inflammatory, antiplatelet, immunomodulatory and immunosuppressive therapeutics (Table 2). This includes non-steroidal anti-inflammatory drugs (NSAIDs), antimalarial agents, glucocorticoids,
disease modifying anti-rheumatic drugs (DMARDs) and other immunosuppressive drugs[55].

**Table 2 Therapeutics in SLE**(56, 57)

<table>
<thead>
<tr>
<th>NSAIDs</th>
<th>Immunosuppressive agents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucocorticoids, Cyclophosphamide, Methotrexate, Azathioprine, Mycophenolate mofetil, Tacrolimus and calcineurin inhibitors</td>
</tr>
<tr>
<td></td>
<td>Modulators of B cell function or numbers</td>
</tr>
<tr>
<td></td>
<td>B cell depletion (Rituximab)</td>
</tr>
<tr>
<td></td>
<td>B-lymphocyte stimulator inhibitor (Belimumab)</td>
</tr>
<tr>
<td></td>
<td>Inhibition of Toll-like receptors (TLRs)</td>
</tr>
<tr>
<td></td>
<td>Hydroxychloroquine and related drugs</td>
</tr>
<tr>
<td></td>
<td>Avoidance of exogenous oestrogen and/or progesterone</td>
</tr>
</tbody>
</table>

**Table 2. Therapeutics in SLE.**

### 2.2.2 Primary Sjögren’s syndrome

Primary Sjögren’s syndrome (pSS) is a systemic autoimmune disease characterised by lymphocytic infiltration of the secretory glands[56]. Sicca syndrome is the central diagnostic feature which presents as dryness of the eyes, oral cavity, pharyngolarynx and/or vagina. Extra-glandular manifestations may be concurrent in patients with pSS including cutaneous, musculoskeletal, pulmonary, renal, haematological and neurological involvement. Constitutional symptoms such as fatigue, glandular swelling and night sweats are common features. The syndrome may present secondary to another autoimmune disease such as SLE or rheumatoid arthritis (RA) in which case it is referred to as secondary Sjögren’s syndrome (sSS). The prevalence of sSS is highest in RA and estimated to be approximately 20%[57]. The incidence of pSS is approximately 4 per 1000 patients per year and overall prevalence of pSS in Europe is between an estimated 0.1-4.8%[58].

#### 2.2.2.1 Primary Sjögren’s syndrome pathogenesis

As in SLE, the pathogenesis of pSS is multifactorial and not fully understood. Autoreactive Th1 cells against variants of the major histocompatibility complex class 2 molecule is thought to have a key role in the pathogenesis of pSS, with autoreactive T helper 17 (Th17) cells[59], Th1 cell cytokines (IL-1b, IL-6, TNF-α and IFN-γ)[60], IL-17 (a Th17 cytokine)[61], and autoreactive B cells contributing to the maladaptive response. The innate immune system is considered to have an initiating role in the autoimmune process of pSS. One of the key mechanisms is increased TLR activity and the excessive expression of IFN type I and II by pDCs[62, 63]. It has been suggested that certain viruses (i.e. EBV) and immune complexes activate the maladaptive innate immune processes, although the evidence is indirect[64, 65]. B cell activating factor (BAFF) is stimulated in the presence of type I and type II IFN and this is thought to contribute to the B cell autoreactivity and consequential expression of
hyperglobulinaemia and immune complex formation in pSS[66]. In pSS patients, 55% have an increased IFN type I activity in CD14 monocytes compared to 4.5% in healthy controls[67]. Increased IFN-I activity in pSS has been correlated with higher EULAR Sjögren’s Syndrome Disease Activity Index (ESSDAI), increased levels of IgG and/or hypocomplementaemia, and raised levels of BAFF mRNA in monocytes[63, 67].

2.2.2.2 Primary Sjögren’s syndrome diagnostic and classification criteria

Diagnostic criteria have not been developed for pSS or sSS however there are classification criteria which have a principle role in supporting participant homogeneity in pSS research. Primary Sjögren’s syndrome can be classified in accordance with the 2002 American-European Consensus Criteria for Sjögren’s syndrome [68] (Table 3). These criteria include the presence of dry ocular or oral symptoms (item I and II), supportive ocular signs based on either an abnormal Schirmer’s test or a positive vital dye staining of the eye surface (item III); a lip biopsy showing focal lymphocytic sialoadenitis (item IV); supportive oral signs of either unstimulated whole salivary flow or abnormal parotid sialography (item V); or the anti-SSA (Ro) or anti-SSB (La) or both autoantibodies (item V). For a pSS diagnosis, any four of the six criteria, including item IV (histopathology) or VI (autoantibodies) must be present, or any 3 or the objective criteria (item III, IV, V or VI) in the absence of exclusion criteria. For a sSS diagnosis, the presence of a well-defined major CTD and one symptom (either item I or II) plus two of the three objective criteria is indicative (Item III, IV and V). In 2016 ACR and EULAR jointly developed a new set of classification criteria for primary Sjögren’s syndrome[69]. The classification of SS is based on five objective items and is met in patients who have a score of ≥4 [70].

Table 3 American-European Consensus Criteria for Sjögren’s Syndrome and the ACR Classification criteria for Sjögren’s syndrome[69-71]

<table>
<thead>
<tr>
<th>American-European Consensus Criteria for Sjogren’s Syndrome</th>
<th>ACR Classification criteria for Sjögren’s syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Item I: Ocular symptoms (≥1):</strong> Symptoms of dry eyes for ≥3 months, a foreign body sensation in the eyes, or use of artificial tears ≥3 times per day</td>
<td>1. Labial salivary gland biopsy exhibiting focal lymphocytic sialadenitis with a focus score ≥1 focus/4 mm² (weight 3)</td>
</tr>
<tr>
<td><strong>Item II: Oral symptoms (≥1):</strong> Symptoms of dry mouth for ≥3 months, recurrent or persistently swollen salivary glands, or need for liquids to swallow dry foods</td>
<td></td>
</tr>
<tr>
<td><strong>Item III: Ocular signs (≥1):</strong> abnormal Schirmer’s test, (without an aesthesia; ≤5 mm/5 minutes) or positive vital dye staining of the eye surface</td>
<td></td>
</tr>
<tr>
<td><strong>Item IV: Histopathology:</strong> Lip biopsy showing focal lymphocytic sialadenitis (focus score ≥1 per 4 mm²)</td>
<td></td>
</tr>
<tr>
<td><strong>Item V: Oral signs (≥1):</strong> Unstimulated whole salivary flow (≤1.5 mL in 15 minutes) or abnormal parotid sialography, abnormal salivary scintigraphy</td>
<td></td>
</tr>
<tr>
<td><strong>Item VI: Autoantibodies (at least one):</strong> Anti-SSA (Ro) or Anti-SSB (La), or both</td>
<td></td>
</tr>
<tr>
<td><strong>Exclusion criteria</strong></td>
<td>Past head and neck radiation treatment, hepatitis C infection, acquired immunodeficiency syndrome (AIDS), pre-existing lymphoma, sarcoidosis, graft versus host disease, and/or current use of anticholinergic drugs</td>
</tr>
</tbody>
</table>
Table 3. Classification criteria for pSS.

**2.2.2.3 Primary Sjögren’s syndrome treatment pathways**

Table 4 lists some of the local and systemic treatment options in SS[72]. Most of the agents recommended for the treatment of sicca symptoms are supported by Grade A evidence (meta-analyses or at least one randomised controlled trial) whilst systemic therapies are supported by either Grade C or D level evidence (one well-designed descriptive study or case-control study; or expert option)[73].

Table 4 Therapeutic options in Sjögren’s syndrome(72)

<table>
<thead>
<tr>
<th>Site</th>
<th>Therapeutic options</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratoconjunctivitis sicca</td>
<td>Tear substitutes, secretagogues, cyclosporine A eye drops, topical corticosteroids or punctal plugs</td>
</tr>
<tr>
<td>Xerostomia</td>
<td>Patient education, avoid xerostomia-inducing drugs, secretagogues, saliva substitutes or stimulants</td>
</tr>
<tr>
<td>Parotid swelling</td>
<td>Corticosteroids or antibiotic treatment if required</td>
</tr>
<tr>
<td>Arthritis</td>
<td>NSAIDs, corticosteroids, DMARDs</td>
</tr>
<tr>
<td>Interstitial lung disease (ILD)</td>
<td>Corticosteroids, cyclophosphamide</td>
</tr>
<tr>
<td>Tubulointerstitial nephritis</td>
<td>Potassium and bicarbonate replacement</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>Corticosteroids, cyclophosphamide, mycophenolate</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>Corticosteroids, intravenous immunoglobulin</td>
</tr>
<tr>
<td>Cryoglobulinaemic vasculitis</td>
<td>Corticosteroids, plasmapheresis</td>
</tr>
</tbody>
</table>

Table 4. Management options in SS.

**2.2.3 Systemic sclerosis**

Systemic sclerosis (SSc) is a heterogenous immune-mediated rheumatic disease that is characterised by three hallmarks: small vessel vasculopathy, production of autoantibodies, and fibroblast dysfunction leading to increased deposition of extracellular matrix[74]. It is the fourth most common systemic autoimmune CTD after RA, SLE and pSS[75]. The estimated prevalence of SSc is 1 in 10,000[76, 77]. The clinical prognosis and features vary, however fibrotic skin thickening is the central feature which occurs with variable involvement of the internal organs[78]. Additional features are Raynaud’s phenomenon, digital ulcers, gastro-oesophageal reflux disease, interstitial lung disease, and pulmonary arterial hypertension. Sine scleroderma is a variant of SSc and is recognised as internal fibrosis in the absence of
skin involvement. Systemic sclerosis has a high mortality, especially in the diffuse SSc form and with multiple organ-based involvement[78, 79].

2.2.3.1 Systemic sclerosis diagnostic and classification criteria

There is no diagnostic criterion for SSc however classification criteria exist. The 2013 ACR-EULAR criteria for the classification of systemic sclerosis require a total score of ≥9 for a definite classification[80] (Table 5). Subsets of SSc can be discerned based on the extent of cutaneous sclerosis relative to the knee and elbow. Proximal cutaneous sclerosis is classified as diffuse cutaneous systemic sclerosis (diffuse SSc), whereas the restricted form known as limited systemic sclerosis (limited SSc) affects the limbs distal to the elbows or knees, and can be with or without facial and neck involvement. The importance of the major subsets of SSc is that there are typical expectant features (Table 6) for which specific disease monitoring and treatment is required.

Table 5 2013 ACR-EULAR criteria for the classification of scleroderma(80)

| Proximal skin involvement: | Skin thickening of the fingers of both hands, extending proximal to the metacarpophalangeal joints (9) |
| Skin thickening of the fingers: | Puffy fingers (2); Sclerodactyly of the fingers (4) |
| Fingertip lesions: | Digital tip ulcers (2); Fingertip pitting scars (3) |
| Telangiectasia (2) |
| Abnormal nailfold capillaries (2) |
| Pulmonary arterial hypertension or interstitial lung disease: | Pulmonary arterial hypertension (2); Interstitial lung disease (2) |
| Raynaud's phenomenon (3) |
| Systemic sclerosis-related autoantibodies: | Anti-centromere (3), Anti-topoisomerase I (3), Anti-RNA polymerase III (3) |

Table 5. SSc classification criteria. Score in (brackets).

Table 6 Typical features of the major subsets of scleroderma(80)

| Limited SSc |
| Distal skin sclerosis |
| Long history of Raynaud’s phenomenon |
| Late-stage complications frequent |
| Pulmonary arterial hypertension and severe gut disease frequent |

| Diffuse SSc |
| Proximal limb or trunk involvement, with skin sclerosis |
| Short history of Raynaud phenomenon |
| Increased risk of renal crisis and cardiac involvement |
| High frequency of severe lung fibrosis |

Table 6. Major SSc subsets.

2.2.3.2 Systemic sclerosis pathogenesis

The pathogenesis of SSc is complex and multifactorial. A combination of causal factors, genetic susceptibility and epigenetic alterations probably underlies the initiation and continuation of the disease. Several immune-regulatory genes have been identified that might contribute to the susceptibility of SSc and its specific associations[76, 77].
Case reports and series have linked certain organic chemicals and pesticides as environmental risk factors in the development of SSc, in particular vinyl chloride, chemotherapy drugs, such as taxanes, and radiotherapy exposure[81]. The evidence is conflicting for silicone breast implants, suggesting no association or that the association occurs however only as a rare event in susceptible individuals[82]. Many studies have found an association between the onset of SSc and a range of solid and non-solid organ malignancies, most commonly in association with the anti-RNA polymerase antibody, and particularly in patients with rapid-onset, severe disease[83].

A growing body of literature is providing insight into the molecular and biological mechanisms of SSc. The current concept is that SSc is the manifestation of persistent and self-sustaining myofibroblast activation, resulting in skin and organ fibrosis of variable severity. A milieu of cells are involved including monocytes, T helper cells, local fibroblasts and myofibroblast precursors together with localised induction and overexpression of a range of cytokines and chemokines. Mechanical stress in the extracellular matrix, autocrine stimulation and epigenetic changes result in persistence of myofibroblasts through failure of apoptosis and ongoing extracellular matrix overproduction[84]. Current strategies to manage SSc include general immunosuppression with more recent therapies specifically targeting cytokines and growth factors implicated in disease pathogenesis. However, none of these approaches have been shown to be curative and all have consequential off-target effects.

### 2.2.3.3 Systemic sclerosis treatment pathways

Modern management of SSc relies on detailed baseline assessment and follow-up tailored to the early identification of specific and important complications of SSc[74]. Immune-modulating therapies is used in accordance with the site of affected tissue or organ. Haematopoietic stem cell transplantation (HSCT) has evidence as a treatment option for interstitial lung disease or severe organ involvement which has failed to improve with conventional immunosuppressive agents. The treatment-related mortality for HSCT is a limiting factor in its use. Other agents include drugs to improve perfusion in digital vasculopathy, anti-reflux treatment, pulmonary hypertension therapies, and prokinetic, cyclical antibiotics and dietary adjustment in gastrointestinal disease.

### 2.2.4 The inflammatory myopathies

Inflammatory myopathies (IM) are a heterogeneous group of autoimmune disorders characterised by muscle inflammation and variable extra-muscular involvement of the skin, lung and joints. Distinct subsets are recognised including dermatomyositis (DM), immune-mediated necrotising myopathy (IMNM), inclusion-body myositis (IBM), overlap myositis (including the anti-synthetase syndrome), and polymyositis (PM). The
discovery of a wide range of autoantibodies has advanced the understanding and identification of these rare systemic autoimmune conditions.

2.2.4.1 Inflammatory myositis diagnosis and classification criteria

There are no diagnostic criteria for IM. The 1975 Bohan and Peter criteria are the most widely used classification criteria but were recently superseded by the 2017 EULAR/ACR classification criteria[85] for adult and juvenile IM[86, 87]. The Bohan and Peter criteria classified DM from PM as either ‘definite’, ‘probable’ or ‘possible’ and incorporated qualifying criteria from electromyography, biopsy and biochemistry investigations while importantly including certain exclusion criteria to eliminate IM mimickers (Table 7) [86, 87]. These criteria were revisited following the description of new clinical subsets of IM and deeper understanding of IM pathophysiology. The EULAR/ACR criteria for adult and juvenile IM published in 2017 (Table 8) aim to categorise IMs into the major subgroups using highly discriminatory clinical and laboratory criteria[88]. A calculated score based on the weighting of 16 variables presents the probability of an individual to have a particular subtype of IM. An online calculator for the 2017 EULAR/ACR criteria for adult and juvenile IM is available online [89].

There are differences in publications as to which auto-antibodies are to be included in myositis-specific autoantibody (MSA) and myositis-associated autoantibody (MAA) definitions, however MSAs are considered to be relatively specific autoantibodies for myositis, while MAAs may be associated with other or overlap forms of CTD[90]. Once the phenotype of myositis is confirmed, and organ involvement defined, the management is tailored towards the disease features and severity. This includes the cancer-associated subtype of IM which requires concurrent investigation for malignancy[91].

Table 7 1979 Bohan and Peter criteria for DM and PM[86, 87]

<table>
<thead>
<tr>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Symmetrical weakness, usually progressive, of the limb-girdle with or without dysphagia and respiratory muscle weakness</td>
</tr>
<tr>
<td>2. Muscle biopsy evidence of myositis: necrosis of type I and type II muscle fibres; phagocytosis, degeneration, and regeneration of myofibers with variation in myofiber size; endomysial, perimysial, perivascular, or interstitial mononuclear cells</td>
</tr>
<tr>
<td>3. Elevation of serum levels of muscle-associated enzymes</td>
</tr>
<tr>
<td>4. Electromyography triad of myopathy</td>
</tr>
<tr>
<td>a. Short, small, low-amplitude polyphasic motor unit potentials</td>
</tr>
<tr>
<td>b. Fibrillation potentials, even at rest</td>
</tr>
<tr>
<td>c. Bizarre, high-frequency repetitive discharges</td>
</tr>
<tr>
<td>5. Characteristic rashes of DM</td>
</tr>
<tr>
<td>- Definite PM: all first 4 elements, probable PM: 3 of first 4, possible PM: 2 of first 4.</td>
</tr>
<tr>
<td>- Definite DM: characteristic rash plus 3 others, probable DM: rash plus 2 others</td>
</tr>
<tr>
<td>- Possible DM: rash plus 1 other</td>
</tr>
</tbody>
</table>

Table 7. Classification criteria for IM.
Table 8 Components of the 2017 EULAR/ACR criteria for adult and juvenile IM[88]

<table>
<thead>
<tr>
<th>Variable</th>
<th>Score</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No muscle biopsy</td>
<td>With muscle biopsy</td>
</tr>
<tr>
<td>Age of onset of first symptom ≥ 18 and &lt; 40 years</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Age of onset of first symptom related to the disease ≥ 40 years</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Objective symmetric weakness, usually progressive, of the proximal upper extremities</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>Objective symmetric weakness, usually progressive, of the proximal lower extremities</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Neck flexors are relatively weaker than neck extensors</td>
<td>1.9</td>
<td>1.6</td>
</tr>
<tr>
<td>In the legs, proximal muscles are relatively weaker than distal muscles</td>
<td>0.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Heliotrope rash</td>
<td>3.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Gottron’s papules</td>
<td>2.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Gottron’s sign</td>
<td>3.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Dysphagia or oesophageal dysmotility</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Anti-histidyl-tRNA synthetase autoantibody present</td>
<td>3.9</td>
<td>3.8</td>
</tr>
<tr>
<td>Elevated serum levels of muscle-associated enzymes</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Endomysial infiltration of mononuclear cells surrounding, but not invading, myofibres</td>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td>Perimysial and/or perivascular infiltration of mononuclear cells</td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>Perifascicular atrophy</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Rimmed vacuoles</td>
<td></td>
<td>3.1</td>
</tr>
</tbody>
</table>

Table 8. EULAR/ACR Classification criteria of IM.

The heterogeneity of the inflammatory myopathies lends itself to a long and complex discussion of IM pathogenesis. As IM pathogenesis is not the focus of this thesis, this chapter will provide a concise description of pathogenic processes for each of the major subtypes of IM. Similar to other conditions on the CTD spectrum, a combination of genetic risk and environmental exposures is thought to trigger and potentiate the disease process through immunological and non-immunological processes[92]. With the exception of IBM, female gender appears to be a risk factor in reflection of the increase rates in women compared to men[93].

The pathognomonic skin features of DM include a violaceous periorbital rash (heliotrope rash) and erythematous lesions on the extensor surfaces of the joints (Gottron's papules)[94]. There is a form of DM called sine DM dermatitis that manifests with cutaneous involvement in the absence of muscle involvement. In classical DM, muscle enzymes are usually elevated and electromyography (EMG) demonstrates a myopathic pattern. A highly specific feature of DM is perifascicular atrophy on muscle biopsy (specificity >90%, sensitivity 25 - 50%). Perifascicular human myxovirus resistance protein 1 and retinoic acid-inducible gene 1 (sensitivity 71%, specificity 50%) can also be detected in addition to histological changes of cellular infiltrates consisting of pDCs, B cells, CD4 T cells, and macrophages in >80% of samples. The cellular infiltrate tends to aggregate around medium-sized blood vessels and the perimysium, which is a hallmark finding in DM. Approximately 70% of patients with DM will have a detectable dermatomyositis-specific autoantibody[92]. In the case of positivity for anti-
NXP2 or anti-transcription intermediary factor antibodies there is an associated increased risk of malignancy that manifests in approximately 20% of individuals within three years of diagnosis. Cancer-associated DM is proposed to have an off-target immune mechanism related to molecular mimicry[95]. In terms of risk factors in DM, certain class-2 HLA alleles and exposure to ultraviolet light have been implicated[94].

IMNM is a distinct subtype of IM characterised by proximal muscle weakness, extreme muscle enzyme concentrations, myopathic EMG findings, and muscle biopsies showing necrosis or regeneration with minimal lymphocytic infiltrate in the absence of perifascicular atrophy[94]. Typical histological changes include type 1 major histocompatibility complex upregulation, macrophage infiltration, and membrane attack complex deposition on non-necrotic muscle fibres. Around 60% of IMNM patients have autoantibodies recognising either signal recognition particle (SRP) or 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) in which case 20% of these cases are positive for lymphocytic infiltrates on muscle biopsy. Autoantibody-positive IMNM is clinically indistinguishable from autoantibody-negative IMNM with the exception that anti-HMGCR IMNM is associated with statin exposure, and anti-SRP IMNM tends to have more severe muscle weakness, higher rates of ILD (12-22% versus <5%) and tissue specimens showing a higher density of necrotic fibres. A cancer association exists for both anti-HMGCR myositis and antibody-negative myositis, unlike anti-SRP myositis. Regarding immunogenetic risk factors in IMNM, class-2 HLA-allele DRB1*08:03 is associated with anti-SRP myopathy, and DRB1*11:01 with anti-HMGCR myopathy. Given these observations, increased HMGCR production has been proposed to contribute to breaking tolerance for the development of HMGCR myopathy. Statins, which inhibit the enzymatic activity of HMGCR and increase the production of HMGCR, are proposed to induce myopathy through this mechanism or alternatively through the generation of a neo-epitope that triggers an immune response.

Similar to other types of inflammatory myopathies, the presentation of IBM is a combination of elevated muscle enzyme concentrations and myopathic EMG features[94]. Severe involvement of the anterior compartment of the thigh is a distinctive MRI characteristic. IBM is a unique disease however in that the proposed mechanistic process may not be autoimmune but rather a degenerative process. IBM differs to other myopathies in that it has a comparatively older age at onset (>50 years), slow progression over years (not weeks or months), an asymmetrical pattern of muscle involvement, and selective knee extensor and distal weakness especially of the deep finger flexors, wrist flexors, ankle dorsiflexors (compared to proximal involvement). IBM is not associated with any MSA although antibodies against cytosolic 5'-nucleotidase 1A (anti-NT5C1a) are present in 30 - 60% or patients in IBM compared with 5-10% of patients with PM, 15-20% of patients with DM, 10% of patients with SLE and 12% of patients with SS[96, 97]. The collective disposition however is
that immunosuppression does not benefit patients with IBM, compared to the other IMs[93]. The histological characteristics pathognomonic of IBM is co-existing inflammation, mitochondrial dysfunction and abnormal protein aggregation. The inflammatory infiltrate is dominated by CD8 T cells that surround and invade non-necrotic muscle fibres. There is an increased presence of ragged-red and cytochrome oxidase-negative muscle fibres, supporting that theory that mitochondrial damage has an important role in IBM. Other distinctive histological hallmarks include rimmed vacuoles, which remain unknown in aetiology, and cytoplasmic inclusions which are representative of amyloid protein and give rise to the name inclusion-body myositis.

Although not specific to IBM, anti-NT5C1a autoantibodies have been associated with increased severity and mortality in patients[98, 99]. It lends support to the idea of an autoimmune origin for IBM, together with the T cell infiltrate seen on histopathology. However the presence of cytoplasmic inclusions and the observation that immunosuppression is ineffective suggests an underlying degenerative process for which to decipher future investigations are necessary.

Overlap myositis is a subtype of inflammatory myopathy that represents the co-existence of myositis with another CTDs[93, 94]. Myositis may coincide with features of another autoimmune disease such as SLE, pSS or SSc and their associated autoantibodies. Anti-synthetase syndrome is the most representative form of overlap myositis and typically manifests with autoantibodies directed against aminoacyl tRNA synthetase enzymes. Patients with anti-synthetase syndrome usually present with one or more of the following features: inflammatory myopathy, ILD, arthritis, Raynaud syndrome, fever, or hyperkeratotic radial fingers lesions known as mechanics hands. Muscle enzyme levels and EMG findings are similar to DM. The muscle biopsies of these patients however reveal both perivascular atrophy and necrosis with nuclear actin aggregation, an electron microscopy feature, that is not seen in other IMs. However little is known about what triggers and maintains autoimmunity in anti-synthetase syndrome, and further research is warranted.

Polymyositis has the presence of muscle weakness, raised muscle enzymes, myopathic EMG findings, and CD8 T cell inflammatory infiltrates on muscle biopsy with none of the hallmark characteristics of the other inflammatory myopathies[94]. PM often remains a diagnosis of exclusion and surveillance for clinical features to suggest an alternative subtype of inflammatory myositis is often recommended.

2.2.4.2 Inflammatory myositis treatment pathways

The challenge of IM is identifying a suitable treatment. Supportive research for treatment selection is limited in light of the low prevalence and wide phenotypic heterogeneity of IM[94]. As previously mentioned, the literature does not support immunosuppressive treatment for IBM and a recent Cochrane review highlighted this in
addition to the scarcity of high-quality randomised controlled trials for the treatment of non-inclusion body myopathies. Leading treatment pathways currently follow expert consensus or case-series and historical clinical practice. Individualised physical exercise programs under the supervision of a physical therapist is supported by two randomised controlled trials as a way to improve strength and reduce disability. A summary of treatment modalities is provided in Table 9.

Table 9 Treatment options for inflammatory myopathies other than IBM[92]

<table>
<thead>
<tr>
<th>Immunosuppressant or immunomodulatory agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosteroids, Azathioprine, Methotrexate, Ciclosporin, Tacrolimus, Mycophenolate mofetil, Cyclophosphamide, Intravenous immunoglobulin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biological agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rituximab, Abatacept, Tocilizumab</td>
</tr>
</tbody>
</table>

Table 9. Treatment options in IMs other than IBM.

2.2.5 The non-traditional CTDs and CTD-variants

The categorisation of CTDs into SLE, SS, SSc and IM has the purpose of defining distinct disease entities and standardising CTD practice pathways. The classification systems of SLE, SS, SSc and IM differ in intent however, in that their purpose is to facilitate homogeneous and reproducible clinical research such that results are transferrable to a specific population. However, on occasion, the clinical features of a patient with systemic autoimmune rheumatic disease may be suggestive of a CTD, and warrant treatment along the pathway of a CTD, but defy categorisation or classification into any one defined entity. In this instance, the variation in presentation and evolution of the CTDs is apparent and highlighted are the short-comings of the current CTD classification and nomenclature systems. This chapter will discuss the non-traditional CTDs and CTD-variants including MCTD, OCTD and UCTD.

2.2.5.1 Mixed connective tissue disease diagnosis and classification

Mixed connective tissue disease (MCTD) is a term used to describe a subset of overlap CTD that is characterised by the presence of high-titre autoantibodies against ribonucleoproteins (anti-RNPs)[28]. The includes autoantibodies directed against the whole protein complex (U1-snRNP), the U1-RNA subcomponent, core Sm proteins, or U1-specific proteins (U1-70K, U1-A and U1-C). There is still no consensus regarding the disease definitions, the classification criteria, or the relationship of MCTD with other CTDs. Some authors argue that MCTD is a distinct disease entity, while others consider it to be an overlap syndrome or an early underdeveloped phase of a more distinct CTD, and few even still disregard the entire concept of MCTD[100].

There are no uniform guidelines for the definition of MCTD. The requirement for RNP autoantibodies in itself is contentious as the literature provides conflicting data on the relevance of the autoantibody. Anti-RNP antibody titres have been correlated with
activity in MCTD[101], including the regression of activity following treatment (p<0.046)[102]. The RNP autoantibody however is not exclusive to MCTD and may be detected in patients meeting criteria for SLE (20 - 40%), SSc (2 - 14%) and IM (6 - 9%). The original report of the concept of MCTD also lends itself to controversy. A review of the case series published in 1972 revealed inconsistencies in data reporting[101]. The anti-RNP antibody was absent in 3 of the 25 cases; excluded organ systems were evident and steroid requirements were high (and not low, as per definitions) in some cases; and prognosis could be seen to mirror SLE.

The clinical features of MCTD include pulmonary hypertension, interstitial lung disease, oesophageal dysmotility, arthritis, "puffy hands" (diffuse hand oedema), leucopoenia, myositis, serositis, glomerulonephritis and Raynaud phenomenon[100]. Some authors propose the minimal diagnostic features should include anti-RNP autoantibody in a patient presenting with Raynaud phenomenon and puffy hands in addition to at least two of the aforementioned organ or tissue-based inflammatory features[100]. Diagnosing MCTD in clinical practice is therefore often based on pattern recognition and a clinical decision that is rich with potential for differences of opinion and practice across rheumatology communities.

Four sets of classification criteria have been developed for MCTD which further complicate description of the disease entity. Shown in Table 10, the criteria by Sharp et al[103], Kasukawa et al[104], Alarcon-Segovia and Villareal[103], and Kahn and Appelboom[103] are distinctly different and do not capture the same patients according to Gunnarsson et al.[100]. Previous literature has reported the highest sensitivity and specificity in the Alarcon-Segovia and Villareal criteria[105] and the lowest in the Sharp et al criteria[103]. A longitudinal study of 161 MCTD patients over a mean period of 7.9 years shed interesting light on the criteria[105]. The highest sensitivity in criteria was rather found to be apparent for Kasukawa et al. (75%), followed by Alarcon-Segovia and Villareal (73%) and then Sharp (42%)[106]. On longitudinal analysis rates were found to decrease when comparing the meeting of at least one of the three classification criteria from the time of diagnosis to the time of study exit. In detail, the rates for Kasukawa et al were 75% vs 53%, Alarcon-Segovia and Villareal 73% vs 44%, and Sharp 42% vs 32%. Of those that evolved, the rate included 17.3% progression into SSc, 9.1% into SLE, 2.5% into RA and 11.5% into reclassified UCTD. The rate of evolution was seen highest in patients with disease durations >5 years than 0 - 5 years. This suggests that MCTD may misclassify an early, in development CTD. Although this conclusion markedly contrasts another longitudinal study of 280 MCTD patients for which new symptoms were observed however the classification remained unchanged[107]. The conflicting results only add to the uncertainty as to whether MCTD has a place in CTD nomenclature.
Table 10 Overview of the items included in the four published MCTD criteria sets[103, 105]

<table>
<thead>
<tr>
<th>Items</th>
<th>Sharp</th>
<th>Kasukawa et al.</th>
<th>Alarcon-Segovia and Villareal</th>
<th>Kahn and Appelboom</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunological</strong></td>
<td>anti-U1RNP</td>
<td>anti-RNP</td>
<td>anti-RNP</td>
<td>anti-RNP</td>
</tr>
<tr>
<td><strong>Raynaud phenomenon</strong></td>
<td>-one of four major criteria</td>
<td>-one of two obligatory criteria</td>
<td>-one of five clinical criteria</td>
<td>-obligatory criteria</td>
</tr>
<tr>
<td><strong>Swollen/puffy hands</strong></td>
<td>-one of four major criteria</td>
<td>-one of two obligatory criteria</td>
<td>-one of five clinical criteria</td>
<td>-one of three clinical criteria</td>
</tr>
<tr>
<td><strong>Joint</strong></td>
<td>Arthritis</td>
<td>Polyarthritis</td>
<td>Synovitis</td>
<td>Synovitis</td>
</tr>
<tr>
<td><strong>Muscle</strong></td>
<td>Myositis (mild)</td>
<td>≥1 of muscle weakness, elevated CK, or myogenic EMG -disease category (2/3 needed)</td>
<td>Myositis (laboratory or biopsy proven) -one of five clinical criteria</td>
<td>Myositis -one of three clinical criteria</td>
</tr>
<tr>
<td><strong>Peripheral sclerosis</strong></td>
<td>Sclerodactyly</td>
<td>Sclerodactyly</td>
<td>Acrosclerosis (+/- scleroderma) -one of five clinical criteria</td>
<td>Not included</td>
</tr>
<tr>
<td><strong>Lung</strong></td>
<td>DLCO &lt;70%, PAH or lung biopsy with proliferative vascular lesions -one of four major criteria</td>
<td>Pulmonary fibrosis, vital capacity &lt;80% or DLCO &lt;70% -one of three SSc-like findings</td>
<td>Not included</td>
<td>Not included</td>
</tr>
<tr>
<td><strong>Oesophageal disease</strong></td>
<td>Hypomotility</td>
<td>Hypomotility or dilatation -one of three SSc-like findings</td>
<td>Not included</td>
<td>Not included</td>
</tr>
<tr>
<td><strong>Serositis</strong></td>
<td>Pleuritis or pericarditis -two of 11 minor criteria</td>
<td>Pericarditis or pleuritis -one of five SLE-like findings</td>
<td>Not included</td>
<td>Not included</td>
</tr>
<tr>
<td><strong>Haematology</strong></td>
<td>Leukopaenia, anaemia, or thrombocytopaenia -three of 11 minor criteria</td>
<td>Leukopaenia or thrombocytopaenia -one of five SLE-like findings</td>
<td>Not included</td>
<td>Not included</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>Alopecia, trigeminal, neuropathy, malar rash, or history of swollen hands -four of 11 minor criteria</td>
<td>Lymphadenopathy, or facial erythema -two of five SLE-like criteria</td>
<td>Not included</td>
<td>Not included</td>
</tr>
</tbody>
</table>
2.2.5.2 Mixed connective tissue disease pathogenesis

The proposed mechanisms of MCTD centres on a pathogenic role for anti-RNP antibodies. The autoantibody can bind to endothelial cells and cause endothelial cell activation and damage leading to vascular dysfunction[108]. The antibodies can also aggregate to form immune complexes and activate complement similar to other antibody-related diseases. B cell and plasma cell activity has been correlated with disease activity in MCTD[102]. A potential role for CD4+ IL-10+ regulatory T-cells and epitope spreading through B and T cell interactions and apoptosis-induced modifications has also been proposed[109].

The strongest evidence in support of MCTD as a distinct disease entity is the identification of unique HLA profiles in patients with MCTD that are distinctly different from the HLA profile of ethnically-matched healthy controls and the profile of SLE, SSc and IM[100]. There are, however, no functional data to explain how the HLA*B08 and DRB1*04:01 alleles may contribute to the formation of anti-RNP antibodies or MCTD pathogenesis. The generic mechanism of CTD development where a genetically “at risk” individual is exposed to a triggering environmental stimulus leading to the initiation of CTD is considered to apply to MCTD, however data to identify an environmental risk factor is yet to published.

2.2.5.3 Mixed connective tissue disease treatment pathways

No randomised controlled trials of therapies for MCTD have been performed. Therapeutic selection is directed by the clinical manifestations and evidence base and experience from the treatment of other CTDs. The use of biological disease modifying anti-rheumatic in MCTD is based on case reports of the successful use of Rituximab. The initial case reports on TNF-alpha inhibitors indicated severe adverse effects but anecdotal expert recommendations suggest it is an option in treatment-resistant arthritis[100].

2.2.6 Overlap CTD (OCTD)

The OCTDs have been identified as entities that satisfy the classification criteria of at least two CTDs and can occur either concurrently or sequentially in the same patient[110]. There is no standardised definition or classification criteria has been accepted for OCTD. Some authors argue that the presence of at least one CTD if coincident with another autoimmune rheumatic disease is sufficient for the diagnosis. SLE, SSc, IM and SS are generally accepted in definitions for OCTD. The confusion for SS relates to the redundancy in nomenclature given that sSS is established as a co-existent disease in CTD. The same applies for MCTD and the
anti-synthetase syndrome which by nature are syndromic partnerships of CTD features. Certain autoantibody profiles lend towards OCTD (Table 11). Rhupus, a clinical condition in which in the same patient clinical signs and symptoms of both SLE and RA occur, is sometimes labelled as an overlap CTD. Whether RA is a true CTD and eligible under the umbrella of overlap CTD is a matter of debate.

Table 11 Proposed classification of OCTDs(110)

<table>
<thead>
<tr>
<th>Associated with specific autoantibody profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCTD (anti-RNP)</td>
</tr>
<tr>
<td>Anti-synthetase syndrome (anti-tRNA synthetase)</td>
</tr>
<tr>
<td>PM and SSc (anti-PM/ScI)</td>
</tr>
<tr>
<td>SLE and sSS (anti-La/SSB)</td>
</tr>
<tr>
<td>Not associated with specific autoantibody profile</td>
</tr>
<tr>
<td>Rhupus syndrome; SSc/SS; SSc/RA; SLE/SSc; RA/SS; IM/SS</td>
</tr>
</tbody>
</table>

Table 11. Proposed classification criteria of OCTDs.

The identification of OCTD is useful to clarify the disease prognosis and facilitate management. By the influence of genetic and environmental factors, it is well known that autoimmune diseases tend to associated with each other. The therapeutic options in the overlap syndromes are as for the individual diseases and usually lead by specific organ features.

2.2.7 Undifferentiated connective tissue disease

Undifferentiated connective tissue disease (UCTD) is a term used to refer to an unclassifiable CTD which shares the clinical and serological manifestations of CTD but which does not fulfil any existing classification criteria[111]. It is a unique clinical entity and a potential forerunner of well-established CTD. Approximately 50% of patients presenting with CTD will have an unclassifiable profile at presentation[112]. The expectation is that a proportion of these patients will remain unclassifiable, others will regress into remission, and a further still will evolve into a definable CTD. The concept of UCTD replaces assumptive terminology such as “incomplete”, “atypical” or “evolving” which fails to acknowledge transient disease cases and may lead the clinician towards misclassification and overtreatment[113]. It is well recognised that early diagnosis, prognostic profiling, and the timely initiation of therapeutic intervention are critical steps in the management of CTDs. The idea of UCTD as an entity is a means to facilitate these steps while avoiding misplaced management and enabling a period of observation.

There are no standardised classification criteria or definitions for UCTD. Proposed criteria with short duration case definitions (i.e. less than 1 year) present the risk of
including transient diseases or diseases that are evolving into definite conditions, while longer case definitions fail to capture the UCTD population. There is no agreement on the accepted minimum symptom duration for case entry into UCTD and still no consensus on how best to identify patients with UCTD after disease onset. Mosca et al. in 1999 proposed a preliminary set of classification criteria for the identification of UCTD. This included (1) signs and symptoms suggestive of a CTD but not sufficient to meet the criteria of a defined CTD; (2) positive ANA; and (3) a disease duration of at least 3 years[114]. It was suggested that the term ‘early UCTD’ could be adopted to refer to presentations with a disease duration of <3 years. The criticism of the criteria is that while they allowed the exclusion of transitory conditions and early forms of developing CTD, the criteria were restrictive in that they excluded early UCTD at its onset and incomplete or overlap forms of CTD[115]. The Mosca group clarified that the criteria should be applied to define stable UCTD and offered to improve specificity with the introduction of exclusion criteria listing features specific for defined CTD[116] (Table 12). However the Mosca classification criteria have not been subject to validation and remain without wide acceptance within the rheumatology community.

**Table 12 Proposed exclusion criteria for UCTD(114)**

<table>
<thead>
<tr>
<th>Clinical criteria</th>
<th>Laboratory criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malar rash</td>
<td>Anti-dsDNA</td>
</tr>
<tr>
<td>Subacute cutaneous lupus</td>
<td>Anti-Sm</td>
</tr>
<tr>
<td>Discoid lupus</td>
<td>Anti-protein P</td>
</tr>
<tr>
<td>Cutaneous sclerosis</td>
<td>Anti-Scl70</td>
</tr>
<tr>
<td>Heliotrope rash</td>
<td>Anti-centromere</td>
</tr>
<tr>
<td>Gottron papules</td>
<td>Anti-La/SSB</td>
</tr>
<tr>
<td>Erosive arthritis</td>
<td>Anti-Jo1 or Anti-Mi2</td>
</tr>
</tbody>
</table>

Table 12. Proposed exclusion criteria for UCTD.

In terms of disease characteristics, stable UCTD is generally considered to be a benign disease which consequently requires only mild therapeutic intervention. It has a favourable prognosis and there is usually an absence of internal organ involvement. Life-threatening conditions and severe organ manifestations (such as renal or neurological disease) have been reported however these are not considered common within the disease spectrum[117, 118]. The manifestations of UCTD are described with wide variation in the literature (as would be expected) however most descriptions list Raynaud phenomenon, arthralgia/arthritis, skin rashes, photosensitivity, fatigue, sicca and mild cytopaenia as common features of the disease[118, 119]. Disease-specific ANA autoantibodies are less common and if
present are more likely to signify a developing definite CTD. Agreement exists on the fact that the majority of UCTD patients (80-99%) are female, with a mean age at disease onset ranging from 32 to 44 years[112]. The non-progression rate among UCTD cohorts appears to be approximately 30%, even in observational studies with ten year follow-up periods[114, 120-122]. The late evolution of UCTD into definite disease has been described however this is rare within the literature[112]. In those that progress, the highest rate of evolution into a definable disease is seen in the first and second year after the onset of symptoms. In UCTD cohorts with five year follow-up periods, approximately 34% of patients develop a well-defined rheumatic disease and 12.3% regress into complete remission[118]. In one study the diagnoses of the progressed cases were RA in 13.3%, pSS in 6.8%, SLE in 4.2%, SSc in 2.8%, MCTD in 4%, systemic vasculitis in 3.3% and IM in 0.5%; however, a range of autoimmune rheumatic conditions that are both within and not within the CTD spectrum have been reported by groups. This finding of diagnostic migration to another class of autoimmune rheumatic disease from the CTD spectrum has been reported in other cohorts and highlights the limitation of diagnostic imprecision in the UCTD concept[123].

Several predictive factors for the evolution of UCTD into definite CTD have been identified. This offers insight into the features that may secure a definitive diagnosis and guides management and prognostication. Reports have been contrasting among the different cohorts however, and some studies have found negative results on analyses for prognostic factors however this could be a reflection of narrow cohort selection and short follow-up duration[112, 124]. The strongest predictors of evolution into a definitive CTD are high and multiple ANA autoantibody specificities and their accrual over time, as well as the presentation of symptoms and signs or laboratory abnormalities that are unique to a definitive CTD (i.e. Gottron’s papules, anti-Scl70 antibodies, puffy fingers). Evolution into SLE has been more specifically predicted by anti-dsDNA, Farr assay dsDNA antibody detection, Coomb’s test positivity, a positive test for syphilis, leucopaenia, African-American ethnicity, alopecia, discoid lupus, serositis, homogenous ANA, and antibody specificity for anti-Sm[125-131] (Table 13). The latter four factors were shown in a multivariate analysis to be independent predictive factors for SLE progression[125]. In terms of SSc, Danieli et al in 1998 in univariate analysis reported significant predictive factors in SSc to include sicca, Raynaud phenomenon, sclerodactyly, oesophageal dysfunction and ANA nucleolar pattern[126]. In the same study the prediction of SS was significant for Raynaud phenomenon, xerostomia, and anti-Ro/SSA. New pathological nailfold capillaroscopy pattern compared to baseline was also shown to be predictive of progression to defined CTD (p=0.01) and the retention of CTD
compared to remission (p=0.03)[132]. One group reported that “haemosiderin deposition/microhaemorrhages” (OR=8.32) and “elongated capillaries” (OR = 12.16) were independent variables especially predictive of progression to SLE (p<0.05).

Table 13 Predictors of evolution of UCTD to define CTD

<table>
<thead>
<tr>
<th>Disease</th>
<th>Predictive factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>High avidity anti-dsDNA detected on Farr assay[130]</td>
</tr>
<tr>
<td>SLE</td>
<td>Age, African-American ethnicity, alopecia, serositis, discoid lupus, Coomb's test, anti-dsDNA, anti-Sm, ANA (homo)[125]</td>
</tr>
<tr>
<td>SLE</td>
<td>Leukopaenia, anti-dsDNA[128]</td>
</tr>
<tr>
<td>SLE</td>
<td>Anticardiolipin antibodies and multiple antibody specificities[129]</td>
</tr>
<tr>
<td>SLE</td>
<td>Age, fever, serositis, photosensitivity, ANA (homo) &amp; anti-dsDNA[118]</td>
</tr>
<tr>
<td>SLE</td>
<td>Accumulation of autoantibodies[131]</td>
</tr>
<tr>
<td>SLE</td>
<td>Malar rash, oral ulcerations, anti-dsDNA (Farr assay), low C4[127]</td>
</tr>
<tr>
<td>SLE</td>
<td>Fever and anti-dsDNA antibodies[126]</td>
</tr>
<tr>
<td>SSc</td>
<td>Sicca, Raynaud phenomenon, sclerodactyly, oesophageal dysfunction, ANA nucleolar pattern[126]</td>
</tr>
<tr>
<td>SS</td>
<td>Raynaud phenomenon, xerostomia, anti-Ro/SSA[126]</td>
</tr>
</tbody>
</table>

Table 13. Predictors of evolution of UCTD to CTD. Homo, homogenous.

Triggering factors for UCTD and the evolution of UCTD into definite CTDs have limited evidence in the literature. Epidemiological reports indicate a significant association between Vitamin D deficiency and UCTD, as well as lower levels of Vitamin D in those who progress to well-defined CTD[133, 134]. In a study on the immune effects of Vitamin D insufficiency in patients with UCTD, supplementation with Vitamin D was reported to have the effect of reversing IL-17 expression and dampening T regulatory cell inhibition[135]. A dose-response study on the effects of Vitamin D supplementation on IL-17 and Th17 imbalance in UCTD patients with severe Vitamin D deficiency (<30 ng/ml) reported a dose threshold of 1.0 micrograms/day over five weeks as the optimal therapeutic regime[136]. This data requires validation with a larger cohort however before their clinical incorporation.

Implanted medical devices including silicone-containing implants and the non-silicone-containing devices of artificial joints and orthopaedic metallic fixation devices were significantly associated with UCTD (OR 2.81, OR 5.01 and OR 1.95 respectively) in a large case-control cohort of women[137]. In a small case-control study on obstetric patients with UCTD, pregnancy was associated with an increased risk of flare in UCTD and progression to a well-defined CTD[138].

The clinical course of UCTD is usually stable and with a favourable long-term prognosis. However, a recent study by Ludici M et al. showed that UCTD patients experienced an impaired quality of life in both functional and mental domains, similarly to SSc patients[10]. In this study, glucocorticoid exposure was significantly associated with improvements in physical and mental impairment (p < 0.001 and p =
0.043 respectively), although response to DMARDs was only observed for the mental domain ($p = 0.037$). In terms of obstetric morbidity, adverse pregnancy outcomes are reported within UCTD cohorts at an approximate rate of 20 - 40%, similar to conventional CTDs[138, 139]. Increased rates of low birthweight, spontaneous miscarriage, neonatal heart conduction disturbance, neonatal lupus, pre-eclampsia and medical intervention are reported across several observational and case-control cohort studies[139]. This data enriches the disease descriptions of UCTD and highlights the importance of quality of life and obstetric impact considerations.

The literature suggests that careful management of UCTD patients is reasonable, but not always simple. Consideration of the severity of symptoms, stage of disease, the potential for organ damage, intervening factors for aggravation, and potential responses or side effects to therapeutic intervention must be weighed[113]. Management paradigms draw from the evidence base of the CTD spectrum. It is generally accepted that hydroxychloroquine is the mainstay of treatment in UCTD, however that a proportion of patients may require stronger immunosuppression and a further still no treatment at all[119]. There is low-level evidence to support the detection and supplementation of Vitamin D as a preventative strategy in CTD evolution[133].

2.3 The pathogenesis of CTD: pre-clinical to definite CTD

2.3.1 Introduction

The developmental model of CTD is underpinned by a progressive process of autoimmune dysfunction that begins with an initiating event in a genetically pre-disposed individual, to an asymptomatic pre-clinical phase, and then to early and established CTD[140] (Figure 3). The process is generally considered to be long, over several years[141]. Numerous genetic and environmental risk factors for CTD have been identified, and many of these are proposed to act as initiators or propagators of immune dysfunction before the clinical appearance of tissue injury. The preclinical phase of CTD is characterised by abnormalities in disease-related biomarkers including autoantibodies, cytokines and immune cell numbers and function. It represents a window of opportunity in which biomarkers to predict the risk of development of autoimmune disease and the introduction of preventative strategies may bear influence on outcomes. This chapter discusses the theoretical model of development of the CTDs and includes discussion of genetic and environmental factors as well as the preclinical stage of disease.
2.3.2 Genetics in the development of CTD

Established and emerging data demonstrate that the autoimmune process in CTD is a continuum that starts with genetic risk and progresses through a series of environmental interactions to phases of preclinical and clinical CTD (Figure 3)[140, 141]. There is a strong genetic component in the pathogenesis of the CTDs; however, genetic risk alone is not enough to manifest the systemic rheumatic autoimmune diseases. Established and emerging data demonstrate that the autoimmune process in CTD is a continuum that starts with genetic risk and progresses through a series of environmental interactions to phases of preclinical and clinical CTD[140, 141]. There is a strong genetic component in the pathogenesis of the CTDs; however, genetic risk alone is not enough to manifest the systemic rheumatic autoimmune diseases.

2.3.2.1 Twin concordance

Family and twin studies on genetic risk where available have shown moderate to low concordance rates throughout the spectrum of CTDs. The heritability of SLE has been estimated at 66% with rates of concordance between 24% to 56% in monozygotic twins and 2% to 4% in dizygotic twins[142, 143]. The overall concordance in SSc in monozygotic and dizygotic twins has been reported at 4.7%
and the familial risk in first degree relatives is estimated to be 1%. Familial clustering of SS has been reported but the level of evidence is low and limited to case studies[144]. The inflammatory myopathies are a group of rare diseases and genetic risk studies reveal both HLA and non-HLA associations however studies on familial risk have not been published[145].

The discordance between monozygotic twins in among the CTDs reinforces the idea that genetics alone is not responsible for the development of systemic autoimmune rheumatic disease. Many additional factors are likely to contribute including environmental exposures and random somatic events occurring in early embryonic development such as genomic imprinting or X chromosome inactivation that is recognised to result in phenotypic differences[143].

### 2.3.3 The pre-clinical period: autoantibodies are detectable in SLE, SS, SSc and IM

The period before clinical disease where abnormalities in immune function is detectable however signs and symptoms of tissue injury are absent has been defined as the 'preclinical' period[140]. In this stage of disease, genetic and environmental risk factors are thought to interact to initiate and propagate immune dysfunction which eventually evolves to a state of detectable tissue inflammation and damage[146, 147]. Increasing data support the existence of the preclinical phase across the range of CTDs which can be identified using biomarkers of autoimmunity and inflammation.

#### 2.3.3.1 Preclinical SLE

Autoantibodies that characterise SLE are detectable in the serum of patients years before the onset of symptoms [148, 149]. A large prospective study of United States military personnel by Arbuckle et al demonstrated a high prevalence of preclinical ANA positivity at a titre of ≥1:120 in 78% of individuals with SLE at a mean of approximately 3 years before the clinical diagnosis. This compared with 0% ANA positivity at this level in matched healthy controls[131]. A remarkable observation in this study was the finding that the earliest sample was ANA positive in many cases, suggesting that the duration of preclinical autoimmunity exceeded the 3 year duration reported in this study. Indeed, SLE-associated autoantibodies have been reported >9 years prior to the diagnosis of classifiable SLE[150]. Arbuckle et al also showed that autoantibody specificities for ANA may materialise at different time points prior to the onset of SLE[131]. Autoantibodies against dsDNA, Smith, and RNP antigens were found to appear closer to the onset of SLE compared to autoantibodies against anti-phospholipid, anti-Ro/SSA and anti-La/SSB. The former
three autoantibodies were detected an average of 1 - 2 years prior to the onset of symptoms of SLE, compared with 2 - 3 years for ANA, anti-phospholipid, and anti-Ro/SSA and anti-La/SSB autoantibodies[150]. Of all the autoantibodies, the anti-RNP antibody demonstrated the shortest interval between positive testing and the onset of SLE symptoms and diagnosis, with duration intervals of 0.20±0.47 years and 0.88±0.32 years respectively. Compared with positive ANA testing, this interval was shorter by approximately 2 years and 2.5 years respectively.

The evidence for evolving immune dysregulation in SLE has been further supported by Arbuckle et al through the finding of autoantibody specificity accrual in the lead up to SLE[131]. Arbuckle et al demonstrated an increase in the average number of autoantibody specificities prior to diagnosis, from 1.47 per person six years before diagnosis, to 2.58 at the onset of symptoms, and 3.01 at the time of diagnosis of SLE. The number of autoantibody specificities detected at SLE diagnosis appeared to remain static at 5 years. This suggests that SLE tends to arise in asymptomatic persons with accumulating positive immunologic tests, and that immunosuppression may halt the evolving immunodysregulation.

Similar results have been reported by Eriksson et al who found that antibodies to Ro/SSA, RNP, histone, La, and dsDNA and ANA were detectable in 63% (n = 38) of patients with SLE approximately 4.2 years before the onset of the disease[150]. In this study, anti-Ro was detected first in the serum of these patients, 6.6 years before the onset of symptoms and 8.1 years before the diagnosis of SLE.

### 2.3.3.2 Preclinical SSc

A preclinical stage of autoimmunity has also been suggested for SSc. In this disease, the two most common autoantibody specificities are the anti-centromere (ACA) and the anti-topoisomerase I (anti-Scl70 Ab) which are highly specific for the diagnosis of SSc and are rarely found in healthy people[151]. The approximate sensitivity of these autoantibody subtypes is 30% and specificity is 99%[151]. It is known that these autoantibodies can precede the clinical signs of SSc in years. Raynaud phenomenon is usually the first manifestation of the disease and may antedate the onset of definite SSc by years[152]. The triad of Raynaud, SSc-specific autoantibodies, and SSc-specific nailfold capillaroscopic changes, even in the absence of other signs of definite SSc, identifies a subset of individuals at highest risk of progression to SSc, referred to as early SSc[153, 154]. Prospective studies of early SSc have reported a mean time interval of 4.6 ± 4.5 years from diagnosis of early SSc to evolution to classifiable SSc[153]. On multivariate analysis, Trapiella-Martinez et al in a prospective study of early SSc identified digestive involvement as
an independent risk factor for the progression of early SSc to definite SSc with an OR of 17.0 (95% CI 6.1 - 47.2, p <0.001)[155].

2.3.3.3 Preclinical SS

The pre-clinical stage of SS is well-recognised. Anti-Ro and Anti-La autoantibodies are commonly found in the circulation of patients prior to SS. In addition, these antibodies appear in the serum of mothers who have given birth to babies with congenital heart block or neonatal lupus[148]. Most of these women are healthy however a proportion of mothers have been observed to go on to develop SS or SLE, in some cases several years after delivery[156, 157]. A small prospective study of women (n = 23) detected to be Ro or La autoantibody positive during pregnancy reported that two patients developed SS after a pre-clinical period of 5 - 9 years[148]. In another cohort of pregnant women, almost all participants were shown to develop SS during a 9-year follow-up period (mean 4.5 years)[156]. Other longitudinal population studies have reported evidence of a preclinical phase in up to 66% of patients with SS, based on the presence of ANA, Rheumatoid Factor (RF), anti-La or anti-Ro antibodies approximately 5 years before the onset of SS, and in some individuals up to 18 years before diagnosis of the condition[158]. This finding was confirmed in a more recent cohort study (n = 117) of pre-clinical SS where autoantibodies were detectable up to 20 years (median 4.3 - 5.1 years) before the diagnosis of SS in 81% of patients, and for some cases possibly earlier, since the earliest sample analysed was positive[159]. The most common autoantibodies were ANAs followed by RF, anti-Ro60/SSA, anti-Ro52/SSA, and anti-La/SSB. Anti-Ro60/SSA and anti-Ro52/SSA were found to have the highest positive predictive values of SS development, at 25% and 100% respectively.

2.3.3.4 Preclinical IM

The rarity and heterogeneity of the IMs makes investigation of the pre-clinical period of the IMs difficult to establish. Pre-clinical reports on the prevalence of asymptomatic myositis-specific and myositis-associated autoantibodies are lacking for this group of conditions. From a genetic perspective, there are few reports on the familiar occurrence of these diseases, hence the heritability of the inflammatory myopathies is unknown[92, 160, 161]. Higher prevalence of autoimmune rheumatic disease, such as SLE, autoimmune thyroid disease and type 1 diabetes mellitus have been reported in patients with IM, as well as in first-degree relatives of both adult and juvenile patients with IM[162, 163]. This aggregation of autoimmunity supports the idea that a shared genetic and/or environmental factor is responsible for the development of the condition. Infectious agents, physical exertion,
malignancy, bovine collagen implants and smoking have all been identified as possible environmental risk factors for IM, however the quality of evidence is low and based on small cohorts or case-control studies[92, 164]. Myositis-specific and myositis-associated autoantibodies however do show clear associations with distinct clinical phenotypes of IM, and disease prognosis, and serum titres have previously been shown to correlate with disease activity which suggests a direct pathogenic role of the autoantibodies[165]. MSAs and MAAs furthermore are highly specific and rarely are detectable in healthy subjects. In one small study, the reported range of specificity for IM for the MSA/MMA antibody panel was 76 - 100%, with the highest specificity found for anti-Jo-1, anti-PL-7, anti-PL-12, and anti-PM/Scl[166]. This compares with a detection rate of MSA/MAAs in 4% of healthy sex- and aged-matched subjects (n = 2/50) and 39% of similarly-matched disease controls who represented various rheumatic and muscle disorders (n = 70). Importantly, in this study, the control group were mostly positive for the Ro/SS-A antibody which is recognised for its low specificity across the spectrum of autoimmune rheumatic diseases. This data collectively suggests a direct pathogenic role for the MSA/MMA autoantibodies and a probable short period of preclinical autoimmunity.

2.3.4 The risk of benign autoimmunity

As discussed earlier in this chapter, ANAs are detectable in a considerable proportion of the general population, and despite an association with an increased risk of CTD, are on their own insufficient to lead to the development of autoimmune rheumatic disease[167-169]. The overall prevalence of ANA in the general population is estimated at 13.8% (95% CI 12.2 - 15.5%) and rises in prevalence with older age, female gender, chronic infections and chronic medical morbidities[170]. Healthy relatives of individuals with autoimmune rheumatic disease are also recognised to have high rates of asymptomatic autoantibody positivity, which supports the idea of a shared environmental and genetic origin in the development of immune aberrancies[171]. The prevalence of systemic autoimmune rheumatic disease in the community however is lower in comparison at approximately 0.1%, and rises to 1% in women over the age of 45 years[172]. The overall odds of development of autoimmune rheumatic disease in an individual with antinuclear autoantibodies is estimated between 10- to 30-fold[150]. The discrepancy between autoantibody positivity and the onset of autoimmune rheumatic disease suggests that immune dysfunction is an evolving process and that environmental and genetic interactions play an important role.

Some authors consider the state of asymptomatic ANA detection to be indicative of early autoimmunity or “preclinical disease”, and have used the term “benign
autoimmunity” to describe this stage of early immune dysfunction[173]. During this stage, genetic and environmental risk factors are assumed to interact to initiate and propagate the development of autoimmunity, resulting in detectable tissue inflammation and injury in select individuals. Autoantibodies are markers for autoreactive B cells and plasma cells. Autoantibodies have the ability to form immune complexes and/or participate in auto-antigen presentation which is the initial phase of innate and adaptive immune system activation and autoimmune disease. An unaddressed question in the concept of benign autoimmunity however is when and how does it become pathogenic? Recent data suggests that benign autoimmunity in itself is a risk factor in cardiovascular disease (CVD) development[173].

2.3.4.1 Preclinical autoimmunity: a risk factor for cardiovascular disease?

Individuals with systemic autoimmune rheumatic disease have a higher risk of cardiovascular disease. This is well-established in RA where the risk is 1.5 to 2 fold and comparable with the risk in type II diabetes mellitus[174, 175]. RA-related autoantibody positivity has been associated with an increased prevalence of CVD events in patients with RA, and antibodies to anti-cyclic citrullinated peptide have been correlated with carotid intima media thickness in early RA[176-178].

In SLE, CVD is the leading cause for mortality[179]. The spectrum of anti-phospholipid antibodies have been correlated with an increased risk of cardiovascular events and subclinical atherosclerosis in patients with SLE. In one large US prospective study, the prevalence of MI in lupus anticoagulant-positive SLE patients was reported at 22% compared with 9% (p=0.04) in non-positive patients[180]. In 182 Swedish patients with SLE, the presence of either the anti-cardiolipin or anti-beta2 glycoprotein antibody was strongly associated with an increased risk of first CVD event (HR 4.9, 95% CI 1.76 - 17.72)[181].This finding was confirmed in a follow-up study of 208 patients in the same inclusion cohort over 12 years, where the auto-antibodies were predictive of cardiovascular mortality (HR 2.8, 95% CI 1.1 - 1.7)[182]. Levels of anti-cardiolipin antibody and anti-beta2 glycoprotein have been correlated with myocardial perfusion defects in SLE patients, as detected by single-photon emission computed tomography, Interestingly, the defects were not within the distribution of the major coronary arteries, suggesting that the auto-antibodies may contribute to microvascular thrombi in the cardiac microcirculation[183]. Recently, An increased risk of coronary artery calcification, a marker of overall atherosclerotic burden, was reported in patients with SLE and antiphospholipid antibodies in two separate studies[184, 185]. In contrast, anti-Smith, anti-dsDNA, and anti-RNP have shown positive correlations
with cardiovascular disease in SLE, however did not reach statistical significance[181].

An increased rate of CVD events has been reported in patients once considered to have had benign autoimmunity to suggest a pathogenic role for the auto-antibodies beyond clinical autoimmune rheumatic disease. This has been demonstrated in several large pre-clinical RA studies where both RF positivity and anti-CCP positivity have been associated with an increased risk of ischaemic heart disease and CVD mortality[186-190]. There is a growing number of studies to demonstrate the same association in pre-clinical CTDs. Positivity for ANA without autoimmune rheumatic disease has been associated with increased rates of triple-vessel coronary artery disease (OR 11.67, 95% CI 3.91 - 31.82)[191], and increased risk of myocardial infarction (HR 1.29 95% CI 1.03 - 1.61)[187]. Several studies have demonstrated a clear association between pre-clinical antiphospholipid antibodies and increased cardiovascular events[192-199]. The autoantibodies have been detected in the serum and plaques of pre-clinical cases, and have shown positive correlations with coronary intima media thickness and acute myocardial infarction[194, 200, 201]. Although one pre-clinical study drew a negative association between antiphospholipid antibodies and coronary arterial calcification, the association has been reported in subgroup analysis of the CARDIA (Coronary artery risk development in young adults) study[196]. Antiphospholipid antibodies were associated with subclinical coronary arterial sclerosis in African-American and white young adults after 15 years follow-up, with anti-β2GP1 IgG OR 6.4 (95% CI 2.4–16.8); anti-β2GP1 IgA: OR 5.6 (95% CI 2.3–13.2), anti-β2GPI IgM OR 1.7 (95% CI 1.0–3.1), and aCL IgG OR 5.1 (95% CI 1.4–18.6)[190].

Associations between autoantibodies and subclinical and clinical atherosclerosis in individuals with and without rheumatic disease suggests a model in which pre-clinical autoantibodies are not only a risk factor for rheumatic disease, but also for CVD, which may even develop in parallel with the condition. The proposed mechanisms are considered to be subclinical inflammation and non-thrombotic functions of antiphospholipid antibodies such as aberrant oxidisation of lipoprotein molecules and triggering of foam cell formation[202, 203]. There is a paucity of published data on this issue in preclinical SS, SSc and IM, making it an area of research need.

### 2.3.5 Environmental factors in preclinical autoimmunity

Genetic and environmental factors are thought to interact to progress the pre-clinical state of autoimmunity to a state of overt tissue damage and connective tissue disease. The prior subsection discussed the evidence for genetic contributions to
this process. This subsection will discuss the evidence for environmental factors in the instigation and progression of the preclinical state. Most of the known environmental factors have been identified through case control and cohort studies and there are no data that quantify the relative contributions of these environmental risk factors to disease development[204]. The environmental risk factors will be discussed according to the subset of preclinical disease.

2.3.5.1 Environmental factors in preclinical SLE

Various environmental factors have been implicated in the induction and acceleration of SLE. New theory in the model of autoimmune development in SLE holds that the first hit or initial break in tolerance may originate at the epithelial surface[147]. A strong body of evidence is available for RA for this process, which is beyond the remittance of discussion in this thesis, however, the evidence is growing in the CTDs. In RA, the mucosal surfaces have been identified as the primary initiating site, namely the lung, oral mucosa, and gut mucosa. In contrast, in the CTDs, the skin is postulated to be a primary initiating site.

Ultraviolet (UV) light is an established trigger of SLE and SLE activity. Experimental studies have shown a significant immunomodulatory role for UV radiation and evidence of induction of SSA/Ro60 and anti-dsDNA autoantibodies[205-211]. UV light in proposed models of SLE is postulated to induce apoptosis and immune interaction of keratinocytes in the skin[208, 212]. Apoptotic keratinocytes express nuclear material in apoptotic blebs which have the ability to enter the circulation as microparticles. Circulating particles of nuclear material are thought to stimulate the expression of autoantibodies against ANA and activate innate and adaptive autoimmunity[147]. The site and sequence of events in this model however have not been subject to prospective study. It is supported by observations that cutaneous disease is one of the most common features of SLE[213]. In the skin of lupus patients photo-provocation by UV light has been shown to increase numbers of apoptotic keratinocytes compared to healthy controls[208]. Keratinocytes activated by UV light have also been shown to produce damage-associated molecular patterns (DAMPs) and inflammatory mediators that lead to the recruitment of lymphocytes and antigen-presenting cells, including peripheral dendritic cells.

The gastrointestinal interface has been implicated in the pathogenesis of CTD. The relationship is still not well-characterised. Two small studies have demonstrated a low Firmicutes/Bacteriodes ratio in patients with SLE compared with healthy controls[214, 215]. In murine lupus models Zhang et al 2014 reported marked depletion of Lactobacilli and an increase in Lachnospiraceae and Clostridiaceae
during the phase of lupus progression[216]. Dietary introduction of Retinoic acid (Vitamin A) restored the down-regulated Lactobacilli, and this correlated within an improvement in disease control[216]. Lactobacillus reuteri supplementation in the diets of mouse models of SLE has also been shown to prevent progression to lupus[216]. Levels of T regulatory cells were observed to be impaired within the pre-supplementation model, and it was postulated that administration of Lactobacilli was protective due modification of the gut microbiota composition that favoured T regulatory cell induction. A decrease in regulatory T cells suggests a skewing in inflammatory and regulatory immune mechanisms which promotes disease development[217]. While the data supports a cause-and-effect model between the gut microbiota and SLE, more work is needed to fully understand the connection. Future research efforts should include therapeutic studies in human subjects.

Dietary and nutritional factors have been proposed to play a role in the development of SLE[218-220]. Vitamin D deficiency has been associated with autoreactive immune abnormalities in healthy individuals, and the onset of SLE[221]. This is supported by epidemiological studies which identify high rates of Vitamin D deficiency in cohorts of undifferentiated CTD and SLE[221-228]. Alcohol consumption has been correlated with an increased risk of SLE in genetically susceptible individuals[229]. As a subject of controversy, alcohol has been shown to have a protective effect when used in moderation[230]. There have been no studies to date that correlate how alcohol affects the gut microbiota in models of autoimmune disease.

Cigarette smoking and environmental air pollutants including the use of marijuana has also been shown to be a risk factor in the onset of SLE[229-237]. The biological effects of cigarette smoke are thought to arise from effects on HLA-DR3 alleles[238]. Silica as well as industrial pollution and solvents have also shown this association[239-244].

Several viruses have been proposed as factors that influence the development and progression of pre-clinical lupus[245-247]. EBV has been shown to induce the formation of ANA autoantibodies[248-250]. EBV and other viral antigens are thought to promote the generation of an initial autoimmune response and antibodies against nuclear antigens like SSA/Ro60[251], SmD1[252] and SmB0/B[250] through molecular mimicry[253, 254] and innate immune activation, which is then amplified through epitope spreading and positive feedback systems[251, 255]. Cytomegalovirus (CMV), retroviruses, human T-lymphocytic virus type 1, human herpesvirus-7, herpes simplex virus 2, hepatitis C, BK virus and parvovirus have also been implicated in the onset of SLE[140, 245-247, 256].
On the converse, some infectious agents have been reported to be protective against the development of SLE and arrest progression towards severe forms of the disease. This may be related to the ‘hygiene hypothesis’ and could explain the greater incidence of autoimmune disease in developed countries where higher hygiene standards and systems are in operation[257]. *Helicobacter pylori* was associated with a lower risk of early onset SLE in African Americans[258]. *Hepatitis B virus* was identified as a protective factor in Chinese SLE patients who were found to have a lower prevalence compared to healthy controls[257]. In murine models of SLE, a decreased disease severity was observed in mice infected with *Toxoplasma gondii*[259], *Plasmodium chabaudi*[260], and *lactate dehydrogenase elevating virus*[261].

Medications have an established connection with the induction of CTD[262]. Although the evidence is limited to case reports and cohort series, drug-induced forms of SLE, SS, SSc and IM have been recognised and may be managed through withdrawal of the offending drug. Environmental factors in preclinical SS

Infectious agents, particularly viruses, have been considered to be involved in the priming or triggering of SS[263]. It is thought that viruses promote autoantibody production through a process of molecular mimicry that leads to epitope spreading. EBV, CMV, chronic Hepatitis C, human T-cell leukaemia virus, and coxsackieviruses are commonly implicated in the pathogenesis on pSS, based on reports of viral antigen detection in the saliva or glandular biopsies of humans and animal models[264-268]. Re-activation of EBV and CMV has been suggested in the induction and maintenance of the disease[267, 269]. Defective viral clearance from salivary gland epithelial cells is postulated to lead to viral persistence, chronic lymphocytic sialadenitis and subsequent glandular dysfunction[270, 271]. Infection of C57BL/6-lpr/lpr mice with murine CMV resulted in sialadenitis which persisted after clearance of the virus and was associated with high levels of anti-Ro, anti-La, RF and dsDNA autoantibodies[272]. A different group reported similar outcomes with CMV-infected NZM2338 mice[273].

Foreign antigen immunisation with components of the Ro/La particle has been reported by several groups to trigger the onset of SS in murine models[274, 275], with reports of epitope spreading. An illness similar to SS was reported in BALB/c mice immunised with short peptides from the sequence of the 60 kD Ro antigen[276]. The mice were observed to develop salivary gland lymphocytic infiltrates and salivary gland dysfunction. The investigators applied the same protocol in different mouse strains and observed differences in the development of SS. Development of the disease was interrupted at different stages, ranging from
immune response to the peptide, epitopes spreading, systemic autoimmunity and lymphocytic infiltration of the salivary glands, to dysfunction of the gland. This study highlights the role of genetics in the overall pathway of disease pathogenesis, suggesting genetic control in immune reactivity, epitope spreading, and disease manifestations[277].

Cigarette smoking has been demonstrated to be a risk factor for pSS-associated ILD (OR 12.84, 95% CI 1.71 - 96.53)[278]. A large cross-sectional multicentre study conducted identified that the risk of pSS was significantly associated with a high cumulative occupation exposure to toluene (OR 4.69, 95% CI 1.42–15.45), white spirit (OR 3.30, 95% CI 1.07–10.26), aromatic solvents (OR 2.50, 95% CI 1.06–5.91) and any types of solvents (OR 2.25, 95% CI 1.20–4.22)[279].

Oestrogen deficiency has been correlated with the development of pSS, and may explain the predominance of the disease in women. Aromatase gene inactivated mice modelled for oestrogen deficiency were reported to develop autoimmune disease resembling SS[280]. In another study, retinoblastoma-associated protein 48 transgenic mice were found to develop glandular dysfunction only in conditions of deficiency in oestrogen[281]. Human cohort studies are conflicting on the association of sex hormones and SS[282]. The data at present is circumstantial and more needs to be undertaken before a clear association can be made.

2.3.5.2 Environmental factors in preclinical SSc

A spectrum of inciting stimuli have been associated with the onset of SSc[283]. Chemical agents have been the most cited environmental factors in SSc development, and includes silica, solvents, silicone breast implants, epoxy resins, welding fumes, pesticides and hair dyes[284]. Several occupations are recognised to be at higher risk of SSc due to higher contact intensity[284]. A scleroderma-like disorder has been described following exposure to bleomycin, a chemotherapeutic agent often administer in cancer[285]. In rat and mice models, infiltration of bleomycin into the lungs was observed to lead to the development of fibrosis in a dose-dependent manner[286-288]. Many other drugs have been associated with the onset of SSc[283]. Moreover, several reports have described the occurrence of pregnancy-related SSc. It is postulated that foetal cells enter the maternal circulation through placental transfer and induce a graft-versus-host reaction with likeness to SSc in the mother[289, 290]. This is theory is supported by reports of the detection of foetal DNA and cells in the peripheral blood and skin biopsies of women with SSc, in some cases years following the puerperium[289, 291, 292]. However robust evidence of a mechanistic role of foetal-to maternal antigenic transfer in the
pathogenesis of SSc is lacking, and the theory would not explain the occurrence of SSc in men or nulliparous women.

2.3.5.3 Environmental factors in preclinical IM

Alike other autoimmune diseases, the environmental risk factors of IM have been identified from animal models, case reports and/or case series; and seem to vary between phenotypes of IM[92]. Associations have been made with several viral[293-300], bacterial and parasitic infections[301], UV radiation[302, 303], smoking[92, 304], collagen and silicone implants[305, 306], birth date[307], seasonal variations[307, 308], occupation exposures to gases, dust and fumes[309], and an expansive list of chemical and drug agents and dietary supplements[92]. Case reports and animal models have identified several specific possible infectious triggers of IM. These include hepatitis B virus (DM[294], PM[293]), hepatitis C virus (IBM[295]); retroviruses such as HIV and human T-lymphotrophic virus-1 (PM[296], DM[297], IBM[298, 299]), influenza, picornavirus, echovirus (DM, PM), and Toxoplasma and Borrelia spp[300, 301] (DM, PM). A multitude of drugs have been reported as possible causative agents, including D-penicillamine, an older style for RA drug now rarely used[310], and statins, a drug often associated with necrotising myopathy[311] and the induction of anti-HMGCR antibodies[312], however reported to initiate the onset of DM and PM[311]. Systemic and localised forms of IM have been associated with vaccination based on animal studies and case reports[313]. This has plausibility given that the risk of IM seems to be increased after any infection (OR 1.5, 95% CI 1.2 - 1.9), especially gastrointestinal (OR 1.9, 95% CI 1.1 - 3.5) and lower respiratory tract infections (OR 2.3, 95% CI 1.8 - 3.3), with the exception of DM/PM where it appears to be decreased following upper respiratory infections according to epidemiological studies[314]. One epidemiological study however reported no association between vaccines and PM and DM[315]. In contrast to the aetiological theory of infectious initiation of IM, parvovirus appeared to be protective against the occurrence of juvenile DM (OR 0.35, 95% CI 0.14 - 0.9).

The epidemiological literature identifies an increased risk of DM and PM after excessive physical exertion (OR 3.9, 95% CI 1.8 - 8.2)[315]; tobacco is a risk factor for anti-Jo1-related IM (OR 3.94, 95% CI 1.54 - 9.89)[316]; bovine collagen dental implants (OR 5.05, 95% CI 2.31 - 9.59) are linked with DM[305]; and group A streptococcal infections have been associated with juvenile DM (OR 2.73, 95% CI 1.14 - 6.53)[317]. Maternal exposure to air pollution, smoking, and occupational dusts and solvents have been raised in a small study to have potential associations with juvenile DM[304].
2.3.5.4 Defining nomenclature in preclinical CTD

There is a growing effort to develop methodical terminology to describe the various stages of the pre-classification period of CTD[140, 318, 319]. Authors have motioned for terminology that can distinguish individuals at every stage of development within the CTD continuum. This includes individuals with increased genetic and environmental risk (high-risk for CTD), pre-clinical autoimmunity and immune dysfunction (pre-clinical CTD), early forms of CTD (incomplete CTD), fully developed but non-classifiable CTD (unclassifiable CTD), and classifiable CTD. Terminology is further required to distinguish between individuals who are high-risk versus low-risk for disease progression during the pre-clinical period. Some terms introduced into the literature are misleading due to lack of broad descriptive characteristics. Terms such as “pre-clinical SLE” are misleading since many individuals with features of SLE do not go on to develop lupus[320]. Other terms such as for example “latent SLE”, “probable SS”, “evolving IM” and “incomplete SSc” raise similar bias and confusion, and are increasingly disregarded in favour of more neutral terms such as UCTD or “at risk” of CTD. These terms are broad enough to describe the disease manifestations suggestive of CTD however do not commit to a definitive diagnosis. This is particularly important given that the first three years of autoimmune disease are often marked by transient or developing disease features.

2.3.6 Problems with ANA testing

ANA positivity is common in the general population, and while the actual frequency of positive assays varies with methodology, about 20% of individuals in epidemiology studies express borderline levels, corresponding to immunofluorescence titres of 1:40 titres or greater[169, 321, 322]. This figure decreases to 5% at significantly elevated titres of 1:160 and 3.3% at 1:320[323, 324]. The basis of this seropositivity is unclear. ANAs are present in nearly all cases of diagnosed disease of CTD. However ANA positivity is neither specific nor prognostic, because most individuals with these antibodies will never develop an autoimmune disease. Indeed the dense fine speckled pattern of nuclear fluorescence (anti-DFS), which corresponds to autoantibodies targeting the dense fine speckled 70kDa protein, has been reported in approximately 9% of healthy individuals and has been proposed as a marker against the presence of autoimmunity[325]. However the autoantibody has been detected in up to 3% of patients with SLE and in patients with Hashimoto’s thyroiditis. This raises doubts regarding the reliability of anti-DFS as a negative of biomarker ANA-associated autoimmune disease, or autoreactivity in general, given the evidence of association with definite autoimmune disease.
Epidemiological studies profile ANA positivity at titres of 1:40 or greater to be most prevalent within the female gender[326]. Associations between the presence of ANA and age are less well described, however are highest in those aged 40-49 years, at least according to one study[170]. Other studies have reported increasing prevalence of ANA expression with age (p = 0.01), with the highest levels seen in individuals aged >70 years[327]. However other studies have shown no correlation between ANA positivity and age[328].

Ethnic associations with ANA expression have also been examined in epidemiological studies. Higher prevalence has been reported in the non-Hispanic black population[170] and in African American[169] individuals compared with other ethnic groups. As higher rates of SLE are found in these population, this relationship is suggestive of a link between the development of autoantibodies in health and the development of CTD[329]. The same link applies to the associations observed for the female gender, where SLE constitutes 80 - 90% of patients with SLE[330].

It is possible that these results represent limitations of ANA assays and the detection of either low titre or low avidity autoantibodies which may never cause autoimmunity[322]. Alternatively, it may represent cross-reactivity with other non-nuclear antigens[322]. This is becoming less of an issue due to the increasing use of automated solid phase multiplex assays, which are less sensitive but generate more false-negative results[322, 331]. It is beyond the scope of this thesis however for this reason the immunofluorescence Hep-2 (human epithelial cells) platform has been recommended by an ACR taskforce as the preferred ANA screening method over direct assays[332].

Another explanation for the frequency in ANA expression in the general population may relate to intrinsic derangements in immune function[322]. ANA positivity in healthy individuals may represent the beginnings of subclinical autoimmunity, as previously discussed in Chapter 2.3.3, although this could be debated, given that one study showed greater lupus-related gene upregulation in some healthy individuals who were ANA negative than in those who were ANA positive [322, 328]. Further work is needed to identify the determinants of disease progression in ANA-related rheumatic diseases, and the relationship between gene expression, serology and disease[322]. This would be particularly valuable to the effort put forward to reduce unnecessary ANA testing and relieve healthcare costs[333].

2.3.7 Biomarkers in CTD

Biomarker research is an area of increasing interest in CTD. A biomarker is defined as a measurement whose alterations correlate with the pathogenesis and/or
manifestations of a disease and can be evaluated through quantitative or qualitative methods in laboratories[19]. It can include, but not have limitation to, a genetic, biological, biochemical, molecular or imaging event. Biomarkers can be prognostic, diagnostic, predictive, pharmacodynamic and surrogate[19]. Prognostic biomarkers identify a specific disease feature, individuals at risk of a disease, or those likely to experience future disease activity[19]. Diagnostic biomarkers confirm the presence or subtype of a disease. Predictive biomarkers are used to anticipate therapeutic response[19]. Pharmacodynamic biomarkers guide therapeutic drug dosing. Surrogate biomarkers are substitutes for clinical end points[19].

Many biomarkers have been identified for CTD however no single biomarker has emerged as surrogate for disease activity or the prediction of disease[18, 19, 334]. Biomarkers have included cell surface proteins, autoantibodies, cytokines, and protein components of the immune system[19, 20]. Double-stranded DNA and complement are routine clinical diagnostic and predictive biomarkers for SLE and compromise components of disease activity indices[19]. Elevation in dsDNA autoantibodies correlate with the onset, activity and impending activity of SLE and LN[18, 19]. Low complement system C1q, C3 and C4 has shown similar correlative ability in terms of SLE activity and flare prediction[19]. Other rheumatological diseases, malignancies, infections and endocrine disorders however can influence either biomarker and sometimes the biomarker is not featured and/or a reactive component of an individual’s lupus disease[19]. Although in routine use, dsDNA autoantibodies and complement are not universal features of lupus and so their application is limited within the lupus population[19].

Emerging biomarkers in lupus are established on cell signalling pathways and involve cytokines, chemokines, growth factors and acute phase reactants[19]. These include serum IFN-alpha, BLyS/BAFF, APRIL, TNF-alpha, IL-6, IL-12, IL-21, IL-23, IL-1, IL-17, TGF-beta, urinary TWEAK, Axl, Fas, ferritin, insulin-like growth factor binding protein 2 (IGFBP-2), insulin-like growth factor binding protein 4 (IGFBP-4), sialic acid-binding Ig-like lectin 5 (siglec-5), anti-mutated citrullinated vimentin antibody (anti-MCV), erythrocyte-bound C4d (E-C4d), B cell bound C4d (B-C4d), Vascular cell adhesion molecule-1, monocyte chemoattractant protein-1 (MCP-1) and sTNFRII[18, 19, 334]. BLyS (also known as B cell activating factor or BAFF) is a B cell growth and survival promoter and has been correlated with SLE disease activity, serum immunoglobulin and dsDNA levels. Manifestations of discoid rash, renal disease, serositis and lymphopaenia have been associated with elevated levels of BLyS however the biomarker failed in longitudinal studies to correlate with disease flare[19, 20]. Despite molecular similarities to BLyS, a proliferation-Inducing ligand (APRIL) negatively correlated with lupus disease activity and anti-dsDNA
levels[18, 19]. APRIL also showed no significant inability to discriminate between pre-flare and non-flaring SLE[18, 19].

TNF-alpha, a cytokine with B cell regulation and T cell stimulation properties, has traditional associations with drug-induced lupus and dsDNA antibody induction when blockaded by TNF-alpha inhibitors[18, 19]. Conversely, high serum TNF-alpha has been shown to correlate with flares and activity in lupus subjects. IL-6, a pro-inflammatory cytokine, has been demonstrated to correlate with lupus diagnosis, activity and impending activity[18, 19]. Raised serum IL-12 and IL-23 cytokines have correlated with SLE diagnosis and higher levels have been demonstrated in pre-flare SLE compared to no-flare SLE[18, 19]. Serum IL-23 was also associated with proliferative LN and renal lupus activity[18, 19]. Serum IL-1 is higher in SLE patients and active SLE patients compared to healthy controls[18, 19].

TGF-beta is a fibrotic cytokine involved in wound healing and angiogenesis[18, 19]. Elevated urinary TGF-beta mRNA levels were higher in diffuse proliferative LN and reduced in LN patients responsive to therapy[18, 19]. IL-21 is a pro-inflammatory cytokine that influences the generation of autoantibody-secreting plasma cells. Increased IL-21-producing peripheral CD4+ T cells in SLE correlate with a concurrent increase in memory B cells and Th17 cells and reduced Treg cells[18, 19]. IL-17 is a pro-inflammatory Th cellular pathway cytokine and has been correlated with disease activity in non-renal and renal SLE patients and pre-flare SLE verses no-flare SLE[18, 19].

IGFBP-2 showed higher levels in LN, and correlation with disease activity and clinical and histological response[18, 19]. Axl, sTNFRII, ferritin and IGFBP-2 strongly correlated with active SLE compared to inactive SLE and healthy controls[18, 19]. IGFBP-4, a biomarker of diabetic nephropathy, was shown to be detectable at increased levels in LN patients compared with non-lupus renal disease and healthy controls[18, 19].

TNF-like weak inducer of apoptosis (TWEAK) is a member of the TNF family capable of inducing IL secretion, apoptosis and cell differentiation[18, 19]. Urinary TWEAK correlated with disease activity in LN. MCP-1 is a recruiter of monocytes, memory T lymphocytes and NK cells to inflammatory sites. LN patients compared with patients without LN and HC showed higher levels of serum MCP-1 and urinary MCP-1 which correlated with flares, urine protein and response to treatment[18, 19]. Neutrophil gelatinase-associated lipocalcin (NGAL) is released from following renal injury and inflammation[18, 19]. Levels are higher in SLE patients with LN than in SLE patients without LN and are correlated with disease activity. Urinary neutrophil gelatinase-associated lipocalcin levels correlated with renal flares if elevated on a
proceeding visit. Vascular cell adhesion molecule-1 is central to immune cell recruitment into tissues. Increased levels correlate with SLE disease activity and active LN compared with non-renal and inactive SLE[18, 19].

Similarly, for SSc, SS and IM clinical and research biomarkers are under increasing attention. ANA autoantibody specificities are central to clinical practice and are prognostic and diagnostic biomarkers in their own right for each of the CTDs[90, 111, 149, 204, 332]. In SSc, there are a wide range of candidate biomarkers under evaluation, including circulating miRNA, proteins derived from collagen and the extracellular matrix, markers of angiogenesis and end-organ damage, and many cytokines, chemokines and growth factors[326]. Scleroderma-affected skin has been subject to biomarker exploration using gene expression and cytokine profiling[27, 74, 153, 155, 335]. In SS, a more restricted range of biomarkers are under investigation in serum, saliva and tears[56, 60, 64, 72, 159, 326]. A particular focus has been on markers of B cell function and activity in SS given their central role in disease pathogenesis[56, 59, 62-64, 66, 72, 158, 273, 281, 336]. In DM, PM and IBM candidate biomarkers include microRNA, chemokine, cell subset and cytokine assays of the serum, muscle, lung and skin[88, 90, 91, 96, 161, 164, 165, 312, 316, 326, 337].

The emerging list of biomarkers have been identified through cross-sectional studies and/or small patient cohorts and require validation in larger cohorts before their clinical role is established[18, 19]. Given the heterogeneous nature of CTD, it is not unexpected for some of these biomarkers to yield conflicting results or fail to fulfil their potential within validation studies[18, 19]. For example, in SLE, drug studies have identified a dichotomy between patients in terms of IFN status (i.e. high/low), which may influence response to IFN-blocking therapy[338, 339]. This concept has led some biomarker researchers to propose the development of composite biomarker panels to overcome this issue of immune dysfunction heterogeneity within and between CTD subsets[18, 19]. To date no validated biomarker panel however has gained widespread use in clinical settings, which is probably a reflection of their cost and processing demands[18, 19]. Interferon however has gained increasing interest as a biomarker in CTD and will be discussed further in the next subheading[20].

2.3.8 Interferon

Interferons (IFNs) are a group of intercellular signalling proteins that have antiviral, immunomodulatory and anti-tumour properties [340, 341]. There are three subtypes of interferons: type I, type II and type III IFN[342]. The classification of IFN is based on differences in receptor binding, molecular structure and source of cellular
production[342]. The type I IFN (IFN-α) family includes IFN-α, IFN-β, IFN-ε, IFN-κ
and IFN-ω and bind to a single receptor, INF-α receptor (IFNAR)[343]. Type II and
type III IFN includes IFN-γ and IFN-λ respectively[342].

Although many types of cells can produce IFN, the primary source of IFN-I appears
to be the pDCs[22, 344]. pDCs uptake immune complexes containing DNA/RNA
through the Fc-γ receptor IIA and in turn express type I IFNs through the activation
of intracellular nucleic acid-sensing TLRs[345]. Dysregulation of the IFN-I pathway
has a well-established role in the pathogenesis and activity of multiple autoimmune
diseases and is of increased interest due to evidence that it may define clinical
phenotypes as well as the potential to respond to IFN-blocking therapy[20, 340].

A central pathogenic role for IFN-I has been established across the entire CTD
spectrum. In lupus, serum IFN-I assays have been correlated with disease flares
and serological and clinical features[20]. This includes neuropsychiatric disease,
histological severity in glomerulonephritis, fever, rash, arthralgia and
leukopenia[346-351]. Smaller studies have reported correlations between IFN
expression, disease flares and severe lupus features (e.g. internal organ
involvement)[352]. A recent prospective study determined that IFN assays were
predictive of progression to CTD in a preclinical cohort[25]. Besides SLE,
upregulation of IFN-I has been detected in the peripheral blood cells of patients with
pSS, DM, PM, RA, MCTD and SSc and positively correlated with disease activity in
DM, PM, RA, pSS and SSc [335, 336, 353-357]. This includes lung fibrosis, digital
ulcers and digital loss in SSc [358]. Myositis in DM and PM with concordant
decrease in IFN expression following immunomodulatory therapy and disease
regression[337]. Complement and hypergammaglobulinaemia (but not other
ESSDAI domains) in pSS[357]. Over-expression of IFN-I has also been observed in the
skin and synovial tissue of patients with lupus, labial salivary biopsies of patients
with pSS, muscle of patients with IM, skin of patients with scleroderma, and
positively correlated with peripheral IFN blood levels [22, 354, 358-366]. Positive
correlations have also been made with anti-dsDNA and ENA antibodies,
hypocomplementaemia and elevated serum BAFF[344].

A major barrier in the characterisation of IFN-I activity is the reliability of current IFN
measurement systems [19]. IFN molecules measured directly in serum or plasma
can be intermittent due to localised expression and uptake, or very low and hard to
detect[19]. IFN-stimulated gene expression (ISGs) (known as IFN signatures) and
weighted IFN-inducible gene scoring systems (known as IFN scores) are alternative
methods of determining IFN expression [343]. IFN signatures represent the
detection of pre-determined sets of ISGs and yield an output of “high” or “low”
expression (qualitative)[24]. IFN scores refer to a continuous parameter of ISG transcriptional activity as derived from quantitative PCR detection[20].

While IFN-I signatures and scores have been shown to correlate with disease features and flare risk in many cross-sectional studies, results are inconsistent and have received negative validation in longitudinal studies[367-369]. Given over 100 genes are induced during type I IFN pathway activation, differences in ISG selection and weighing are likely to account for the performance differences among the IFN signatures and scores[368]. Contribution of type-II or type III IFN and other non-IFN cytokines to the IFN-I signature; ethnicity, the pathogenic heterogeneity of lupus, and the limitations of data-reduction in gene selection are additional confounders in these results[25]. Gene expression may also vary between circulating cell populations such that assays that use whole blood or unsorted PBMCs may show apparent differences in level of ISG expression[25]. Changes in the size of cell populations may also influence results; for example, leucopaenia as is characteristic of autoimmune disease and when transient during autoimmune activity[25].

This complex relationship may be addressed through the alternative investigation of non-gene-based surrogate markers of IFN-I, such as interferon-induced proteins (e.g. IFIT4) and chemokines (e.g. CCL2, CXCL10)[370, 371]. Tetherin, an interferon-induced anti-viral membrane protein, has a physiological role in inhibiting enveloped viral particle release from the surface of infected cells[372]. Associations have been shown between serum flow cytometric tetherin and lupus severity and activity[373]. As a flow cytometric biomarker tetherin has convenience in terms of negating the need for complex RNA extraction and analysis.

Given its role in disease pathogenesis, blockade of IFN-I has the potential to become a treatment option for autoimmune rheumatic disease. However studies on the neutralisation of IFN-α (sifamumab, rontalizumab) or blockade of the IFNAR1 receptor (anifrolumab) have had mixed results in phase II clinical trials[20]. In a phase IIb study, sifalimumab met its primary end point of a reduction in global disease activity score in patients with SLE. Efficacy was reported in the high IFN signature group but not the low IFN signature group, which may be a reflection of IFN expression or related to cohort size[339]. Surprisingly, rontalizumab in a post-hoc analysis showed superior response in patients with a low baseline IFN signature[374]. This may be explained by the lower serological activity in the IFN low signature group, which suggests a milder disease cohort, or the higher trough concentrations of rontalizumab which may be cause for the difference[374]. Anifrolumab, an IFNAR1 monoclonal antibody antagonist, reduced global disease activity in a phase II trial in moderate-to-severe SLE[338]. The IFN high signature
group showed superior response which was clearly associated with increased anifrolumab concentrations[338]. Preliminary results from a recent phase III trial on anifrolumab failed to meet the primary end point of reduction in disease activity in patients with SLE, as measured by the SLE Responder Index 4 (SR14)[375]. Specific organ responses and subgroup analysis of the IFN high signature subgroup is yet to be published[375].

IFN Score A and IFN Score B are validated continuous 2-score systems for the measurement of IFN status[24]. The systems were developed using Factor Analysis of 31 ISGs as expressed within sorted PBMCs from SLE, RA and Healthy controls[24]. Score A represents a weighted composite expression score as derived from 12 co-clustering ISGs (ISG15, IFI44, IFI27, CXCL10, RSAD2, IFIT1, IFI44L, CCL8, XAF1, GBP1, IRF7, CEACAM1)[24]. Score B represents a weighted composite expression score from 14 co-clustering ISGs (LAMP3, IFIH1, PHF11, SERPING1, IFI16, BST2, SP100, NT5C3B, SOCS1, TRIM38, UNC93B1, UBE2L6, STAT1, TAP1)[24]. Score A and Score B differ between PBMC cell subsets, highlighting the importance of cell selection[24, 25]. There is a comparable distribution in range between SLE and healthy controls, but with marginal yet statistically higher values observable in SLE[24]. It is thought that the system may have greater discrimination in characterisation of IFN status over bimodal (i.e. high/low) assays[24]. Score A and Score B have been shown to correlate with lupus serology and features of disease activity (BILAG domains) in adjusted models[24].

In a small retrospective analysis Score A was significantly associated with lupus flares and Score B with severe lupus features (e.g. internal organ involvement)[352]. Score A and Score B have been shown to be predictive of progression to autoimmune connective tissue disease in an at risk cohort[25]. Memory B cell tetherin (tetherin) was shown to be predictive of disease severity and future activity in both preclinical and established SLE cohorts[352, 373].
Chapter 3  General methodology

3.1  Introduction

This chapter outlines the methodology that underpins the IFN assays and patient-reported outcome measures described in this manuscript.

3.2  IFN Score A and IFN Score B

A two-score system of ISGs was briefly described in Chapter 2.3.8. IFN Score A and IFN Score B was calculated without knowledge of the participant’s clinical status.

3.2.1  Gene probe selection and gene expression

Ten genes were selected from each IFN-annotated module (M1.2, M3.4, M5.12) of a previous microarray study as reported by Chiche and colleagues (2014), with addition of other common IFN ISGs[25, 376]. This summated to a panel of 31 ISGs. The reference gene peptidyl prolyl isomerase A (PPIA) was selected due to non-response to IFN-I.

Using density gradient method (LymphoprepTM, Alere Technologies, Norway), peripheral blood mononuclear cells (PBMCs) were separated ethylenediaminetetraacetic acid (EDTA) anticoagulated blood. Total ribonucleic acid (RNA) was extracted from PBMCs and sorted cell subsets using the total ribonucleic acid purification kit (Norgen Biotek, Canada). Fluidigm® Reverse Transcription Master Mix buffer was mixed with random primers and oligo dT for priming to obtain the complementary DNA (cDNA) synthesis from total RNA acquired. TaqMan assays (Applied Biosystems, Invitrogen) were used to perform the quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) for the selected 31 ISGs. The instruments used were the BioMark™ HD System with appropriate cycling protocols for the 96.96 chip. Data were normalised to the reference gene PPIA to calculate ∆Ct.

3.2.2  Factor analysis

Factor analysis (FA) was performed to reduce the 31 ISGs into a smaller number of factors[25]. Proceeding this, undetected ∆Ct values were singly imputed using the R package non-detects. The Kaiser-Meyer-Olkin measure was incorporated for verification of the sampling adequacy of the analysis. Principle factor extraction (without rotation) was used to determine the optimum number of factors, which was firstly calculated according to a parallel analysis (Monte Carlo simulation using 1000 replications). This indicated the maximum number of factors however if a smaller number was required to explain >80% of the variance and resulted in lower levels of
cross loading (genes loaded by ≥2 factors at >0.4), the selection was then of a simpler structure. Following identification of the number of factors present, the final factor solution was determined from oblique (promax; kappa = 4) rotation.

This study calculated the factor scores for each participant using median gene expression loaded at ≥0.4 by each factor provided that there was no greater cross load than one factor. The strength of this approach is in its reflection of the variability of the data and the avoidance of compromise of the within-participant ordinal scaling of ∆Ct values.

As described in the literature, 84% if the variance with limited cross-loading could be explained by two factors among the ISGs [24]. The ISGs that contributed to each factor are shown in the table (Table 14) and are distinguished by the names IFN Score A (12 co-clustered genes) and IFN Score B (14 co-clustered genes).

**Table 14 IFN Score A and IFN Score B**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Modules from previous study using microarray</th>
<th>Rotated Factor Loading</th>
<th>Factor 1: IFN-I Score A</th>
<th>Factor 2: IFN-I Score B</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISG15</td>
<td>1.2</td>
<td>0.96*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFI44</td>
<td>1.2</td>
<td>0.80*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFI27</td>
<td>N/A</td>
<td>0.77*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL10</td>
<td>1.2</td>
<td>0.71*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSAD2</td>
<td>1.2</td>
<td>0.70*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFIT1</td>
<td>1.2</td>
<td>0.67*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFI44L</td>
<td>1.2</td>
<td>0.66*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL8</td>
<td>3.4</td>
<td>0.58*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XAF1</td>
<td>1.2</td>
<td>0.54*</td>
<td></td>
<td></td>
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<tr>
<td>IFI6</td>
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<td>0.51</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>GBP1</td>
<td>3.4</td>
<td>0.46*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF7</td>
<td>3.4</td>
<td>0.46*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEACAM1</td>
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<td>0.45*</td>
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<td>HERC5</td>
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<td>0.43</td>
<td>0.59</td>
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<tr>
<td>EIF2AK2</td>
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<td>0.42</td>
<td>0.64</td>
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<td>MX1</td>
<td>1.2</td>
<td>0.40</td>
<td>0.56</td>
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</tr>
<tr>
<td>LAMP3</td>
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<td>0.40*</td>
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<td></td>
</tr>
<tr>
<td>IFIH1</td>
<td>3.4</td>
<td>0.45*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHF11</td>
<td>5.12</td>
<td>0.58*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERPING1</td>
<td>1.2</td>
<td>0.60*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFI16</td>
<td>5.12</td>
<td>0.64*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BST2</td>
<td>5.12</td>
<td>0.74*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SP100</td>
<td>5.12</td>
<td>0.74*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT5C3B</td>
<td>5.12</td>
<td>0.80*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gene</td>
<td>Score</td>
<td>P-Value</td>
<td></td>
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<tr>
<td>----------</td>
<td>-------</td>
<td>---------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOCS1</td>
<td>3.4</td>
<td>0.84*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRIM38</td>
<td>5.12</td>
<td>0.87*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNC93B1</td>
<td>5.12</td>
<td>0.88*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UBE2L6</td>
<td>3.4</td>
<td>0.89*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STAT1</td>
<td>3.4</td>
<td>0.94*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAP1</td>
<td>5.12</td>
<td>&lt;0.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CASP1</td>
<td>5.12</td>
<td>&lt;0.40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 14. *Indicates genes included in the factor scores

3.3 Memory B cell tetherin

3.3.1 Introduction

Memory B cell tetherin was briefly introduced in Chapter 2.3.8. It is a flow cytometric membrane bound protein (also known as bone marrow stromal cell antigen 2 / BST2 / CD317) responsive to interferon activity and measurable on the cell subset of choice, in the case of this study, memory B cells[373]. Memory B cell tetherin was calculated without knowledge of the participant's clinical status.

3.3.2 Sample preparation

PBMCs were separated using density gradient method (Lymphoprep®, Alere Technologies, Norway) from EDTA-anticoagulated peripheral blood. Isolated cells were twice washed by Dulbecco’s Phosphate-Buffered Saline (DPBS) and were labelled with a panel of monoclonal antibodies for immunotyping or FACS cell sorting.

3.3.3 Antibody clones

The antibody clones used in this study were: CD19 (clone HIB19). The following antibody clones were used in this study; CD19 (clone HIB19); CD69 (clone FN50); CD56 (clone B159), CD3 (clone SK7), CD4 (clone RPA-T4); CD8 (clone SK1); CD27(clone M-T271), all from BD Biosciences (Oxford, UK); CD14 clone TÜK4); CD16 (clone Clone VEP13), CD38 (clone DX9); CD64 (clone 10.1.1), CD169-Siglec-1 (clone 7.239), all from Miltenyi Biotec (Bisley, UK) and BST2/tetherin/CD317 (clone 26F8) from eBiosciences (Hatfield, UK).

3.3.4 Flow cytometry and cell sorting

A multflow analysis was applied to detect and quantify the tetherin on PBMCs. A gating strategy was used to define and sort cells into T-cells, NK-cells, monocytes as well as B cell subsets: naïve, memory and plasmablasts using a BD Influx™ cell sorter (Figure 4). For each population, mean fluorescence intensity (MFI) of tetherin BD was determined using FACSCanto flow cytometer and BD FACSDiva software.
The memory B cell tetherin MFI of CD19+CD27+CD38- lymphocytes was selected for this study.

**Figure 4 Flow cytometry for memory B cell tetherin**

![Flow cytometry for memory B cell tetherin](image)

**Figure 4.** Memory B cell tetherin flow cytometry

### 3.4 Patient reported outcome measures

#### 3.4.1 Introduction

Many patient-reported outcome measures (PROMs) have been validated for use in adults with CTD.[377] The instruments are usually in the form of a questionnaire and can be used to assist health professionals and researchers to gain insight into a patient’s perspective of health. PROMs may be generalised or have a specific focus on matters relating to the patient’s experience. In light of the heterogeneity of CTD, this research program included a variety of PROMs for the purpose of gaining an in-depth understanding of the patient’s perspective. Indices were administered to participants for completion at the time of the study visit and were analysed in accordance with published scoring rules, as described as follows.

#### 3.4.2 Visual analogue scales (VAS)

Visual analogue scales (VAS) for pain, morning joint stiffness, arthritis, fatigue, and global health was administered to measure symptom severity and general health. Each VAS was a single item that measured response on a 100 mm continuous horizontal scale. Endpoints were no symptoms or best health (0) and worst symptom or worst health (100). Participants were directed to mark on the scale at a site that corresponded with the severity of their symptoms in the past month. Missing data was excluded in analyses.
3.4.3 RAND® 36-Item Health Survey version 1.0 (SF-36)

The RAND® 36-Item Health Survey version 1.0 (SF-36) was administered to measure general health and quality of life. The instrument reports on eight health concepts: physical functioning, bodily pain, role limitations due to physical health problems, role limitations due to personal or emotional problems, emotional well-being, social functioning, energy/fatigue, and general health perceptions. The responses were scored in accordance with RAND® Instrument scoring rules[378]. Missing data was omitted in analyses.

3.4.4 5-level EQ-5D version (EQ-5D-5L)

The 5-level EQ-5D version (EQ-5D-5L) consists of the EQ-5D descriptive system and the EQ visual analogue scale (EQ-VAS)[379]. The EQ-5D-5L is a standardised measure of health status that relates to the respondent's situation at the time of completion. The descriptive system is comprised of five dimensions: mobility, self-care, usual activities, pain/discomfort and anxiety/depression. Each dimension has a response option of either no problems, slight problems, moderate problems, severe problems or extreme problems. The EQ-VAS records the respondent's self-rated health on a 20cm, 100 point vertical VAS, where the endpoints were labelled ‘The worst health you can imagine’ (or 0) and ‘The best health you can imagine’ (or 100). Respondents mark an X on the scale to indicate how their health is today, and write the number they marked on the scale within an adjacent box. Responses for the EQ-5D descriptive system were coded as either 0 for no problems, or 1 for slight, moderate, severe or extreme problems. The EQ-VAS was scored according to the number in the box, or if missing, scored according to the site marked with X on the scale. Missing values were excluded from analyses.

3.4.5 Work productivity and Activity Impairment Questionnaire: Specific Health Problem version 2.0 (WPAI:SHP)

The Work productivity and Activity Impairment Questionnaire: Specific Health Problem version 2.0 (WPAI:SHP) is a measure of work and activity impairment due to a target health problem[380]. The WPAI:SHP yields four types of scores: absenteeism (work time missed), presenteeism (impairment at work or reduced on-the-job effectiveness), work productivity loss (overall work impairment or absenteeism plus presenteeism) and activity impairment. WPAI:SHP outcomes are expressed as impairment percentages, with higher numbers indicating greater impairment and less productivity. Missing items were excluded from analyses.
3.4.6 ICEpop CAPability measure for Adults (ICECAP-A)

The ICEpop CAPability measure for Adults (ICECAP-A) is a generic measure of capability for well-being in adults[381]. ICECAP-A conceptualises well-being as the capability of the individual to achieve valuable functioning in five attributes: attachment, stability, achievement, enjoyment and autonomy. Previous work on the ICECAP-A suggests that the instrument can comprehensively capture quality of life[381]. Respondents indicate their level of capability for an attribute on a four-tiered Likert scale. ICECAP-A Instrument scoring guidance was used to code and produce respondent tariff values[382]. Only complete case data were analysed.

3.4.7 Functional Assessment of Chronic Illness Therapy - Fatigue Scale (FACIT-Fatigue)

The Functional Assessment of Chronic Illness Therapy - Fatigue Scale (FACIT-Fatigue) version 4 is a 13-item questionnaire that assesses quality of life concerns related to fatigue[383]. It measures an individual's level of fatigue on a four point Likert scale during their usual daily activities over the past week. Responses were scored in accordance with the FACIT-Fatigue Subscale Scoring Guidelines (Version 4)[384]. Missing items were handled using a proration method where the calculation was altered to reflect the actual number of items answered.
Chapter 4 In SLE patients validated interferon assays predict flares and glucocorticoid requirements

4.1 Introduction

Damage accumulates and long-term mortality and quality of life remains impaired despite current therapies in lupus[55]. Disease activity and glucocorticoid use are recognised predictors of poorer long-term outcomes[55]. Treat-to-target strategies are known to improve long term outcomes in other rheumatic diseases[55]. In light of this, the 2010 EULAR treat-to-target guidelines were created for the management of SLE[55]. The guidelines recommended minimisation of glucocorticoids, the achievement of lowest disease activity, and the goal-directed use of validated activity indices and/or organ-specific biomarkers as the benchmarks of lupus best care[55]. Achieving these aims is a challenge, glucocorticoid minimisation in particular, as there are few reliable means to predict future disease activity and therefore guide withdrawal decisions. There is therefore an unmet need for biomarkers that can predict future therapy.

Potential biomarkers in lupus are numerous including cytokines, chemokines, growth factors and acute phase reactants[19, 222, 385]. However, there are inconsistent reports on the performance of many of these biomarkers to discriminate activity, and most have been studied in small cohorts or cross-sectional observational studies[18]. Demonstrating association between a potential biomarker and diagnosis or disease activity suggests that it may contain important information about a disease, but the more critical aspect of biomarker validation is the ability of the biomarker to predict clinical outcomes of value[18]. Data on this are more limited, which prevents the incorporation of many biomarkers into clinical practice[20, 25].

IFN-I plays a fundamental role in the pathogenesis of SLE and is known to be associated with disease activity[20]. IFN-I has numerous pleiotropic effects but remarkably IFN-I stimulates B cell activation which is a pre-requisite for plasmablast and plasma cell differentiation, a key pathway of disease pathogenesis in SLE[20]. IFN-I protein is difficult to detect in the blood or serum directly[19, 343, 376]. IFN-I status is therefore more commonly determined using the expression of IFN-stimulated genes (ISGs). There are over 100 genes induced by type I IFN pathway activation and the choice of which ISGs to include and how to summarise results affects the validity of scores[343, 368]. The metric properties of ISG expression has been described to be improved by (i) analysing ISGs as more than one score, and (ii) describing interferon activity as a continuous score rather than a simple high /
low “signature”[24]. A two-score system IFN Score A and IFN Score B was recently shown to predict future development of SLE[25].

An alternative approach to IFN status determination is the measure of tetherin, a flow cytometric surface marker[373]. Tetherin is a IFN-stimulated cell surface protein expressed on all nucleated cells[373]. It has been validated on memory B cells as a biomarker of disease activity in SLE[24].

The purpose of the present study was to evaluate these IFN biomarkers to examine the therapeutic goals of predicting disease activity and glucocorticoid requirement. We also sought to compare these continuous scores with the more commonly used categorical high / low measure of IFN status used in other studies[339, 347, 374, 386-390]. If appropriately validated, this could allow the clinical application of these biomarkers to guide decisions regarding management of immunosuppression.

4.1.1 Hypothesis

i. Risk of future flares and oral glucocorticoid requirements in SLE can be predicted using IFN Score A, IFN Score B and memory B cell tetherin

4.1.2 Objectives

i. To evaluate disease activity and glucocorticoid requirements relative to IFN biomarker sampling in a cohort of SLE patients

4.2 Methods

4.2.1.1 Ethical approval and methods

The Leeds Human Research Ethics Committee (approval RR10/9608) granted ethical approval for the study (Chapter 9). This study was a retrospective study that used the Connective Tissue Disease and Vasculitis Longitudinal Cohort (CONVAS) database and was conducted at the Leeds Teaching Hospitals NHS Trust. Consecutive patients with SLE meeting SLICC-12 classification criteria who submitted IFN biomarker samples (IFN Score A, IFN Score B and/or memory B cell tetherin) were identified and selected into this cohort study. A total of 166 patients were selected.

Medical records were then used to determine outcome measures. British Isles Lupus Activity Group 2004 index (BILAG-2004) was used to determine disease activity. Average monthly prednisolone dose was calculated for the three months before and after biomarker submission. Glucocorticoids were adjusted for equivalent prednisolone dose (i.e. hydrocortisone was prescribed for one patient which was
recalculated into the equivalent prednisolone dose). The clinical assessors were blinded to IFN assay results.

Case data was divided into two subgroups to address each objective (Figure 5). Group 1: patients with no BILAG-2004 A or B activity in the six months prior to biomarker submission. Group 2: patients with any level of disease activity who had available records for prednisolone dosing for the three months before and after biomarker submission.

**Figure 5 Schema of case selection.**

### 4.2.1.2 Clinical endpoints

The objective of Group 1 was to examine the relationship between biomarker expression and the clinical outcome of flare or no flare. The clinical endpoint in Group 1 was new onset of BILAG-2004 A or B activity in the six month period after biomarker submission. The objective of Group 2 was to examine the relationship between biomarker expression and pattern of prednisolone requirement. The clinical endpoint in Group 2 was change in prednisolone exposure relative to the time of biomarker submission. Change in prednisolone exposure was determined by comparing the total mean monthly prednisolone equivalent dose between the periods pre- and post-biomarker submission. Categories of prednisolone change patterns were: dose increased; dose decreased; no change; or no prednisolone prescribed before or after biomarker sampling.
4.2.1.3 Interferon assays

The development and validation of IFN assays are described in detail in Chapter 3 [24, 373]. IFN Score A and IFN Score B are a 2-score system derived from factor analysis of 31 ISGs selected from three IFN annotated modules as measured by TaqMan[25]. Briefly, RNA was extracted from PBMCs and a custom TaqMan array was used to measure the expression of 31 ISGs normalised to PP1A. These were then used to calculate Score A and Score B. For memory B cell tetherin, PBMCs were analysed fresh with conventional surface staining. MFI of CD317 was measured on CD19^+CD27^+CD38^- lymphocytes.

IFN Scores were initially calculated as an untransformed delta cycle threshold (dCT) normalised to the reference gene PP1A. Untransformed scores lead numerically higher scores for a patient with lower ISG expression. For clarity of presentation we therefore presented the reflected dCT (dCT x -1) in graphs and tables.

4.2.2 Statistical analyses

Two analyses were performed to examine the clinical associations of the three IFN assays. For Group 1, the onset of new disease activity (defined as new BILAG A or B activity) in patients in sustained low disease activity before biomarker submission (no BILAG-2004 A or B in the six months prior to IFN biomarker sampling was used as the definition of sustained low disease activity). For Group 2, the pattern of prednisolone change relative to the time of biomarker submission. Since memory B cell tetherin is measured on B cells we excluded patients who were B cell depleted due to rituximab exposure in the 6 months prior to biomarker submission. Independent samples t-test was used to compare IFN biomarkers between groups. Prediction of flare including age, gender, pre- and post-sampling prednisolone dose was performed using multivariate logistic regression.

4.2.3 Results

4.2.3.1 Overall clinical outcomes

166 patients meeting SLICC 2012 criteria for SLE were identified from the database. The study schema is shown in Figure 5. In Group 1, 99 patients were in sustained low disease activity prior to biomarker sampling and had complete data on flares and interferon status. New BILAG A or B activity occurred within 6 months in 11/99 patients (11.1%). In group 2, 144 patients had complete data on glucocorticoid usage and IFN-I status. Mean monthly prednisolone doses were increased in 12 (7.2%), decreased in 13 (7.8%), no change in 34 (20.5%), and were not prescribed in 85 (51.2%).
4.2.3.2 Baseline characteristics

Baseline characteristics for the patient population and each group are shown in Table 15. A notable characteristic is that prednisolone exposure was lower in Group 1 compared to Group 2 however this could be expected for a group of patients in low disease activity. Numerically higher rituximab use was found in Group 2, but there was no difference in internal organ involvement or cyclophosphamide exposure. Patients in Group 1 appeared to have well-controlled disease at the time of biomarker sampling, but historically had not always had mild SLE.

Table 15 Baseline characteristics of the 166 SLE patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Population N = 166</th>
<th>Group 1 N = 99</th>
<th>Group 2 N = 144</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Age (Range), Years</td>
<td>46 (18, 76)</td>
<td>42 (18, 74)</td>
<td>45 (18, 74)</td>
</tr>
<tr>
<td>F : M</td>
<td>156 : 10</td>
<td>92 : 7</td>
<td>137 : 7</td>
</tr>
<tr>
<td>Met SLICC 2012 criteria</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Internal organ involvement</td>
<td>59.4%</td>
<td>55.6%</td>
<td>58.3%</td>
</tr>
<tr>
<td>No internal organ involvement</td>
<td>40.6%</td>
<td>44.4%</td>
<td>41.7%</td>
</tr>
<tr>
<td>2 or more organs</td>
<td>18.7%</td>
<td>16.1%</td>
<td>18.8%</td>
</tr>
<tr>
<td>Cyclophosphamide (ever)</td>
<td>28.3%</td>
<td>27.3%</td>
<td>27.8%</td>
</tr>
<tr>
<td>Rituximab exposure (ever)</td>
<td>48.8%</td>
<td>39.4%</td>
<td>48.6%</td>
</tr>
<tr>
<td>On prednisolone (pre-biomarker)</td>
<td>54/144 (37.5%)</td>
<td>29/99 (29.2%)</td>
<td>54/144 (37.5%)</td>
</tr>
<tr>
<td>Median dose (range)</td>
<td>5mg (1, 22.5)</td>
<td>1.8mg (1, 22.5)</td>
<td>8.1mg (1, 22.5)</td>
</tr>
<tr>
<td>On prednisolone (post-biomarker)</td>
<td>58/144 (40.3%)</td>
<td>31/99 (31.3%)</td>
<td>57/144 (40%)</td>
</tr>
<tr>
<td>Median dose (range)</td>
<td>5.6mg (1, 25)</td>
<td>1.62mg (1, 15)</td>
<td>7.9mg (1, 25)</td>
</tr>
<tr>
<td>Captured flare types</td>
<td>N/A</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td>Arthritis</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthritis + skin</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haematological</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumonitis</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 15. N/A: not applicable
4.2.3.3 Overall interferon score status

The overall IFN Score A mean expression (95% CI) in delta CT was 3.57 (3.23, 3.91) for the study population. Since many publications have reported a bimodality to IFN Score distribution, we re-classified the continuous IFN Score A into the two groups: high or low; as described in a previous paper from our group [24, 347, 365]. Briefly, the mean interferon score of samples from patients with SLE, RA, UCTD and healthy controls (n = 328) plus two standard deviations (SD) above the mean was calculated. Plus two SD was chosen as a conservative approach to the analysis of the data. Therefore, a value of ≥2.32 was designated as IFN Score A high[391]. For IFN Score B, overall mean expression (95% CI) in delta CT was 3.18 (3.01, 3.34).

100% of SLE patients had high IFN Score B expression. Mean MFI for memory B cell tetherin (95% CI) was 1708 (1596, 1820).

In the following analyses, the clinical outcomes and the level of expression of each IFN biomarker has been compared for the whole group and within the IFN Score A high expression subgroup.

4.2.3.4 Prediction of flares (Group 1)

New BILAG A/B activity in the 6 months after biomarker submission (Group 1) was associated with significantly higher expression of IFN Score A, IFN Score B and memory B cell tetherin MFI (Figure 6). The mean difference (95% CI) for IFN Score A was 2.16 (0.369, 3.954, p=0.02), for IFN Score B was 0.694 (0.025, 1.364, p=0.04) and for memory B cell tetherin MFI the mean difference 1045 (1718, 371, p=0.003). Using the bimodal classification, all flares occurred in patients with high IFN Score A status.

Figure 6 Mean IFN Score A, IFN Score B and memory B cell tetherin levels relative to new disease activity in the post-sampling period

![Figure 6](image)

Figure 6. Mean IFN Score A, IFN Score B and memory B cell tetherin levels in lupus patients in low disease activity relative to new disease activity in the six month post-sampling period (left-to-right): IFN Score A, IFN Score B and Memory B cell tetherin levels. Error bars in figure legends represent 95% Confidence interval.

Significant associations with BILAG A or B flares remained for IFN Score A (n=73, OR=1.57/unit (95% CI 1.02, 2.40) p=0.040), and memory B cell tetherin MFI (n=46,
OR=6.46/1000 units (95% CI 1.25, 33.3) p=0.026) after adjustment for age and post-sampling steroid dose in logistic regression. IFN Score B did not produce significance in logistic regression, but showed a positive trend: IFN-I Score B (n=73, OR=1.86/unit (95% CI 0.81, 4.30) p=0.144).

Despite small study numbers, Receiver Operator Curve analysis of memory B cell tetherin was undertaken as a single biomarker for flare prediction (Figure 7) to estimate whether the clinical utility of the test. A threshold of tetherin=2403 units MFI produced 80% sensitivity and 85.4% specificity for flare. Application of this threshold finds that 10.3% of patients would be expected to flare in a Tetherin-low group, and 36.4% of patients would flare in a Tetherin-high group; a group that included 16% of all included patients.

**Figure 7 Receiver operator curve for prediction of flare and tetherin**

![Receiver operator curve](image)

**Figure 7.** Receiver operator curve for prediction of flare (new BILAG A or B) using memory B cell tetherin. A threshold of tetherin=2403 units MFI gave a sensitivity of 80% and specificity of 85.4% for flare.

### 4.2.3.5 Prediction of glucocorticoid use (Group 2)

Analysis of the four steroid pattern groups confirmed clinically meaningful changes in prednisolone dose change within Group 2 (Figure 8-A). The mean steroid dose remained at 5mg per day (SD 4.73) over the 3 months before and after biomarker sampling in the “no change” subgroup. For the “increased” subgroup, the mean steroid dose increased from 5mg per day (SD 6.10) to 10mg per day (SD 5.57). For the “decreased” subgroup, the mean steroid dose reduced from 10mg per day (SD 5.64) to 7.5mg per day (SD 4.57).

Overall trends in IFN status within these subgroups are provided in Figure 8 B-D. These demonstrate a significant association between high IFN biomarker status and
future prednisolone need. Future requirement for the same \((p=0.007)\) or an increased \((p=0.002)\) dose of prednisolone was associated with significantly higher IFN Score A expression compared to a decreased prednisolone dose. IFN Score B \((p=0.022\) and \(p=0.026\) respectively) demonstrated a similar pattern. Compared to individuals not requiring prednisolone, increased prednisolone need was predicted by significantly higher IFN Score A and Score B \((p=0.004\) and \(p=0.047\) respectively). The overall pattern was similar for memory B cell tetherin MFI levels (despite patient numbers being lower). Greater memory B cell tetherin MFI correlated with exposure to the same \((p=0.032)\) or an increased \((p=0.039)\) dose of prednisolone compared to a decreased dose of prednisolone. Statistical data in full are shown in Table 16.
Figure 8 Mean IFN Score A, IFN Score B and memory B cell tetherin levels relative to prednisolone dose change in the biomarker peri-sampling period

Figure 8. From left to right, above to below (A) Prednisolone dose change pattern relative to mean prednisolone dose within the cohort, pre- and post-biomarker sampling; (B) mean IFN Score A level relative to prednisolone dose change pattern; (C) mean IFN Score B level related to prednisolone dose change pattern; (D) mean IFN memory B cell tetherin relative to prednisolone dose change pattern; (E) IFN High Score A group only: mean IFN Score A (high group) relative to prednisolone dose change pattern. X axis denotes subgroups of prednisolone change. Patient numbers in brackets. Error bars in figure legends represent 95% Confidence interval.
Table 16 Comparison between prednisolone dose change categories

<table>
<thead>
<tr>
<th>Comparison between prednisolone dose change categories</th>
<th>Mean Difference</th>
<th>p-value</th>
<th>95% CI Lower</th>
<th>95% CI Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Score A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No prednisolone</td>
<td>Increased†</td>
<td>2.13</td>
<td>0.004</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>Decreased‡</td>
<td>0.75</td>
<td>0.261</td>
<td>-2.07</td>
</tr>
<tr>
<td></td>
<td>No change‡</td>
<td>1.27</td>
<td>0.010</td>
<td>0.31</td>
</tr>
<tr>
<td>Increased</td>
<td>Decreased‡</td>
<td>2.88</td>
<td>0.002</td>
<td>4.67</td>
</tr>
<tr>
<td></td>
<td>No change‡</td>
<td>0.86</td>
<td>0.280</td>
<td>-2.41</td>
</tr>
<tr>
<td>Decreased</td>
<td>No prednisolone†</td>
<td>0.75</td>
<td>0.260</td>
<td>-0.57</td>
</tr>
<tr>
<td></td>
<td>No change†</td>
<td>2.02</td>
<td>0.007</td>
<td>0.57</td>
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<tr>
<td><strong>Score B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No prednisolone</td>
<td>Increased†</td>
<td>0.69</td>
<td>0.047</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Decreased‡</td>
<td>0.28</td>
<td>0.372</td>
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</tr>
<tr>
<td></td>
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<tr>
<td>Increased</td>
<td>Decreased‡</td>
<td>0.98</td>
<td>0.026</td>
<td>1.83</td>
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<td></td>
<td>No change‡</td>
<td>0.16</td>
<td>0.672</td>
<td>-0.90</td>
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<tr>
<td>Decreased</td>
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<td>0.372</td>
<td>-0.35</td>
</tr>
<tr>
<td></td>
<td>No change†</td>
<td>0.82</td>
<td>0.022</td>
<td>0.12</td>
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<td><strong>Memory B cell tetherin MFI</strong></td>
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<td></td>
</tr>
<tr>
<td>No prednisolone</td>
<td>Increased†</td>
<td>333</td>
<td>0.287</td>
<td>-950</td>
</tr>
<tr>
<td></td>
<td>Decreased‡</td>
<td>547</td>
<td>0.068</td>
<td>-41</td>
</tr>
<tr>
<td></td>
<td>No change†</td>
<td>325</td>
<td>0.096</td>
<td>-710</td>
</tr>
<tr>
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<td>No change†</td>
<td>872</td>
<td>0.008</td>
<td>1512</td>
</tr>
</tbody>
</table>

Table 16. † = higher biomarker group; ‡ = lower biomarker group

4.2.3.6 Continuous vs Categorical IFN Status

The question of whether a continuous IFN score could provide clinically meaningful information over and above bimodal high/low categorical IFN scores (as reported in other studies) was tested by repeat analysis using the cases of high IFN Score A status only.[347, 369] This analysis found that more extreme elevations of IFN Score A expression were associated with higher predicted prednisolone needs compared to the rest of the IFN Score A-high group. Level of IFN Score A significantly correlated with remaining on the same dose of glucocorticoid (p=0.041) and increasing the dose of glucocorticoid (p=0.017) compared with a future prednisolone dose decrease. This indicates that continuous IFN score systems may
have an advantage over bimodal IFN score systems in terms of addressing the question of prediction of future glucocorticoid need.

4.2.4 Discussion

Quantification of IFN-I activity in SLE using IFN Score A, IFN Score B and memory B cell tetherin MFI is demonstrated in this study to predict outcomes previously recommended by an international guidelines as targets for the management of SLE. IFN Score A, IFN Score B and memory B cell tetherin may therefore assist with treatment pathways.

The maintenance of low disease activity is strongly evidenced: in longitudinal studies disease activity was associated with eventual damage and mortality, while attaining low disease activity or remission predicted better long-term health status[392-395]. In patients who achieve low disease activity, the future flare rate has been reported as 64-75% in cohorts, with 17-38% of these being severe. Sustained remission is observable in 10-40% of patients. Identifying the individuals at highest risk of flare is an opportunity for prevention through pre-emptive addition of non-glucocorticoid immunosuppression. Although our study included a relatively small number of flares, we have estimated the potential utility of using IFN biomarkers for this purpose. In our data, a level of memory B cell tetherin, the best biomarker, over a critical threshold was associated with a tripling of flare rate, from 10% to 36%. In vivo, if high tetherin were used to trigger escalation of therapy in the 16% of all low disease activity patients approximately 40% of all flares could potentially be prevented.

Apart from disease activity, another major predictor of negative long term outcomes is the use of glucocorticoids, which generates an additional dilemma in patients on long term glucocorticoids when the risk of flare on tapering is unpredictable[396]. If activity was allowed to occur on tapering of prednisolone, as well as potential damage accumulation, the ultimate result may be a higher total cumulative glucocorticoid dose than if withdrawal was never attempted. In our cohort IFN biomarkers were able to differentiate changes in glucocorticoid requirement of a mean increase of 5mg, mean decrease of 2.5mg and clusters of patients whose ultimate mean prednisolone dose was more than 10mg. These levels are clinically significant in terms of long term glucocorticoid toxicity. The hazard ratio for damage is 1.05 per 1mg of prednisolone, and is 1.50 for use of doses over 6-12mg[397, 398]. Our results therefore suggest that IFN biomarkers could identify patients in whom glucocorticoid doses could be safely reduced by a margin that practically alters long term outcome and without increasing the risk of activity.
The challenges in measuring IFN status is well recognised, which is reason for the vast array of potential IFN biomarkers published. Importantly, many of these biomarkers have not been able to establish the same correlations that were found in this study. For instance, Petri et. al. researched the predictive performance of an ISG IFN status assay for prediction of flare and found no correlation[369]. The difference from these results is likely because the results from other studies used semi-quantitative micro-array findings limited to 3 ISGs, while our gene expression results were calculated using 2 fully-quantitative scores that had already been validated against a range of other in-vitro and clinical outcomes. Head-to-head comparisons of the clinical utility of different biomarkers are few. A strength of this study is that 3 biomarkers were analysed in the same population for the same endpoints.

IFN status using two scores has been previously shown to strengthen clinical associations, and each score has been correlated with different clinical characteristics. While patients with SLE have high levels of both IFN Score A and IFN Score B, patients with RA have been shown to have high levels of IFN Score B only[24]. IFN-Score-B has previously only been shown to be a better predictor for the progression to SLE in an at risk population than IFN-Score-A. For the particular endpoints in this study the three assays demonstrated broadly similar characteristics, but with some difference in the strength of correlations.

The differences in expression between IFN Score A and IFN Score B from a biological point of view is not entirely clear. These scores were derived from previously described modules of IFN-stimulated genes; IFN Score A mostly from module 1.2 and 3.4 and IFN Score B mostly from module 3.4 and 5.12[376]. These modules were previously thought to represent response to Type I and Type II interferons. However, changes in gene expression may also reflect other inflammatory cytokines or differences in the cellular composition of the sample. In this respect it is remarkable that tetherin measured specifically on B cells was somewhat more predictive than the other assays.

Another possible clinical application for a predictive IFN assay has been patient selection for IFN-targeting drugs. In the clinical trials of anifrolumab and sifalimumab, IFN-high patients showed a better clinical response in the active arms compared to placebo[20]. However alternatively, this was due to a lower response rate in the IFN-high patients randomised to placebo. Our findings on flares and prednisolone use would match the inability to meet a composite endpoint such as SRI-4 with prednisolone taper. Our results therefore suggest that IFN status could
stratify and dictate the placebo response rate for a clinical trial of any investigational therapy compared to placebo, not just IFN-blocking drugs.

4.2.5 Conclusion

In conclusion, the value of IFN biomarkers has been demonstrated for a specific clinical decision including a head to head comparison of different IFN biomarker subtypes and analyses. These results warrant validation studies to confirm that addition of immunosuppressive therapy, or prednisolone withdrawal based on biomarker results are effective in a clinical study. The Defining Interferon-mediated Connective Tissue Disease (DEFINITION) study is a prospective longitudinal cohort study that is currently investigating this idea.

4.2.6 Key messages

- Validated interferon assays predict activity and glucocorticoid use in lupus and may therefore aid achievement of low disease activity
- IFN stimulated gene expression should be analysed as a continuous variable not a simple high “signature”
- Further validation study of IFN Score A and IFN Score B is necessary to explore their clinical utility.
Chapter 5 The use of IFN assays in distinguishing patients who met CTD classification criteria from a cohort of patients labelled as UCTD

5.1.1 Introduction

Undifferentiated connective tissue diseases (UCTD) is a term used to describe patients that who exhibit signs, symptoms and serological abnormalities suggestive of an underlying autoimmune disorder, but who do not fulfil any classification criteria for definitive CTDs such as SLE, pSS, SSc, IMs and others[116]. Patients with UCTD have detectable ANAs and sometimes positive autoantigen specificities. Arthralgia, arthritis, rash, alopecia, Raynaud’s phenomenon, mucosal ulcers, photosensitivity, sicca and low grade fever are common symptoms in UCTD[115]. Although the term “undifferentiated” sounds uncertain or vague, the condition can substantially affects patients’ quality of life [399]. Moreover, the prognosis of UCTD could be disease stability, regression to remission, or progression to a classifiable form of CTD[112, 116]. Therefore, there is an unmet need for prognostic biomarkers to stratify those likely to progress to the classification criteria of established CTD[116-118, 126, 400].

As described in Chapter 2.3.8, variants in IFN-I pathway are prominent in the genetic susceptibility to CTDs and therefore are a focus for investigation[401-403]. Our group, previously published that IFN Score B was an independent predictor of progression from at risk individual (i.e. defined as ANA positive; ≤1 clinical SLE criterion; symptom duration <12 months and treatment-naïve) to meeting criteria for CTD[404]. However, the role of IFN in UCTD cohort is less well documented and is currently unclear. In one cross-sectional study, of 28 patients recruited, 50% of the patients with UCTD had elevated IFN signature versus healthy controls, as measured by IFN-5 score i.e. summation of the expression levels of five IFN-stimulated genes ([ISGs] - \textit{LY6E}, \textit{OAS1}, \textit{IFIT1}, \textit{ISG15} and \textit{MX1}), which were normalised to \textit{GAPDH} gene. However, its significance has not been reported.

Another important issue is related to the labelling of patients as UCTD. To date, the UCTD classification criteria are only provisional[114] and may be considered outdated in light of several recently published classification criteria for definitive CTDs[71, 80, 405, 406]. Moreover, these criteria can be difficult to use and may contain criterion considered subjective (e.g. alopecia, photosensitivity, mucosal ulceration and myalgia). Criteria need not be present all at the same time, further complicating accurate classification. Consequently, some patients who are labelled as UCTD could indeed meet the classification of definitive CTD, should these criteria
have been rigorously assessed. Biomarkers to support the differentiation between classifiable CTD and UCTD may help identify the former group, who may benefit from close monitoring and stronger immunosuppression based on patterns seen within peers.

5.1.2 Hypothesis

i. The use of IFN Score A and IFN Score B will support identification of patients with classifiable CTD from a cohort of patients labelled as UCTD.

5.1.3 Objectives

i. To evaluate the use of IFN assays in distinguishing patients with classifiable CTD immunologically from a cohort of patients labelled as UCTD.

5.2 Methods

5.2.1 Design and Patients

A cross-sectional study of consecutive patients with UCTD was conducted at a single centre in Leeds between July 2017 till March 2019. Inclusion criteria were (i) ANA positive; (ii) signs and symptoms suggestive of CTD but not fulfilling classification criteria for an established CTD[38, 71, 80, 405] or RA [407]; disease duration of at least 12 months and iv) UCTD diagnosis made by Consultant Rheumatologists.

5.2.2 Ethical approval

All individuals provided informed written consent and this research was undertaken in compliance with the Declaration of Helsinki (Chapter 9). The patients’ blood samples used for this study were collected under ethical approval, REC approval 17/YH/0166, National Research Ethics Committee Yorkshire and the Humber – South Yorkshire. All experiments were performed in accordance with the relevant guidelines and regulations. The University of Leeds was contracted with administrative sponsorship.

5.2.3 Assessment and Clinical data collection

Patients with a Consultant diagnosis of UCTD were recruited from the National Health Service (NHS) Leeds Connective Tissue Disease Clinic. The research assessment took place within 3 months from the last patient visit to this NHS clinic. At this research visit, age, gender, ethnicity, history of first or second degree relative(s) with rheumatic and musculoskeletal diseases (RMDs), smoking history,
presence of fibromyalgia, treatment including DMARDs, corticosteroids, disease features and number of classification criteria met for CTD based on previous clinic letters and medical notes were recorded. Patients were then schematically assessed for classification criteria of the four most common CTDs: SLE[38], pSS[71], SSc[80] and IMs[405]. Any discrepancy in the assessment of UCTD classification between both physicians was recorded.

5.2.4 Laboratory assessment
ANA was tested using a panel of nuclear autoantibodies that included anti-dsDNA and extractable nuclear antigens (including Ro52, Ro60, La, Sm, Chromatin, RNP, Ribosomal P, Jo-1, Centromere and Scl-70) using the Bioplex 2200 Immunoassay. Full blood count, liver function and electrolytes were processed at a single accredited diagnostic laboratory. Complement levels (C3 and C4) were measured by nephelometry. Samples for IFN biomarkers were collected and processed as per Chapter 3.2. IFN Score A and IFN Score B were calculated without knowledge of the participants’ clinical status.

5.2.5 Statistical analyses
Descriptive statistics were summarised using mean with standard deviation or median with interquartile range for continuous variables (where appropriate) and proportion for categorical variables. The independent samples T-test was used to analyse the difference in the IFN Scores between patients with classifiable CTDs and those who remained undifferentiated according to the investigator’s assessment.

5.3 Results
5.3.1 Patient Characteristics
43 patients with a Consultant diagnosis of UCTD were enrolled into the study. Of these, 36 (84%) were females, mean (SD) age was 49.4 (12.4) years, median (range) disease duration was 4 (1-18) years, 7 (16%) had major internal organ involvement and 28 (65%) were on concomitant DMARDs (including anti-malarials). Baseline characteristics are described in Table 17.
Table 17 Baseline characteristics of the 43 patients with UCTD

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD) years</td>
<td>49.4 (12.4)</td>
</tr>
<tr>
<td>Female patients, N (%)</td>
<td>36 (83.7)</td>
</tr>
<tr>
<td>Ethnicity, N (%)</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>30 (71.4)</td>
</tr>
<tr>
<td>Black</td>
<td>4 (9.5)</td>
</tr>
<tr>
<td>South Asian</td>
<td>6 (2.3)</td>
</tr>
<tr>
<td>Positive ANA, N (%)</td>
<td>43 (100)</td>
</tr>
<tr>
<td>Autoantibody specificities</td>
<td></td>
</tr>
<tr>
<td>Anti-dsDNA</td>
<td>9 (20.9)</td>
</tr>
<tr>
<td>Anti-Ro (52 or 60)</td>
<td>12 (27.9)</td>
</tr>
<tr>
<td>Anti-La</td>
<td>4 (9.3)</td>
</tr>
<tr>
<td>Anti-Smith</td>
<td>1 (2.3)</td>
</tr>
<tr>
<td>Anti-RNP</td>
<td>4 (9.3)</td>
</tr>
<tr>
<td>Anti-Scl-70</td>
<td>2 (4.7)</td>
</tr>
<tr>
<td>Anti-Jo1</td>
<td>1 (2.3)</td>
</tr>
<tr>
<td>Anti-Centromere</td>
<td>1 (2.3)</td>
</tr>
<tr>
<td>Anti-Ribosomal P</td>
<td>1 (2.3)</td>
</tr>
<tr>
<td>Family history of autoimmune disease, N (%)</td>
<td>11 (25.6)</td>
</tr>
<tr>
<td>Current smoker, N (%)</td>
<td>7 (17.1)</td>
</tr>
<tr>
<td>Fibromyalgia, N (%)</td>
<td>5 (11.6)</td>
</tr>
<tr>
<td>Disease duration, median (range) years</td>
<td>4 (1-18)</td>
</tr>
<tr>
<td>No of clinical criteria for CTD, N (%)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1 (2.3)</td>
</tr>
<tr>
<td>2</td>
<td>15 (34.9)</td>
</tr>
<tr>
<td>3</td>
<td>13 (30.2)</td>
</tr>
<tr>
<td>4</td>
<td>8 (18.6)</td>
</tr>
<tr>
<td>5</td>
<td>4 (9.3)</td>
</tr>
<tr>
<td>≥ 5</td>
<td>2 (4.7)</td>
</tr>
<tr>
<td>Internal organ involvement, N (%)</td>
<td>7 (16.2)</td>
</tr>
<tr>
<td>ILD</td>
<td>2</td>
</tr>
<tr>
<td>Myositis</td>
<td>2</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>3</td>
</tr>
<tr>
<td>Concomitant DMARDs, N (%)</td>
<td>28 (65.1)</td>
</tr>
<tr>
<td>Anti-malarials</td>
<td>18 (41.9)</td>
</tr>
</tbody>
</table>
5.3.2 Clinical criteria of UCTD as assessed between Consultant diagnosis and the research investigator

The median (range) duration of time when patients with UCTD were reviewed between NHS and research clinics was 4 (1-10) weeks. Following a schematic review of patients’ signs and symptoms against classification criteria for these four CTDs i.e. SLE, pSS, SSc and IMs at the research clinic, 9/43 (21%) patients met classification criteria for an established CTD. Of these, 6 patients were re-classified as SLE, 2 were definite or probable IMs and 1 was SSc. The differences in clinical criteria scored between a Consultant diagnosis and the schematic review by the research investigator are presented in Table 18.

Table 18 Clinical criteria of UCTD as assessed between Consultant diagnosis and the research investigator

<table>
<thead>
<tr>
<th>Clinical criteria</th>
<th>Pre-Study Consultant diagnosis of UCTD (n=43)</th>
<th>Schematic review of UCTD by investigator (n=43)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alopecia, N (%)</td>
<td>2 (22.2)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>Raynaud’s phenomenon, N (%)</td>
<td>4 (44.4)</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>Sclerodactyly, N (%)</td>
<td>1 (11.1)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Sicca, N (%)</td>
<td>2 (22.2)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>Arthritis, N (%)</td>
<td>3 (33.3)</td>
<td>6 (66.6)</td>
</tr>
<tr>
<td>Cutaneous rash, N (%)</td>
<td>4 (44.4)</td>
<td>5 (55.5)</td>
</tr>
<tr>
<td>Raised CK/myositis, N (%)</td>
<td>2 (22.2)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Leucopaenia, N (%)</td>
<td>2 (22.2)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>Thrombocytopenia, N (%)</td>
<td>0</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Mouth ulcers, N (%)</td>
<td>1 (11.1)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>GORD, N (%)</td>
<td>0</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Telangiectasia, N (%)</td>
<td>1 (11.1)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Photosensitivity, N (%)</td>
<td>0</td>
<td>3 (33.3)</td>
</tr>
</tbody>
</table>

Table 18. CK, Creatinine kinase; GORD, Gastro-oesophageal reflux disease.
5.3.3 Treatment characteristics of the UCTD patients in this study

Treatment characteristics of patients with UCTD (as agreed by Consultant diagnosis and the research investigator) and those who reclassified to CTD were compared. The proportions of patients on any concomitant immunosuppressant including glucocorticoids were similar between the two groups. However, the proportions on concomitant anti-malarials were higher in those who were re-classified to CTD compared to those whom UCTD diagnosis was agreed (Table 19).

Following an assessment at the research clinic, treatment for those who were re-classified as CTD was changed in 5/9 patients (2 were started on anti-malarials, 2 = Azathioprine and 1 = Intra-muscular glucocorticoids) as shown in Table 19.

Table 19 Comparison of treatment characteristics between those whom a diagnosis of UCTD was agreed and reclassified to CTD

<table>
<thead>
<tr>
<th>Agent</th>
<th>Agreed UCTD, N = 34</th>
<th>Reclassified to CTD, N=9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any immunosuppressant including glucocorticoids, N (%)</td>
<td>23 (67.6)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Prednisolone, N (%)</td>
<td>2 (5.9)</td>
<td>0</td>
</tr>
<tr>
<td>Anti-malarials, N (%)</td>
<td>13 (38.2)</td>
<td>5 (55.6)</td>
</tr>
<tr>
<td>Methotrexate, N (%)</td>
<td>8 (23.5)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Mycophenolate, N (%)</td>
<td>1 (2.9)</td>
<td>0</td>
</tr>
<tr>
<td>Azathioprine, N (%)</td>
<td>3 (8.8)</td>
<td>0</td>
</tr>
<tr>
<td>Sulfasalazine, N (%)</td>
<td>1 (2.9)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 19. UCTD and reclassified CTD characteristic differences.

5.3.4 IFN Scores were higher in those who re-classified to CTD than whom a diagnosis of UCTD was agreed

Both IFN Scores (Score A and Score B) were compared between those whom a diagnosis of UCTD was agreed (n=34) and re-classified into CTD (n=9) following assessment at the research visit. IFN Score A was higher in the latter versus the former group (fold difference (FD) 2.76 (95% CI 1.11 - 6.88; p=0.030) (Figure 9-A). For IFN Score B, there was a trend to association for higher expression in those whom a diagnosis of UCTD was agreed versus classified to CTD (FD 1.74, 95% CI 0.53 - 3.21; p=0.077) (Figure 9-B).
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Figure 9 Comparison of IFN Scores between those who a diagnosis of UCTD was agreed and re-classified into CTD

Figure 9. A) Mean gene expression of IFN Score A was higher in those whom a diagnosis of UCTD was agreed versus re-classified into CTD. B) There was a trend to higher mean gene expression in the latter versus former groups. Error bars denote upper and lower limits of the mean. CTD; connective tissue disease.

5.3.5 Discussion

In this chapter, a cohort of patients who had a Consultant diagnosis of UCTD were studied in order to phenotype those who had already met classification criteria of an established CTD (through schematic assessment of signs and symptoms against criteria) or remained “unwaveringly” undifferentiated. A substantial proportion of patients could be re-classified clinically, and this was supported by immunological evidence. These results show that the IFN Scores (i.e. particularly IFN Score A) can help to immunologically distinguish patients who may have a more severe disease, although we did not see evidence of this greater severity (e.g. in the form of greater therapy requirement) within this small cohort.

UCTD is an understudied area in rheumatology and currently there is no widely-accepted guideline on the management of this disease. The absence of universally-accepted classification criteria for UCTD is a significant barrier for patient diagnosis and research. This was also acknowledged by the European Reference Network on Rare and Complex Connective Tissue and Musculoskeletal Diseases (ERN ReCONNET) group in their recent publication on UCTD[408]. The 1999 preliminary classification criteria for UCTD by Mosca et al.[114] could be used but might be perceived as outdated in light of the repeated revisions made to several of the classification criteria for definitive CTD since 1999. For instance, classification criteria for SLE has been revised twice i.e. in 2012[38] and 2018[409], while for pSS the criteria has been revised three times i.e. in 2002[68], 2012[410] and 2016[69]. Furthermore, some of these criteria were weighted to clinical significance and
clinicians often had to refer to the criteria glossary sections to learn how these criteria were scored. This can be time-consuming particularly in a busy clinic. Thus, the issues above motivated the exploration of the role of an IFN biomarker in delineating patients with definitive classifiable CTD from the undifferentiated.

Recent advances have elucidated the important role of IFN in the pathogenesis of various CTDs[20, 411]. High expression of ISGs had been widely reported in both the tissues and serum of patients with SLE[24, 412, 413], pSS[414, 415], SSc[416, 417] and IMs[418, 419]. Therefore, findings of higher ISG expression of patients with CTDs versus healthy controls are no longer perceived as novel, although may add to the wealth of the body of evidence. Studies pertaining the role of IFN-I5 in UCTD have been limited. In one study by Wither et al., of the 28 patients with UCTD recruited, none of the patients at baseline with pre-SSc (Raynaud’s phenomenon, oesophageal dysmotility, telangiectasia and digital ulcers, in the absence of scleroderma or sclerodactyly) had high IFN-5 levels, whereas 50% of patients with incomplete lupus erythematosus (arthritis, lupus rashes, vasculitic skin lesions, pleuritis, idiopathic thrombocytopenic purpura, pericarditis and mucocutaneous ulcers) and 100% of patients with sicca symptoms and anti-Ro+ had high IFN-5 levels. 23/28 patients had 1-year follow-up. Of these, 7/23 had progressed to meeting classification criteria of CTD at 1 year. However, there was no difference in baseline IFN-I5 levels between those who progressed to CTD versus those who remained undifferentiated[420]. It might be that IFN biomarkers were not predictive of progression from UCTD to CTD but these findings need to be interpreted with caution since the results could be influenced by the small sample size, short-term follow-up and potentially the performance of IFN signatures used (as discussed in Chapter 2.3.8).

In this study, 9/43 (21%) were re-classified into CTD at the research visit following a schematic review of signs and symptoms against the four established criteria for SLE, pSS, SSc and IMs. The most common symptom for re-classification from the previous Consultant diagnosis was arthritis. The definition of arthritis based on the 2012 SLICC criteria for SLE was synovitis involving two or more joints, characterised by swelling or effusion OR tenderness in two or more joints and thirty minutes or more of morning stiffness [38]. However, assessment of arthritis in the context of CTD can be challenging since patients often present with arthralgia but without obvious clinical synovitis. The use of musculoskeletal ultrasound has the potential to objectively assess this in CTD including SLE[421] but it still not used commonly in clinics compared to other inflammatory disease like RA. Hence this
could lead to discrepancy in the scoring of this element. Importantly, no discrepancy pertaining to major end organ involvement was observed between the two assessments. Although non-major organ threatening signs and symptoms such as leucopaenia, rash and photosensitivity could be overlooked in the previous Consultant diagnosis assessment, these did pose an impact on re-classification of patients to definitive classifiable CTD. This reclassification was supported by the use of novel two-factor for IFN status, as described in Chapter 3.2. IFN Score A which comprised many well-known ISGs that respond to IFN-I (IFN-α, -β, -κ, -ω) was significantly higher in those who were reclassified to CTD compared to those remained undifferentiated. This elevated level was not affected by the use of concomitant DMARDs since more patients were on anti-malarials in the former group versus the latter group. Suppression of endosomal TLR activation by anti-malarials has been attributed to the inhibition of endosomal acidification, which was a prerequisite for the activation of these receptors [422]. Although not statistically significant, there was a trend to increase in IFN Score B in the former versus latter groups. In contrast to IFN Score A, IFN Score-B comprised ISGs that coincided with M3.4 and M5.12 modules of a previous microarray study [376], which were suggested to be responsive to not only IFN-I but also IFN-II (IFN-γ), IFN-III (IFN-λ) as well as other inflammatory mediators which are yet to be discovered[423]. Moreover, following the research assessment, about half of the patients had their immunosuppressive therapies escalated.

This study has some limitations. First, the two assessments of UCTD were not undertaken at the same time which could theoretically contribute to the discrepancy in classifying patients. However, all patients with UCTD were seen in the research clinic within 3 months i.e. median 4 weeks. Since these patients were deemed stable with no change in immunosuppressive therapies in the NHS clinic, the likelihood that these patients would have progressed to CTD was low. Second, although largest in this field to date, the sample size was low, which was also demonstrated by the wide confidence intervals in both IFN scores. Thirdly, the re-definition of UCTD would be achievable through more stringent application of the classification criteria by clinicians. Lastly, the role of these IFN biomarkers in the prediction of progression to CTD could not be deduced in the absence of longitudinal follow-up.

5.3.6 Conclusion

In conclusion, the current proposed classification criteria for UCTD led to discordance in disease classification between a Consultant diagnosis in a routine
clinic and a schematic review against established classification criteria for SLE, pSS, SSc and IM. Patients who were classified to CTD based on the latter method of assessment were supported by the use of IFN assays i.e. IFN Score A. Longitudinal study of the use of IFN biomarkers in patients who are labelled as UCTD may help elucidate their use in prognostic stratification and identify those who may need greater immunosuppression for disease control.

5.3.7 Key messages

- In a cohort of patients labelled as UCTD under a Consultant diagnosis in clinic, 9/43 (21%) were re-classified into CTD using a schematic review against established classification criteria for SLE, pSS, SSc and IM.
- The discrepancy in both methods of disease classification was contributed to by a lack of a formal classification criteria for UCTD.
- IFN Score A was higher in those who were re-classified into CTD than those remained undifferentiated, thus could be used to distinguish between these two groups, and patient’s initially labelled as UCTD.
Chapter 6 Relationship between IFN activity and patient-reported outcomes among individuals at risk and with established CTD, including UCTD

6.1 Introduction

Connective tissue diseases (CTD) are chronic, systemic rheumatic diseases that have the potential to impose a significant impact on patients' well-being and health-related quality of life[35, 424]. Interferons (IFNs) are pro-inflammatory cytokines that play a pivotal role in the pathogenesis and activity of CTD[20, 425, 426]. IFN-I is systemically upregulated in a significant proportion of patients with CTD, and has been detected at higher levels of expression in patients at risk of CTD and with UCTD[20, 25, 427]. Multiple studies have described physician-reported associations between IFNs and the CTDs, such as correlations with disease flares and therapeutic response, however few studies have explored the relationship between IFN and patient-reported outcome measures (PROMs) in the CTDs[20, 426].

Fatigue is a major complaint in patients with CTD[428, 429]. The cause of fatigue is unclear, however likely multifactorial, originating including among others, the pro-inflammatory state, depression, medication effects and medical co-morbidities[428-430]. The concept of higher levels of IFN leading to worse fatigue was researched by Howard-Tripp et al. (2016) in a cohort of patients with pSS [431]. Remarkably, Howard-Tripp et al. found an inverse relationship between many pro-inflammatory cytokines and fatigue, which includes IFN-γ (p=0.022), and no significant relationship between IFN-α and fatigue[431]. These results support a more complex relationship in IFN and fatigue immunophysiology, and possibly one where the initial inflammatory response is driven by IFN, however followed by regulatory mechanisms comprised of other cytokines that are responsible for the sustained fatigue[431]. Alternatively, the study used a cytometric bead array immunoassay to measure cytokine expression. Prior work suggests that immunoassays are sensitive to the metabolic activities of cells in the blood and may misestimate the expression of IFN[25].

The impact of therapeutic IFN when used as an anti-viral, immunomodulatory and antineoplastic therapy in conditions other than CTD may be extrapolated to understand the impact of IFN on patient's quality of life. In a superiority study that compared IFN-containing versus non-IFN-containing regimens for chronic hepatitis C in elderly patients, IFN-free courses of therapy were found to have substantial superiority in PRO data compared to the IFN-containing regimens[432]. Physical functioning, vitality, physical and emotional well-being, fatigue levels, and activity
and energy were significantly decreased in patients who received IFN, which was seen to resolve within 4 weeks after treatment cessation[432]. Superiority in health-related quality of life has been reported in other studies comparing IFN-free and IFN-containing regimens, including in treatment programs for Hepatitis C, multiple sclerosis and renal cell carcinoma[432-436]. Collectively, this data suggests that IFN has a direct impact on quality of life.

Patient reported outcomes (PROs) reflect patients’ perspectives on their illness, health-related quality of life, and well-being, and can be used in screening, monitoring, or evaluating interventions or to stimulate dialogue between patient and clinician[377]. Evidence suggests that patient-reported symptoms have greater importance on health-related quality of life than disease manifestations captured on physician indices[437, 438]. However, to date, there are limited data pertaining to comparison of PROMs between disease subgroups. Our group (for which I am a co-author) had evaluated the use of patients’ VAS score of global health assessment of disease activity in pre-CTD or so called “At-Risk” group[25]. Although this was not independently predictive of progression to CTD in multivariable analysis, there was a trend to increase in VAS score at baseline in those who progressed versus those who remained At-Risk in univariable analyses. Hence, the scores of PROMs in this group could be important. Lastly, studies on the use of PROMs in UCTD are lacking. Therefore, it would be interesting to compare PROMs between these three disease subgroups to evaluate their relationship to a known biomarker of disease activity, namely IFN-I.

6.1.1 Hypothesis
i. Patient-reported outcomes correlate with IFN Score A and IFN Score B in patients with CTD, UCTD and who are At-Risk of CTD

6.1.2 Objectives
i. To determine whether IFN Score A and IFN Score B correlate with patient-reported outcomes in CTD, UCTD and patients At-Risk of CTD

6.2 Methods

6.2.1 Design and Patient

A cross-sectional study of consecutive three groups of patients: (i) Group 1: CTD; (ii) Group 2: UCTD and (iii) Group 3: At-Risk of CTD was conducted at a single centre in Leeds between July 2017 till March 2019.
Inclusion criteria were (i) adults between 18-80 years; (ii) Group 1 (CTD): fulfilling classification criteria for an established CTD[38, 71, 80, 405, 439] including anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)[440]; or Group 2 (UCTD): ANA positive; signs and symptoms suggestive of CTD but not fulfilling classification criteria for an established CTD[38, 71, 80, 405] or RA[407]; disease duration of at least 12 months and UCTD diagnosis was made by Consultant Rheumatologists; or Group 3 (At-Risk of CTD): ANA positive, ≤1 clinical criterion based on SLICC-12 for SLE[38] and not meeting classification criteria for other CTDs[69, 80, 441] or RA[442]; (3) symptom duration <12 months; (4) glucocorticoid, anti-malarial and immunosuppressive treatment-naïve.

6.2.2 Ethical approval

All individuals provided informed written consent and this research was undertaken in compliance with the Declaration of Helsinki (Chapter 9). The patients’ blood samples used for this study were collected under ethical approval, REC approval 17/YH/0166, National Research Ethics Committee Yorkshire and the Humber – South Yorkshire. All experiments were performed in accordance with the relevant guidelines and regulations. The University of Leeds was contracted with administrative sponsorship.

6.2.3 Clinical data

At a single research visit, a comprehensive clinical assessments were conducted. Data collection included age, gender, ethnicity, history of first or second degree relative(s) with rheumatic and musculoskeletal diseases (RMDs), smoking history, presence of fibromyalgia, treatment including DMARDs and corticosteroids. Active disease was defined according to the impression of the evaluating clinician.

6.2.4 Patient-reported outcome measures (PROMs)

At the study visit, the following PROMs were administered to participants for completion on the day of the visit: VAS pain, VAS morning joint stiffness, VAS arthritis, VAS fatigue, VAS global health, RAND® 36-Item Health Survey version 1.0 (SF-36), 5-level EQ-5D (EQ-5D-5L), Work Productivity and Activity Impairment questionnaire: Specific Health Problem version 2.0 (WPAI:SHP), ICEpop CAPability measure for Adults (ICECAP-A), and the Functional Assessment of Chronic Illness Therapy - Fatigue Scale (FACIT-Fatigue) version 4. Details of these PROMs are summarised in Chapter 3.4.

6.2.5 Laboratory and IFN assays
ANA was tested using immunofluorescence and a panel of nuclear autoantibodies including anti-dsDNA, extractable nuclear antigens (ENA, including Ro52, Ro60, La, Sm, Chromatin, RNP, Ribosomal P, Jo-1, Centromere and Scl-70) using the Bioplex 2200 Immunoassay.

Samples for IFN biomarkers were collected and processed as per Chapter 3.2. IFN Score A and IFN Score B were calculated without the knowledge of the participants’ clinical status.

6.2.6 Statistical analyses

Descriptive statistics were summarised using mean with standard deviation or median with interquartile range for continuous variables (where appropriate) and proportion for categorical variables. Associations between categorical variables were tested by Chi-squared test while for continuous variables, either Student’s t-test or ANOVA followed by pairwise Tukey tests. Correlation between two continuous variables was tested using Spearman’s correlation coefficient.

Due to the scope of this thesis as an MSc, it is not possible for me to analyse risk factors for all the PROMs above individually. Therefore, since fatigue and patients’ perception of their global health were considered to be the most impactful to the patients, risk factors for the two PROMs, FACIT-Fatigue and EQ-VAS were evaluated. Some of the clinical data were missing. Imputation by predicted mean matching was used to estimate these missing data since frequency of missing data were less than 5%. For prediction of FACIT-Fatigue and EQ-VAS, initially univariable analyses using linear regression were performed on each of the plausible predictors. Then after, multivariable analyses were performed using linear regression with backwards elimination with a p-value of <0.25 as a criterion for exclusion from the model.

6.3 Results

6.3.1 Patient characteristics

A total of 279 patients were recruited into the study. Of these, 127 patients had a diagnosis of CTD or Vasculitis, 42 UCTD and 110 At-Risk of CTD. The most common subtype of established CTD was SLE (61.4%) followed by pSS (16.5%), EGPA (6.3%), anti-synthetase syndrome (5.5%), MCTD (4.7%), SSc (4.7%), and APLS (0.8%).

The mean age of patients for CTD and Vasculitis, UCTD and At-Risk of CTD groups were 48.6, 49.8 and 50.6 years respectively. The majority of patients were female,
White and had one ANA specificity. Approximately one in ten patients were smokers or had symptoms of fibromyalgia. Over one third of patients in the CTD group had internal organ involvement compared with 11.9% in the UCTD group and none in the At-Risk group. In most cases, the involvement was limited to only one internal organ i.e. nephritis. Active disease at research assessment was detected in 70.9% of the CTD group and 76.2% of the UCTD group. This corresponded with a modest rate of prednisolone use at 36.2% and 7.1% of the CTD and UCTD groups respectively. Baseline clinical and demographic characteristics are presented in Table 20.

**Table 20** Demographic and clinical features of CTD, UCTD and At-Risk cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CTD N=127</th>
<th>UCTD N=42</th>
<th>At-Risk of CTD N=110</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (range)</td>
<td>48.6 (21, 88)</td>
<td>49.8 (22, 72)</td>
<td>50.6 (18, 89)</td>
</tr>
<tr>
<td>Female : Male</td>
<td>107 : 20</td>
<td>35 : 7</td>
<td>94 : 16</td>
</tr>
<tr>
<td>White ethnicity</td>
<td>76 (59.8%)</td>
<td>31 (75.6%)</td>
<td>76 (69.1%)</td>
</tr>
<tr>
<td>Total ANA specificities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>54 (42.5%)</td>
<td>25 (59.5%)</td>
<td>70 (63.6%)</td>
</tr>
<tr>
<td>Two</td>
<td>26 (20.5%)</td>
<td>9 (21.4%)</td>
<td>35 (31.8%)</td>
</tr>
<tr>
<td>Three or more</td>
<td>31 (24.4%)</td>
<td>3 (7.2%)</td>
<td>5 (4.5%)</td>
</tr>
<tr>
<td>IIF only</td>
<td>16 (12.6%)</td>
<td>5 (11.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Internal organ involvement</td>
<td>59 (46.5%)</td>
<td>5 (11.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Number of internal organs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>38 (29.9%)</td>
<td>5 (11.9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Two or more</td>
<td>21 (16.5%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Fibromyalgia</td>
<td>14 (11%)</td>
<td>5 (11.9%)</td>
<td>9 (8.2%)</td>
</tr>
<tr>
<td>Prednisolone use</td>
<td>46 (36.2%)</td>
<td>3 (7.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>csDMARD(s)</td>
<td>101 (79.5%)</td>
<td>28 (66.7%)</td>
<td>3* (2.7%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>17 (13.4%)</td>
<td>8 (19%)</td>
<td>10 (9.1%)</td>
</tr>
<tr>
<td>Active disease</td>
<td>90 (70.9%)</td>
<td>32 (76.2%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CTD diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLE</td>
<td>78 (61.4%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>pSS</td>
<td>21 (16.5%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>EGPA</td>
<td>8 (6.3%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Anti-synthetase syndrome</td>
<td>7 (5.5%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>MCTD</td>
<td>6 (4.7%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SSc</td>
<td>6 (4.7%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>APLS</td>
<td>1 (0.8%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*csDMARDs prescribed for non-rheumatic disease e.g. psoriasis or Crohn’s colitis.

6.3.2 Completion of PROMs

Across the groups, approximately one in five patients failed to complete the PROM assessments in full (Table 22). The highest completion rate was seen for the VAS PROMs at approximately 1 in 9 patients. The WPAI:SHP (35.4% CTD; 35.7%
UCTD; 34.5% At-Risk) and VAS arthritis (38.6% CTD) were the PROMs with the lowest frequencies of full completion.

**Table 21 Full completion rates of PROMs (sub-grouped)**

<table>
<thead>
<tr>
<th>PROM Instrument</th>
<th>CTD</th>
<th>UCTD</th>
<th>At-Risk of CTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAS pain</td>
<td>111 (87.4%)</td>
<td>41 (97.6%)</td>
<td>83 (75.5%)</td>
</tr>
<tr>
<td>VAS morning joint stiffness</td>
<td>86 (67.7%)</td>
<td>36 (85.4%)</td>
<td>69 (62.7%)</td>
</tr>
<tr>
<td>VAS arthritis</td>
<td>49 (38.6%)</td>
<td>30 (71.4%)</td>
<td>48 (43.6%)</td>
</tr>
<tr>
<td>VAS fatigue</td>
<td>111 (87.4%)</td>
<td>41 (97.6%)</td>
<td>80 (72.7%)</td>
</tr>
<tr>
<td>VAS global health</td>
<td>111 (87.4%)</td>
<td>41 (97.6%)</td>
<td>91 (82.7%)</td>
</tr>
<tr>
<td>SF-36</td>
<td>103 (81.1%)</td>
<td>36 (85.7%)</td>
<td>78 (70.9%)</td>
</tr>
<tr>
<td>EQ-5D-5L</td>
<td>107 (84.3%)</td>
<td>34 (80.9%)</td>
<td>93 (84.5%)</td>
</tr>
<tr>
<td>EQ-VAS</td>
<td>107 (84.3%)</td>
<td>34 (80.9%)</td>
<td>93 (84.5%)</td>
</tr>
<tr>
<td>WPAI:SHP</td>
<td>45 (35.4%)</td>
<td>15 (35.7%)</td>
<td>38 (34.5%)</td>
</tr>
<tr>
<td>ICECAP-A</td>
<td>108 (85.0%)</td>
<td>34 (80.9%)</td>
<td>90 (81.8%)</td>
</tr>
<tr>
<td>FACIT-Fatigue</td>
<td>108 (85.0%)</td>
<td>34 (80.9%)</td>
<td>82 (74.5%)</td>
</tr>
</tbody>
</table>

**Table 21.** Completion rates of the PROMs according to subgroup.

### 6.3.3 Comparison of PROMs between disease subgroup

In general, there are no consistent differences in the various PROMs used between disease subgroup. The PROMs with differences are described in detailed below.

VAS scores were measured in 10cm scale. For VAS morning joint stiffness (EMS), there was a difference between groups, ANOVA F=3.21; p=0.043. Within groups, the only difference was that patients with UCTD had higher VAS-EMS score than At-Risk group; mean difference 1.50 (95% CI 0.10 t0 2.90); p=0.033.

For the General Health item of the SF-36 score, there was a difference between groups, ANOVA F=6.15; p=0.002. Within groups, the only difference was that patients with At-Risk had higher SF-36 General Health scores than the established CTD & Vasculitis group (i.e. the higher the item score the better the perception of general health); mean difference 10.69 (95% CI 3.47 to 17.93); p=0.002.

For the FACIT-Fatigue score (ranges between 0-52), the higher the score, the less fatigue reported by the patients. There was a difference between groups, ANOVA F=4.08; p=0.018. Within groups, patients with At-Risk had higher FACIT-Fatigue score than the established CTD & Vasculitis group; mean difference 5.44 (95% CI 0.74 to 10.13); p=0.019.
6.3.4 IFN status between the groups

IFN Score A differed between the groups (ANOVA F=42.31; p<0.001). The gene expression was higher in the CTD and Vasculitis in relation to the At-Risk groups; fold difference (FD) 1.78 (95% CI 1.07 to 2.47); p=0.001. There were no differences in gene expression between CTD and Vasculitis and UCTD groups; p=0.111 and At-Risk and UCTD; p=0.735 (Figure 10-A).

In contrast, there was no difference in IFN Score B between the groups overall (ANOVA F=0.986; p=0.652) (Figure 10-B).

Figure 10 Comparison of IFN scores between patients with CTD & Vasculitis, UCTD and At-Risk

Figure 10. Graph A) IFN Score A differed between groups. There was a higher expression of IFN Score A in CTD & Vasculitis in relation to At-Risk groups. Graph B) IFN Score B did not differ between groups. Error bars denote upper and lower limit of the mean. At-CTD; autoimmune connective tissue disease.

6.3.5 Correlations between PROMs and IFN scores between the groups

Correlations between PROM scores and IFN scores were performed in observed data using reflected delta Ct (ΔCt) so that higher scores represented greater expression for the latter. In general, there was no consistent correlation between IFN scores and the various PROMs evaluated in this study based on disease subgroups. Only variables with that were correlated with IFN scores are described below.

In the UCTD group, there were moderate correlations between IFN Score A and SF-36 Energy Function (n=36, Spearman’s r=0.353, p=0.034), IFN Score A and SF-36 Emotional well-being (n=36, Spearman’s r=0.394, p=0.017), IFN Score A and SF-36 Social Functioning (n=36, Spearman’s r=0.337, p=0.044), and IFN Score A and ICECAP (n=34, Spearman’s r=0.370, p=0.031). and IFN Score A and EQ-VAS (n=34, Spearman’s r=0.363, p=0.035). Next, there were also moderate correlations between both IFN scores and the SF-36 General health and well-being; IFN Score A
(n=37, Spearman’s \( r=0.397, p=0.015 \)) and IFN Score B (n=37, Spearman’s \( r=0.399, p=0.014 \)) respectively.

In the At-Risk group, IFN Score B was weakly correlated the WPAI:SH percent activity impairment scores (n=62, Spearman’s \( r=0.269, p=0.035 \)). Patients who had impairment in the EQ-5D-5L Self-care scores had higher IFN Score B expression compared to those without impairment; FD 0.74 (95% CI 0.55 to 0.92); \( p=0.044 \).

In the established CTD and Vasculitis group, IFN Score A was only weakly correlated with VAS Arthritis (n=51, Spearman’s \( r=-0.330, p=0.028 \)) and SF-36 physical functioning scores (n=112, Spearman’s \( r=0.208, p=0.028 \)).

### 6.3.6 Multivariable analysis of predictors of FACIT-FATIGUE

FACIT-Fatigue scores are inversely representative of the level of fatigue i.e. the higher the score, the less fatigue and better quality of life reported by the patients. In imputed univariable analyses, putative predictors that were associated with worse (i.e. lower) FACIT-Fatigue scores were female, presence of fibromyalgia, concomitant DMARDs, current smoker, current features of active disease and disease subgroups. Older age was associated with better (i.e. higher) FACIT-Fatigue score.

In multivariable analysis, predictors of worse (lower) FACIT-Fatigue scores were female, presence of fibromyalgia, current smoker and current features of active disease. Older age and higher IFN Score A expression was associated with better (higher) FACIT-Fatigue score (Table 22).

### Table 22 Linear regression for predictors of FACIT-FATIGUE score

<table>
<thead>
<tr>
<th>Baseline predictors</th>
<th>Univariable UCEB (95% CI), p value</th>
<th>Multivariable UCEB (95% CI), p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.154 (0.03, 0.27), 0.010</td>
<td>0.132 (0.01, 0.24), 0.022</td>
</tr>
<tr>
<td>Female</td>
<td>-6.386 (-11.41, -1.36), 0.013</td>
<td>-5.790 (-10.5, -1.06), 0.017</td>
</tr>
<tr>
<td>White ethnicity</td>
<td>2.322 (-1.76, 6.41), 0.265</td>
<td>Excluded</td>
</tr>
<tr>
<td>Internal organ involvement</td>
<td>0.406 (-3.85, 4.66), 0.851</td>
<td>Excluded</td>
</tr>
<tr>
<td>No. of ANA-specificities</td>
<td>-0.372 (-2.00, 1.26), 0.655</td>
<td>Excluded</td>
</tr>
<tr>
<td>Fibromyalgia</td>
<td>-14.600 (-20.05, -9.14), &lt;0.001</td>
<td>-10.873 (-16.38, -5.35), &lt;0.001</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>-4.182 (-9.07, 0.713), 0.094</td>
<td>-2.791 (-7.31, 1.73), 0.225</td>
</tr>
<tr>
<td>csDMARD use</td>
<td>-4.162 (-7.78, -0.53), 0.025</td>
<td>Excluded</td>
</tr>
<tr>
<td>Current smoker</td>
<td>-9.243 (-14.19, -4.29), &lt;0.001</td>
<td>-5.602 (-10.34, -0.86), 0.021</td>
</tr>
<tr>
<td>Active disease</td>
<td>-7.759 (-11.30, -4.21), &lt;0.001</td>
<td>-6.996 (-10.55, -3.44), &lt;0.001</td>
</tr>
</tbody>
</table>
Table 22. FACIT-Fatigue. *Analysis was based on reflected ΔCt. Higher values indicate higher gene expression to give positive values for UCEB. UCEB, unstandardized co-efficient B.

6.3.7 Multivariable analyses of predictors of EQ-VAS

EQ-VAS scores were scored on a 100mm scale and are related to the patients’ subjective perception of their health state i.e. the higher the score, the better they felt about their health. In imputed univariable analyses, putative predictors that were associated with lower (worse) EQ-VAS scores were female, presence of fibromyalgia, concomitant prednisolone, concomitant DMARDs, current smoker and current features of active disease.

In multivariable analysis, predictors of lower (worse) EQ-VAS scores were female, presence of fibromyalgia, current features of active disease and higher IFN Score B. Higher IFN Score A was associated with higher (better) EQ-VAS scores (Table 23).

Table 23 Linear regression for predictors of EQ-VAS

<table>
<thead>
<tr>
<th>Baseline predictors</th>
<th>Univariable UCEB (95% CI), p value</th>
<th>Multivariable UCEB (95% CI), p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.980 (-0.09, 0.27), 0.304</td>
<td>Excluded</td>
</tr>
<tr>
<td>Female</td>
<td>-8.041 (-16.08, -0.00), 0.050</td>
<td>-8.503 (-16.40, -0.60), 0.035</td>
</tr>
<tr>
<td>White ethnicity</td>
<td>4.350 (-2.08, 10.78), 0.185</td>
<td>3.674 (-2.79, 10.14), 0.264</td>
</tr>
<tr>
<td>Internal organ involvement</td>
<td>-4.850 (-11.62, 1.92), 0.160</td>
<td>Excluded</td>
</tr>
<tr>
<td>No. of positive ANA-specificities</td>
<td>-0.041 (-2.64, 2.56), 0.975</td>
<td>Excluded</td>
</tr>
<tr>
<td>Fibromyalgia</td>
<td>-20.770 (-29.53, -11.89), &lt;0.001</td>
<td>-16.829 (-26.10, -7.54), &lt;0.001</td>
</tr>
<tr>
<td>Prednisolone use</td>
<td>-10.84 (-18.64, -3.04), 0.007</td>
<td>-10.503 (-18.66, -2.33), 0.012</td>
</tr>
<tr>
<td>DMARD use</td>
<td>-7.009 (-12.67, -1.33), 0.016</td>
<td>Excluded</td>
</tr>
<tr>
<td>Current smoker</td>
<td>-4.580 (-12.66, 3.49), 0.265</td>
<td>Excluded</td>
</tr>
<tr>
<td>Active disease</td>
<td>-9.680 (-15.32, -4.03), 0.001</td>
<td>-10.353 (-17.74, -2.96), 0.006</td>
</tr>
<tr>
<td>IFN Score A (-ΔCt)</td>
<td>0.628 (-1.064, 2.320), 0.465</td>
<td>2.757 (0.533, 4.754), 0.014</td>
</tr>
<tr>
<td>IFN Score B (-ΔCt)</td>
<td>-1.183 (-3.979, 1.614), 0.406</td>
<td>-3.374 (-6.608, -0.140), 0.041</td>
</tr>
<tr>
<td>At-Risk v UCTD v established CTD</td>
<td>-2.814 (-5.88, 0.24), 0.071</td>
<td>1.604 (-2.54, 5.74), 0.446</td>
</tr>
</tbody>
</table>
Table 23. EQ-VAS. *Analysis was made based on reflected ΔCt. Higher values indicate higher gene expression to give positive values for UCEB. UCEB, unstandardized co-efficient B.

6.4 Discussion

In this study, no consistent relationship was found between the IFN Scores and various PROMs. However in a subgroup of patients with UCTD, IFN Score A was moderately correlated with components of the SF-36, the ICECAP-A and the EQ-VAS. In At-Risk individuals, weak correlations were detected between IFN Score B and WPAI:SH percent activity impairment scores and EQ-5D-5L self-care scores. In CTD and Vasculitis patients, the only association found was a weak correlation between IFN Score A and VAS arthritis and the SF-36 physical function. Therefore, it appears that IFN Scores may be unreliably correlated with PROMs. However, when these assays were included with other clinical variables, IFN Score B was independently predictive of worse patient perception of health based on EQ-VAS. In contrast, higher IFN Score A was independently predictive of lower fatigue based on FACIT-Fatigue and better patient perception of health based on EQ-VAS.

This was the first study that compared PROMs between patients with CTD and Vasculitis, UCTD and At-Risk. As expected, patients with CTD and Vasculitis had lower FACIT-Fatigue scores (i.e. more fatigue) compared to individuals At-Risk. Moreover, patients within the CTD and Vasculitis subgroup had worse perception of health based on lower SF-36 General health scores than individuals At-Risk. This agreement could be representative of the burden of disease in established CTD.

In this study, IFN Score A was moderately correlated with a number PROMs in the UCTD group compared to established CTD and At-Risk individuals. The observation that an immunological abnormality could correlate with a patient’s perception of health in this cohort is intriguing. Therefore this study highlights that there are various unanswered questions in the field of UCTD research in relation to the relationship between disease features, immunological disturbances and PROMs, which could be the focus of future research.

In a previous study by Howard-Tripp and colleagues, they found an inverse relationship between IFN-γ and fatigue in pSS, while IFN-α demonstrated no significant relationship[431]. However our findings differed with this in that the higher the expression of IFN Score A (i.e. comprised of ISGs that were responsive to IFN-I), the lower the level of fatigue, and the better the EQ-VAS scores as reported by patients. Furthermore I also found that higher IFN Score B was predictive of worse patient perception of general health score as based on EQ-VAS. One potential
explanation for these observations could be that IFN-I might have a short-lived role in early onset of fatigue, whereas in chronic fatigue this could be mediated by other different cytokines other than IFN[431]. Indeed, as described in Chapter 3 and Chapter 5, IFN Score B comprised a set of ISGs which were mostly responsive to IFN-I, IFN-III and other inflammatory cytokines which have yet to be determined. Another explanation of the observed relationship between IFN and PROMs could be the failure to return to baseline levels of health following active disease. With regards to the inverse relationship between IFN-γ and fatigue, I was not able to confirm this as reported by Howard-Tripp et al. as IFN-γ is a IFN-II which was not evaluated in my study.

The limitations of this study is that not all PROMs were completed by participants. However our incompletion rate was still lower compared to other studies using PROMs, which ranged between 0.3% - 96.4%[434, 436, 443]. Secondly, psychosocial factors beyond immunological processes may have influenced participant responses to the PROMs (i.e. depression). Lastly, since this study was exploratory, various PROMs were administered to participants since it was not known which one was predominant. Hence the collection of questionnaires was quite arduous to patients and could have influenced responses.

6.5 Conclusion

In conclusion, in this exploratory study, we have demonstrated moderate correlation between IFN Score A and several PROMs in UCTD, but an inconsistent relationship between PROMs and At-Risk and established CTD. Our data also suggests that the relationship between IFN activity and PROM was not straightforward, and that other clinical risk factors and potentially other cytokines should be taken into account with respect to PROMs. These findings may be relevant to the subgrouping and assessment of patients for therapy targeting the IFN system.

6.6 Key messages

- Correlation between IFN Scores and PROMs varied widely among diagnoses of CTDs, with the strongest correlation found in patients with UCTD.
- Higher IFN Score B was independently predictive of poor perception of general health based on EQ-VAS.
- The relationship between IFN activity and PROM is not straightforward, and other clinical risk factors and potentially other cytokines should be taken into account with respect to PROMs.
Chapter 7 Discussion

7.1 General discussion of results

The data presented in this thesis highlights the potential applications of an IFN assay in the diagnosis and management of patients with CTD. Given the heterogeneity in the genetics, aetiology, and clinical phenotype of these conditions, it is likely that an IFN assay will not have universal application as a biomarker for prognostic, diagnostic, predictive or surrogate purposes in all individuals within the CTD spectrum. This programme of research was designed to explore the potential applications for an IFN assay such that clinical goals could be achieved for the purpose of improved outcomes for patients with CTD. The programme was focussed on three key clinical areas: (i) the prediction of flares and glucocorticoid requirements in patients with SLE; (ii) the use of IFN assays in distinguishing patients with classifiable CTD immunologically from a cohort of patients labelled as UCTD; and (iii) the relationship of the IFN assays with PROMs in patients At-Risk, with UCTD and with established CTD. By distinguishing the association of the IFN assays in the identification of classifiable CTD from a cohort of patients initially labelled as UCTD; the predictive ability of the IFN assays to identify flares and glucocorticoid requirements in patients with SLE; and the relationship between IFN assays and PROMs across the range of CTD diagnoses, the unifying hypothesis of this thesis which was that the use of interferon biomarkers will aid the prognosis and management of autoimmune connective tissue diseases has been addressed.

Disease identification, prognosis and prevention is often a focus of research and management in rheumatology. However this will continue to operate with uncertainty while the immunopathogenesis of CTD is poorly understood and when reliable prognostic, diagnostic, predictive and surrogate biomarkers remain unidentified[18]. IFNs are essential mediators of autoimmunity but their role is poorly defined. This could be due to variability in pathogenic mechanisms among individuals with autoimmunity, different time points in pathogenic action of IFN within patients and diseases, or variation in sensitivity and specificity of methods for quantifying IFN status and activity. Previous work in this area has sought to improve methods of IFN quantification. Earlier publications from our institution have reported work on two continuous ISG expression scores (IFN Score A and IFN Score B) and a flow cytometric cell specific marker memory B cell tetherin[24, 373]. The ISG expression scores have been shown in combination to be better at identifying clinically meaningful differences in IFN status within a range of autoimmune rheumatic diseases[25]. This includes prediction of progression to CTD in an At-Risk
population and disease severity and activity in an SLE cohort [24, 352]. Memory B cell tetherin levels have been shown to be related to SLE diagnosis, disease severity and flares [352, 373]. Through examination of the performance of these IFN assays in other clinical scenarios, as I have shown, we find that (1) IFN Score A, IFN Score B and memory B cell tetherin may be predictive of future flare and glucocorticoid requirements in an SLE population; (2) that IFN Score A and Score B may identify patients who are eligible for re-classification into established CTD after an initial diagnosis of UCTD; and lastly (3) that IFN Score A has moderate correlation with several PROMs in UCTD, but an inconsistent relationship between PROMs and At-Risk and established CTD. These results have confirmed the importance of IFN in the disease activity and impact of CTD. The findings have the power to inform future research in IFN and the understanding of its role in autoimmunity.

The inconsistent relationship between the IFN Scores and various PROMs was an intriguing finding in this work. As explained, PROM results may be influenced by other clinical risk factors and potentially other cytokines, as well as the failure of the patient to return to baseline levels of health following active disease. Future clinical studies may therefore be better positioned to examine the relationship between IFN and PROMs through prospective methodology, instead of relying on cross-sectional sampling. Exploratory studies on the cytokine milieu during different phases of CTD development could also be of value to broaden our understanding of CTD disease pathogenesis. Composite biomarker panels are an option if heterogeneity in cytokine activity is found across the CTD spectrum.

7.2 Impact of research

This programme of research has importance from an international perspective in terms of improving the diagnosis and management of CTD. It has explored specific clinical applications for IFN score A, IFN Score B and memory B cell tetherin, and although validation studies are required before recommendations can be drawn, these studies support the candidacy of these three IFN assays as future predictive and prognostic biomarkers in CTD. This research also affirms the central role of IFN in disease pathogenesis in CTD. The lack of strong correlation between IFN and multiple PROMs informs future studies that may use these tools as activity or response measures.

7.3 Future perspectives

The available data clearly demonstrate that there is a potential for the three IFN assays to be biomarkers in the prediction and prognosis of clinical aspects of CTD.
However in some patients, the scores were not predictive or prognostic and this is likely due to heterogeneity in cytokine activity between and within individuals and disease subtypes. Although the results are promising, the studies in this body of work were small and not powered or designed as validation studies. In terms of this, a validation study on the prognostic ability of the scores is currently in progress. The Defining Interferon in autoimmune Connective Tissue Diseases study (DEFINITION) is a powered, longitudinal study that will examine the parameters tested in this work over multiple time points and in cases of At-Risk, UCTD and established CTD. At the time of writing, the study had recruited 333 participants and is expected to release a preliminary report in 2020.

### 7.4 Conclusions

Three IFN assays were examined with respect to three clinical scenarios in this program of work. IFN Score A, IFN Score B and memory B cell tetherin was found to be significantly associated with activity and glucocorticoid requirements in SLE. IFN Score A and Score B was detected to identify patients who were eligible for re-classification into established CTD after an initial diagnosis of UCTD. Correlation between IFN Scores and PROMs varied widely among diagnoses of CTDs, with the strongest correlation found in patients with UCTD. These findings together suggest that there may be a role for IFN assays in the prognosis and management of CTDs. This assumption will be addressed through the validation work currently in progress in DEFINITION.
Chapter 8 References

15. Boyle, D.J. How a Rheumatologist Thinks: Cognition and Diagnostic Errors in Rheumatology. The Rheumatologist 2010; Available from:


114. Mosca, M., R. Neri, and S. Bombardieri, *Undifferentiated connective tissue diseases (UCTD): a review of the literature and a proposal for


130. Swaak, T. and R. Smeenk, Detection of anti-dsDNA as a diagnostic tool: a prospective study in 441 non-systemic lupus erythematosus


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Chapter 9 Appendices

9.1 Health Research Authority Letter for RR10/9608

Health Research Authority
Yorkshire & The Humber - Leeds East Research Ethics Committee
Jarrow Business Centre
Roding Mill Road
Jarrow
NE32 3DT
Tel: 0207 1048 028

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

20 February 2017
Mr James Goulding
MSK Trial Management Lead
LRMM, Level 2
Chapel Allerton Hospital
Chapeltown Road
Leeds, LS7 4SA

Dear Mr Goulding

Study title: Leeds Teaching Hospitals Connective Tissue Disease and Vasculitis Cohort Cross-sectional and Longitudinal Clinical & Basic Science Evaluation (CONVAS)
REC reference: 10/H1365/88
Protocol number: RR10/9608
Amendment number: Substantial Amendment v6.0, 08/02/17
Amendment date: 09 February 2017
IRAS project ID: 00762

9.2 Health Research Authority Letter for 17/YH/0166

Health Research Authority

Dr Edward M Vital
Leeds Institute for Rheumatic and Musculoskeletal Medicine
2nd Floor, Chapel Allerton Hospital
Leeds
LS7 4SA

05 July 2017

Dear Dr Vital

Study title: DEFining Interferon mediated autoimmune conditions (DEFINITION)
IRAS project ID: 220254
Protocol number: ESR-16-1186
REC reference: 17/YH/0166
Sponsor: Faculty of Medicine and Health Research Office

I am pleased to confirm that HRA Approval has been given for the above referenced study, on the basis described in the application form, protocol, supporting documentation and any clarifications noted in this letter.