Biomarker profiling in rheumatoid arthritis: an investigation of markers of immune dysfunction and their relationship to disease phenotype

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ABSTRACT

Rheumatoid arthritis is an autoimmune disease in which various mediators of immune function play a vital role in the pathological process, influencing its development, progression and severity.

The aim of this study was to investigate the relationship between markers of immune dysfunction and the disease process. One of the main objectives was to identify key biomarkers involved in early RA (≤1 year) and their association with disease measures and development of established disease (≥5 years). Another objective was to determine the relationship between biomarkers of the peripheral circulation and the synovial joint, and to investigate the association of markers from both environments with disease activity and severity. The influence of smoking on biomarker expression and its relationship with disease activity and severity was also investigated.

Using ELISAs and bead-based multiplex assays, 41 mediators were analysed in the sera of early RA (n=86), established RA (n=77), OA (n=20), PsA (n=34) and ReA (n=26) patients as well as in paired sera and synovial fluid of 52 RA patients. Data were analysed using a variety of statistical methods including hierarchical clustering, principal component analysis, regression analysis and ROC analysis.

This study demonstrated the involvement of numerous interleukins, matrix remodelling mediators and pro-inflammatory mediators in the disease process in both early and established RA, and enabled its differentiation from other arthritides. In addition, biomarkers from both the synovial fluid and the peripheral circulation were found to be

associated with disease activity and severity. Cigarette smoking influenced biomarker expression in both the systemic and local environment, and a smoking associated biomarker profile was associated with worse disease.

This study reveals that RA is an extremely heterogeneous disease involving many mediators in a complex network which may include various pathological routes. Many of these biomarkers could potentially become new targets for disease intervention.

CONTENTS

Declaration……..I

Acknowledgements..…….…...III

Abstract……..……....…….IV

Contents……...….VI

CHAPTER 1. INTRODUCTION……..….1

1.1.1 Arthritis Overview….……...…….2

1.1.2 Rheumatoid arthritis, an introduction……2

1.1.3 History of RA……...…….3

1.1.4 Classification of RA…….……4

1.1.5 Epidemiological morbidity: prevalence and incidence rates…….6

1.1.6 Atieology……..…….7

1.1.6.1 Genetic factors……....……8

1.1.6.2 Environmental factors……8

1.1.6.2.1 Pathogens……...…….…….……8

1.1.6.2.2 Other factors……9

1.1.7 Mortality and Co-Morbidity……10

1.1.8 Physiology of RA……11

1.1.9 Pathophysiology of RA……...……15

1.1.10 RA disease features……..…..……20

1.1.10.1 Synovial Joints…….……..……20

1.1.10.2 Subcutaneous nodules……23

1.1.10.3 Autoantibodies……24

1.1.10.3.1 Rheumatoid Factor……..……25

1.1.10.3.2 Anti-cyclic citrullinated proteins…….……26

1.1.11 Other Arthritides……29

1.1.11.1 Reactive Arthritis……...29

1.1.11.2 Psoriatic arthritis…….…33

1.1.11.3 Osteoarthritis……..……36

1.1.12 RA Treatment……41

1.1.12.1 NSAID treatment……41

1.1.12.2 DMARD treatment…….……43

1.1.12.3 Biologic therapy…….……44

1.2.1 RA genetics…….……47

1.2.1.1 Identification of the genetic influence…….…….……47

1.2.1.2 Association of the HLA genes with RA…….……..48

1.2.1.3 Association of the HLA genes with RA prevalence…….....51

1.2.1.4 Linkage disequilibrium and HLA haplotypes……..52

1.2.1.5 Other RA genetic influences……...…..……53

1.3.1 Cigarette smoking……....……55

1.3.1.1 Association between cigarette smoking and RA susceptibility……55

1.3.1.2 Association between cigarette smoking and RA severity……..…..56

1.3.1.3 Association between cigarette smoking and RA features……...…….58

1.3.1.4 Association between cigarette smoking and autoantibodies…….…...58

1.3.1.5 Association between cigarette smoking duration and frequency and RA...…..……59

1.3.2 A gene-environment interaction……..……62

1.3.2.1 A gene-environment interaction for RA susceptibility……62

1.3.2.2 A gene-environment interaction for RA progression……..…….……63

1.4 Biomarkers…….……66

1.4.1 Biomarker profiling RA disease activity and severity…..……..70

1.4.2 Biomarker profiling for RA disease subtypes……...…….…….73

1.4.3 Biomarker profiling pre-development and onset of RA disease...……..…74

1.4.4 Comparative biomarker profiling between RA disease and different pathologies….75

1.4.5 RA biomarker profiling between the periphery and the synovial joint……...79

1.4.6 Cigarette smoking and biomarker profiling……81

1.4.7 RA drug treatment and biomarker profiling……84

CHAPTER 2. AIMS AND OBJECTIVES……...……89

2.1 Aims……...90

2.2 Objectives……...…90

2.2.1 Early and established RA……....90

2.2.2 Paired RA sera and synovial fluid……..……...……..92

2.2.3 RA and other arthritic diseases…….……...…93

CHAPTER 3. PATIENTS AND METHODOLOGY…….….94

3.1 Patients……...……95

3.1.1 Patient cohorts…….……95

3.1.1.1 Selection criteria and consent……...……95

3.1.1.2 Population cohorts' major characteristics and features……96

3.1.2 Smoking history and classification……..……97

3.1.3 RA measures of disease……...98

3.1.3.1 Markers of Inflammation……..99

3.1.3.2 Markers of Pain……..105

3.1.3.3 Markers of Damage……106

3.1.3.4 Markers of Function……...107

3.1.4 Patient drug therapy regimes……..……..……108

3.2 Methodology……109

3.2.1 Patient sample collection…….……..110

3.2.1.1 Blood collection and serum isolation……..……...…110

3.2.1.2 Synovial fluid extraction……..……..111

3.2.2 Biomarkers investigated and their quantification..…..……..111

3.2.2.1.1 Sandwich based Enzyme Linked Immunosorbance Assay (ELISA)…..…...…..112

3.2.2.1.2 Duoset ELISA methodology……...114

3.2.2.1.3 Quantikine ELISA methodology for the measurement of Endostatin……....116

3.2.2.2 The Muliplex bead based X-multianalyte profiling (-MAP) principle…….….117

3.2.3 Statistical software and data analysis……121

3.2.3.1 Principal Component analysis biomarker profiling……122

3.2.3.2 Genesis hierarchical clustering biomarker profiling…...……...……123

CHAPTER 4. RESULTS AND DISCUSSION…….……125

4.1 BIOMARKER PROFILING IN EARLY AND ESTABLISHED RA……...……126

4.1.1 Relationship of RA features and characteristics……126

4.1.2 Early RA……...…129

4.1.2.1.1 Relationship of RA disease features and patient characteristics with disease activity and severity in early RA……129

4.1.2.1.2 Influence of cigarette smoking……130

4.1.2.1.3 Influence of DMARD treatment……..……134

4.1.2.2 Biomarker profiling in early RA……..……136

4.1.2.2.1.1 Early RA Hierarchical clustering biomarker profiling……..…136

4.1.2.2.1.2 Association with early RA disease activity and severity…….……138

4.1.2.2.1.3 Association with cigarette smoking……..…142

4.1.2.2.1.4 Early RA Hierarchical clustering results summary……...…144

4.1.2.2.2.1 Early RA Principal Component Analysis biomarker profiling.……146

4.1.2.2.2.2 Association with early RA disease activity and severity…….147

4.1.2.2.2.3 Association with cigarette smoking and DMARD treatment……...153

4.1.2.2.2.4 Early RA Principal Component Analysis results summary……..…153

4.1.2.2.3 Composite Biomarker Profiles……154

4.1.2.2.4 Early RA Overall Summary……155

4.1.3 Biomarker profiling for RA prognosis……..158

4.1.3.1.1 Hierarchical clustering prognostic biomarker profiling……..158

4.1.3.1.2 Principal component analysis prognostic biomarker profiling……160

4.1.3.2 Prognostic biomarker profiling for disease remission……162

4.1.3.3 Summary……164

4.1.4 Established RA……165

4.1.4.1.1 Relationship of RA disease features and patient characteristics with disease activity and severity in established RA……..……165

4.1.4.1.2 Influence of cigarette smoking……166

4.1.4.1.3 Influence of DMARD treatment……,,……168

4.1.4.2 Biomarker profiling in established RA……..……169

4.1.4.2.1.1 Established RA Hierarchical clustering biomarker profiling……...……169

4.1.4.2.1.2 Association with established RA disease activity and severity……171

4.1.4.2.1.3 Association with cigarette smoking……..…177

4.1.4.2.1.4 Established RA Hierarchical clustering results summary……179

4.1.4.2.2.1 Established RA Principal Component Analysis biomarker profiling.....179

4.1.4.2.2.2 Association with established RA disease activity and severity……..…..…180

4.1.4.2.2.3 Established RA Principal Component Analysis biomarker profiling results summary……..184

4.1.4.3 Composite Biomarker Profiles……...……185

4.1.4.4 Established RA Overall Summary……..……187

4.1.5 Biomarker profiling RA disease progression ……...……189

4.1.5.1 Association of mediator expression changes and changes in disease activity and severity……...…..…192

4.1.5.2 Association of mediator changes with cigarette smoking & DMARD treatment..196

4.1.5.3 Summary ……...…197

4.2 COMPARISON OF BIOMARKERS IN THE PERIPHERAL CIRCULATION AND THE SYNOVIAL FLUID IN RA ……..……..…….198

4.2.1 Comparison of synovial joint and peripheral circulation biomarker profiles for disease activity and severity ……...…...…...…...…...…...…...…...…...…...……200

4.2.2 Comparison of synovial joint and peripheral circulation biomarker profiles for joint damage and disability ……...……..…202

4.2.3 Association with cigarette smoking……..……204

4.2.4 Association with DMARD treatment……205

4.2.5 Summary……...……206

4.3 RA BIOMARKER PROFILING IN COMPARISON WITH OTHER ARTHRITIDES……208

4.3.1 Arthritides differentiation……..…209

4.3.2 Disease activity & severity biomarker profiling between the different arthritides...215

4.3.3 Summary……..…….222

4.4 Conclusion……....223

4.5 Limitations of the investigations….....224

4.6 Further suggested work……....226

CHAPTER 5. REFERENCES……228

CHAPTER 6. APPENDIX……...……261

CHAPTER 1

INTRODUCTION

1.1.1 Arthritis Overview

Arthritis originates from the Greek word *arthro-* meaning joint, and *–itis* meaning inflammation. It is a group of damaging musculoskeletal disorders affecting the synovial joints through inflammation. There are over 100 conditions of arthritis which include reactive arthritis (ReA), gout, Lyme disease and numerous others. This research focuses primarily on rheumatoid arthritis (RA). However, investigation into reactive arthritis (ReA), psoriatic arthritis (PsA) and osteoarthritis (OA) were also undertaken to make comparisons between different arthritic diseases and RA.

1.1.2 Rheumatoid arthritis, an introduction

Rheumatoid arthritis is an autoimmune disease, where the immune system is in dysfunction and initiates an inappropriate immune response against components of the host body which is termed as "self". This gives rise to the attack of its own targeted cells or tissues which are normally present in their body as it perceives it as a '"non-self" or foreign antigen. RA is a systemic disease which affects many tissues and organs but primarily affects the synovial joints. RA currently has no known cure, however the disease is managed with various drug therapies which will be discussed later (Section 1.1.12 RA Treatment). RA can affect both males and females of all ages and ethnicity. It predominantly occurs in individuals 40-50 years of age, particularly in females. The precise aetiology of the disease is still unknown, though a multi-factorial combination of both environmental and genetic factors is considered to be integral to the onset of RA.

Immune function in RA disease is thought to be highly dysregulated, involving a persistent and excessive expression of immunomodulatory and inflammatory mediators. This results in chronic inflammation which leads to the destruction of the synovial joints in a symmetrical manner. This is a distinguishing characteristic of the disease. The majority of RA cases progressively become more severe over time, which ultimately leads to disability and functional impairment. The disease is said to be extremely heterogeneous in terms of developmental course, disease characteristics, range of outcomes (mild with limited bone damage to severe with deformity, disability and premature mortality), and therapeutic responses, with the majority of cases becoming progressively more severe over time (van der Pouw Kraan *et al.*, 2003). Remission of the disease can occur in rare cases, but disability and deformity of the disease is however permanent. Therefore the inflammatory response is critical to the pathogenesis of RA, as well as its severity and progression.

The specific pathologic mechanisms leading to the onset and development of RA have yet to be understood. Therefore, the role of inflammatory mediators and their complex interaction network is crucial to further understanding of the disease.

1.1.3 History of RA

The presence of RA was first observed in the skeletal remains of Native American Indians dated to 8000 BC, and 4500 BC, in Tennessee (Rothschild and Woods, 1990, Aceves-Avila *et al.*, 2001). Other evidence of RA was discovered (Aceves-Avila *et al.*, 2001), in pre-historic individuals living in 500BC in India, and 100BC in Italy. In both instances, descriptions of the disease by its characteristic deformities and symmetrical arthritis were observed. RA may also have been observed in the late 15th Century where Flemish artists

illustrated swelling and deformity of the metacarpophalangeal (MCP) joints of their subjects' hands. In 1578 the first distinction of RA was identified in Mexico (Aceves-Avila *et al.*, 2001). In 1676 a joint disease was described by characterizing its chronicity and deformities (Aceves-Avila *et al.*, 2001). In 1800, Dr Landre-Beauvias published the first definitive description of RA (Landré-Beauvais, 2001). Finally, in 1859, Sir Alfred Garrod documented a case in an individual and gave the disease its current name of Rheumatoid arthritis.

1.1.4 RA Classification

Since the first recording of RA, the definition of the disease is now more detailed and specific compared to Garrod's initial description. Until 2009, the accepted classification of RA was the 1987 American College of Rheumatology (ACR) revised criteria (Arnett *et al.*, 1988), where a minimum of 4 out of 7 criteria must be met to be diagnosed as Rheumatoid arthritis (Table 1.1.4a).

Table 1.1.4a: The 1987 American College of Rheumatology (ACR) revised criteria set for the classification of rheumatoid arthritis (RA).

Criteria	Qualifying parameters
1. Morning stiffness	Duration > 1 hour before maximal improvement for 6 weeks
2. Arthritis of > 3 joints*	Soft tissue swelling over 6 weeks determined by a physician
3. Arthritis of hand joints	Swelling of the wrist, PIP or MCP joints over 6 weeks
4. Symmetrical arthritis	Same joints affected on both sides of the body over 6 weeks
5. Rheumatoid nodules	Subcutaneous nodules determined by a physician
6. Rheumatoid factor	Positive by a method where 95% of normal control subjects are negative
7. Radiographic changes	Bone erosion or decalcification observed on posteroanterior hand and wrist radiographs

* Possible joints: left or right PIP (proximal interphalangeal), MCP (metacarpophalangeal), wrist, elbow, knee, ankle and MTP (metatarsophalangeal) joints. Table reproduced from (Arnett *et al.*, 1988).

In 2010 a new classification criteria for RA was set (Table 1.1.4b) (Aletaha *et al.*, 2010), which focuses on early disease characteristics that are associated with chronic or erosive RA, rather than defining it using established disease charactersitics. This system is based on an algorithm scoring system, where individuals must score ≥6 out of 10, with at least 1 "B" and 1 "C" criteria to be defined for the presence of definite RA. This new RA classification criteria can confirm early unconfirmed RA diagnoses, and thus effective drug therapy can be administered at an earlier stage of their disease.

Table 1.1.4b: The 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) criteria for RA.

The 2010 ACR/EULAR criteria for RA	Score
Target population, patients who:	
1. Have at least 1 joint with definite clinical synovitis	
2. with synovitis not explained by another disease	
Classification criteria for RA (score-based algorithm)	
A. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without large joint involvement)	2
4-10 small joint (with or without large joint involvement)	3
>10 joints (with at least 1 small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF and negative anti-CCP	0
Low-positive RF or low positive anti-CCP	2
High-positive RF or high-positive anti-CCP	3
C. Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
>6 weeks	1

Reproduced from (Aletaha *et al.*, 2010).

1.1.5 Epidemiological morbidity: prevalence and incidence rates

Rheumatoid arthritis can affect both males and females of any age, although it predominantly occurs between the ages of 40 to 50 years old. The disease is also more prevalent in females than in males, with a 3:1 ratio (Majithia and Geraci, 2007). Females also exhibit a more severe and progressive disease along with a worse long-term outcome. This appears to be increased further in female smokers (Criswell *et al.*, 2002). The reason for females commonly having worse disease than males is unknown, although it has led to the suggestion that sex hormones may be involved (Da Silva and Hall, 1992).

It is estimated that up to 1% of the world population is affected with RA. Although the disease can affect any ethnicity, the prevalence rate can differ quite significantly between some populations. A few Native American tribes have a 5-6% higher prevalence rate of RA, such as the Pima Indians (5.3%), and the Chippewa Indians (6.8-7.1%) (Silman and Pearson, 2002, Ferucci *et al.*, 2004). The high prevalence rates in Native American Indians suggest a major genetic influence in these populations (Peschken and Esdaile, 1999, Ferucci *et al.*, 2004).

Low prevalence of RA are found in southern Europe with a rate of 0.3-0.7%. Similarly, populations in south-east Asia (China and Japan), show also low prevalence rates (0.2-0.3%) (Alamanos and Drosos, 2005). Reports from developing countries also show low rates, ranging from 0.1-0.5% and where African ancestry populations (Carribean, Nigerian and South African) indicate very little presence of RA (Silman and Pearson, 2002). Though genes play a major role in RA, environmental and lifestyle factors also contribute to the onset of the disease.

The mean annual incidence rate of RA is estimated to be 0.02-0.05% in North American and North European countries (Alamanos and Drosos, 2005). In the UK, a study in 1994 determined the annual disease incidence to be 0.014% in males and 0.036% in females (Symmons *et al.*, 1994). Another study in 1999 estimated that the annual rate of RA incidence in the UK could be in the region of 0.012% for males and 0.03% for females (Wiles *et al.*, 1999). These small numbers of RA incidence studies, along with a lack of incidence rate studies in developing countries make its very difficult to determine precise incidence figures (Alamanos *et al.*, 2006).

1.1.6 Aetiology

The exact aetiology of RA is still currently unknown, however it is a general consensus that the disease is thought to be triggered by a combination of both environmental and genetic factors. The contribution of the genetic component is thought to constitute approximately up to 60% towards RA susceptibility. This was shown from a study of monozygotic and dizygotic twins, using two published nationwide population studies (Macgregor *et al.*, 2000). Additionally, familial inheritance also increases the risk of RA (Deighton *et al.*, 1992, Grant *et al.*, 2001). Therefore a large proportion of RA susceptibility, approximately 40%, arises from environmental factors influencing disease development.

1.1.6.1 Genetic factors

Comprehensive RA genetic research has demonstrated a genetic susceptibility for the development of the disease in certain individuals. The genetic contribution to RA is extensive and will be discussed in a separate section (Section 1.2.1 RA genetics). This section of RA aetiology will continue to discuss the role of environmental factors.

1.1.6.2 Environmental factors

As of yet, no specific environmental factors have been conclusively identified, although there are numerous environmental factors that have been suggested that contribute to RA onset and development such as pathogenic infections.

1.1.6.2.1 Pathogens

A number of studies have proposed the role of micro-organisms as a potential RA onset trigger. A few viruses and bacteria have been detected within RA patients, including the Epstein Barr Virus (EBV), rubella, parvovirus B19, cytomegalovirus (CMV), bacterial DNA and peptidoglycan (PG) -a major bacterial cell wall component in gram-positive bacteria. Additionally, the association of bacteria as the cause of onset and development of Reactive Arthritis as well as the various animal models of arthritic disease induced by bacteria, supports the notion that bacteria may play a role in disease development, although most probably within a specific subset of patients.

Although the mechanism behind the roles of pathogens in RA aetiology is unclear, there have been two suggested mechanisms. One is "molecular mimicry", where a viral or bacterial antigen mimics a structure within the joint, creating an immune response towards the antigen but also the synovium as a consequence. Another possible mechanism is the "carrier effect" mechanism. A foreign antigenic peptide cross-reacts and conjugates with an endogenous synovial peptide. The immune system directs its response towards the foreign antigen, as well as inappropriately recognizing the host peptide, initiating autoimmunity.

1.1.6.2.2 Other factors

Numerous other environmental factors have been investigated, including coffee consumption, climate, atmospheric toxins, pollution and cigarette smoking. Of these, cigarette smoking has been identified to be the most important contributing factor to RA in terms of both onset risk and disease severity (Heliovaara *et al.*, 1993, Gorman, 2006). However the extent of cigarette smoking needed to trigger RA onset is unclear, as well as the mechanisms behind it.

It is perhaps not surprising that cigarette smoking is involved in RA pathogenesis as it is widely known that smoking is hazardous to health, contributing to other diseases such as cancer, chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD) and asthma. Tobacco smoke contains over 4000 chemicals of which 50 are carcinogenic. Therefore it is quite plausible that any of these toxins could be responsible for the development of RA. The effect of cigarette smoking on RA patients and its interactions will be discussed further in detail in Section 1.2.2.

1.1.7 Mortality and Co-Morbidity

Rheumatoid arthritis is a systemic inflammatory disease that affects many organs and tissues, though the joints are the principal targets of attack. The mortality rate is increased a minimum of 2 fold in RA compared to the general population, and increases with duration of the disease (Wolfe *et al.*, 1994, Myllykangas-Luosuj~irvi *et al.*, 1995, Naz, 2007). As the disease develops, life expectancy of RA patients is reduced by 3-18 years, where patients with a more severe disease have an increased risk for earlier mortality (Reah, 1963, Reilly *et al.*, 1990, Myllykangas-Luosuj~irvi *et al.*, 1995). However, patients who respond to therapy have a better survival rate, and thus mortality rate is quite variable (Naz, 2007).

RA patients are associated with an increased risk to CVD such as atherosclerosis, stroke, mycocardial infarctions (MI) and ischaemic heart disease (IHD) (Wolfe *et al.*, 1994, Aviña-Zubieta *et al.*, 2008, Gupta and Fomberstein, 2009). These types of diseases occur in approximately 50% of RA patients (Aviña-Zubieta *et al.*, 2008). Other co-morbid diseases and disorders include hypertension, Sjogren's syndrome, Felty's syndrome, neuropathy, pericarditis, fibromyalgia, lymphadenopathy, non-Hodgkin's lymphoma, pleural effusion, renal amyloidosis, diabetes, carpal tunnel, respiratory diseases such as lung cancer, fibrosis and pulmonary disease to name a few (de Groot, 2007, Naz, 2007). Co-morbidity is a common extra-articular aspect associated with RA and is a major contributor to mortality (Naz, 2007).

The increased risk of mortality from co-morbid diseases may be explained by cigarette smoking, autoantibodies and the Human Leukocyte Antigen (HLA) genes, all of which

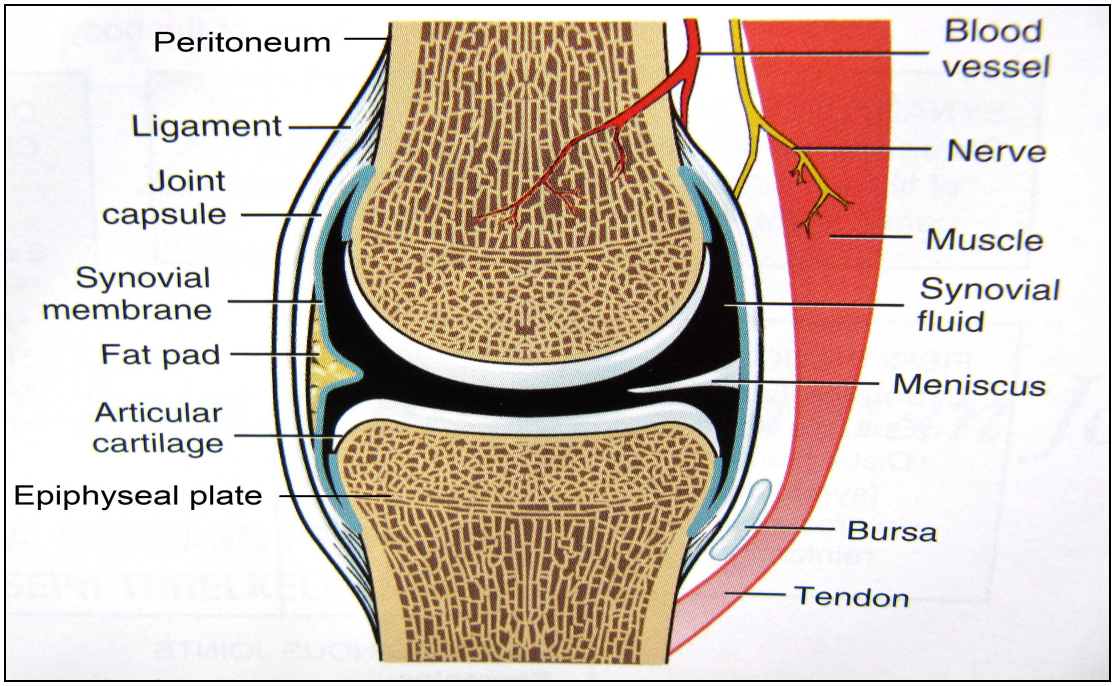
have been shown to be RA associated factors. Apart from a role in predisposing patients to RA disease, these factors could possibly render RA patients more susceptible to co-morbid diseases (Myllykangas-Luosuj~irvi *et al.*, 1995, Mattey *et al.*, 2007, Turesson *et al.*, 2007). Another explanation for increased mortality rate could be due to the reduced functionality and mobility of the joints. Physical activity would be subsequently decreased, which may in turn contribute to higher CVD mortality due to the lack of fitness through cardiorespiratory exercise.

Along with co-morbid diseases, RA patients can also experience flu like symptoms, such as fatigue, fever, anaemia, weight loss, morning joint stiffness, loss of appetite and a general feeling of unwell. These are all common systemic symptoms and can be quite pronounced, especially in people who have acute onset RA.

1.1.8 Physiology of RA

The synovial joint consists of a fibrous capsule surrounding two adjoining bones. The inner layer of the capsule is the synovial membrane, also known as the synovium (Figure 1.1.8a). Under normal healthy conditions, synovial like-fibroblasts and macrophage-like cells (synoviocyte A and B respectively) within the synovium produce and secrete hyalaronan (hyaluronic acid) and lubricin into the joint space (Bresnihan, 1999). These two constituents, along with trapped water, are the major components of the synovial fluid, which is responsible for the nourishment and lubrication of the articular cartilage and bone. This allows for fluidic movement, with the synovial membrane keeping the fluid contained within the joint space (Unsworth *et al.*, 1971, Bresnihan, 1999).

Figure 1.1.8a: The joint and its structural components



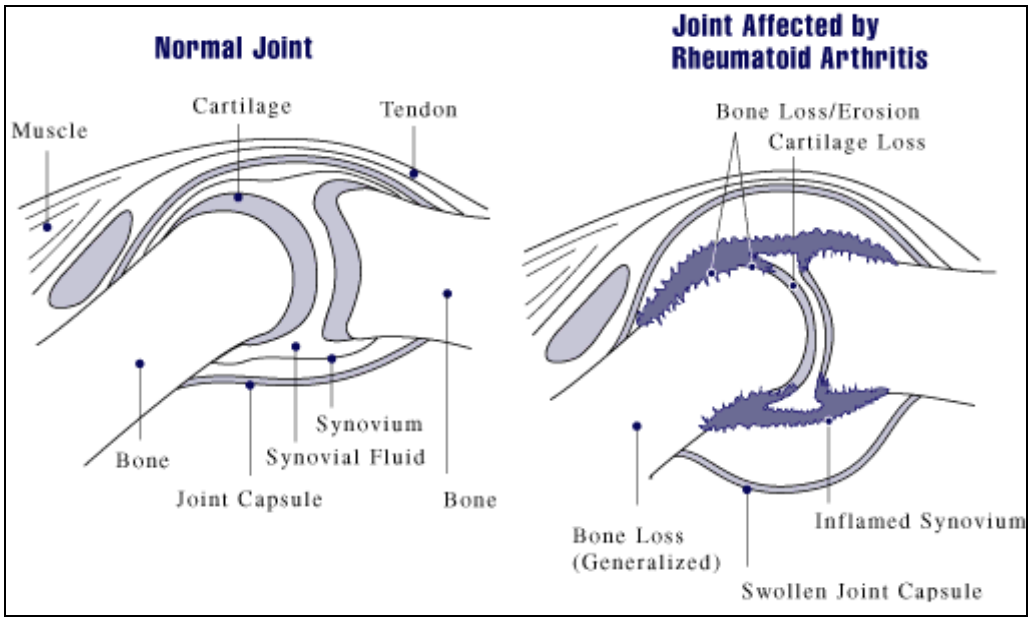
Reproduced from (Neumann, 2010) via http://ittcs.wordpress.com/2010/05/24/anatomy-and-physiology-facet-joints-of-the-spine/, adapted by S. Judah 2014.

In RA however, the synovial joint undergoes extensive degradation and damage due to excessive inflammation of the synovium (synovitis) and is used as a distinguishing factor of RA. This inflammation causes the joint capsule to swell (edema), due to an excess of synovial fluid (effusion) from the inflamed synovium (Figure 1.1.8b). Synovitis causes joint tenderness, swelling, and thus the pain felt by patients. In addition, inflammatory damage is not limited to just the joint, but also the surrounding tissue including the bursae, the tendon sheath and the ligaments. This subsequently weakens the ligaments ability to stretch, causing tendons to rupture and muscles to spasm and atrophy, leading to contracture (Unsworth *et al.*, 1971, Bresnihan, 1999).

Initially, synoviocytes A and B proliferate extensively (hyperplasia), increasing the thickness of the synovium from 2-3 cell layers up to 10 cell layers thick. In addition, the

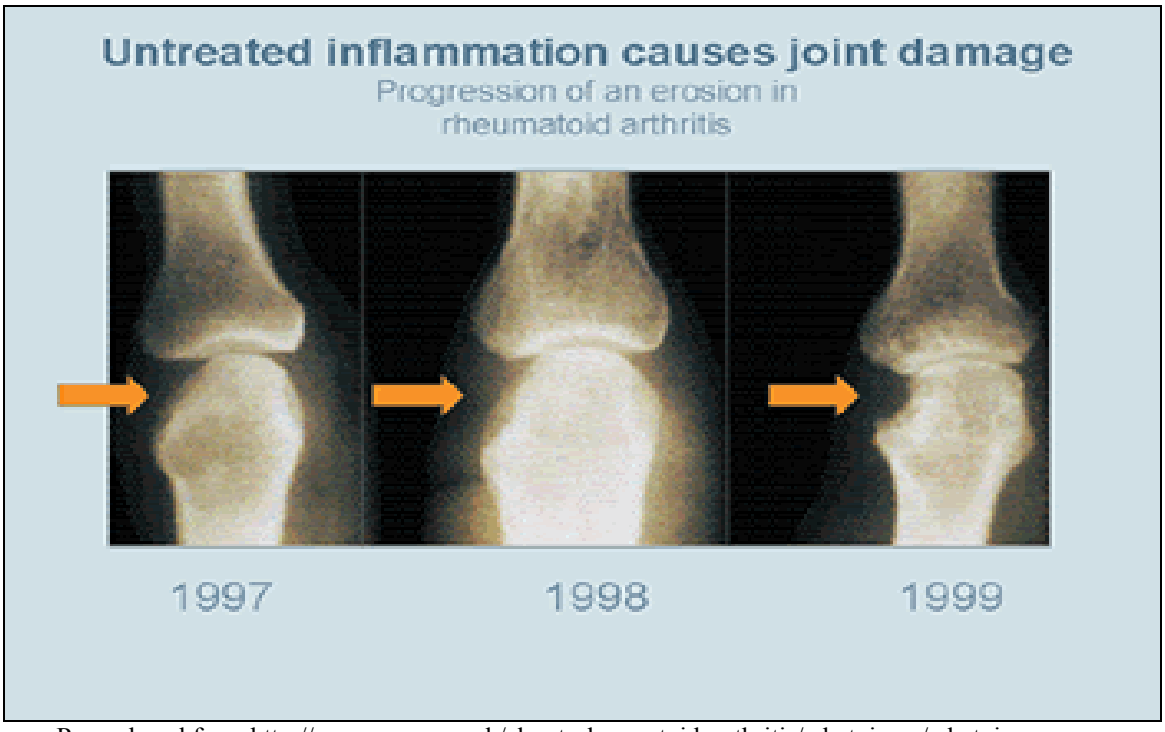
number of blood vessels plus its permeability increase within the synovium, allowing an excess of fluid to enter the joint tissue resulting in swelling. In addition, an influx of leukocytes infiltrate the synovium which also secrete pro-inflammatory and degradative markers (eg. Matrix Metalloproteinases and degranulating neutrophilic enzymes), causing inflammation and destruction of the cartilage (Unsworth *et al.*, 1971, Bresnihan, 1999). As the disease develops, the granuloma synovium becomes heavily inflamed, and makes this tissue protrude and extend out (the pannus) towards the cartilage and the bone surface (Figure 1.1.8b). The pannus adheres and infiltrates the articular cartilage surface, eroding it with leukocyte inflammatory mediators and numerous enzymes from osteoclasts, replacing it with soft fibrous connective tissue (Figure 1.1.8b). As the cartilage is degraded, the fibrous tissue left by the pannus is ossified by osteoblast cells which turn the tissue into bone. This ultimately fuses the two bones of the joint together, leading to crippling and immoveable affects. The destructive nature of the pannus is also involved in the destruction of the bone at the epiphysis corners, the ligaments, the joint capsule, the extracellular matrix (ECM), and the surrounding tissues. In addition, the excess synovial fluid creates a barrier for nutrients diffusing into the cartilage, which results in the starvation and necrotic death of the cartilage cells. The persistent inflammation and the resulting joint destruction cause joint swelling, tenderness and pain. This leads to the impairment of motility and functionality in varying degrees, as well as to deformity and disability (Unsworth *et al.*, 1971, Bresnihan, 1999). Figure 1.1.8c demonstrates the progression of RA in an untreated patient over a period of 3 years, where it clearly depicts the thinning of the cartilage, fusion of the bones, and most dramatically, the erosion at the epiphysis corners of the bone (Figure 1.1.8c).

Figure 1.1.8b: Comparison of a normal joint and a joint affected by RA



Reproduced from http://www.niams.nih.gov/Health_Info/Rheumatic_Disease/default.asp.

Figure 1.1.8c: Progression of joint damage in an RA patient over 3 years by radiographic imaging



Reproduced from http://www.nras.org.uk/about_rheumatoid_arthritis/what_is_ra/what_is_ra.aspx.

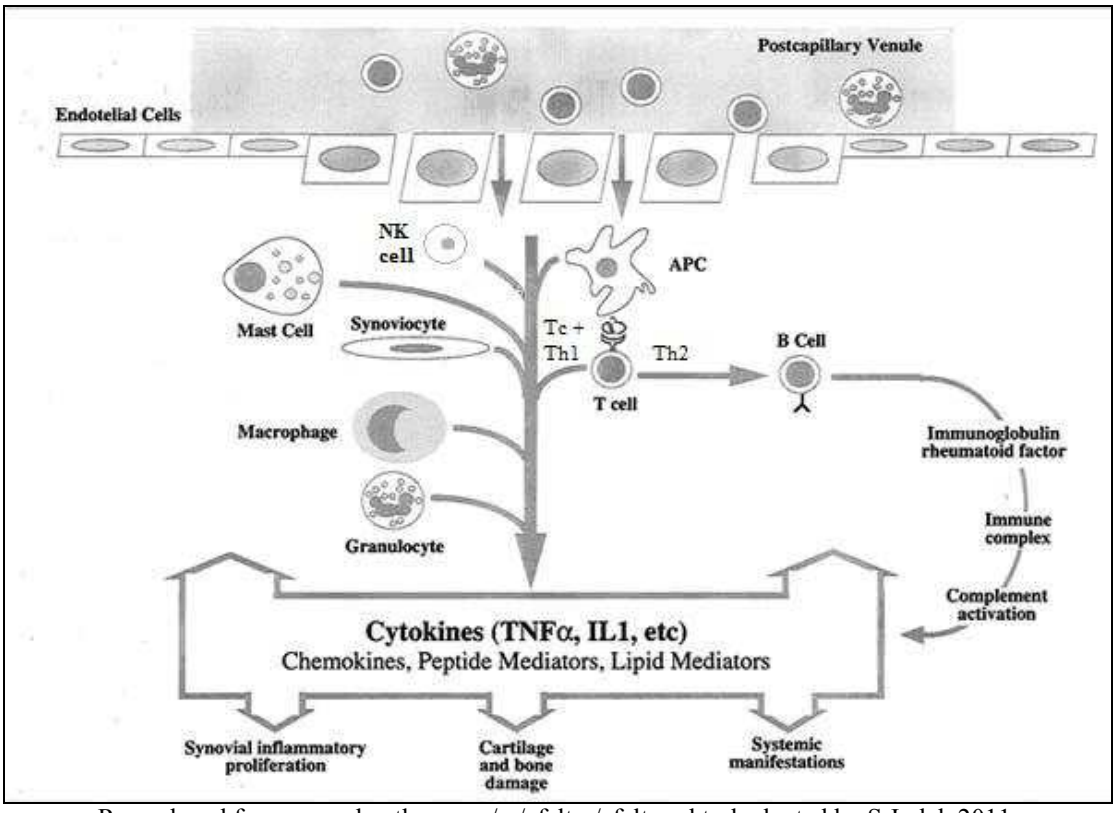
1.1.9 Pathophysiology of RA

Within a healthy individual there is a natural equilibrium which regulates the balance of the pro and anti-inflammatory response. The pathology of RA is due to an imbalance of this equilibrium with an excess of pro-inflammatory mediators, which is critical for the development, severity and progression of RA. There are various mediators involved in RA disease pathology including cytokines, chemokines, acute phase proteins including CRP, matrix metalloproteinases (MMPs), A Disintegrin And Metalloproteinases/ with Thrombospondin motifs (ADAM/Ts), degrading enzymes (collagenases, aggrecanases, cathepsin K, granulocyte enzymes) (Kojima and Ishiguro, 2012), prostaglandins, calcium binding protiens including calgranulin, T regulatory cell suppression, antibodies, serine proteases (neutrophil elastase), reactive oxygen species and numerous others that both directly and indirectly affect the destruction of the joint (Figure 1.1.9a) (Hutchinson *et al.*, 2002, Liao *et al.*, 2004). However pro-inflammatory and immunomodulatory markers are the major mediators responsible for the pathogenesis of RA (Choy and Panat, 2001). RA pathophysiology is typically described as a T helper (Th) 1 type (CD4+ T cell) disease as a strong presence of Th1 type cytokines are found within the inflamed joint comparatively to other pro-inflammatory markers (Panayi *et al.*, 1992, Fox, 1997, Miossec and van den Berg, 1997). Specifically, tumor necrosis factor-α (TNF-α), interleukin (IL) -1 and IL-6 are the most commonly known Th1 cytokines associated with RA and are key mediators in its pathology (Panayi *et al.*, 1992, Fox, 1997, Choy and Panat, 2001). Although these cytokines are generally regarded as the main drivers in RA disease, there are numerous other inflammatory mediators at the joint site such as interferon (IFN)-γ, IL-12, IL-7, IL-8 IL-17, IL-18, monocyte chemotactic protein (MCP) -1 and granulocyte macrophage-colony stimulating factor (GM-CSF) to name a few, that are integral to the pathological

process. Not only are they conducive to directly inducing inflammation and joint degradation, but they also stimulate various cells within the joint to induce the expression of additional inflammatory markers (van Roon *et al.*, 2003). This type of positive feedback loop creates and sustains the persistent inflammation found and characterised by this disease and hence termed as a chronic inflammatory disease.

The pro-inflammatory response in the joint primarily arises due to the significant infiltration of leukocytes into the thickened synovium from the peripheral circulatory system, particularly macrophages and CD4+ T lymphocytes (Panayi *et al.*, 1992, Fox, 1997, Choy and Panat, 2001). These leukocytes migrate to the joint by passing through the blood vessels via adhesion molecules into the tissue, which are recruited in response to inflammatory chemoattractant stimuli from localized chemokine production from the joint (chemotaxis). The increased cellular infiltration of leukocytes, secreteing TNF-α and IL-1 as well as other mediators promotes the induction of angiogenesis within the synovium and surrounding tissues. With the formation of new blood vessels, the number of immune effector cells migrating into the synovium is increased, including CD8+ cytotoxic T cells (Tc), granulocytes (neutrophils, basophils and eosinophils), mast cells, natural killer cells (NK), B cells and dendritic cells (DC) to the joint (Figure 1.1.9a). In addition, synovial macrophages and dendritic cells also function as APCs wtihin the joint, thereby contributing to further inflammation and establishing a localized immune response in the tissue. These various cells all contribute to disease pathology, increasing the swelling and the pain felt by patients as they secrete a vast spectrum of inflammatory mediators, degranulating enzymes, histamines and various other mediators such as prostaglandins and autoantibodies (Chaiamnuay and Louis Bridges Jr, 2005). This ultimately leads to the destruction of all the components within the joint (Choy and Panat, 2001).

Figure 1.1.9a: An overview of the main cells involved in RA disease



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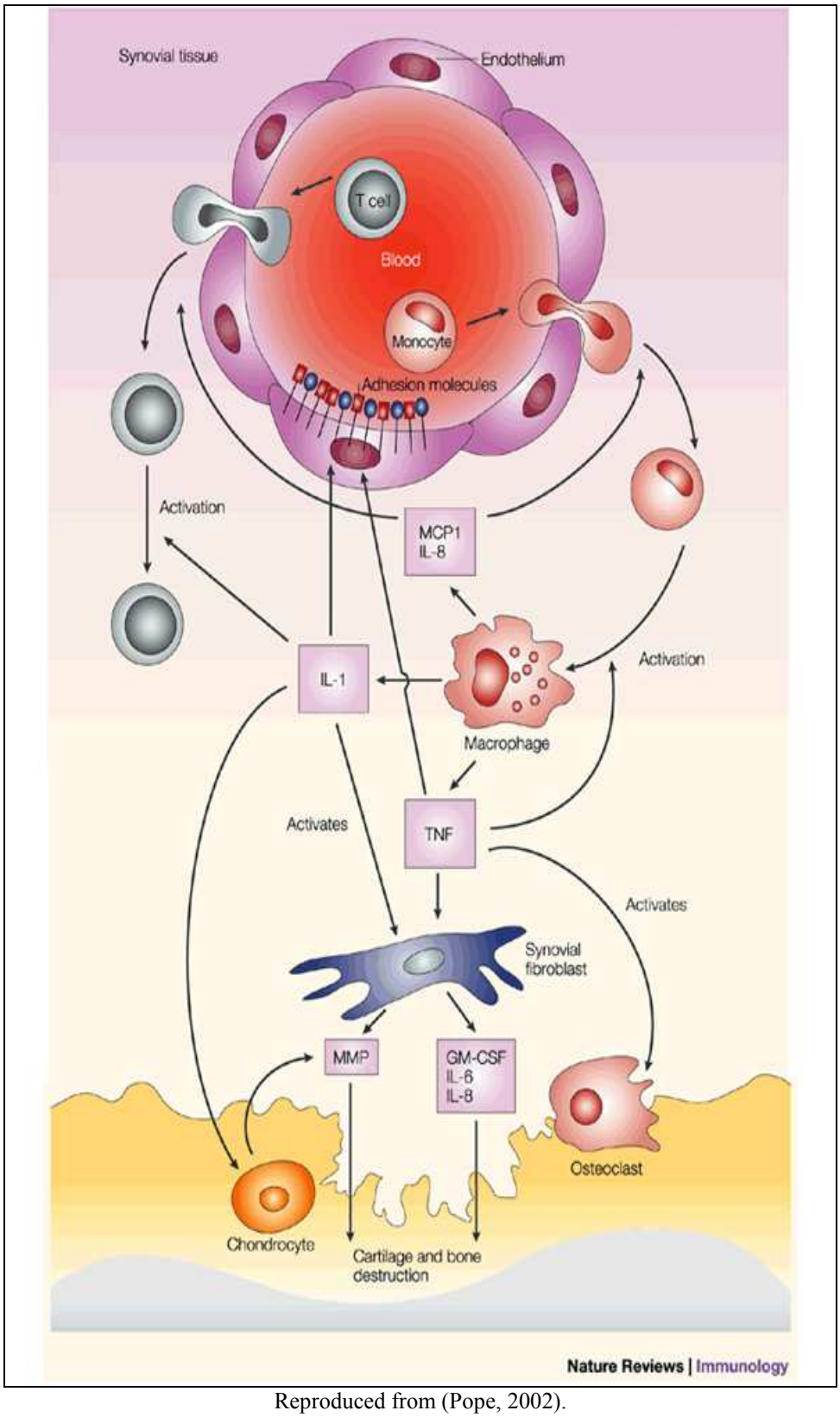
Although RA pathophysiology is predominately based upon the chronic stimulation of pro-inflammatory mediators attacking the joint, non-immune cell types are also involved in RA disease. These include fibroblasts, chondrocytes, osteoclasts and osteoblasts. Resident fibroblasts stimulated by TNF-α and IL-1 from recruited T cells and macrophages, in turn secrete MMPs and various pro-inflammatory mediators which not only target the cartilage and bone for destruction, but recruit and promote additional leukocytes into the joint, such as neutrophils and monocytes.

Chondrocytes reside in the cartilage and are responsible for maintaining the cartilaginous extracellular matrix (ECM) consisting of collagen and proteoglycans. With persistent stimulation and over expression of key pro-inflammatory mediators such as TNF-α and IL-

1, chondrocytes alter to a pathological role secreting matrix degrading enzymes, such as MMP-1 and MMP-3. These ECM degrading enzymes target and attack the components of the cartilaginous matrix damaging the cartilage. The pro-inflammatory mediators secreted by T cells also stimulate and activate osteoclasts, which are responsible for bone resorption. Furthermore, TNF-α and IL-1 mediators simultaneously inhibit osteoblasts, which suppresses bone formation as type II collagen and proteoglycans are not synthesized (Figure 1.1.9b) (Goldring, 2003). The activation of osteoclasts and suppression of osteoblasts in concert with chronic inflammation and degrading enzymes therefore pernamently damage the bone, as well as inhibits the ability to repair the bone structure.

Figure 1.1.9b: The general pathophysiological process of RA



Reproduced from (Pope, 2002).

1.1.10 RA disease features

RA is diagnosed and characterized by various disease features as classified by the 1987 ACR revised criteria. These include early morning stiffness and the symmetrical arthritis of synovial joints, specifically the hands, bone erosion, nodules and rhematoid factor (RF).

1.1.10.1. Synovial Joints

Rheumatoid arthritis can gradually occur or manifest suddenly with a severe attack. In the early stages of disease, RA typically presents itself in the smaller peripheral synovial joints of the hands and feet. In particular, the distal and proximal interphalangeal (DIP and PIP respectively) and the metacarpophalangeal (MCP) joints of the fingers and the metatarsophalangeal (MTP) joints of the toes are primarily observed to be swollen and stiff (typically in the morning). It is unknown why in the initial symptomatic stages of the disease the smaller joints are affected first. However, they are not exclusively the only joints to show signs of early disease.

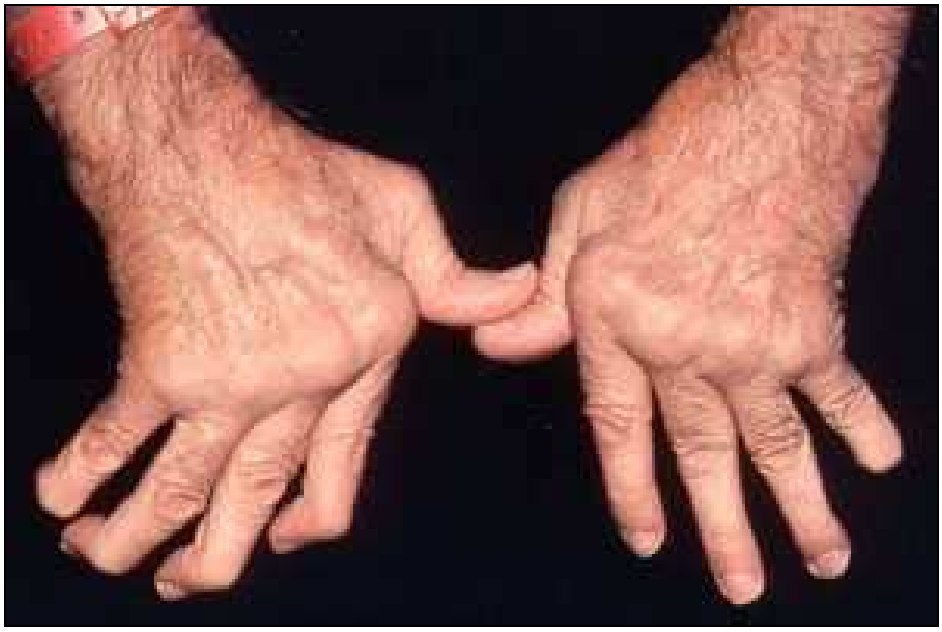
As RA disease progresses in severity, the number of joints affected also increases as it is polyarticular, manifesting in the larger synovial joints such as the wrists, elbow, knees and ankles, along with the shoulders, neck, spine and jaw. It is not yet understood what governs the reason for disease development in these specific joints. The progression of the disease differs greatly for each patient, with the number and particular joints affected varying for each patient. Some patients only have acute short term symptoms, but for the majority of people, it is a progressive and painful disease for life, with flare exacerbations and remissions of joint swelling, stiffness and pain. Joint damage usually begins within the first

two years of the disease. As such, the first two years of the disease is classified as early RA, with particular emphasis on the first year of disease (Scott, 2007).

Another clear indication of symptomatic disease of the joints is the erosion of the bone and cartilage. This is not always evident in recently diagnosed patients due to the lack of disease severity in the early stages where only 10-26% of RA patients develop erosions within 3 months from onset of the disease. Joint degradation has a very distinct morphology compared to a healthy joint and is assessed by radiographic techniques. In severe cases, the destruction of the joint can be so extensive that deformity and disability occur as a result, requiring the possibility of a joint replacement. Figure 1.1.10.1a illustrates the hands of an RA patient with severe deformity, while Figure 1.1.10.1b shows a radiographic comparison between a healthy individual hand and that of a RA patients' hand, demonstrating that RA disease can affect the bone structure greatly. Eighty percent of these severe RA patients are disabled after 20 years of the disease.

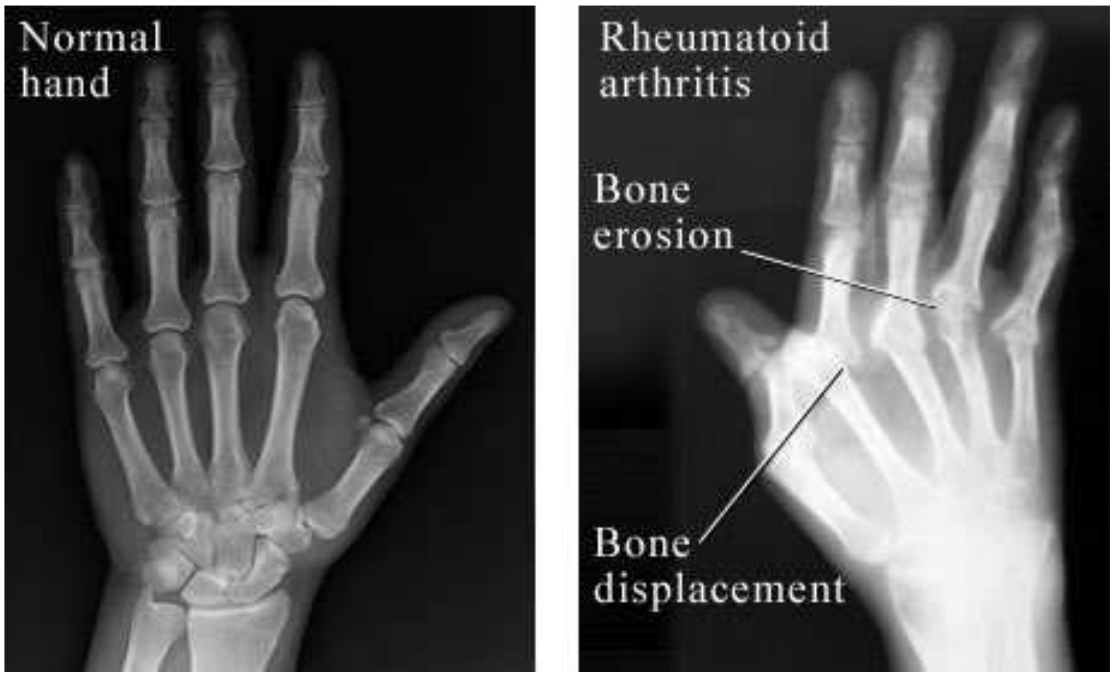
Although RA is a systemic disease, the characterization of disease severity is determined wholly upon the joint. This is based on the presentation of symptoms (joint pain, stiffness, tenderness and swelling), damage, physical function performing daily activities, deformity and disability.

Figure 1.1.10.1a: An RA patient with extrememly deformed hands.



Reproduced from http://www.cedars-sinai.edu/5234.html.

Figure 1.1.10.1b: Radiographic images of a healthy individual hand compared with a deformed hand of a RA patient.



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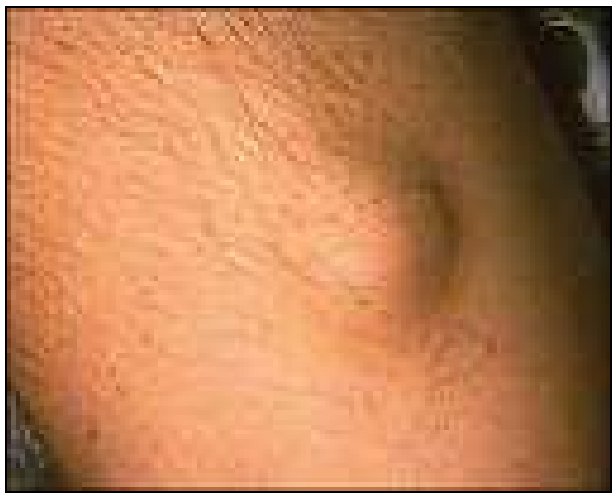
1.1.10.2 Subcutaneous nodules

Another feature of RA is the manifestation of extra-articular ("outside the joints") features such as subcutaneous nodules (Turesson *et al.*, 2003). These nodules are granuloma-like formations, ranging from a few millimetres to a few centimetres in diameter. This extra-articular feature is a common characteristic of RA and is associated a severe erosive arthritis and mortality (Kaye *et al.*, 1984). Nodules develop underneath the skin in the subcutaneous tissue overlying the bone (Figure 1.1.10.2). They are frequently observed at sites of mechanical pressure and stress, such as around the joints, as well as the hands, the back of the head, ears, heels, arms and legs (Ziff, 1990). Suppression of such stress may promote the disappearance of the nodule within a few days (Veys and De Keyser, 1993). Nodules can also develop in connective tissue of systemic organs, such as the valves of the heart, the meninges and the pleura. However this rarely occurs at these diverse sites except in patients with a very severe disease (Ziff, 1990). Five percent of patients develop nodules withing the 1st 2 years, which increases to 20-35% of patients with longer disease duration (Turesson *et al.*, 2003).

The development of nodules is unknown. However, it may come about through a piece of a bone that has become separated from the main bone fixture, which migrates and settles within the subcutaneous tissue (Ziff, 1990). Histologically, subcutaneous nodules have an appearance of a granuloma, which matures and consists of 3 distinct zones. These zones comprise of a central zone of necrotic tissue, a surrounding layer of palisading macrophages plus fibroblast mononuclear cells, and an outer layer of vascular connective tissue with infiltrating lymphocyte and plasma cells (Ziff, 1990, Baeten *et al.*, 2004). The pathologic process of nodule formation is unknown, although it may be similar to joint

synovitis. This is due to the presence of various leukocytes and a Th1 pro-inflammatory cytokine profile (TNF-α, IFN-γ and IL-1β) which have been found within the nodule (Wikaningrum *et al.*, 1998, Hessian *et al.*, 2003).

Figure 1.1.10.2: A subcutaneous nodule in an RA patient.



Reproduced from (Hahn *et al.*, 2005).

1.1.10.3 Autoantibodies

Another characteristic of RA is the development of autoantibodies. There are two commonly recognised autoantibodies present in RA disease: rheumatoid factor (RF) and anti-cyclic citrullinated protein (anti-CCP). The specific role for either autoantibody in RA pathology has not been determined. Although, they do contribute indirectly to RA disease pathology by tissue opsonisation, leukocyte mediated cytokine production, immune complex formation, apoptosis induction and initiating the complement system (Chaiamnuay and Louis Bridges Jr, 2005).

1.1.10.3.1 Rheumatoid Factor

Rheumatoid factor (RF) is an autoantibody directed against the *Fc* region (the constant region) of immunoglobulins (Ig) to form immune complexes which contribute to disease. RF is important in RA pathology, as studies have found it to be independently associated with disease onset, severity, bone erosion, co-morbid diseases and increased mortality risk (Reilly *et al.*, 1990, Myllykangas-Luosuj~irvi *et al.*, 1995, Másdóttir *et al.*, 2000, Wolfe, 2000, Mattey *et al.*, 2001b, Vencovsky´ *et al.*, 2003, Greiner *et al.*, 2005, Lindqvist *et al.*, 2005, Sihvonen *et al.*, 2005, Mewar *et al.*, 2006, Mattey *et al.*, 2007, Turesson *et al.*, 2007, Nielsen *et al.*, 2012). High seropositive RF titres are also independently shown to be strongly associated with the presence of nodules which occur in approximately 20-30% of Caucasian RA patients. This would suggest that RF may be involved in the formation of subcutaneous nodules (Kaye *et al.*, 1984, Mattey *et al.*, 2002b). RF is thus used as a marker for disease severity, progression and outcome, and are additionally useful markers for prognosis since the majority of studies have shown elevated expression of RF are associated with a more destructive articular outcome (Vencovsky´ *et al.*, 2003, Greiner *et al.*, 2005, Lindqvist *et al.*, 2005).

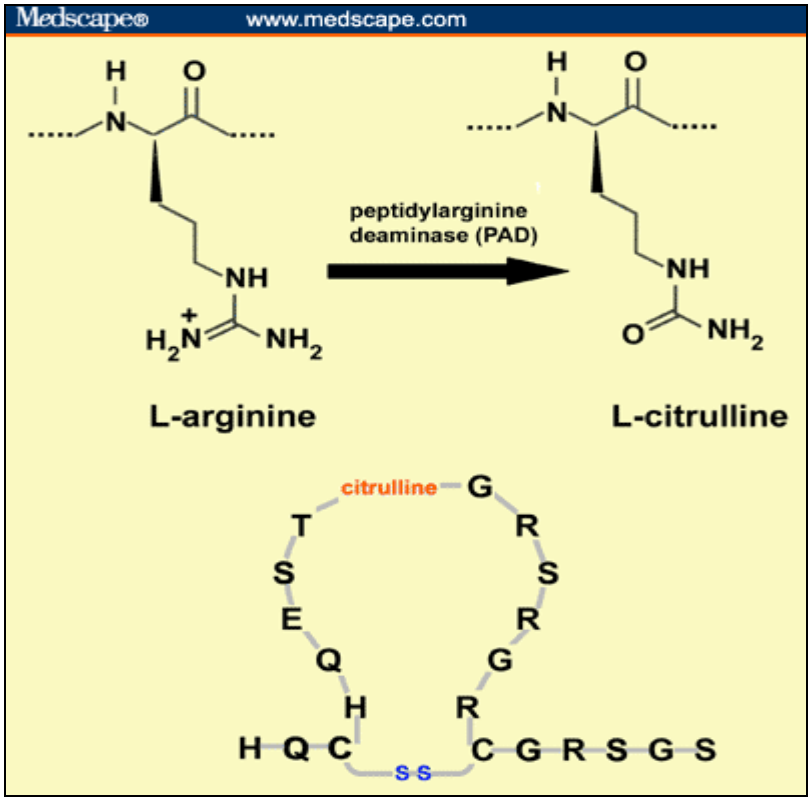
RF is found to be present in approximately 60-90% of RA patients (van Gaalen *et al.*, 2005). RA patients are frequently seronegative within their first year of onset, but the frequency of seropositivity increases with disease duration, with 80% of patients developing RF over time. However there are a few RA patients that do not present with RF (10-40%). Rheumatoid factor is not restricted to RA, as it has been found to be present in other diseases such as Sjogren's syndrome, systemic lupus erythematosus (SLE), and mixed connective tissue disease, as well as being present in 5-10% of normal healthy

individuals. RF has been shown to have approximately 60-70% sensitivity rate for RA disease (Nishimura *et al.*, 2007), with a specificity rate of 70-80% but is still routinely used as an aid in the diagnosis and characterisation of RA (Westwood *et al.*, 2006, Song and Kang, 2009).

1.1.10.3.2 Anti-cyclic citrullinated protein

Anti-cyclic citrullinated proteins (anti-CCP) are autoantibodies directed against cyclic peptides containing the amino acid citrulline. Citrulline is enzymatically converted in the post-translational modification of arginine residues in proteins by peptidylarginine deiminase (PAD) enzymes during inflammation and apoptosis (van Gaalen *et al.*, 2005). Commercial assays for detecting antibodies to citrullinated proteins use a citrullinated peptide containing a dilsulphide bond which allows formation of a loop that transforms the synthetic peptide into a cyclic peptide (Figure 1.1.10.3.2) (Chaiamnuay and Louis Bridges Jr, 2005).

Figure 1.1.10.3.2: The generation process of anti-CCP autoantibodies.



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The specific role of anti-CCP autoantibody in RA disease is unknown, although if the shape of the peptide is sufficiently altered, the crulline containing peptide could be seen as an antigen by the immune system thereby invoking an immune response. Studies have shown however that anti-CCP is independently associated with severity, bone erosion, co-morbidity, nodules and increased mortality (Forslind *et al.*, 2004, Greiner *et al.*, 2005, Lindqvist *et al.*, 2005, Sihvonen *et al.*, 2005, van Gaalen *et al.*, 2005, Avouac *et al.*, 2006, Mewar *et al.*, 2006, Turesson *et al.*, 2007). This indicates that this autoantibody is important in the pathology of RA, and is used as a marker for disease severity, progression and outcome as well as a useful marker for prognosis (Vencovsky´ *et al.*, 2003, Forslind *et al.*, 2004, Avouac *et al.*, 2006, Turesson *et al.*, 2007, Valesini and Alessandri, 2009). Anti-CCP is a very specific autoantibody to RA (88-96%), although rarely, it can be found in

other diseases such as psoriatic arthritis, SLE, Sjogren syndrome and scleroderma, as well as in healthy normal individuals, allowing for a sensitivity rate of 70-77% (Coenen *et al.*, 2007). Anti-CCP therefore has become a more reliable and effective tool for RA diagnosis, as it is more precise than RF but with similar accuracy (Greiner *et al.*, 2005, van Gaalen *et al.*, 2005, Nishimura *et al.*, 2007).

Since both autoantibodies are strongly associated with disease pathology (Mewar *et al.*, 2006, Agrawal *et al.*, 2007), a combination of both anti-CCP and RF are found to be the most accurate prediction of severity and joint damage in RA patients (Vencovsky´ *et al.*, 2003, Forslind *et al.*, 2004, Song and Kang, 2009). Both autoantibodies can also manifest in patients many years prior to the onset of RA, and before any joint symptoms are felt by the patient. Studies have revealed that this is associated with an increased risk for developing RA, as well as early disease onset (Rantapää-Dahlqvist *et al.*, 2003, Vossenaar and van Venrooij, 2004, Niewold *et al.*, 2007, Diaz *et al.*, 2011, Nielsen *et al.*, 2012). Despite the presence of both autoantibodies can occur before onset of RA, no evidence has been shown to suggest that they are the initial onset cause of disease, but rather as part of the pathological process. As no immediate effect is observed when autoantibodies develop, this suggests that additional aspects such as genetic and environmental factors are required for the onset of RA. As such, both these autoantibodies are considered as prognostic markers for RA development (Chaiamnuay and Louis Bridges Jr, 2005).

Despite their strong association with disease pathology, these autoantibodies are not solely responsible for the pathological process of RA. As mentioned above, some RA patients are negative for the presence of either autoantibody, and in contrast, healthy individuals negative for RA can be positive for either autoantibody (<1%). This suggests that various

inflammatory mediators other than these autoantibodies are also driving and contributing to the disease pathological process.

1.1.11 Other arthritides

Many studies have investigated other autoimmune diseases as well as different arthritides in comparison with RA to determine differences and gain insights into the different processes between the different patholgies. In the present study, comparisons were made between RA patients and patients with two other types of inflammatory arthritis (psoriatic arthritis, reactive arthritis) and a "non-inflammatory" arthritis; osteoarthritis. These three different forms of arthritis' are generally considered to have a less severe disease pathology outcome compared to that of RA. A brief description of each these arthritides is provided below.

1.1.11.1 Reactive arthritis

Reactive arthritis is an asymmetrical inflammatory oligoarthritic disease that arises in response to an infection in a distant part of the patients' body. The infection triggers an immune response against the infection as well as subsequently against the joints, gradually developing 1-4/6 weeks after the initial infection (Kim *et al.*, 2009, Kwiatkowska and Filipowicz Sosnowska, 2009). It is unclear why these "reactive" symptoms develop in areas of the body that were not infected by the foreign antigen, though a number of possible explanations have been suggested. It is postulated that the immune system hyper-responds to the initial infection, which creates an abundance of the inflammatory response, resulting in the destruction of surrounding tissues, thereby inducing autoimmunity (Hill

Gaston and Lillicrap, 2003). Studies have found that joint culture results are negative in the majority of patients, making it unlikely that a viable infection can exist within the joint space. However non-viable fragments of bacterial antigens such as nucleic acids and proteins have been identified in the synovium, which may cause the immune response, and so trigger the onset of disease (Pacheco-Tena *et al.*, 2001, Singh *et al.*, 2007b). These infection fragments created through the destruction of the antigen could circulate in the bloodstream and migrate, and subsequently deposit in the joint, creating a persistent presence of foreign material within the joint, triggering the onset of disease and the inflammatory cascade. A third possibility is the presence of an arithrogenic type peptide sequence within the foreign antigen protein structure. In such a situation, the immune system would recognize and target the arthritogenic peptide within the joint, as well as the invading antigen (Hill Gaston and Lillicrap, 2003).

The invading organism responsible for onset of ReA can occur at any site in the host, but is usually present in the gut or urethra. Infections that are in the gut could be responsible for the onset of ReA include shigella, salmonella, streptococcus, campylobacter and yersinia (Ugrinovic *et al.*, 1997). Additionally, Chlamydia, neisseria gonorrhoeae, HIV and viral infections that cause sore throat, coughs and skin rashes can sometimes be the antigenic trigger to disease. It is very difficult in determining what the initial infection is, as it is usually treated and cured before the symptoms of reactive arthritis develop and are observed. However, arthritic symptoms can sometimes appear before the initial infection is resolved (Hill Gaston and Lillicrap, 2003).

Reactive arthritis is also known as Reiter's syndrome, as it was first discovered by the German doctor, Dr Reiter. It is a systemic extra-articular disease, with three common

symptoms- urethritis, conjunctivitis and uveitis. Patients can also express additional symptoms including skin rashes, mucocutaneous lesions, circinate balantis, keratoderma blennorrhagica (Figure 1.1.11.1), mouth ulcers, gastrointestinal and genitourinary problems, fever, weight loss, nail changes similar to psoriasis, nodules primarily on the soles of their feet, tiredness, and in rare instances heart and kidney problems. Certain features of reactive arthritis resemble rheumatoid arthritis, with inflammatory swelling and pain affecting the lower and peripheral joints. These include joints such as the knee, ankles, toes and the hands, wrists and feet, but also can affect the spine, making it additionally a spondylarthropathic type condition. Joint arthritis develops very quickly (typically within a day), though in some cases it develops gradually. Like RA, synovitis affects the surrounding tissue such as the tendons (tendonitis) and ligaments, which become inflamed and swollen with enthesitis and dactylitis common features of ReA. Diagnosis of the disease is determined by the presence of these clinical measures for more than one month, plus microbial culture tests to determine presence of infection (Hill Gaston and Lillicrap, 2003, Kim *et al.*, 2009, Kwiatkowska and Filipowicz Sosnowska, 2009).

Reactive arthritis, unlike RA which is a chronic disease, is usually self-limiting. Once arthritis develops, it will run its full course, ranging from a few weeks to years. Most patients experience symptoms lasting 2-6 months, and these then dissipate without leaving any long-term problems termed as acute ReA. Patients with ReA lasting longer than 6 months are termed as chronic, and are likely to have bone erosion and disability as a result, as well as manifestation of extra-articular features. Chronic ReA is observed in approximately 4-19% of patients. ReA disease duration varies between patients, possibly due to the genetic disposition of the individual, as well as the type of triggering pathogen

within the host (Hill Gaston and Lillicrap, 2003, Kim *et al.*, 2009, Kwiatkowska and Filipowicz Sosnowska, 2009).

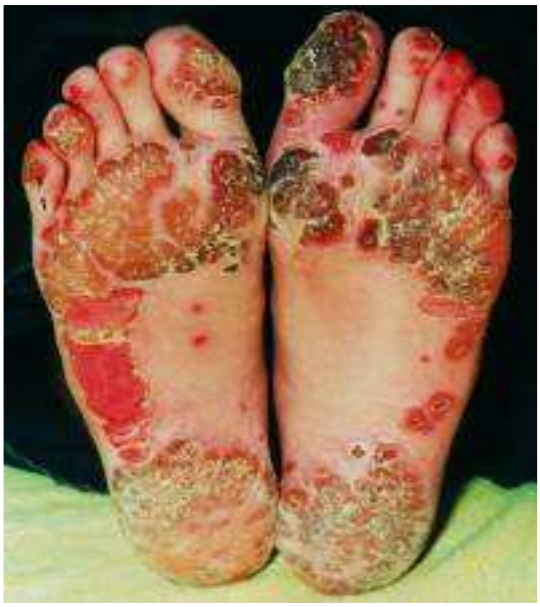
ReA is typically observed in 4% of the population. It can occur at any age but is mostly seen between the ages of 20-40 (Kvien *et al.*, 1994). In contrast to RA, ReA is more common in males than in females (3:1), and has a more severe pathology in males (Bas *et al.*, 2001). The disease affects individuals worldwide but is more prevalent in Caucasian individuals due to its strong association with the presence of the HLA-B27 molecule which is predominantly found in white populations. Studies have shown that about 70% of ReA patients are positive for HLA-B27 and are more likely to have severe and prolonged episodes of ReA (Kim *et al.*, 2009, Kwiatkowska and Filipowicz Sosnowska, 2009).

There is no cure for reactive arthritis, as in the majority of cases the disease will disappear in 3-4 months. Antibiotics are only prescribed to eliminate the initial bacterial infection that triggered the disease. There are treatments that relieve disease symptoms, including the use of analgesics and non-steroidal anti-inflammatory drugs which reduce inflammation and pain. Patients with severe ReA can be prescribed immunosupproesive drugs (methotrexate and sulphalzaxzine), corticosteroids or biologic therapy if they cannot be controlled by other drugs (Hill Gaston and Lillicrap, 2003, Kim *et al.*, 2009, Kwiatkowska and Filipowicz Sosnowska, 2009).

A number of patients (15-50%) after recovery have symptoms that can re-emerge months or even years after the first onset. This may be due to a spontaneous flare-up or a reaction as a result of a new infection, as the patient is more susceptible to repeated infections. Repeated attacks over many years are common and can develop into a chronic condition

that ultimately leads to joint erosion, disability and systemic extra-articular manifestations (Hill Gaston and Lillicrap, 2003, Kim *et al.*, 2009, Kwiatkowska and Filipowicz Sosnowska, 2009).

Figure 1.1.11.1: Pustules on the feet of a Reactive arthritis patient.



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1.1.11.2 Psoriatic arthritis

Psoriatic arthritis (PsA) is a systemic autoimmune inflammatory type arthritis which commonly affects the joints of the hands and feet causing swelling, stiffness and pain. This disease can also affect the larger joints of the body including the wrists, knees, elbows, shoulders, hips and at times the spine (spondyloarthropathic). Psoriatic arthritis typically presents as a mild asymmetrical oligoarticular disease, however with time it often progresses and develops to a symmetrical polyarticular pattern. This disease is said to resemble RA symptomatically although the majority of patients experience a milder form of severity than RA patients (McGonagle *et al.*, 2011, Mease, 2011).

Other symptoms experienced by patients with PsA are general tiredness, swelling of the hands, feet and digits (dactylitis), along with the muscle, entheses (enthesitis) and tendons (tendonitis), further limiting movement (Carneiro *et al.*, 2011). Patients also experience shortening of the digit due to bone lysis, bone fusion and digit telescoping where the bone overlaps another. As a result of disease progression, 20% of these patients will develop a severe disease outcome with increasing inflammation, pain, joint destruction and disability. Psoriatic arthritis patients who initially present polyarticular disease, have a higher risk for disease progressing into a severe phenotype with joint deformity, plus an increased mortality rate due to the development of co-morbid diseases (Gladman *et al.*, 2005).

This disease not only affects the joints, but a skin rash of raised red skin with scales or plaques also develop in parallel (Figure 1.1.11.2a). This rash is similar to patients with psoriasis (PS) alone. Approximately 10-30% of PS patients develop PsA due to the persistence of the pro-inflammatory response. Usually, the more severe presentation of skin rash, the greater the likelihood that PsA develops, which occurs approximately 10 years after the first signs of psoriasis. There is a subset of patients, where the arthritic symptoms develop prior to the presence of the skin rash. Psoriatic arthritis is typically described as a RF sero-negative arthritic disease. However RF has been detected in approximately 13% of patients with PsA. Unlike RA, the presence of extra-articular manifestations such as nodules are not present in PsA (Mease, 2011). Other manifestations of the disease include nail pitting, which are round lesions on the surface of the nail plate and occur in 80% of PsA patients (Figure 1.1.11.2b). Onycholysis can occur in extreme severe cases, which is the complete loss of the nail itself.

Psoriatic arthritis disease can manifest at any age, although it does tend to develop between the ages of 30-50 years old. The prevalence of the disease is estimated to be between 2-3% of the general population, and 7% of the total arthritic population. Furthermore, people who have psoriasis have a higher rate of arthritis than the general population. Disimillar to RA, both males and females are equally susceptible to the disease, although females tend to have a worse PsA outcome compared to males (Gladman *et al.*, 1987, Gladman *et al.*, 2005).

The exact cause of PsA is unclear, though a number of associations with particular genes have been identified. These include the HLA- class 1 type MHC molecules, including those of the B and C classes, as well as IL-23R and IL-12B SNPs (Rahman and Elder, 2005, Liu *et al.*, 2008). Unfortunately, both psoriatic arthritis and psoriasis are life-long conditions, with no cure as yet. The treatment of the disease is similar to that of RA where NSAIDs and DMARDs are used, and if necessary biologic therapy (Gottlieb *et al.*, 2008). Remission of the PsA disease can occur, though this tends to be observed in male patients with a low number of inflamed joints. Additionally, flare-ups of the disease can occur at any time point after the initial treatment. Complete remission of the disease is rare (6% of patients), and a majority of patients are on some type of permanent drug therapy to manage the disease.

Figure 1.1.11.2a: Skin psoriasis rash. Figure 1.1.11.2b**:** Nail pitting.

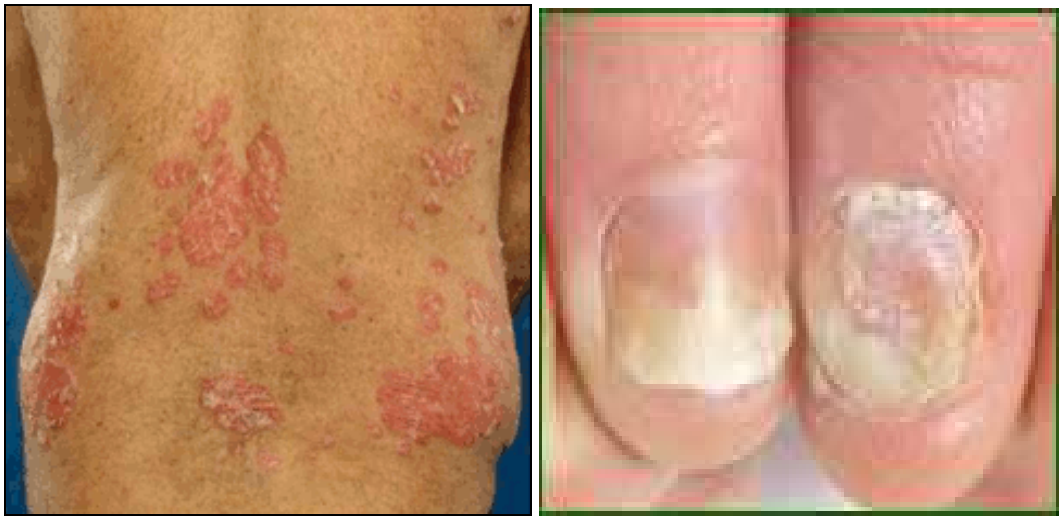


Figure 1.1.11.2a reproduced from (Goedkoop *et al.*, 2004).
Figure 1.1.112b reproduced from http://www.psoriasis-tablets.com/psoriasis_skin.html

1.1.11.3 Osteoarthritis

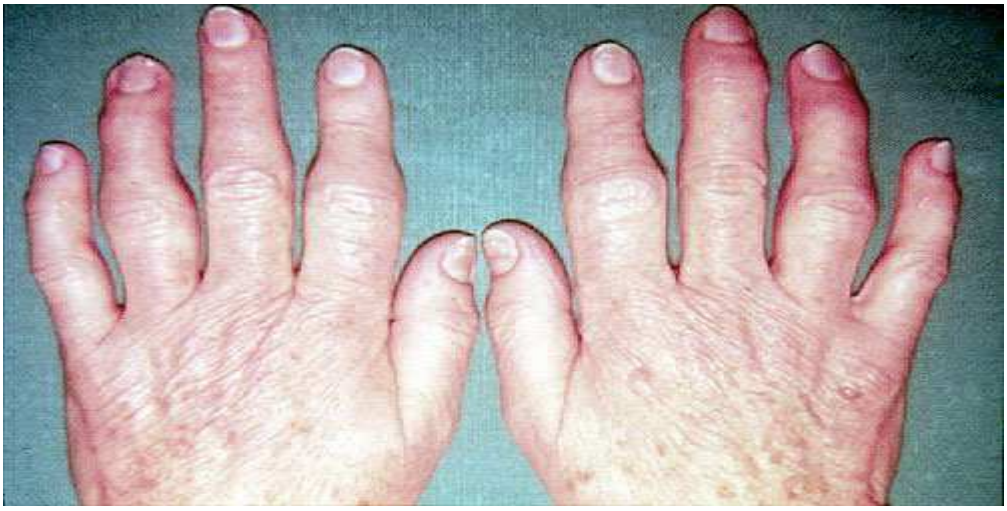
Osteoarthritis (OA) is a non-inflammatory disease that involves degradation of the joint. It is the most frequent type of arthritis in the UK, affecting approximately 8.5 million people, and more prevalent in females than in males (Brandt *et al.*, 2009).

This degenerative arthritis typically affects joints which are load bearing and under the most mechanical activity such as the knees, hips, spine and the small joints of the hands and feet. These joints are the most motile and therefore more prone to be affected, although any joint can be afflicted by the disease. The disease involves degradation of the joint where the cartilage thins down until the bone ends are exposed for erosion. OA is considered to be a non-inflammatory disease especially when compared to RA, although mild inflammation of the joint does occur. This arises due to the break down products of the cartilage which are deposited into the synovial space. Synoviocytes within the synovial membrane increase and consequently attempt to remove the break down products. Some

patients experience a synovial effusion of the joint due to the amount of fluid that builds up in the joint space. As the disease progresses, the joint space between the bones narrows, due to thickening and creation of new bone to compensate for the lack of protection from the worn down cartilage. This leads to bone contact and locking with creation of a fused joint, where the ends rub together causing friction and further bone damage (Kean *et al.*, 2004, Dieppe and Lohmander, 2005, Abramson and Attur, 2009, Brandt *et al.*, 2009).

A distinguishing feature of OA is the formation of osteophytes which develops around the edges of the joint as an attempt to repair disease damage unlike in RA. This type of bone outgrowth however can be quite debilitating for articular movement and create another friction point which can cause pain to the patient. During the progression of OA, bone nodes which are hard bony enlargements, develop particularly on the DIP and PIP joints. Nodes, although not directly painful to the patients, do significantly limit the movement of the peripheral joints (Figure 1.1.11.3a). OA at the toes leads to the development of bunions (Kean *et al.*, 2004, Dieppe and Lohmander, 2005, Abramson and Attur, 2009, Brandt *et al.*, 2009). As a result of decreased movement, muscles, tendons and ligaments begin to atrophy and relax.

Figure 1.1.11.3a: Presentation of OA in the hands.



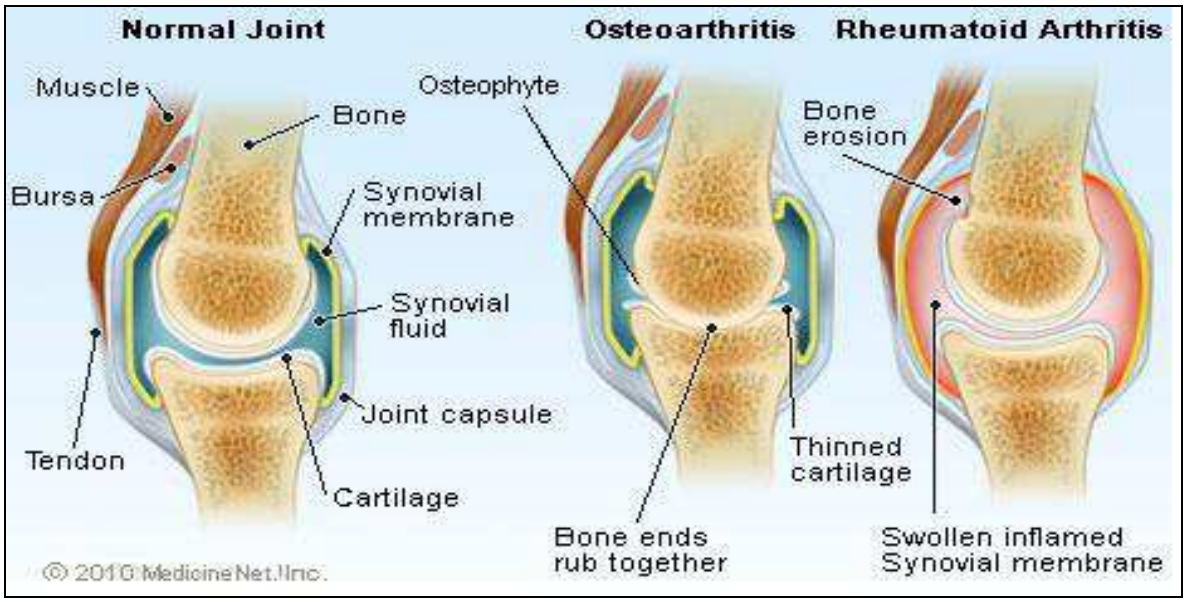
Reproduced from http://www.hopkins-arthritis.org/arthritis-info/osteoarthritis/clinical_4.html

Patients with OA experience joint pain, tenderness and stiffness but not typically swelling as a non-inflammatory type arthritis. Symptoms can differ greatly between patients ranging from mild to severe, with the number of joints and the amount of damage. In addition, OA patients feel worse through the day due to use of their joints, while RA patients often feel better after motility. Unlike RA which progressively worsens in severity, OA plateaus over time but patients experience persistent stiffness and pain. In some individuals, symptoms are alleviated as the body repairs itself, while in others the damage is too severe for joint repair- such patients will always experience stiffness and pain (Kean *et al.*, 2004, Abramson and Attur, 2009, Brandt *et al.*, 2009).

Most cases of OA have no known cause and are referred to as primary OA, which is characterized as chronic and degenerative. Primary OA is associated with aging due to the "wear and tear" process, and is most prevalent in individuals over 50 years old. OA develops gradually over many years as the protein content (collagen) within the cartilage decreases over time, and thus is susceptible to degradation. The 'wear and tear' action is not the direct cause of the condition, as some elderly people do not suffer from OA (Radin

et al., 1972). Other factors such as infection, injury, hereditary factors, pathological and congenital disorders, obesity, developmental causes plus metabolic and mechanical stresses are considered to be causes of secondary OA. Mechanical stress is the most common cause of osteoarthritis. This includes bone misalignment, injury, obesity, loss of supporting joint muscle strength, and peripheral nerve impairment that lead to movements that overstress the joints (Kean *et al.*, 2004, Valdes and Spector, 2008, Brandt *et al.*, 2009). Regardless of the cause of OA, the resulting pathology is the same in primary and secondary OA. A sub-set of primary OA is termed erosive OA (EOA) which has an aggressive pro-inflammatory response compared to normal OA, and results in high bone erosion of the DIP joints. This condition though is not a common form of OA (Belhorn and Hess, 1993).

Figure 1.1.11.3b: Differences between the various joint states.



There is currently no cure for OA. The symptoms are managed through the use of analgesics, to reduce the amount of pain and inflammation. Modifying lifestyle aspects, such as losing weight and physical therapy also help, as it alleviates the pressure on the

joint and keeps it motile. NSAIDs are prescribed if conventional pain relief is not sufficient. A number of other treatments such as glucocorticoids and antibodies are also available for moderate to severe patients. In severe OA patients, joint replacement surgery or resurfacing can be undertaken if previous treatments were ineffective (Dieppe and Lohmander, 2005, Abramson and Attur, 2009, Brandt *et al.*, 2009).

The table below (Table 1.1.11) summarizes the major similarities and differences between the different arthritides.

Table 1.1.11: Comparison of arthritides

	OA	**PsA**	**ReA**	**RA**
Cause/origin	natural joint degradation, "wear and tear"	autoimmune	A cross-reaction in response to infection	autoimmune
Disease pattern	• oligo and polyarthritic • peripheral & large joints • load bearing & high mechanical activity joints • asymmetrical	• oligoarthritic progressing to polyarthritic • peripheral & large joints • asymmetrical progressing to symmetrical	• oligoarthritic • peripheral, large & lower joints • asymmetrical	• polyarthritic • peripheral, large & lower joints • symmetrical
Disease span	chronic & progressive (gradual), which plateaus over time	chronic & progressive	acute & self limiting	chronic & progressive
Disease features	• finger bone nodes & osteophytes (bone spurs)	• skin rash with raised scale plaques • finger digit shortening • nail pitting	• feet & palm pustules/nodules, skin rashes & lesions • nail pitting • urethritis, conjunctivitis & uveitis •HLA-B27 positive (70%)	• subcutaneous nodules
Autoantibodies	RF seronegative	typically RF seronegative (87%)	RF seronegative	typically anti-CCP & RF seropositive (60-90%)
Age range	50+	30-50	20-40	40-50
male:female ratio	1:2	1:1	3:1	1:3
Prevalence	3.8%	2-3%	4%	1%

OA=Osteoarthritis, PsA=Psoriatic arthritis, ReA=Reactive arthritis, RA=Rheumatoid arthritis.

1.1.12 RA Treatment

Complete and permanent remission of RA disease is very rare, with the majority of patients suffering from the disease until mortality, which is often premature. Patients can experience periods of regression, followed by flare-ups which are exacerbated symptoms. As such, the disease is managed by reducing inflammation and the destructive activity of RA with the use of various chemical agents. Drugs for the treatment of RA can be classed into two categories: non-steroidal anti-inflammatory (NSAID) and disease modifying anti-rheumatic drugs (DMARD) (Green, 2001).

1.1.12.1 NSAID treatment

NSAIDs have 2 major actions which include an analgesic which reduces the pain felt by the patient as well as acting an anti-inflammatory agent which reduces stiffness and swelling. This occurs through the inhibition of cyclooxygenase (COX) type enzymes which subsequently reduce prostaglandin levels from damaged tissue and thus decreases the level of inflammation and symptoms felt by the patient (Vane, 1996). There are various NSAIDs used to treat RA patients (asprin, ibuprofen, celecoxib, diclofenac, diflunisal, etodolac etc.) as not all NSAIDs are effective for each patient. However, they all have similar anti-inflammatory and analgesic effects (Majithia and Geraci, 2007). As every individual is different, their response to NSAID treatment will also differ between them. NSAIDs do not actually effect the pathophysiology of RA, but aid more in the management of its symptoms. NSAIDs therefore are commonly used in conjunction with more potent RA drugs such as disease modifying anti-rheumatic drugs (DMARDs) and biologics that help slow disease development and progression.

NSAIDs vary in strength and thus the level of COX enzyme inhibition and ultimately expression of prostaglandins. Prostaglandin not only stimulates inflammation but also aids in the protection of the stomach lining and kidneys. High strength NSAIDs therefore can inhibit prostaglandin synthesis excessively which can cause negative side effects termed as adverse drug reactions (ADR), which are undesired secondary responses of the intended drug effect. Other ADRs include stomach pain, skin rash, constipation, diarrhoea, dyspepsia, drowsiness, nausea, anaemia, dizziness, leg swelling, headaches, ear ringing, skin bruising (Giercksky *et al.*, 1989, Bjarnason *et al.*, 1993, Rainsford, 1999). NSAIDs can also have toxic effects which are any outcomes that are harmful to the body generally resulting from high doses of the drug. Toxic effects of NSAIDs include anaphylaxis, abnormal liver enzyme levels, increased heart attack and stroke risk, increased blood pressure, kidney (fluid retention) and liver damage. NSAID toxicity can also induce stomach ulcers and gastrointestinal bleeding and therefore a strong stomach acid blocker is also prescribed to prevent stomach problems. Due to the number of ADRs and toxicity, NSAIDs should be prescribed with caution to patients if they have kidney or liver disease, heart failure, high blood pressure, diabetes, asthma or ulcers. As a consequence the COX-2 inhibitor NSAIDs (eg. Celecoxib and etoricoxib) were developed. These function in a similar way to the original NSAIDs but were believed to have reduced ADRs. However, numerous ADRs similar to those of the original NSAIDs, have been reported (Giercksky *et al.*, 1989, Bjarnason *et al.*, 1993, Rainsford, 1999). Corticosteroids are prescribed to RA patients when traditional NSAIDs fail (Süleyman *et al.*, 2007). Corticosteroids reduce pain, stiffness and swelling in RA patients. This drug is used on a short term basis as long term use of the drug creates ADRs such as weight gain, acne, excess hair growth, menstrual irregularities, insomnia, skin bruising, muscle weakness and skin thinning. This treatment can cause ulcers, stomach bleeding, osteoporosis, cataracts, glaucoma, diabetes, fluid

retension, infection susceptibility, hypertension, capillary fragility as well as making existing conditions such as diabetes and glaucoma worse (Süleyman *et al.*, 2007).

1.1.12.2 DMARD treatment

In patients where NSAIDs are not effective, DMARDs are used instead. DMARDs alter the disease course as they slow down the progression and improve long term outcome as well as relieve symptoms like inflammation, stiffness and swelling. They do not however reverse the damage already presented (Nandi *et al.*, 2008). DMARDs work by dampening down the functional activity of immune cells. This includes reducing the production of inflammatory molecules, as well as cell division rate by inhibiting enzymes responsible for producing DNA and RNA. There are numerous DMARDs currently used with varying modes of action and efficacy in patients. They are slow acting with a delay of 3-6 months before a response is observed (Capell *et al.*, 2007). Early administration of DMARDs is therefore more effective for RA patients to limit the progression and damage of the disease (Emery, 1995). However, this is not always implemented, as patients must meet certain criteria to move onto DMARDs (Nell *et al.*, 2004). The most commonly prescribed DMARDs are Methotrexate (MTX), leflunomide, hydroxychloroquine and sulphasalazine. In particular, MTX is the preferred drug of choice, especially when active RA has been diagnosed. However it does not work in every patient so a trial and error based method of DMARD treatment is undergone until the most suitable drug is determined. DMARDs lose their effectiveness over time (2-5 years) as patients develop resistance to them but are most likely to be discontinued in patients first due to the high level of toxicity (Alarcóan *et al.*, 1989, Pincus *et al.*, 2004, Donahue *et al.*, 2008). DMARDs are usually well tolerated, though there are a few ADRs as a cause of their action. These include nausea, vomiting,

headaches, dizziness, abdominal pain, mouth ulcers, diarrhoea, hair loss or thinning, skin rashes (Pincus *et al.*, 2004, Donahue *et al.*, 2008). In addition, DMARDs have toxic effects and cause liver and kidney damage, decreased WBC count, susceptibility to infections (due to alteration of the immune system from DMARDs), high blood pressure, pancreas gland inflammation, lung disease and increases the risk of birth defects (Donahue *et al.*, 2008). Combinations of DMARDs are often used together but at lower doses, which allows the toxicity and ADRs to be kept at a reduced level and provide increased efficacy in patients who do not respond to just one DMARD (Capell *et al.*, 2007, Majithia and Geraci, 2007). Additionally, NSAIDs can also be used in conjunction with DMARD therapy to target both aspects of RA disease.

1.1.12.3 Biologic therapy

With an increased understanding of pathology in the joint, drug development has shifted away from chemical agents and directed to the development of biological agents. Increased knowledge of cells and molecules that have significant roles in RA have been used as specific targets for therapeutic intervention. Biologic therapy is a new form of treatment for RA which targets specific molecules, both soluble and cell-bound and competitively binds to them. This alters their functional ability either by inhibiting them or acting as decoys by binding to cell receptors thereby activating cell processes. There are now a few biologic therapies available with varying modes and sites of action: either cytokine or cell mediated. Table 1.1.12.3 summarizes these biologic therapies.

Table 1.1.12.3: Summary of Biologic drugs currently available.

Drug	Primary action	Structure	Administration	Half Life
Infliximab	TNF-α antagonist, activate complement & destroy TNF-α expressing cells	Chimeric anti-TNF monoclonal antibody	Intravenous (IV) infusion	9 days
Etanercept	TNF-α & β (lymphotoxin) antagonist	Recombinant heterodimeric TNF receptor II fusion protein	Subcutaneous injection	4 days
Adilumimab	TNF-α antagonist, activate complememt & destroy TNF-α expressing cells	Chimeric anti-TNF monoclonal antibody	Subcutaneous injection. Self administered	2 weeks
Certolizumab	TNF-α antagonist	Chimeric anti-TNF-α monoclonal antibody	Subcutaneous injection	2 weeks
Anakinra	IL-1 Receptor antagonist (Ra)	Recombinant non-glycosylated human IL-1ra	Subcutaneous injection	4-6 hours
Rituximab	Binds to B cell CD20 inducing down-regulation of B cell receptor, sheds CD23 IgE receptor, increases HLA II & adhesion molecules plus induce B cell destruction	Chimeric murine anti-CD20 monoclonal antibody	Intravenous infusion	1-16 days
Abatacept	Binds to B7, CD80/86 on APC inhibiting CD28 T cell co-stimulation of naïve T cell	Cytotoxic T lymphocyte-associated antigen 4-Ig (CTLA4Ig) fusion protein.	Intravenous infusion	13 days
Tocilizumab/MRA	IL-6 Receptor antagonist	Anti-IL-6 receptor monoclonal antibody	Intravenous injection	-

Biologic therapy supersedes conventional therapy of DMARDs, although a third of patients do not respond to this type of therapy. Clinical studies where patients do respond to therapy, have shown it to be effacious with complete and sustained suppression of inflammation (Majithia and Geraci, 2007). However, only a 50% reduction in symptoms is observed, with patients still functionally disabled. This type of therapy must be continuous as relapses can occur if treatment is ceased, although most patients will stop responding to treatment eventually. Interestingly, response to biologic therapy decreases if patients had previous similar biologic treatment in the past limiting its efficacy. Biologic therapy is well

tolerated but can induce side effects including nausea, abdominal pain, fever, malaise, headaches, rashes at injection site, vomiting, diarrhoea and respiratory and urinary tract infection. It also has toxic effects, liver disease, malignancies, high blood pressure, serious susceptibility to infections (tuberculosis etc), convulsion, anaphylaxis, blood cell damage, with larger doses increasing the risks of side effects. As it is still a relatively new type of treatment the long term side effects are unknown (Majithia and Geraci, 2007). Despite these limitations, biologic therapy is still a very useful treatment strategy and further development and understanding in this area will ultimately lead to better responses in patients. Due to the relative new advancement of biologic therapy, it is a very expensive treatment and patients must fulfil specific criteria including failure on MTX and another DMARD before being prescribed biologic therapy. Even though studies indicate that early referral and early treatment in patients have improved outcomes, diagnosis and presentation usually take longer and early treatment is usually too late (Song and Kang, 2009).

Combination of all 3 drug categories provides the best approach for patients against RA disease as it would reduce inflammation and symptoms, slow disease progression and limit immune activity. Combination therapy is also useful for limiting ADRs and toxicity since using a combination approach allows lower doses of drugs to be administered. Biologic therapy in conjunction with DMARDs and NSAIDs therefore has the best potential to dramatically reduce RA activity and severity. A study showed where a combination of anti-TNF-α biologic therapy and DMARDs improved outcome in RA patients more than just a single treatment therapy (Hyrich *et al.*, 2006). As each individual patient is different and experience RA differently, it takes time to determine the best combination of drugs by trial and error process.

1.2.1 RA genetics

There have been a number of genes that have been identified with RA disease. These include the Human Leukocyte Antigen (HLA), protein tyrosine phosphatase non-receptor 22 (PTN22) and the glutathione S-transferase theta/Mu (GST T/M1) gene. The following sections details the genes associated with RA that are involved in its onset, development, severity, progression and outcome.

1.2.1.1 Identification of the genetic influence

Identification of a genetic component in RA aetiology was determined through inheritance studies. In a number of families, the prevalence of RA was higher compared to the general population. Additionally, first- and second-degree relatives also had an higher incidence of RA than normal healthy individuals, indicating family history as a risk factor (Grant *et al.*, 2001). From twin studies the concordance for RA showed increased rates in monozygotic twins comparatively to dizygotic twins (Aho *et al.*, 1986, Silman *et al.*, 1993). As the concordance rate is not fully complete between twins, it indicates that RA aetiology is not solely genetic, and an interaction with environmental factors must occur. From these studies, the genetic component was calculated to be approximately responsible for 60% of RA susceptibility and an integral part for RA onset (Macgregor *et al.*, 2000).

As RA is driven by an excessive pro-inflammatory response, analyses of genes coding for proteins that are involved in the immune response and its regulation are of high importance for identifying genetic aetiological and pathological factors. The HLA has been thoroughly investigated in RA, as this genetic locus has over 200 genes coding for proteins involved in

the function of the immune system (Aguado *et al.*, 1999). As a result, it has been determined that the HLA is a major factor in RA in both its susceptibility, onset, development, severity, progression and outcome and was calculated to contribute 37% to the disease (Deighton *et al.*, 1989).

1.2.1.2 Association of the HLA genes with RA

The HLA also known as the Major Histocompatability Complex (MHC) in all mammals are cell surface antigen presenting proteins. They are expressed on antigen presenting cells (APC) primarily on macrophages and dendritic cells which present antigenic peptides to T helper (Th) cells to activate the adaptive immune response and aid in the innate immune response. The genetic HLA locus is highly polymorphic with over 6000 HLA alleles identified, the majority of which are contained within HLA class I subtypes (HLA-A to –G, excluding HLA-D). The remaining polymorphic variability are contained in the class II subtypes (HLA-DM, -DN, -DO, -DP, -DQ and -DR) (IMGT/HLA-Database, 2011). A number of these subtypes have been found to be associated with RA susceptibility as well as its onset, development, progression, severity and outcome, specifically the –DR subtype (Reveille, 1998). In particular, specific alleles pertaining to the first beta chain gene (B1) out of four isoforms of the class II -DR subtype (HLA-DRB1) have been shown to be the most associated with RA disease. This gene is encoded at several loci positions for different subtypes of the beta 1 chain: HLA-DR1 to HLA-DR17. In particular, alleles of the DR1 and especially those of the DR4 subtypes, give the greatest susceptibility for RA as well as its severity (Jaraquemada *et al.*, 1986).

The most consistent and strongest HLA-DRB1 RA association are the alleles of the shared epitope (SE) (Gregersen *et al.*, 1987). The shared epitope is a highly conserved sequence of 5 amino acids in the 3rd hypervariable region in certain HLA-DRB1 subtypes and represents codons 70-74 of the HLA-DRB1 molecule. Over 15 RA DRB1 alleles posses the SE, encoding the amino acids QKRAA, QRRAA and the rare RRRAA sequence, where the amino acids specifically differ at position 70-71 in the molecule (Jameson, 1998). Some SE alleles are greater risk factors than others, eight of which are shown to be associated with RA: HLA-DRB1*0101 and *0102 of the DR1 subtype, *0401, *0404, *0405 and *0408 of the DR4 subtype, *1001 of the DR10 subtype and *1402 of the DR6 subtype. Studies have shown that SE alleles are associated more with severity and disease progression than with RA onset (Weyand *et al.*, 1992). Despite this, the SE is still a leading risk factor in the development for RA disease, as a strong positive association was observed with patients positive for SE and the risk of RA development (Criswell *et al.*, 2006).

Many studies have demonstrated various HLA-DRB1 associations with numerous RA features and disease measures. However, those of the DR4 subtype and of the SEs in particular have been the most consistent association (Deighton *et al.*, 1989). Patients carrying the homozygous SE HLA-DRB1*0401 genotype were shown to be independently associated with the presence of subcutaneous nodules (Mattey *et al.*, 2002b). Other studies have shown that patients carrying alleles of the shared epitope to be associated with anti-CCP positivity and concentration, rapid disease progression, worse outcome including the presence of erosions as well as early mortality, especially from co-morbid diseases independent of RF (Mattey *et al.*, 2001b, Irigoyen *et al.*, 2005, Klareskog *et al.*, 2006, Mattey *et al.*, 2007, Farragher *et al.*, 2008, Kapitány *et al.*, 2008, Morgan *et al.*, 2009,

Rojas-Villarragaa *et al.*, 2009, Uçar *et al.*, 2011). Rheumatoid factor has had conflicting studies, debating its association with HLA-DRB1 genes. A number of studies have shown no significant association with this particular disease feature, while other studies have demonstrated a positive association (Weyand and Goronzy, 1997, Van Jaarsveld *et al.*, 1998, Mattey *et al.*, 2002a, Irigoyen *et al.*, 2005, Morgan *et al.*, 2009, Uçar *et al.*, 2011). Additionally, it has been suggested that the SE may have non-antigen specific roles in RA. It can activate production of nitric oxide (NO), a known vasodilator and reactive oxygen species, in increased amounts from B cells and synovial fibroblasts by acting as a signalling ligand to the NO-mediated pro-oxidative pathway, and so contribute to RA pathogenesis, as well as enhancing inflammation (Ling *et al.*, 2006).

The presence of these particular SE alleles either singularly or in combination, on one or both chromosomes respectively, is independently associated with varying degrees of progression, severity and early mortality as well as susceptibility and so can be used as a possible prognostic factor (Weyand *et al.*, 1992, Mattey *et al.*, 2007, Farragher *et al.*, 2008). For example, an individual with a homozygous DR4 genotype SE like *0401 and *0404 has considerably more RA severity than someone with a homozygous DR1 genotype, a heterozygous SE or with only one SE such as a *0101, however this mechanism is still not understood (Weyand *et al.*, 1992, Wordsworth *et al.*, 1992, Mattey *et al.*, 2007). It is thought that the allelic variants are thought to have different peptide binding specificity clefts due to the differing amino acids and so form different antigen binding pocket specificities to other HLA-DRB1 molecules. This may allow the presentation of arithrogenic peptides to occur and consequently initiate the immune response.

While HLA-DR4 and DR1 show positive association with RA susceptibility, progression and severity, the HLA has also been shown to display negative associations to disease development and thus are considered to be protective against RA disease. These alleles are of the subtypes DR2, DR3, DR5, DR7 and DR8 and are also observed to be less in frequency compared to controls (Young *et al.*, 1984, Larsen *et al.*, 1989, del Rincón and Escalante, 1999). In particular, it was shown that alleles encoding the amino acid sequence DERRA in the SE region, are protective against RA development (Wagner *et al.*, 2003). The amino acid sequence of the SE is not the only factor to determine association with RA susceptibility. Alleles that encode an aspartic acid residue at position 70 within the β_1 domain protect against RA development (Mattey *et al.*, 2001a).

1.2.1.3 Association of HLA genes with RA prevalence

HLA genetic studies have identified that particular SEs are a major genetic risk factor in different ethnic populations. Specific RA alleles are observed more prevalently in certain ethnic populations and thus a greater risk than others, with some patients suffering more severe forms of RA within the ethnicities (Ferucci *et al.*, 2004). The HLA-DR4 subtype gene, alleles DRB1*0401 and *0404 are more prevalent in North American and Western European Caucasian populations compared to other ethnic populations (50% and 30% respectively) (Silman and Pearson, 2002). High frequencies of HLA-DRB1*1402 were observed in American Indians and Alaskan natives (Ferucci *et al.*, 2004).

1.2.1.4 Linkage disequilibrium and HLA haplotypes

The contribution of the HLA genes to the development of RA is complicated, as a number of genes appear to be "linked" with close or distant genes on the same 6th chromosome. Certain alleles have been shown to be commonly observed in conjunction with several other alleles from different loci and are inherited together in a block on the same chromosome (haplotypes). A number of haplotypes are more frequently observed than a random haplotype formation and are said to be in linkage disequilibrium. In addition, the two HLA homologous chromosomes are inherited polygenically, meaning that an individual will carry and co-dominantly express a heterozygous or a very rare homozygous HLA genotype, due to the variability of inheritance (Jirholt *et al.*, 2001).

It has been estimated that ~40% of the HLA genes have a role in the immune system, and the others are still of unknown function (Aguado *et al.*, 1999). The carriage of specific HLA-DRB1 genes are not the only susceptibility factor for RA, as 10-15% of RA patients do not carry any of the specific RA HLA-DRB1 molecules. This was observed in a study where the majority of African-American RA patients did not express the SE (McDaniel *et al.*, 1995). A study of Greek patients also demonstrated that just over half of patients (57%) lacked the HLA-DRB motif (Boki *et al.*, 1992). In particular populations, patients can be independent of the presence and dose effect of the SE to the predisposition and severity of RA development (McDaniel *et al.*, 1995, González *et al.*, 1997). These studies suggest that other immune related gene (a heterogeneous immunogenicity), are involved in disease susceptibility within these patients. Conversely there are many individuals that carry the RA associated HLA-DRB1 molecules who do not present RA disease, indicating that these HLA genes are not the only driving factor for disease development (O'Dell *et al.*, 1998).

1.2.1.5 Other RA genetic influences

While the HLA is the primary genetic locus associated with RA development accounting for approximately 30-50% of the disease genetic contribution, there are polymorphisms outside the HLA region that are also associated with RA disease both with susceptibility and severity (Table 1.2.1.5), although, none have shown as strong and consistent association as the HLA. Other gene polymorphisms have been identified through the use of gene association studies and genome wide association studies (GWAS) (Barton and Ollier, 2002, Lettre and Rioux, 2008). These type of investigations have brought the total number of confirmed RA risk loci to 31 among individuals of European ancestry (Stahl *et al.*, 2010). Patients expressing a number of risk factor alleles including those of the SE therefore may have an increased risk of early onset, disease severity and earlier mortality (Lee *et al.*, 2005, Karlson *et al.*, 2008, Orozco *et al.*, 2008).

RA is a polygenic and genetically heterogeneous disease. Thus, a number of different genes predispose to RA, which may differ from one patient to another. Furthermore, certain combinations of genetic polymorphisms can increase the risk of RA onset (Chen *et al.*, 2002). Due to the gentic background of RA involving multiple genes, allelic polymorphisms can only partially explain the variable course of RA (Jawaheer and Gregersen, 2002). The contribution of genes to RA disease can therefore be used as prognostic factor for severity and outcome in patients.

Table 1.2.1.5: A selection of identified polymorphic genes that influence RA susceptibility and severity

Polymorphism Gene	GWAS/Gene Association study sources
Autoimmune regulator (AIRE)	(Terao *et al.*, 2011)
Protein tyrosine phosphatase non-receptor 22 (PTPN22)	(Begovich *et al.*, 2004, Plenge *et al.*, 2005)
N-acetyltransferase 2 (NAT2)	(Tanaka *et al.*, 2002)
TNF receptor-associated factor 1 (TRAF1)	(Plenge *et al.*, 2007, Toms *et al.*, 2011)
Complement Component 5 (C5)	(Plenge *et al.*, 2007, Toms *et al.*, 2011)
Signal Transducer and Activator of Transcription 4 (STAT4)	(Sugino *et al.*, 2010, Kunz and Ibrahim, 2011, Toms *et al.*, 2011),
Methylenetetrahydrofolate reductase (MTHFR)	(Naoko *et al.*, 2007)
Serum amyloid A1 (*SAA1*) gene promoter	(Naoko *et al.*, 2007)
Manganese Superoxide Dismutase (MnSOD)	(Mattey *et al.*, 2000)
Peptidyl-arginine deiminase I4 (PADI4)	(Suzuki *et al.*, 2003, Sugino *et al.*, 2010)
Solute carrier family 22 member 2 (SLC22A2)	(Tokuhiro *et al.*, 2003, Sugino *et al.*, 2010)
Cytotoxic T-Lymphocyte Antigen 4 (CTLA4)	(Gregersen *et al.*, 2009, Sugino *et al.*, 2010)
Cluster Determinant 40 (CD40)	(Raychaudhuri *et al.*, 2008, Kunz and Ibrahim, 2011)
Protamine 1 (PRM1)	(Kunz and Ibrahim, 2011)
Tumor necrosis factor alpha-induced protein 3 (TNF-AIP3)	(Julià *et al.*, 2008, Kunz and Ibrahim, 2011)
Krueppel-like factor 12 (KLF12)	(Julià *et al.*, 2008)
IL-6 Signal Transducer (IL-6 ST)	(Stahl *et al.*, 2010)
Sprouty-related, EVH1 domain-containing protein 2 (SPRED2)	(Stahl *et al.*, 2010)
Recombining binding protein suppressor of hairless (RBPJ)	(Stahl *et al.*, 2010)
Interferon regulatory factor 5 (IRF5)	(Stahl *et al.*, 2010)
PX domain containing serine/threonine kinase (PXK)	(Stahl *et al.*, 2010)
V-rel reticuloendotheliosis viral oncogene (REL)	(Gregersen *et al.*, 2009)
Protein kinase C theta type (PRKCQ)	(Gregersen *et al.*, 2009)
B lymphoid tyrosine kinase (BLK)	(Gregersen *et al.*, 2009)
AF4/FMR2 family member 3 (AFF3)	(Stahl *et al.*, 2010)

1.3.1 Cigarette smoking

Cigarette smoking is widely accepted to be hazardous to health in humans due to the numerous chemical toxins secreted through tobacco inhalation and second-hand smoke (Solomon *et al.*, 1999). There are over 4000 identified chemicals contained in cigarettes, with 50 of the components being carcinogenic in nature and include benzene, cadmium, lead, acetone and arsenic (Harder, 2002). It is commonly known that cigarette smoking contributes to the development of many diseases including cancer, COPD, asthma, crohn's disease and numerous others (Madretsma *et al.*, 1996b), as well as to be involved in RA (Solomon *et al.*, 1999). A study on RA monozygotic and dizygotic twins discordant for cigarette smoking, identified a significant association between patients who had ever smoked and RA disease (Silman *et al.*, 1996). This indicates that cigarette smoking regardless of genes is in an important factor in the development of RA.

1.3.1.1 Association between cigarette smoking and RA susceptibility

A number of studies demonstrated cigarette smoking to be associated with RA disease, being more common in smokers both past and current than in non-smokers (Hazes *et al.*, 1990, Heliovaara *et al.*, 1993, Silman *et al.*, 1996, Symmons *et al.*, 1997, Uhlig *et al.*, 1999, Hutchinson *et al.*, 2001, Costenbader *et al.*, 2006, Criswell *et al.*, 2006). This association between smoking exposure and increased risk of RA was also observed in a study performed by Criswell *et al*, 2006, in patients independent of genetic factors of the SE and null GSTM1 gene where patients are unable to detoxify cigarette compounds (Criswell *et al.*, 2006). In addition, the effect of smoking increased the risk of RA development and was found to be much stronger in individuals positive for RF than RF

negative (Costenbader *et al.*, 2006). As such, smoking is considered a major risk factor for RA disease susceptibility. However, the mechanism of how cigarette smoking influences susceptibility is still unclear, although there are a number of mechanisms that have been suggested. One such mechanism is that smoking may modify potential autoantigens such as citrullinated proteins, that are then recognized by T cells (particularly via the HLA-DRB1 molecule on APCs) (Klareskog *et al.*, 2006). Another mechanism is the delivery of autoantigens to the HLA by cigarette smoke. A third mechanism is that cigarette smoke substances may act as an adjuvant, and subsequently trigger the pro-inflammatory immune response. Cigarette smoking is also considered as an immunosuppressant as it affects the function and numbers of inflammatory cells including T cell unresponsiveness and anergy, as well as other tissues, such as the epithelium in blood and respiratory vessels (Geng *et al.*, 1996, Sopori and Kozak, 1998, McCue *et al.*, 2000, Singh *et al.*, 2000, Mehta *et al.*, 2008). This could allow the presentation of arithrogenic peptides, and initiate the immune response rather than enable immunotolerance of the molecule.

1.3.1.2 Association between cigarette smoking and RA severity

Numerous studies have revealed smoking to be associated with increased disease severity and progression (Heliovaara *et al.*, 1993, Saag *et al.*, 1997, Másdóttir *et al.*, 2000, Wolfe, 2000, Mattey *et al.*, 2002c). The mechanisms of how smoking influences disease progression and severity is still not fully understood. However, the various toxic compounds present in cigarette smoke have been implicated in the process. This may partly explain modulate and alter immune cell function and quantities in RA smokers (Ginns *et al.*, 1982, Hughes *et al.*, 1985, Tollerud *et al.*, 1989, Adams *et al.*, 1997, Mehta *et al.*, 2008). Consequently, such alterations therefore may effect which mediators are

secreted and their level of expression (Ginns *et al.*, 1982, Hughes *et al.*, 1985, Tollerud *et al.*, 1989), as smoking is known to induce apoptosis and angiogenesis via the the endothelial nicotinic acetylcholine receptor, collagen synthesis and cytokine production (Ginns *et al.*, 1982, Hughes *et al.*, 1985, Tollerud *et al.*, 1989, Tappia *et al.*, 1995, Madretsma *et al.*, 1996a, Nakamuraa *et al.*, 1998, Anderson *et al.*, 1999, Sher *et al.*, 1999, Singh *et al.*, 2000, Knuutinen *et al.*, 2002, Lei *et al.*, 2002, Barua *et al.*, 2003, Nordskog *et al.*, 2003, Cozen *et al.*, 2004, Raitio *et al.*, 2005, Glossop *et al.*, 2006, Karimi *et al.*, 2006, Cooke, 2007, Harel-Meir *et al.*, 2007, Cooke and Ghebremariam, 2008, Shizu *et al.*, 2008, Egleton *et al.*, 2009, Valavanidis *et al.*, 2009).

As cigarette smoke is a foreign agent within the host, it may be able to act as an adjuvant and induce an immune response against it. RA patients who had a history of smoking were observed to have more active and severe disease compared to non-smokers (Söderlin *et al.*, 2011). In contrast another study did not demonstrate any association with disease severity variables (ESR, pain, joint counts or functional ability) and smoking (Wolfe, 2000). Furthermore, one study revealed that RA patients showed no significant difference in disease activity scores (DAS 28) between current smokers and non-smokers with past smokers in either RF positive or negative patients (Westhoff *et al.*, 2008). This suggests that cigarette smoking may not contribute to alterations in disease activity measures, but does play a role in disease severity. Additionally, the effects of smoking have been shown to be independently associated with co-morbidity and mortality in RA patients (Reilly *et al.*, 1990, Wolfe *et al.*, 1994, Myllykangas-Luosuj~irvi *et al.*, 1995, Turesson *et al.*, 2003, Wolfe *et al.*, 2003, Sihvonen *et al.*, 2005, Mattey *et al.*, 2007).

1.3.1.3 Association between cigarette smoking and RA features

As cigarette smoking is associated with the severity and progression of RA, it is therefore not surprising that several studies have shown it to be significantly associated with various features of RA. This includes the manifestation of subcutaneous nodules which has been shown to be independently linked with smoking in RA patients (Másdóttir *et al.*, 2000, Mattey *et al.*, 2002b). In addition, patients with a history of smoking were associated with increased joint damage and bone erosions, independently of RF (Wolfe, 2000). Another study revealed that female patients who had ever smoked (past and current), as well as past and current smokers individually, was associated with worse radiographic outcome and HAQ score than RA patients who had never smoked, but this may be influenced by the GSTM1 null gene and RF presence in smokers (Mattey *et al.*, 2002c). However, there are a number of studies that do not reveal an association between smoking and joint damage. A study by Finckh *et al*, 2007 showed that radiographic damage progressed at a similar rate in current smokers and non-smokers (Finckh *et al.*, 2007), which is similar to another study that showed no differences in radiographic damage between current smokers and never smokers along with past smokers, in either RF positive or negative patients (Westhoff *et al.*, 2008).

1.3.1.4 Association between cigarette smoking and autoantibodies

Numerous studies have reported the independent association of smoking and the presence of RF and anti-CCP autoantibodies in RA patients (Mattey *et al.*, 2002a, Morgan *et al.*, 2009). A Finnish study in RA, revealed that men who smoke were more likely to be RF seropositive than non-smokers (Heliovaara *et al.*, 1993). Another study demonstrated that

patients with any smoking history had an increased risk for RF seropositivity in both genders, although this was only shown in heavy smokers (Stolt *et al.*, 2003). Although the presence of RF can be observed in RA non-smokers, RF production is found to be at higher titres in the serum of smokers compared to non-smokers, with current smokers significantly more RF positive than past or never smokers (Wolfe, 2000, Mattey *et al.*, 2002a, Mattey *et al.*, 2002c, Westhoff *et al.*, 2008). Cigarette smoke may modify B cell function and give rise to RF. The mechanism is still unclear but may invovled the CD91 scavenger pathway (Jónsson *et al.*, 1998, Newkirk *et al.*, 2012).

1.3.1.5 Association between cigarette smoking duration and frequency and RA

Not only is the status of cigarette smoking in patients (never, past and current) important in RA but also the duration and frequency (quantity per day) of smoking over the years (pack year history). The risk for the development of RA was found to be significantly elevated in patients who smoked ≥10 pack years and increased significantly with the increased number of pack years (Costenbader *et al.*, 2006). In addition, this study also demonstrated that increased risk of RA disease remained elevated in past smokers until 20 years or more after patient cessation (Costenbader *et al.*, 2006). A study in post-menopausal women showed that both the duration and frequency of smoking were associated with the development of RA. This was still found to be significant when adjusted for age, menopausal age, oral contraceptive use and hormone replacement therapy (Criswell *et al.*, 2002). Another study demonstrated an increasing association between increased pack years and the development of RA. This particular study also showed that heavy smoking (41-50 pack years) was strongly associated with RA development (Hutchinson *et al.*, 2001).

Various investigations have demonstrated that the more an individual smokes, the more severe the disease in RA patients, thereby indicating a dose-dependent response. This has been shown in autoantibody positive patients, where a dose-dependent effect was shown between increased frequency of smoking and the occurrence of anti-CCP autoantibodies (Klareskog *et al.*, 2006). A study by Masdottir *et al*, 2000 revealed that heavy smoking RA patients who smoked more than 20 cigarette packs a year showed an association with the presence of rheumatoid nodules, more radiological damage as well as worse functional ability (a higher HAQ and lower grip strength severity score).

In addition, a positive correlation was observed between smoking quantity and the level of RF as well as its positivity (Másdóttir *et al.*, 2000, Morgan *et al.*, 2009). Another study by Saag *et al*. also demonstrated similar results where pack years were significantly associated with RF seropositivity, radiographic erosions and nodules in a dose-dependent manner (Saag *et al.*, 1997). Furthermore, patients who smoked more than 25 pack years were over 3 times more likely to be seropositive and over 2 times more likely to have the presence of erosions than patients who had never smoked. However analysis revealed that smoking was associated with less severe radiographic disease rather than worse radiographic scores than patients who have never smoked (Saag *et al.*, 1997). It was also found that the number of years smoked was associated with the presence and level of RF as well as the formation of nodules, radiographic progression and pulmonary disease, with the latter 3 effects shown to be independent of RF (Wolfe, 2000). Interestingly, this particular study demonstrated an association between overall disease severity and smoking, but not with disease process activity measures (ESR, pain, joint count, global severity or functional ability) (Wolfe, 2000). Interestingly, while RA patients with any smoking history were associated with an increased risk for RF in both genders, this was only observed in subjects

who had smoked more than 20 years and evident with a moderate frequency of smoking (6-9 cigarettes per day). The risk also increased in a dose-dependent fashion, with the cumulative addition of smoking. In addition, this risk for seropositivity remained for up to 10-19 years after smoking cessation (Stolt *et al.*, 2003). In contrast, a particular study of RA non-smokers and those who smoked less than 20 pack years were twice as likely to reach ACR improvement compared to RA patients who smoked ≥20 pack years (Westhoff *et al.*, 2008). These type of studies illustrate that the pack year history is related to various aspects of RA disease, including its development, progression and outcome as well as development for co-morbid diseases.

Although the majority of studies illustrate an association with RA disease and increased number of pack years, a study performed by Finckh *et al*, suggested that cigarette smoking does not accelerate progression of RA disease, as an inverse dose-dependent response was observed. RA patients who smoked more than one pack a day (heavy smokers) progressed significantly less than either moderate smokers and non-smokers as well as demonstrating better functional scores, suggesting that smoking does not accelerate disease progression and have protective properties (Finckh *et al.*, 2007).

As smoking has been found to be predominantly associated with RA development, severity and progression, further studies are required to determine if there is a direct relationship, and what mechanisms may be involved, such as the alteration of various factors such as the production and secretion of inflammatory mediators, proteins, anti-oxidants as well as interactions with various genes.

1.3.2 A gene-environment interaction

Both smoking and genes have been shown to strongly influence RA disease, a combination of these factors specifically a gene-environment interaction is thought to be involved in the susceptibility, development, progression and outcome of RA.

1.3.2.1 A gene-environment interaction for RA susceptibility

The combination of gene and environmental factors has been found to be responsible for the onset of RA disease. This has been demonstrated in several studies where the HLA-DRB1 gene (shared epitope alleles in particular), and cigarette smoking is associated with RA susceptibility (Klareskog *et al.*, 2006, Morgan *et al.*, 2009). The combination of these two factors may trigger immune reactions which are involved in the development of RA (Gorman, 2006, Källberga *et al.*, 2007). A study with a population of older Caucasian women, found that those who smoked and carried a SE allele had a significant risk of developing RA (Criswell *et al.*, 2006).

Other genes with cigarette smoking have also been demonstrated to be associated with disease susceptibility. A significant risk of RA development was found in patients who smoked and had the GSTM1 null genotype (Criswell *et al.*, 2006). One study demonstrated a combination of a 2 gene-environment interaction and RA, where risk substantially increased with the combination of the PTPN22 gene (another gene highly associated with RA susceptibility), the HLA-DRB1 SE and cigarette smoking to the development of RA (Morgan *et al.*, 2009). This illustrates that a combination of multiple genes and an environmental factor further increases the risk of RA disease.

1.3.2.2 A gene-environment interaction for RA severity and progression

A gene-environment interaction has also been demonstrated to be associated not only with susceptibility to RA, but also with severity and progression of disease. A major interaction between cigarette smoking and the HLA-DR genes was evident for anti-CCP positive RA compared to negative anti-CCP RA patients. Furthermore, patients with a smoking history and the presence of SEs on both chromosomes, increased the risk 21 fold for RA positive anti-CCP compared to non-smokers without the presence of SE genes (Klareskog *et al.*, 2006).

In addition, a gene-environment interaction of patients carrying double SE genes and cigarette smoking combined with anti-CCP autoantibodies was shown to be associated with a greater risk of mortality from CVD (Farragher *et al.*, 2008). This type of combination of various factors can indicate a worse type of RA, as it is linked with co-morbidity.

In some studies the GSTM1 genotype and cigarette smoking has been shown to be associated with increased RA severity, while in others no interaction was found (Mattey *et al.*, 2002c, Bohanec Grabar *et al.*, 2009). A study in 2002, performed by Mattey *et al*. demonstrated the effects of smoking on radiographic damage in RA could be modulated by the GSTM1 polymorphism. Patients who were GSTM1-null and smoked, had worse disease outcome than non-smokers. While in contrast, patients who had a functional GSTM1 and smoked, had less severe radiographic outcome than null GSTM1 patients. Interestingly, this study found that smoking was associated with RF production in patients carrying the GSTM1 null allele only (Mattey *et al.*, 2002c). RF is closely associated to

smoking, therefore true correlation between GSTM1 and smoking is difficult to determine. As this study found that GSTM1 null associated with RF, it can be partly explained by the relationship between GSTM1 and RF in production in smokers.

Another study investigating the GSTT1 genotype revealed an interaction with the polymorphisms of this gene and cigarette smoking. In a group of RA smokers, patients carrying the homozygous deletion of GSTT1 (GSTT1 null) had over an 8 fold higher risk for developing high disease activity scores (DAS 28) than patients carrying the functional GSTT1 genotype (Bohanec Grabar *et al.*, 2009). Therefore those RA patients that carry GSTT1 have less severity then those who do not carry this gene.

Rheumatoid factor has been shown to be independently associated with both cigarette smoking and HLA-DRB SE polymorphisms, but adjusting for previous or current smokers, the significance was reduced or lost respectively (Mattey *et al.*, 2002a). Particularly patients that carried the *0401 SE and smoked, increased the likelihood of RF production (Mattey *et al.*, 2002a). This suggests that RF synthesis occurs through a different mechanism in smokers compared with non-smokers who carry the SE allele. A study by Padyukov *et* al, showed nearly a three fold increased risk for RF positive RA in the presence of SE alleles in non-smokers, and over a two fold increased risk for RF positive RA in current smokers without carriage of SE genes (Padyukov *et al.*, 2004). The combination of current smoking and the carriage of a SE showed a seven fold increased risk for RF positive RA. In addition, smokers carrying SE alleles on both chromosomes displayed nearly a sixteen fold increased risk for RF positive RA. This study illustrates significant interaction between smoking and the SE genes, where the additional presence of SE genes increases the likelihood of RF positive RA (Padyukov *et al.*, 2004).

There are studies however that demonstrated no association between smoking and the PTPN22 gene. One study found no association in patients carrying the PTPN22 gene and smoking for the development of anti-CCP (Källberga *et al.*, 2007). Another study which investigated the combination of PTPN22 and smoking found that it did not affect the risk of mortality (Farragher *et al.*, 2008).

Although a gene and environment interaction influences RA disease in both its development and severity, the relative contribution of genetic and environmental influence upon the development of RA and its severity differs between individual patients.

1.4 Biomarkers

Biomarkers typically refer to biological molecules that are used as measurements to reveal indications of a biological state or condition. Biological molecules particularly those involved in biochemical pathways or physiological processes (eg. proteins, hormones, enzymes, growth factors, steroids, metabolites, cytokines etc.), are termed as mediators and are used to assess normal and pathogenic activity.

A number of factors are able to influence mediator expression including environmental toxins (pollution and smoking), drugs (medicinal and recreational) as well as induction of the immune response though diseases and infections (bacteria and viruses). Such alterations to mediator expression generate downstream effects through the complex cellular-mediator network by intracellular signalling which subsequently changes the mediator environment. RA is thought to arise through the dysfunction of the immune system, where immune regulation is lost and leads to an inbalance of excess production of inflammatory and modulatory response mediators (markers of immune dysfunction). Measurement of these biomarkers is therefore crucial to assess immune activity and determine what pathological processes are occurring in RA disease.

Mediators are classically measured in the blood but also other bodily fluids, such as the synovial fluid, tissue, urine and saliva, by the use of protein based assay systems like the enzyme linked immuno-sorbent assay (ELISA), liquid chromatography, flow cytometry, protein microarray, mass spectrometry and the multiplex suspension array system.

There are a number of qualities that make an ideal biomarker, such as a long half-life, easily and safely measured, non-invasive, reproducible, economical as well as being proportionately representative of the investigating outcome. In addition, any potential biomarker needs to be able to identify the investigating outcome accurately. This is achieved by evaluating its validity (test accuracy), measuring its level of sensitivity and specificity. Sensitivity relates to its ability to correctly identify an individual of the particular outcome, whereas specificity relates to its ability to correctly identify an individual that is free of the investigating outcome. Therefore, it needs to be sensitive enough to include anyone presenting with the outcome in any displayed fashion, but specific enough to not incorporate anyone displaying outcomes of a similar but different nature. Furthermore, potential biomarkers are evaluated in terms of their reliability of identified individuals actually having the investigated outcome (result accuracy). This is achieved by measuring its positive and negative predictive value (PPV and NPV respectively). PPV relates to how many outcome identified individuals actually have the outcome, whereas NPV relates to how many outcome-free identified individuals are actually free of the outcome. Both these performance evaluations are calculated by equations using true positive, true negative, false positive and false negative values (Parikh *et al.*, 2008).

With the advancement of technology, an increasing number of studies have been able to investigate multiple biological mediators in parallel in RA patients. This allows a much more extensive and comprehensive analysis of the mediators responsible and its interactions involved in RA disease rather than singular mediators, which can give insights into the RA altered environment and help elucidate the comprehensive cellular-mediator network in RA. In addition, sets of mediators termed as profiles or signatures can also be

identified and illustrate relationships to specific disease measure indices. These can become specific biomarkers used to assess the level of disease activity and severity in RA, and can provide greater accuracy (sensitivity and specificity) rather than singular biomarkers. One clinical biomarker profile has been recently characterized and validated is the multi-biomarker disease activity (MBDA) (Cavet *et al.*, 2010, Centola *et al.*, 2013). This new assessment measure is a composite of 12 mediators (EGF, VEGF-A, leptin, IL-6, serum amyloid A (SAA), CRP, VCAM-1, MMP-1, MMP-3, TNF-RI, human cartilage glycoprotein 39 (YKL-40) and resistin) and evaluated through a specifically designed algorithm (Centola *et al.*, 2013). This novel assessment measure complements current RA disease activity measures (Bakker *et al.*, 2012, Curtis *et al.*, 2012, Eastman *et al.*, 2012, Hirata *et al.*, 2013). It should be noted that this measure of disease activity was not used in this project, as the MBDA incorporates mediators which were not investigated at the time of this study.

Biomarker profiling investigations can also give insights into the heterogeneity of RA and aid in the characterization of phenotypes and classification of sub-types. Biomarker profiling can also elucidate the extent of immune activity occurring in RA as well as provide useful indications into its disease pathological process (onset, development, progression and outcome), which ultimately can assist in RA diagnosis as well as identify stages of disease. Identification of biomarker profiles can eventually become useful clinical markers in the management of RA to improve patient care and outcome as well as becoming potential new targets for therapeutic intervention. In addition, biomarkers also have the potential to become a valuable clinical prognostic tool for diagnosing disease early as well as predicting its activity, severity, outcome, re-occurrence potential and the response individuals may expect to treatment intervention for newly developed arthritic

patients. This allows for more appropriate management and treatment early in the course of the disease which may limit the level of destruction of RA and possibly drive it to a state of remission.

With further understanding of the various mediators involved in RA and its complex network of interactions, this can give insights into the immune dysfunction and pathology of RA. Ultimately this can help elucidate the mechanisms and molecular pathways involved in RA disease, which may provide potential novel targets for therapeutic intervention. This could eventually develop into targeted personalized medicine based on the unique biomarker profile presentation of the patient. Biomarker drug therapy could become specific to the individual and be selected to enable the best type of response based on the presentation of the individuals' unique biomarker profile, rather than employing general drug treatments which require multiple administrations. Ultimately, research into mediators may lead to prognostic biomarkers that could indicate disease risk and susceptibility in undiagnosed asymptomatic individuals, and therefore prevention of the disease before onset and symptoms of the disease could be halted before they even develop. Thus, early therapeutic intervention as well as more specifically targeted drug development will ensue.

Biomarker profiles that are specific for disease pathologies and their symptoms would provide many advantages to pharmaceutical companies, as it could be determined with high likelihood what drug treatments would be successful. In addition, which patients would best respond to the treatment due to their biomarker profile, as well as have clues into the potential side effects of the drug. This would ultimately significantly reduce the cost of developing effective drugs, clinical trials, insurers and hospitals.

Initially, understanding of the relationship between biomarkers and RA disease activity and severity is required first to fully optimize their potential in both the research and clinical setting. This would provide the first steps to creating biomarker diagnostic and therapeutic applications. There have been a number of studies which have investigated multiple mediators (≥5 mediators) in RA patients to identify profile signatures specific to RA disease, as well as relationships to disease activity and severity measures.

1.4.1 Biomarker profiling RA disease activity and severity

A number of studies have demonstrated a particular type of biomarker profile of various interleukins and other notable mediators to be linked with RA autoantibodies. In particular, several studies have shown that IL-1β, IL-4, IL-10, IL-12, TNF-α and GM-CSF in particular, specifically distinguished RA patients positive for RF and/or anti-CCP autoantibodies (Hitchon *et al.*, 2004, Alex *et al.*, 2007, Hueber *et al.*, 2007, Jørgensen *et al.*, 2008, Kokkonen *et al.*, 2010, Meyer *et al.*, 2010, Chandra *et al.*, 2011, Hodkinson *et al.*, 2011). This type of biomarker profile and autoantibody expression was also illustrated in pre-disease onset patients, where autoantibodies have been found prior to disease development (Jørgensen *et al.*, 2008, Deane *et al.*, 2010, Kokkonen *et al.*, 2010). Interestingly, inflammatory regulators sTNF-R1 (Jørgensen *et al.*, 2008) and IL-1ra (Jørgensen *et al.*, 2008, Kokkonen *et al.*, 2010) were also found in patients with high autoantibody expression prior to their disease onset (Jørgensen *et al.*, 2008, Kokkonen *et al.*, 2010). However one study revealed a completely contrasting biomarker profile with autoantibody expression. From a set of 163 immune related mediators which included various interleukins and cytokines, a unique profile of high M-CSF, TNFRSF9, myeloid progenitor inhibitory factor-1/macrophage inflammatory protein (MPIF-1/MIP-3), TGF-α

and B lymphocyte chemoattractant (BLC) expression correlated with RF expression in RA patients (Rioja *et al.*, 2008). This type of profile of various interleukins and other notable mediators therefore could predict a more severe phenotype as autoantibodies have been associated with worse disease and poorer outcome.

Acute phase proteins (CRP and SAA) and the ESR are common markers of peripheral inflammation, where a number of studies revealed IL-6, MIP-1α, M-CSF, TNFRSF9, MPIF-1/MIP-3, TGF-α and BLC in particular to be linked with these inflammatory processes (Hueber *et al.*, 2007, Rioja *et al.*, 2008, Meyer *et al.*, 2010). This type of profile therefore would be a good indicator of systemic active disease in RA.

Disease activity composites are useful indicators of general disease activity and severity status in RA patients. Many mediators including interleukins, growth factors, RA commonly regarded mediators, chemokines and TNF receptors from a number of studies have been shown to be associated with assorted disease activity composites such as the DAS28, active-quiescent index and the simplified disease activity index (SDAI) (Paramalingam *et al.*, 2007, Rioja *et al.*, 2008, Meyer *et al.*, 2010, Hodkinson *et al.*, 2011). However, IFN-γ, VEGF, TGF-α, MPIF-1/MIP-3, M-CSF and TNFRSF9 were shown in particular to be predominantly linked with disease activity composites, and thus could be a profile for worse disease activity (Paramalingam *et al.*, 2007, Rioja *et al.*, 2008, Meyer *et al.*, 2010, Hodkinson *et al.*, 2011).

A range of mediators have been identified with bone erosions in RA patients, including elevated expression of IL-1α, IL-2 (Fong *et al.*, 1994), MMP-1, MMP-3, MMP-8, MMP-9 and TIMP-1 (Yoshihara *et al.*, 2000), and decreased IL-4, IL-18 (Uppal *et al.*, 2008).

Interestingly RA patients positive for nodules, expressed a profile of elevated IL-2, IL-7, IL-12, VEGF and IFN-γ also presented with larger amounts of bone damage compared to nodule negative RA patients (Hodkinson *et al.*, 2011). Another study demonstrated 30 mediators incorporating serum proteins (CRP, α_2-plasmin inhibitor, glutathione transferase), metabolic enzymes (triosephospate isomerise, G3PDH), calcium binding S100 proteins (S100A4, A8, A9, A11, A12, S100P), matrix-degrading cysteine proteinase cathepsin B and its inhibitor cystatin B, cellular signalling proteins (14-3-3 protein, RhoGD12) and various other less characterized proteins to be more abundant in erosive positive RA patients with low kininogen, apolipoprotein C-II and vitamin K-dependent protein C expression in addition (Liao *et al.*, 2004). This diverse range of mediators identified from these various studies could be a marker for a more destructive disease outcome, although further investigation should be undertaken to better characterize mediators involved in joint damage. Biomarker profiling studies have also identified a possible prognostic profile for bone erosion, where increased IL-6 (Knudsen *et al.*, 2008), MMP-8, MMP-9 (Yoshihara *et al.*, 2000), GRO- α and RANTES (Boiardi *et al.*, 1999) as well as decreased eotaxin (Syversen *et al.*, 2008) in early RA patients have been shown to be associated with increased radiographic progression.

Other joint specific indices have also been identified with specific mediator profiles, where one study demonstrated high IL-6, M-CSF, TNFRSF9, MPIF-1/MIP-3 and TGF-α correlated with the 68 swollen joint score (Rioja *et al.*, 2008). Similarly this profile with the addition of BLC also correlated with the tender joint score (Rioja *et al.*, 2008). Interestingly another study in seropositive RA patients, revealed IL-4 was correlated with a smaller number of joint involvements (3-6 joints), illustrating that this anti-inflammatory cytokine is linked with less joint activity (Uppal *et al.*, 2008).

1.4.2 Biomarker profiling for RA disease subtypes

As RA is a heterogenic disease, a number of studies have used grouping methods in an attempt to characterize biological subtypes of the disease. Characterizing RA disease will not only be useful in elucidating the diverse pathological pathways of RA, but also may be important in clinical use as they may have also differential responses to therapy.

Two studies were able to identify patient clusters based on their relative mediator expression level (ie. low, moderate, high etc. mediator expression) (Alex *et al.*, 2007, Hueber *et al.*, 2007). In particular, relative expression of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/70, IL-13, TNF-α, MIP-1α and GM-CSF were shown to correspond to the level of disease activity of their respective cluster, where autoantibodies, ESR, CRP and the HAQ were either relatively low, moderate or high to their respective patient cluster (Alex *et al.*, 2007, Hueber *et al.*, 2007). This indicates that these patients had a relatively homogenous disease type that varied in phenotype severity rather than in functional disease subtypes.

Another study identified 2 clusters within early RA patients where a profile of IL-4, IL-12, IL-13 and TNF-α was shown to significantly distinguish between the 2 clusters. Furthermore, the relatively higher expression cluster had low expression of IL-2, IL-5, IL-10 and IL-12 but high IL-4, IL-6, IFN-γ and GM-CSF levels comparatively to the lower expression cluster, and interestingly demonstrated significantly higher autoantibody presence (Hitchon *et al.*, 2004). However, these 2 clusters displayed similar scores of CRP, ESR, HAQ, VAS and swollen joint counts suggesting that this patient cluster could be an autoantibody specific subtype.

Furthermore, one study revealed a subset of seronegative patients at early RA disease with elevated IL-1α, IL-1β, IL-6, IL-12 p40 and p70 subunits, IL-15, IL-17, eotaxin, FGF-β, IP-10, MCP-1, TNF and GM-CSF, which were more reflective of a seropositive RA disease. This could illustrate a subtype of RA more clinically and immunologically aggressive than previously assumed, although it should be noted that RF may not yet have developed in these early RA seronegative patients (Chandra *et al.*, 2011).

1.4.3 Biomarker profiling pre-development and onset of RA disease

A few studies have identified specific profiles which were characterized in RA patients before their onset and diagnosis of disease (pre-RA undifferentiated arthritis), where IL-10, IL-13, TNF-α, FGF-β and IP-10 were found to be predominantly linked with pre-RA patients compared to healthy individuals or established RA patients (Raza *et al.*, 2005, Jørgensen *et al.*, 2008, Deane *et al.*, 2010, Kokkonen *et al.*, 2010). This suggests that these are important mediators involved in the initial development of RA and could be a prognostic indicator for individuals who may potentially develop disease pathology. Specific mediators of this profile were also shown to be elevated in pre-RA patients who were closer to their time of diagnosis and increased as time of diagnosis approached, and were also predictive of decreased time to diagnosis (Deane *et al.*, 2010). A distinct profile of elevated IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-12p70, sTNF-RI and RII plus low TGF-β expression was found however to be associated with RA disease onset (Jørgensen *et al.*, 2008, de Paz *et al.*, 2010), whereas another study distinguished pre-RA developed patients from RA onset patients with a profile of IL-4, IL-6, IL-17, G-CSF, VEGF, PDGF-ββ, MIP-1β and MIG (Kokkonen *et al.*, 2010). It should be noted that classification of pre-RA patients is dependant on how quickly rheumatologists are able to

diagnose undifferentiated arthritides, as patients could be characterized with unclassified polyarthritis for years rather than recognising that they were actually in the early stages of RA. Interestingly, patients with raised IL-4 and IL-18 in PBMC both correlated with an earlier age disease onset in seropositive patients and IL-8 correlated with established RA disease (Uppal *et al.*, 2008). These studies give insights into the development and progression process in the different stages of RA.

1.4.4 Biomarker profiling RA in comparison with other disease pathologies

Various studies have investigated multiple mediators in RA patients compared with healthy individuals to further understand the pathology of RA. Studies revealed predominantly IL-4, IL-6, IL-10, TNF-α and MCP-1 that distinguished RA patients against from those without disease (Butrimiene *et al.*, 2004, Hitchon *et al.*, 2004, Stabler *et al.*, 2004, Havemose-Poulsen *et al.*, 2005, Alex *et al.*, 2007, Hueber *et al.*, 2007, Paramalingam *et al.*, 2007, Jørgensen *et al.*, 2008, Knudsen *et al.*, 2008, Rioja *et al.*, 2008, de Paz *et al.*, 2010, Kokkonen *et al.*, 2010, Meyer *et al.*, 2010). Interestingly, a number of studies revealed IL-10 to be either elevated (Butrimiene *et al.*, 2004, Hitchon *et al.*, 2004, Stabler *et al.*, 2004, Havemose-Poulsen *et al.*, 2005, Jørgensen *et al.*, 2008, Kokkonen *et al.*, 2010, Meyer *et al.*, 2010), or decreased in comparison with healthy people (de Paz *et al.*, 2010). This contributes further to the well known pleiotropic roles of IL-10 with both pro- and anti-inflammatory effects, as well as further illustrating the heterogeneous developmental pathways of RA. In addition, metastasin (S100A4); a calcium binding protein involved in regulation of cellular processes including cell cycle, has been shown to be reduced in RA patients compared to healthy individuals (Liao *et al.*, 2004).

Multi-biomarker profiling has also been performed in other arthritides for comparison with RA to identify profiles that distinguish between the different arthritides. This type of profiling will not only be useful in aiding differential diagnosis, but may also help in potentially being able to direct aggressive destructive articular diseases like RA to milder arthropathic forms.

Reactive arthritis is a self-limiting arthritis and so has been compared with RA to identify ways able to restrict the chronicity of RA. There have only been a few multi-mediator profiling studies between these arthritides, each revealing a different profile that distinguished RA from ReA. One such study demonstrated a profile of high IL-4, IL-8, IL-10 and sTNFR-II along with a low expression of IFN-γ in RA patients compared to patients with ReA (Ribbens *et al.*, 2000). Another study revealed a profile of significantly lower IL-6, IL-17, TGF-β and IFN-γ expression in patients with RA compared to patients with ReA disease (Singh *et al.*, 2007a). One study also illustrated elevated levels of IL-10 in RA patients, but interestingly within acute ReA patients in comparison to chronic ReA patients, revealing the diversity of this cytokine as mentioned previously (Butrimiene *et al.*, 2004). This particular study also demonstrated RA patients had higher expression of the pro-inflammatory cytokine TNF-α than in both acute and chronic ReA patients (Butrimiene *et al.*, 2004).

Distinct profiles were also found in a few studies distinguishing RA from psoriatic arthritis (PsA), where IL-1β, IL-8, IL-10, TNF-α, TNF-β (lymphotoxin) were shown to be highly expressed in RA patients with low IL-4 compared to those with PsA (Partsch *et al.*, 1997, Partsch *et al.*, 1998). Another study demonstrated elevated IL-1α, IL-12p40, IL-13, TNF-α, IP-10, eotaxin and MCP-1 with low IL-8 in early RA patients against PsA patients (Hueber

et al., 2007). Whereas a study in the synovial fluid of early RA patients demonstrated a profile of IL-2, IL-4, IL-13, IL-15, FGF-β and EGF that differentiated RA from early PsA patients (Raza *et al.*, 2005). Interestingly this profile also discriminated RA from both crystal- and non-crystal related resolving arthritis (Raza *et al.*, 2005).

Chondrocalcinosis also known as pseudogout is a crystal oligarticular arthritis through the deposition of calcium pyrophosphate (CPP) in the synovial joints. It is an inflammatory arthritis, typically afflicting the larger joints of the body including the wrist, elbow, ankle and knees with pain. One study identified IL-8, IL-10 and sTNFR-II to be largely expressed in RA disease compared with acute crystal arthritis (Ribbens et al., 2000).

Systemic lupus erythematosus (SLE) although an autoimmune disease that targets nuclear material and thus affects any part of the body, commonly afflicts the joints in patients due to the excessive accumulation of auto-antibody complexes. A sera profile of 12 mediators; IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-12p40, TNF-α, GM-CSF and MCP-1 were found to be specific to RA compared with SLE (Szodoray *et al.*, 2004, Alex *et al.*, 2007). This type of profiling can give insights into RA pathology as SLE is also an autoimmune disease and includes auto-antibody complexes like RA, but is a less destructive and disabling disease of the joints than RA.

Another autoimmune disease; relapsing polychondritis (RP), is a chronic reoccurring inflammatory disorder of the cartilage. It inflames and destroys cartilage particularly in the ear, nose, windpipe, spine and any other place with cartilage. It also affects predominantly joints of the hands, feet, knees, ankles, wrists plus elbows and therefore sometimes is misdiagnosed as RA especially in the early stages of disease. Despite that RP and RA are

both autoimmune diseases that target the cartilage, a study illustrated the 2 disease states are distinctly different as IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IFN-γ, TNF-α, G-CSF, GM-CSF and MCP-1 were found to be higher in RA than in RP patients while IL-8, IL-13, and MIP-1β were shown to be significantly lower in RA patients (Stabler *et al.*, 2004). These differences in mediator expression may in part explain why RP is a seronegative, non-deforming and non-bone destructive polyarthalgia comparatively to RA.

Osteoarthritis has been commonly compared with RA as it is regarded as a non-inflammatory arthritis. Studies have shown that a profile of IL-1β, IL-4, IL-6, IL-8, IL-10, TNF-α and IFN-γ in particular discriminated RA disease from OA (Schlaak *et al.*, 1996, Partsch *et al.*, 1997, Partsch *et al.*, 1998, van den Ham *et al.*, 2009, Moura *et al.*, 2010). Furthermore, extracellular matrix degrading enzymes were also shown to differentiate between the arthritides, where particularly MMP-1, -2, -3, -8 and -9 were elevated in RA patient synovial fluid compared to OA patients (Yoshihara *et al.*, 2000, Tchetverikov *et al.*, 2004).

These studies illustrate the diverse process pathways between the different pathologies and demonstrate biomarker profiles that may be able to distinguish RA from different arthritides.

1.4.5 RA biomarker profiling between the peripheral circulation and the synovial joint

Multi-biomarker profiling studies have been predominantly performed in either the peripheral circulation or the synovial joint with the former particularly investigated due to the ease of procuring samples. However the peripheral circulation may not reflect the same level of immune activity as within the joint, the main site of inflammation. There are a limited number of biomarker profiling studies that have investigated differences between the peripheral circulation and the active joint. Commonly associated RA pro-inflammatory cytokines IL-6, TNF-α and IFN-γ have been shown to be abundantly expressed in the synovial fluid compared to the plasma in RA patients (Steiner *et al.*, 1999), as well as extracellular matrix degrading enzymes MMP's -1, -2, -3, -8 plus TIMP-1 (Tchetverikov *et al.*, 2004). Another study revealed a profile of just under 30 other inflammatory mediators of various interleukins (IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18), chemokines (IL-8, MCP-1, MIP-1α, MIP-4, eotaxin, TARC, MIG, IP-10, CCL22) and other mediators (TNF-α, IFN-γ, OPG, MIF, OSM, sRANKL, sCD54, sCD106) that were elevated in the synovial fluid which was not reflected in the plasma (van den Ham *et al.*, 2009). This raised mediator pattern was similarly shown with IL-6, IL-8, IL-10, IL-23, OPG, APRIL (A Proliferation Inducing Ligand) and BAFF (B cell activating factor) which were distinctly elevated in the joint of RA patients compared with the sera (Moura *et al.*, 2010). Although in contrast, TGF-β an anti-inflammatory cytokine was shown to be higher in the sera than in the active joint (Moura *et al.*, 2010). These studies illustrate that the mediator environment is uniquely diverse between these 2 sites and that the joint has specific mediators in comparison to the periphery which can reflect their respective site.

In contrast, similar profile patterns have also been identified between the peripheral circulation and the synovial joint where IL-1α, IL-2 (Fong *et al.*, 1994) and calcium binding proteins calgranulin A, B and C (Liao *et al.*, 2004) were shown to be elevated in both the active joint and the peripheral circulation of erosive RA patients in comparison to non-erosive RA patients. These studies suggest not only a more destructive outcome of RA disease in both the periphery and the active joint, but also a relationship between the 2 sites as indicated by the strong correlation found in MMP-8 and MMP-9 expression between the serum and synovial fluid of RA patients (Tchetverikov *et al.*, 2004). Similarities between the 2 environments are not unexpected since the synovial joint recruits inflammatory cells and mediators from the peripheral circulation to promote disease activity within the active joint. However calcium binding proteins S100A4, A11 and S100P were found to be highly expressed in the synovial fluid of erosive RA patients but were not reflected in the sera compared to non-erosive patients (Liao *et al.*, 2004). This suggests that this joint biomarker profile specifically relates to bone erosion and joint damage.

Additional comparative investigation in RA patients is warranted to identify specific peripheral or joint originating profiles which contribute to RA activity and severity respectively. It should be noted, that the majority of these studies were performed with serum/plasma and synovial fluid samples procured at different time points as synovial effusions do not always coincide with time of blood attainment. Therefore further profiling investigations using paired RA samples would be useful for accurately comparing between the 2 environments.

1.4.6 Cigarette smoking and biomarker profiling

Cigarette smoking exacerbates all aspects of RA pathology (onset, development, activity, progression, severity, outcome), by influencing the production and secretion of mediators. However there is a paucity of multi-biomarker profiling studies in RA smokers with the exception of one study which demonstrated TNF-α to be higher in RA patients with a smoking history compared to those who never smoked, and correspondingly increased with duration of smoking as well as quantity of cigarettes smoked (Glossop *et al.*, 2006).

A few studies however have investigated the influence of cigarette smoke upon mediators in healthy individuals, which can provide insights into how smoking may effect immune function and give indications in relation to RA disease. Past studies have demonstrated a number of mediators (IL-1α, IL-1β, IL-5, IL-6, IL-8, IL-13 and TNF-α) to be raised with cigarette smoke (Tappia *et al.*, 1995, Wang *et al.*, 2000, Lei *et al.*, 2002, Cozen *et al.*, 2004, Karimi *et al.*, 2006, Shizu *et al.*, 2008). MMPs (MMP-2, -8, -9) have also been shown to be raised in individuals who smoke compared to non-smokers suggesting that smoking can also effect extracellular matrix degradation and turnover (Nakamuraa *et al.*, 1998, Knuutinen *et al.*, 2002, Raitio *et al.*, 2005). The actual mechanism of how cigarette smoke can alter mediator expression is unknown, although it is a foreign toxic compound and therefore has the ability to induce an inflammatory immune response. Cigarette smoke has also demonstrated downstream effects as cigarette smoke condensate (CSC) was shown to augment the effects of TNF-α to induce interleukins IL-1α, IL-1β, IL-6 and IL-8 (Shizu *et al.*, 2008). Such modulations to mediator expression can also affect the mediator environment which consequently can contribute to disease pathologies.

The mechanism of how cigarette smoke modulates mediator expression seems to reflect a dose-dependent response. Healthy heavy smokers (>20 cigarettes/day) were shown to express IL-5 and IL-13 significantly higher than that of light smokers (<20 cigarettes/day) or non-smokers (Cozen *et al.*, 2004). Levels of TNF were also found to be increased in both smokers and non-smokers PBMC alike after incubation with nicotine at incremental concentrations (Lei *et al.*, 2002). Furthermore, cigarette smoke has been shown to alter mediator expression in a time-dependent manner, where individuals who had smoked for more than 20 years, expressed higher MMP-9 compared to those who smoked for less than 20 years (Nakamuraa *et al.*, 1998). Therefore individuals who smoke both heavily and for multiple years are likely to have a very altered mediator environment as shown in a CSC treated synovial fibroblast cell line, where protein levels of IL-1α, IL-1β, IL-6, IL-8 were up-regulated in both a time- and dose-dependent manner (Shizu *et al.*, 2008). This dual relationship was similarly observed in a study with RA patients, where increased TNF-α expression corresponded with increased smoking quantities and length of time (Glossop *et al.*, 2006). Therefore cessation of cigarette smoking is also likely to alter mediator expression and environment as shown in healthy individuals who had ceased smoking for more than 6 months, had significantly lower levels of MMP-9 than current smoking individuals (Nakamuraa *et al.*, 1998), illustrating the quick-turn around effects of stopping this mediator influencing stimulus.

Cigarette smoke has also been shown to inhibit mediator expression, such as smokers were found to have decreased expression of IL-2, IL-10, IFN-γ , human beta defensin-2 (hBD2) and TIMP-1 (cell proliferator with anti-apoptotic abilities as well as MMP inhibitor) compared to healthy non-smoking individuals (Madretsma *et al.*, 1996a, Madretsma *et al.*, 1996b, Ouyang *et al.*, 2000, Lee *et al.*, 2007, Choi *et al.*, 2008), as well as in a dose-

dependent manner (Madretsma *et al.*, 1996a, Choi *et al.*, 2008). This suggests that cigarette smoke may have pleiotropic effects with inhibitory activities also which could suppress immune and downstream responses (Lee *et al.*, 2007). This immunosuppressive activity could account for the increased susceptibility of microbial infections in individuals linked with cigarette smoking (Sopori *et al.*, 1998, Raza *et al.*, 1999, Choi *et al.*, 2008), as well as the contribution of a worse pathophysiology in diseases, including RA. Although this is not the case for all pathologies as cigarette smoking seems to have a beneficial effect in individuals with ulcerative colitis (UC), a colon autoimmune disease, which could be brought about by the immunosuppressive properties of cigarettes (Madretsma *et al.*, 1996a, Madretsma *et al.*, 1996b). Although another suggested theory is hydrogen cyanide within cigarette smoke reacts with hydrogen sulphide from the UC gut flora to produce non-toxic isothiocynate thereby inhibiting sulphides from disrupting the colon-mucosa balance (Levine *et al.*, 1998).

In contrast, other studies have demonstrated an opposing expression of specific aforementioned mediators including IL-1β, IL-8 and particularly TNF-α to be inhibited in the presence of cigarette smoke (Madretsma *et al.*, 1996a, Ouyang *et al.*, 2000, Lei *et al.*, 2002, Lee *et al.*, 2007). This could be due to the paradoxical ability that has been observed with cigarette smoking, of being able to both promote and inhibit mediator expression, where one study demonstrated raised TNF expression in individuals which corresponded to incrementing concentrations of nicotine, until TNF expression was inhibited (Lei *et al.*, 2002), this suggests a possible immunoregulatory threshold limit within individuals.

Interestingly, some mediators such as IL-2, IL-4, IFN-γ, TNF-α and MMP-1 have shown no differences in expression levels with or without the presence of cigarette smoke in

healthy individuals (Knuutinen *et al.*, 2002, Cozen *et al.*, 2004). This could be attributed to the suggestion that cigarette smoke appears to modulate mediator expression within certain specific cells, as demonstrated in a study with RA patients where TNF-α from lymphocytes, not monocytes, were significantly higher in patients who had ever smoked in their lifetime than those who had never smoked (Glossop *et al.*, 2006). This is similarly demonstrated with specific mediators from certain types of cells in other studies (Knuutinen *et al.*, 2002, Cozen *et al.*, 2004), including IL-8 which was highly secreted from both monocytes and macrophages in comparison to other peripheral blood mononuclear cells (PBMC) (neutrophils and lymphocytes) upon cigarette smoke stimulation (Karimi *et al.*, 2006). Therefore comprehensive biomarker profiling in RA smokers is necessary to elucidate what mediators are modulated by cigarette smoke and how this influences RA disease.

1.4.7 RA drug treatment and biomarker profiling

Therapeutic drug agents intervene in the disease process and progression of RA, making it difficult to identify the true mediator environment in RA and what immune activity processes are actually occurring. This is particularly true of DMARDs compared to biologics, as they are in greater use and are administered at an earlier stage of RA, which would influence the development of disease. Multi-biomarker profiling in RA patients receiving treatment allows for the identification of profiles specific to therapy responsive patients. RA disease therapy like biologics not only affects the specifically targeted mediator but also consequently generates downstream effects, which ultimately influences the mediator environment. One such example is the restoration of T regulatory cellular function after anti-TNF-α treatment, and its subsequent inhibition of cytokine production

and suppression of T helper cells (Ehrenstein *et al.*, 2004). This type of biomarker profiling can help better understand the mediator-cellular network involved in RA as well as gain insights into the pathways involved in disease pathology.

Various studies have demonstrated a range of mediators have their expression altered in response to infliximab, etanercept or adalimumab RA anti-TNF-α treatment, including decreased OPG, sRANKL (Ziolkowska *et al.*, 2002), BLC (CXCL13), MIP-3/MPIF-1, TFG-α (Rioja *et al.*, 2008), IL-7 (van Roon *et al.*, 2007), IL-6 and IL-13 but increased M-CSF (Tokayer *et al.*, 2002), sIL-2R (Amital *et al.*, 2007), MCP-1, EGF (Fabre *et al.*, 2008) and interestingly TNF-α, despite responding to treatment (Amital *et al.*, 2007). Another study which investigated a combination of 3 RA geographical independent cohorts also demonstrated TNF-α as well as IL-15 to be significantly elevated (Hueber *et al.*, 2009). Rituximab, a B cell antagonist biologic drug treatment also demonstrated changes in mediator expression in responsive RA patients, where IL-6 was revealed to be decreased but MCP-1 and EGF were elevated (Fabre *et al.*, 2009). This differential mediator expression further reveals the discrete downstream pathways of the cellular-mediator network in RA as well its variable heterogeneity. Patients are distinct individuals and so respond uniquely, which may explain why a third of patients do not respond to biologic treatment (refractive patients).

As the mediator environment is consequently altered in RA responsive patients, the level of disease activity and inflammation also changes, and severity and joint damage alter as a result. A number of studies have illustrated that the level of inflammation and autoantibodies were decreased after the administration of biologic therapies (Alessandri *et al.*, 2004, Ehrenstein *et al.*, 2004, Rioja *et al.*, 2008, Sennels *et al.*, 2008, Fabre *et al.*,

2009). A profile of reduced IL-6 and IL-13 but increased M-CSF was shown to have a clinical improvement in RA patients (Tokayer *et al.*, 2002), while a decrease in IL-7 correlated with a decrease of ESR and DAS (van Roon *et al.*, 2007). Another study revealed a profile of IL-6, BLC, MIP-3 (CCL23) and M-CSF positively correlated with the DAS28 score in anti-TNF-α treated patients (Rioja *et al.*, 2008).

DMARDs suppress the immune system to inhibit leukocyte functional activity, although the exact mechanism is still unknown, so the expression of mediators changes as a result. A large range of mediators have been found to be altered after DMARD treatment in RA patients including reduction in various interleukins, ECM related mediators, adhesion molecules, cartilage glycoproteins, chemokines and growth factors (Barrera *et al.*, 1994, Boiardi *et al.*, 1999, Kraan *et al.*, 2000, Alex *et al.*, 2007, Knudsen *et al.*, 2008, van Roon *et al.*, 2008, Maillefert *et al.*, 2010), plus the upregulation of soluble TNF receptors (Barrera *et al.*, 1994). A profile of IL-1β, IL-6, TNF-α, and MCP-1 in particular were downregulated after treatment with DMARD therapy. Mediator expression after DMARD treatment also seem to reflect a dose-dependent relationship where one study revealed reduced levels of interleukins, cytokines, chemokines and growth factors which corresponded to incremented methotrexate (MTX) concentrations (Alex *et al.*, 2007) Furthermore, one study demonstrated IL-1β was no longer reduced after long term treatment of MTX (Barrera *et al.*, 1994). This could be because chronic stimulation leads to RA patients developing a resistance to DMARD efficacy leading to a loss of effect on mediator expression (Donahue *et al.*, 2008).

As DMARDs are able to effect the mediator environment this ultimately influences the level of disease activity and severity in RA, where peripheral inflammation (ESR, CRP)

and joint disease activity (SWJ, TJ) have been shown to be reduced (Knudsen *et al.*, 2008, van Roon *et al.*, 2008). A few studies have identified specific profiles with clinical improvement where decreased IL-2, IL-4, IL-6, IL-8, IL-13, IL-17, TNF-α, G-CSF, GM-CSF and MCP-1 was shown to correlate with a reduction in both the HAQ and the DAS 28 score (Alex *et al.*, 2007). Another study singularly demonstrated a decrease of IL-7, an important mediator in T-cell activity to correlate with ESR, swollen joint and tender joint count reduction (van Roon *et al.*, 2008) while a reduction of GRO-α and RANTES has been shown to halt the progression of joint erosion (Boiardi *et al.*, 1999). As each RA patient is a unique individual, DMARD suppression is not always complete and thus the alleviation of symptoms felt by patients can differ considerably between them.

Multi-biomarker profiling also can be useful in identifying patients who are more likely to respond to RA treatments and thus a better use of resources as well as earlier administration and more appropriate treatment to the specific patient. This was demonstrated in a study where a prognostic profile of IL-1α, IL-1β, IL-6, IL-12-p40 and p70, IL-15, GM-CSF, FGF-β, MCP-1, eotaxin, TNF-α and IP-10 as well as a number of autoantigens predicted a positive response to etanercept (Hueber *et al.*, 2009).

The efficacy of RA treatments can also be influenced by various factors like toxins, physiological factors such as stress as well as cigarette smoking. RA smokers have a poorer outcome despite responding to anti-TNF-α therapy (Hyrich *et al.*, 2006), and smokers generally feel worse and have a higher need for increased DMARDs to control disease activity. This may reflect alterations in pharmacokinectics (Westhoff *et al.*, 2008).

Overall these profiling studies illustrate the wide range and the complex network of mediators involved in RA, although further investigation is needed to fully elucidate the mediators specifically connected to RA and their role in the disease process. A number of these studies investigated a restricted set and limited number of mediators (due to the expensive nature of multi-mediator profiling in terms of both cost and mediator target availability) which limits profiling analysis as well as not being able to give a complete reflection of the broad range and extensive network of mediators in RA disease. Furthermore a few studies were performed with PBMC, specific cells of tissues which do not accurately reflect the various cells and tissues involved in RA. Appropriate samples such as the synovial fluid and sera or plasma should be used alternatively as they better encompass the extensive mediators involved in RA.

CHAPTER 2

AIMS AND OBJECTIVES

2.1 Aims

The main aim of this research is to investigate the relationship between markers of immune dysfunction and the disease process in RA, as well as their relationship to disease severity and therapeutic response. Research in this area is important for further understanding the pathological process and the molecular pathways involved in the development of RA, and to identify potential molecular targets for therapeutic intervention. Such information could help in the development of new strategies for manipulation of immune function in patients with RA, and provide alternative therapies to those currently available.

2.2 Objectives

Three patient cohorts were investigated in this study; an early RA cohort with 5 year follow-up data, RA patients with paired serum and synovial fluid and a cohort which allowed comparison between patients with RA with other arthritic pathologies. These are summarized in further detail below.

2.2.1 Early and established RA

The purpose of this research is to investigate markers of immune dysfunction which contribute to RA disease development and severity. RA has a variable developmental course, which results in a wide variety of outcomes, ranging from mild disease, with limited bone damage to severe RA including disability, deformity and premature death. The measurement of biomarkers in serum from patients with early RA could provide valuable clues to the pathways by which RA develops. This investigation will also

determine whether specific measures of disease activity and severity are associated with particular markers of immune dysfunction, both in early and established RA. This is only possible because of the unique patient database available which contains an extensive and comprehensive range of data at baseline and 5 year follow-up. It includes a broad set of demographic and lifestyle factors as well as clinical data on disease activity and severity which will allow detailed analysis of the various factors associated with specific disease characteristics, disease progression and disease outcome.

The availability of serum samples at baseline and after 5 year follow-up in RA patients is a unique resource which will allow investigation of changes in biomarkers and their relationship to changes in disease activity and severity during the progression of RA. This contrasts with most previous studies which have examined biomarkers at only one time point and have not investigated such a wide range of markers as this study. Another objective is to identify prognostic biomarkers which are predictive of disease outcome in established RA, which could provide clues to the pathways in RA development. Clinically, the use of biomarkers may have a significant impact on the ability to predict disease outcome and the type of response to treatment that might be expected. This may in turn make it possible to determine what type of treatment to use in the early stages of the disease to achieve a better response.

Smoking has repeatedly been shown to be significantly involved in the onset and development of RA as well as being strongly associated with worse disease progression and severity. While it is unlikely that smoking is the only environmental factor involved in the development of RA, it may provide important clues as to how changes in immune regulation lead to disease development. Therefore, a secondary objective of this

investigation is to determine whether cigarette smoking in RA patients is associated with any biomarkers which may be related to alterations in immune regulation. Such investigations can elucidate the role of cigarette smoking in RA as well as help understand the mechanisms involved in the effects of smoking in RA.

2.2.2 Paired RA sera and synovial fluid

Investigation into the relationship between paired serum and synovial fluid biomarkers is another objective of this study. The availability of paired serum and synovial fluid samples from the same patient, alongside extensive clinical information, is another novel feature of this study. No previous studies have reported on analysis of multiple biomarkers in such paired samples. Such investigations are important since the peripheral circulation may not necessarily reflect the same level of immune activity as within the joint, the main site of inflammation. Comparison of markers between the 2 sites may provide unique insights into immune activity in the different environments within RA and provide further clues into the pathological process. In addition, investigation of biomarkers can determine whether there are specific markers which results in the destruction of the joint. As the active joint recruits biomarkers from the peripheral circulation, this can elucidate the relative contribution of markers from the different environments involved in joint damage.

Additionally, investigation into the relationship between cigarette smoking and biomarkers in both the joint and the peripheral circulatory system will also be undertaken. This will determine whether cigarette smoking is associated with any effects on immune activity in the joint and how this compares with any effects it has upon immune activity in the

peripheral circulatory system. Such information would be important in helping to understand the mechanisms involved in the effects of smoking in RA.

2.2.3 RA and other arthritic diseases

Another objective of this study is to investigate the expression of biomarkers in sera from other arthritic diseases and to compare this with their expression in RA. These other arthritides include 2 inflammatory diseases: reactive arthritis and psoriatic arthritis, along with a non-inflammatory disease: osteoarthritis. Comparisons between the different arthritides may indicate whether there are particular markers which may explain the different pathologies of these arthritides. Such information may be useful in diagnosis, where a particular profile of markers could be indicative of the type of arthritis, and may provide clues to the different routes involved in development of inflammatory arthritis compared with less/non-inflammatory types of arthritis. In addition, investigation of the relationship of biomarkers with disease activity and severity in the different arthritides may determine whether any markers could explain the more destructive and damaging outcomes of RA compared to other arthritides.

CHAPTER 3

PATIENTS AND METHODOLOGY

3.1 Patients

All the patients used in this study were selected from a patient population database that had been previously generated at the Staffordshire Rheumatology Centre at the Haywood Hospital.

3.1.1 Patient cohorts

All the patients used in this study were selected from a patient population database that had been previously generated at the Staffordshire Rheumatology Centre at the Haywood Hospital. Three major patient cohorts were studied in total: early RA patients with a 5 year follow up, RA patients with paired serum and synovial fluid, and patients with different types of early arthritic diseases: osteoarthritis (OA), psoriatic arthritis (PsA) and reactive arthritis (ReA). The latter cohort was used as comparative groups with early RA.

3.1.1.1 Selection criteria and consent

Diagnosis of RA was based on the 1987 American College of Rheumatology (ACR) revised criteria (Arnett*, et al.*, 1988). Patients with early stage RA characterized by a disease duration of $\leq$ 2 years at time of first admittance to the clinic (baseline) were included in the study. These patients were then invited back to the centre after 5 years for a follow up. This gave a unique database that charted the progress of RA patients through using numerous parameters.

The selection criteria for the paired serum and synovial fluid RA cohort was based on patients having available both serum and synovial effusion sample obtained at the same time point. The disease duration for the procurement of the paired samples were not limited to a specific time frame.

The third cohort included a selection of patients with other arthritic diseases for comparative investigations with baseline RA. Patients with confirmed OA, PsA and ReA were selected for study as representative of other types of non-inflammatory (OA) or inflammatory arthritis (PsA, ReA).

All the patients included in this project were confirmed to have ReA, OA, PsA or RA by a consultant rheumatologist at the centre. All the patients in this study had previously given written informed consent for their data and samples to be used for research purposes. This study was also approved by the Medical Ethics Committee of the North Staffordshire Hospital Trust.

3.1.1.2 Population cohorts' major characteristics and features

The patient database contained comprehensive data for every individual. This included information on a broad set of demographic and lifestyle factors such as age, gender, cigarette smoking as well as data on disease measures and features. The major characteristics for each cohort are illustrated in Table 3.1.1.2 below to demonstrate the extensive range of the data. Although the database encompasses a large amount of data, not all the information on the patients were complete as some were either unavailable or recorded. In addition, some patients were either lost or their patient sample was no longer

available at time of 5 year follow-up and thus could not be investigated. Thus an indication of the number of patients which were available for each analysis will be mentioned.

Table 3.1.1.2: The major characteristics and features for each of the 3 patient cohorts

	Cohort 1		Cohort 2	Cohort 3		
Characteristic and Lifestlye Information	RA Baseline	5 year Follow-up	Paired RA	OA	PsA	ReA
Number of patients	86	77	52	20	34	26
Male/Female (No.)	39/47	34/43	24/28	9/11	17/17	18/8
Age, mean ±SD, years (total *n*)	53 ±13.10 (85)	52 ±13.0 (77)	57.0 ±16.0 (52)	61.0 ±10.6 (20)	41.3 ±14.2 (34)	34.4 ±13.0 (25)
Disease duration, mean ±SD, years (total *n*)	0.58 ±0.38 (86)	5.54 ±0.30 (77)	6.6 ±9.0 (52)	1.1 ±1.0 (20)	1.7 ±1.9 (33)	0.32 ±0.53 (26)
RF positive % (total *n*)	52.3% (86)	52% (77)	43.2% (44)			
Anti-CCP positive % (total *n*)	72.1% (86)	72.7% (77)	67.3% (52)			
Nodule positive % (total *n*)	3.5% (85)	2.63% (76)	9.8% (51)			
Synovial effusion % (total *n*)	18.6% (86)		100% (52)			
Erosions % (total *n*)	65.8% (76)		75.7% (37)			
Ever Smoked positive % (total *n*)	68.6% (86)	66% (77)	60% (40)	70.6% (17)	53.3% (30)	52.2% (23)
Smoke status i.e. non-smoker: past smoker: current smoker counts (total *n*)	27:35:24 (86)	26:34:17 (77)	16:10:14 (40)	5:6:6 (17)	14:7:9 (30)	11:3:9 (23)
DMARD treatment % (total *n*)	14% (86)	91% (76)	45% (51)		9% (32)	

3.1.2 Smoking history and classification

Cigarette smoking has been repeatedly shown to be important in the development and severity of RA as discussed earlier in the section 1.3. Therefore each patient had their smoking history recorded when attending the clinic and used in analyses in this study (Appendix figure 3.1.2 for patient smoking questionnaire). Patients were asked if they had

smoked at any time in their lives (ever smoked) as well as their current smoking status; if they were a current smoker (having smoked $\geq$ 1 year), a past smoker (cessation $\geq$ 6 months) or a never smoker. These patient numbers are included in the Table 3.1.2 above. In addition, the quantity, frequency and duration of cigarettes smoked was also recorded. This allowed the determination of the intensity patients smoked and is described as patient smoking pack year history. This was calculated by the formula below, where a cigarette pack has 20 cigarettes in each pack, therefore 1 pack year=20 cigarettes per day for a year.

$$\frac{\text{Number of cigarettes per day smoked x Number of years smoked}}{\text{Number of cigarettes in a pack}}$$

3.1.3 RA measures of disease

Assessment and monitoring of RA is integral to determine the activity and severity of the disease in patients. There are numerous assessment measures for RA such as Simplified Disease Activity Index (SDAI), Clinical Disease Activity Index (CDAI), Global Arthritis Score (GAS), Rheumatoid Arthritis Articular Damage (RAAD) etc. However the patient database already held a large set of clinical and laboratory assessment measures for disease activity and severity for each patient. These various disease measures encompass markers of inflammation, pain, function and joint damage parameters, which illustrate the process and outcome of RA disease (Table 3.1.3). The following section details an explanation of each disease measure used and its implementation in RA monitoring and assessment.

Table 3.1.3: Clinical and laboratory measures of disease activity and severity parameters

Disease Processes (Disease Activity)		Disease Outcomes (Disease Severity)	
Inflammation	Pain	Damage	Function
CRP	VAS	Bone Erosion	HAQ
ESR	TJ	OSRA-D	EMS
SJ	RI	Larsen Hand Score	Grip strength
WBC			
CGDA			
DAS44CRP			
DAS44ESR			
OSRA-A			
SI			
PIPS			
Platelets			
Anaemia			
Rheumatoid Factor			
Anti-CCP			
Synovial effusion			

CRP (C-reactive protein), ESR (erythrocyte sedimentation rate), DAS (Disease Activity Score), SJ (Swollen joints), TJ (Tender joints), RI (Ritchie Index), SI (Stoke Index), OSRA-A/D (Overall Status in RA-Activity/Damage), CGDA (Clinician's Global Disease Activity), VAS (Visual Analogue Score), PIPS (Proximal Interphalangeal Score), HAQ (Health Assessment Questionnaire), EMS (Early Morning Stiffness).

3.1.3.1 Markers of Inflammation

The erythocyte (red blood cell) sedimentation rate (ESR) is a common haematology test. It is the measurement rate of red blood cells sedimenting in a period of one hour (mm/h). The ESR is dependent on a balance between pro-sedimentation factors particularly fibrinogen versus anti-sedimentation factors mainly erythrocyte negative charge. In an inflammatory process, high levels of fibrinogen produced from the liver causes red blood cells to stick to

one another and form stacks which sediment faster. Thus ESR rate is a useful tool in the monitoring of disease activity and thus commonly used as a marker of inflammation.

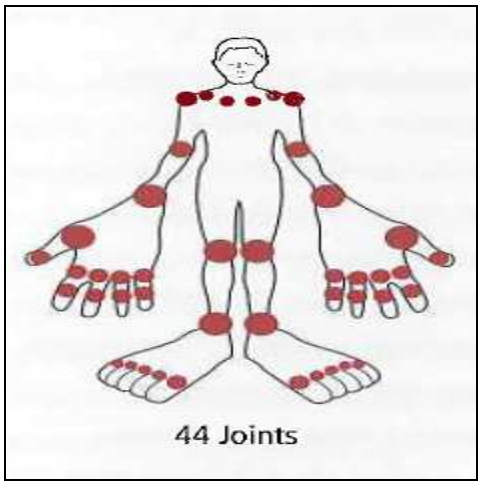
Another commonly used marker of the inflammatory process is C reactive protein (CRP). This protein is rapidly synthesized in the liver in response to inflammation after the onset of either trauma (injury and burns), infection, diseases or tissue necrosis. It is part of the acute phase response which is an early defence system in innate immunity as it enhances phagocytosis and activates the complement system. This acute phase protein is measured by testing the blood and is highly expressed in individuals depending on the type of infection, disease or trauma. A level of more than 10mg/L is considered abnormal and indicates inflammation. This protein therefore is useful in monitoring disease activity as it better reflects short-term changes and.more sensitive than the ESR as it is less susceptible to disturbing factors.

The platelet count is another marker of the inflammatory process where the number of platelets in the patient's blood are counted and used to indicate the level of inflammation, as excess amounts of platelets are rapidly sent to sites of inflammation and aid in the secretion of inflammatory cytokines by interacting with leukocytes which can reflect disease activity of a few weeks. The normal of platelets in the blood is between 150,000-400,000 platelets per microlitre (mcL), and above this is considered abnormal and an indication of inflammation. Similar to that of platelet count is the white blood cell count, another measure of inflammation, where approximately 4,000-11,000 cells per cubic millimetre of blood is the normal range whereas anything above this is considered leukocytosis and an indication of an immune response.

Anaemia which is a reduction in the number of erythrocytes or a deficiency of haemoglobin (Hb): the iron-containing oxygen-transport metalloprotein in the blood, and is also used as an inflammatory marker. There are several types of anaemia each with a different cause, although one type of anaemia is present in chronic illnesses due to persistent infection, constant immune activation or malignancy which is known as anaemia of chronic disease (ACD) (Beutler, 2003, Weiss and Goodnough, 2005). This condition may be largely due to the increased production of hepcidin: a master regulator of iron metabolism (Nemeth and Ganz, 2006). Hepcidin is synthesized in the liver as a response to inflammatory cytokines which in turn prevents the release of iron from iron stores (Beutler, 2003, Nemeth and Ganz, 2006). Inflammatory cytokines also decrease the release of iron directly as well as shorten the survival of circulating erythrocytes and decrease the production of red blood cells in the bone marrow (Beutler, 2003, Means Jr, 2004). As such, anaemia is therefore a good marker for inflammation in chronic diseases such as RA, where Hb levels lower than 13.3 in males and lower than 12 in females is characterized as anaemic.

The 44 swollen joint count is a clinical measure of inflammation used in assessing RA activity and severity. It is a quantitative assessment of the joint, where the number of swollen joints incorporating also soft tissue joint margin swelling, synovial effusion, joint fluctuation and joint swelling are counted in a set of 44 joints. These joints include those of the hands, wrists, ankles, feet, elbows, knees, shoulders and collar bone (Figure 3.1.3.1). It should be noted that there is also a 28 swollen joint count, although this clinical measure does not include the joints of the collar bone, feet, ankles which are affected in RA and thus the original measure of 44 joints was used in this study.

Figure 3.1.3.1 Diagram of the 44 joints counted for joint swelling



Reproduced from (Fleischmann, 2006).

The Clinician's/Physician's Global Disease Activity (C/PGDA) score is a measurement of overall activity and severity of RA disease as judged by the clinician or physician. This incorporates patient physical examination and laboratory results, such as swollen joints, functionality, ESR, CRP, autoantibodies etc and rated by a qualified evaluator by marking vertically on a 10cm line according to a set visual analog scale: left end of line = no evidence of disease activity, midpoint of line = moderate disease activity and right end of line = extremely active/severe disease activity. In addition global disease activity is also rated on a 5-point Likert scale: 0 = none, 1 = mild activity, 2 = moderate activity, 3 = severe activity, 4 = extremely severe activity. Although this RA assessment measure is somewhat biased, it is performed by qualified skilled professionals and is an established measure in assessing RA disease.

The Proximal Interphalangeal Synovitis (PIPS) score is an assessment of the synovial thickening of the proximal interphalangeal joints which are between the proximal (first) and intermediate (second) phalanges (0=nil, 1=possible, 2=definite, 3=gross). Due to the

measurement of synovial thickening it therefore is used as an indicator of inflammation and used in the assessment of RA.

The Overall Status of Rheumatoid Arthrits (OSRA) is a summary measure based on patient history and physical examination. The OSRA questionnaire incorporates 4 components: demographic information, the OSRA-disease activity (OSRA-A), the OSRA-damage (OSRA-D) and a treatment category (Harrison *et al.*, 2007). In this study the OSRA-A and OSRA-D scores were used independently of the complete OSRA. The OSRA-A score was developed to represent the potentially reversible aspects of RA whereas the OSRA-D score aimed to capture the irreversible impact of the disease. The OSRA-A and OSRA-D scores both are based on 5 different components of activity (well being, pain, EMS, active joints (swollen and tender), extra-articular disease) and damage (function, social life, number of destroyed or replaced large joints, small joint damage, organ impairement) respectively. Both these measures require a clinician to assess the patient mobility and disability as well as to complete a questionnaire on the patients' functional ability. Below are the 5 varying components of the OSRA-A and OSRA-D to determine overall activity and overall damage of patients. These measures provide a good overall summary of the disease activity and damage and thus are broad markers of inflammation and damage respectively. These scores are widely established in clinics and were used in this study as well.

Some disease measures used in this study are composites of other formulated disease measures. The Disease Activity Score (DAS) is one of such composite disease measures and is calculated through the use of 4 parameters. This incorporates the number of swollen joints out of a set of 44, the Ritchie Index and either the ESR or CRP to allow the formulation of the DAS44ESR or DAS44CRP respectively. The mathematical formulas

below show how they are calculated and where the higher the DAS score the worse disease activity of RA.

$$(0.54 \times \sqrt{\text{Ritchie Index}}) + (0.065 \times 44 \text{ swollen joint count}) + (0.33 \times \text{Ln/Log natural ESR}) + 0.224 = \text{DAS44ESR}$$

$$(0.54 \times \sqrt{\text{Ritchie Index}}) + (0.065 \times 44 \text{ swollen joint count}) + (0.17 \times \text{Ln/Log natural (CRP+1)}) + 0.65 = \text{DAS44CRP}$$

There are a number of validated formulas for the DAS score which also incorporate the VAS for pain, general health, tender joint score etc to accommodate what data is available at the time. As this is a composite measure, therefore this gives an overall assessment of RA disease as it uses multiple measures and thus is a good indicator of activity and severity (van der Heijde *et al.*, 1993, Fransen *et al.*, 2003, Anderson *et al.*, 2011).

Another composite measure is the Stoke Index which is a Staffordshire validated measure. It is a global measure of RA disease activity and comprises of a complex hierarchical formula involving the PIPS, ESR, EMS, CRP and the Ritchie Index. It is an algorithm developed into a flow diagram to classify patients into 1 of 17 ranked classes of RA activity (Davis *et al.*, 1990)**.** This measure is therefore another useful inflammatory marker and was used in the study.

Disease measures which are composited from independent inflammatory measures are overall global assessors of the disease process and thus can be separated into phenotypes of severity. The Stoke Index is divided into 4 phenotype categories based on patient scores. These phenotype categories are: minimal (0-3), mild (4-7), moderate (8-11) and severe (12-17). The DAS44ESR and DAS44CRP can also be categorized into severity

phenotypes. These phenotypes are separated by patient scores into: low (≤2.4), moderate (>2.4-3.7) and high (>3.7). In addition, a score of ≤1.6 in either the DAS44ESR or DAS44CRP in patients are characterized as remission, although this is used when disease is an established stage (reproduced from http://www.das-score.nl/downloads.html).

Another marker for inflammation is the manifestation of a synovial effusion where an excess of synovial fluid in the joint arises due to inflammation and is demonstrated by swelling of the joint capsule and must be drained.

Autoantibodies RF and anti-CCP are also used as markers of inflammation, and are described in section 1.1.10.3.1 Seropositivity of RF is considered to be levels above 60IU/mL, while levels above 5 units/mL is considered positive for anti-CCP.

3.1.3.2 Markers of Pain

There are three measures of pain in the patient database and used in this study, one of which is the visual analogue scale (VAS) for pain. This measurement of pain is subjective as it based on the patient, and involves the patient marking on a 10cm horizontal line similar to that of the CGDA according to a set visual analog scale: left end of line = no pain felt, midpoint of line = moderate pain felt and right end of line = severe pain felt. Although this is a biased measure of the disease process, and each individual patient has a different resistance threshold, only the subject can indicate if the joint is painful or not, and thus is a realistic indicator of pain and therefore included in this study.

The number of tender joints is also a measure of pain and is the total count of the same set of 44 joints as detailed in the swollen joint counts. It is assessed by the clinician by applying pressure to the joint or with movement and determining if the patient is in pain, and as such is a good marker for the amount of pain felt.

Another measure of pain is the Ritchie Articular Index (RAI). It is based on the sum of applying pressure to 26 joints (shoulders, elbows, wrists, MCP, PIP, hips, knees, ankles, talocalcaneal, midtarsal, metatarsals, temporomandibular, cervical spine, sternoclavicular and acromioclavicular) and is graded on a 0-3 scale of pain intensity: 0= no tenderness, 1 = pain felt, 2 = pain felt and expressed wincing, 3 = pain felt, expressed wincing and withdrew (Ritchie *et al.*, 1968). RAI is a more detailed assessment of pain as it measures the intensity of pain. This score therefore is a reliable indicator of pain and is not biased on subject perception, and as such is used in this study.

3.1.3.3 Markers of Damage

A strong marker for joint damage is the presence of any bone erosion in joints. This is determined by a radiologist through radiograph examination of the joints. As such this marker is very useful in the assessment of damage and outcome in RA. It should be noted that erosive data in most patient cases was not collected at baseline or at 5 year follow-up but recorded at any time point and is indicative of an erosive disease.

The Larsen score is also a marker for damage, where the joints of the hands, wrists and feet are examined and graded on a scale of increasing damage where the total is used to asses full extent of the patients' bone damage (Larsen *et al.*, 1977). The scoring method is a

combination of bone erosion presence, soft tissue swelling, cartilage thinning and narrowing of the joint space and is determined by a radiologist.

The OSRA-D is used as a marker for damage and one parameter of the OSRA, and has been previously discussed.

3.1.3.4 Markers of Function

The Health Assessment Questionnaire (HAQ) is a patient questionnaire to clinically measure health-related quality of life for RA patients. It assesses the mobility and functional status of patients in daily activities such as walking, grooming etc. It is scored by a clinician and graded on an increasing scale of 0-3 of worse mobility (Appendix figure 3.1.3.4 for HAQ questionnaire). The HAQ has become an established clinical assessment for health outcome and thus included in this study.

The Early Morning Stiffness (EMS) disease measure is a marker of function. Joints are commonly stiff, sore and painful after inactivity for a long period of time or a nights sleep in RA patients. After moving a while, the joints “loosen up” as they are move around and are used. In RA, the lubrication of the cartilage with joint fluid is inadequate during rest periods as there is less cartilage, less fluid and cartilage is poorer in quality and thus feels stiff. EMS is difficult to quantify, where the length of time (minutes) until joints are moveable is recorded. EMS is one of the parameters for the ACR classification of RA, and thus also used in this study.

Grip strength is a test to measure the maximum isometric strength of the hand. A sphygmomanometer cuff inflated to 30mmHg (millimetre of mercury) is used, where the

patient squeezes the apparatus and the pressure achieved is recorded. This disease measure is therefore a good indicator of functional ability in patients and thus used as a marker of function and mobility outcome.

These measures of disease described above are all valid indicators of RA activity and severity and were all included in the study to assess the overall process and outcome of RA, not just particular aspects of the disease. In addition, many patients have exceptions to the expected standard, where some patients have swollen joints, but little pain, whereas others may have considerable pain but few swollen joints. Therefore using multiple measures of disease for assessing the RA process and outcome encompasses the extensive range of severity and activity of RA rather than relying on one clinical measure to indicate the activity and severity of RA disease.

3.1.4 Patient drug therapy regimes

Regardless of the type of disease, most patients were receiving various anti-inflammatory and/or anti-rheumatic therapy drugs, either singularly or in combination. The drugs administered to the patients included analgesics, NSAIDS, DMARDs and steroids. None of the patients in this study were receiving biologic therapy. Table 3.1.4 below describes the percentage of patients on the various drug therapy regimes in each patient cohort at both baseline and at 5 year follow up. Approximately half of the RA, PsA and ReA patients were receiving NSAIDs at baseline. The figure lowers for patients with OA (35%), although more of these patients were taking analgesics (40%). The paired RA cohort had a large number of patients receiving both NSAIDs and DMARD therapy. This can be

explained by the longer disease duration for this group. Unsurprisingly, for patients with RA or PSA, the percentage receiving DMARDs had greatly increased at 5 years follow up.

It is should be noted that it is unknown at what time point patients were started on analgesics or NSAIDs since General Practitioners could have prescribed treatment before attendance at the clinic. Patient drug therapy may also have changed during the 5 years prior to follow up data collection. However no data was available for the time points when any therapy changes occurred.

Table 3.1.4: The percentage of patients on DMARD treatment at baseline and 5 year follow-up

	Cohort 1		Cohort 2	Cohort 3		
Patient Groups	Baseline RA	Five year follow-up RA	Paired RA	OA	PsA	ReA
DMARD positive % (total n)	14% (86)	91% (76)	45% (51)	0% (20)	9% (32)	0% (26)

3.2 Methodology

The measurement of numerous mediators was investigated in the serum and synovial fluid by use of ELISAs (Enzyme Linked Immunosorbance Assay) and multiplex bead assays. The following sections describe the methodological protocols performed for investigation into mediator quantification in the various cohorts.

3.2.1 Patient sample collection

Patient sample collection was performed by a qualified nurse practitioner at the clinic both at baseline and at subsequent 5 year follow up. These patient samples of synovial fluid and blood for serum isolation were used to perform the number of investigations discussed in this project.

3.2.1.1 Blood collection and serum isolation

Blood was collected by drawing 22mls of peripheral blood from patients from either arm. This was achieved by tying a tourniquet around the patients' upper arm to increase the blood pressure in the veins of the arm. The skin over the blood vessel, usually the median cubital vein on the inside of the elbow is cleaned with antiseptic. A sterile needle is then inserted into the vein while the opposite end of the needle is inserted into a sterile vacutainer subsequently drawing up blood. All blood samples were processed within 1 hour of collection and stored in a -80°C freezer. The standard procedure for isolating serum from whole blood samples was performed by a clinical technician within 1 hour of blood collection. Serum was removed from whole blood by allowing the sample to clot for 30 minutes at room temperature. This sample was then spun in a 5°C centrifuge for 15 minutes at 4000rpm. The serum at the top of the vacutainer was then drawn off by a glass pasteur pipette and into a sterile microcentrifuge tube to be stored in a -80°C freezer until use for either ELISA or multiplex assays.

3.2.1.2 Synovial fluid extraction

Synovial fluid is extracted from patients by arthrocentesis, which involves a practitioner inserting a needle into the affected inflamed joint. A syringe is then attached to draw up the synovial fluid. A local anaesthetic is administered before the needle and syringe are applied. Synovial fluid samples are also stored in the -80°C freezer until use in ELISA and multiplex assays. Synovial fluid after extraction from the joint did not require any additional preparation for mediator investigation.

3.2.2 Biomarkers investigated and their quantification

Forty one different mediators in total were investigated in this study. Osteoprotegerin (OPG), osteopontin (OPN), Dkk-1 and endostatin were measured by the use of ELISAs. The remaining 37 mediators were investigated by using 3 types of multiplex systems: a 27-plex from Bio-Rad, a MMP-plex and a TIMP-plex both from R&D Systems. The Luminex system is designed to facilitate measurement of different assay plates from different manufacturers. The 3 multiplex systems are each able to detect a specific set of mediators. The specific set of mediators for each system is listed in Table 3.2.2.

Table 3.2.2: A list of mediators investigated

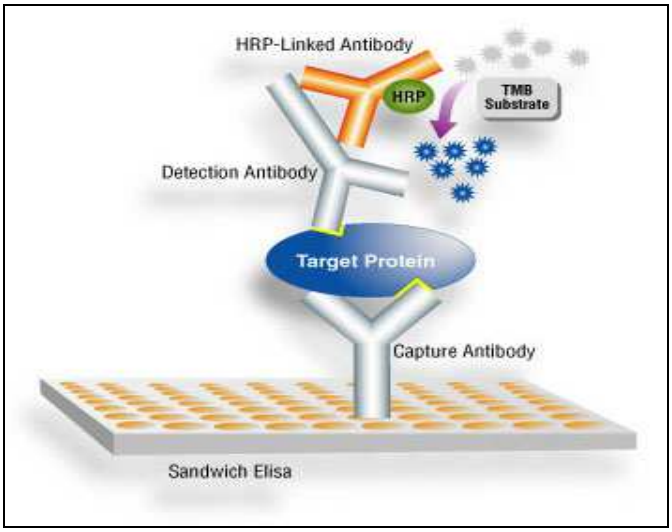
27-plex Multiplex (Bio-Rad)		MMP Multiplex (R&D Systems)	TIMP Multiplex (R&D Systems)	Duo Set/Quantikine* ELISA (R&D Systems)
IL-1β	G-CSF	MMP-1	TIMP-1	OPG
IL-1ra	GM-CSF	MMP-2	TIMP-2	OPN
IL-2	IFN-γ	MMP-3	TIMP-3	Dkk-1
IL-4	PDGF-bb	MMP-8	TIMP-4	Endostatin*
IL-5	TNF-α	MMP-9		
IL-6	VEGF	MMP-12		
IL-7	IL-8			
IL-9	Eotaxin			
IL-10	IP-10			
IL-12p70	MCP-1			
IL-13	MIP-1α			
IL-15	MIP-1β			
IL-17	RANTES			
FGF-β				

3.2.2.1.1 Sandwich based Enzyme Linked Immunosorbance Assay (ELISA)

Following serum isolation from blood and collection of synovial fluid, quantification of specific mediators in both the serum and synovial fluid samples were performed through the use of the sandwich Enzyme Linked Immunosorbance Assay (ELISA) system. The basic principle of the sandwich ELISA procedure is based on a two antibody immunoassay system. This involves the molecule of interest bound between the capture antibody and the detection antibody, hence its given name "sandwich". The capture antibody is permanently attached to the well plate. The target molecule binds to the specific capture antibody and the specific detection antibody in turn binds to the target molecule. A third enzyme conjugated antibody is designed to bind to the constant region of the detection antibody. Enzymes commonly linked to these antibodies are alkaline phosphatase or horseradish peroxidise (HRP). An alternative to using an enzyme conjugated third antibody is to use a

Streptavidin-HRP conjugate which will bind to a biotinylated detection antibody. With the addition of Tetramethylbenzidine (TMB) substrate the HRP enzyme is catalyzed which leads to a change in colour. The absorbency of the plate wells as measured by the optical density determines the presence and quantity of the target molecule. The figure below (Figure 3.2.2.1.1) shows simply the procedure of the most common sandwich ELISA process.

Figure 3.2.1.1: The mechanism of a common-sandwich ELISA system



Reproduced from http://www.synchronium.net/wpcontent/uploads/2009/09/sandwich_elisa.jpg.

The above procedure is one of the most common methods of sandwich ELISAs. However there are other sandwich ELISA methods, that use different enzymes instead of horseradish peroxidise (HRP) for colour development. There are also sandwich ELISAs that completely bypass the use of a third antibody or a streptavidin protein. In these cases the colour changing enzyme is conjugated instead to the detection antibody directly. This was the case with endostatin assay, where a specific Quantikine ELISA kit was purchased from R&D Systems which utilized this method. This specific protocol is discussed in detail in section 3.2.2.1.3.

The investigation into OPG, OPN and Dkk-1 were performed by the classic sandwich ELISA methodology. These ELISAs were performed using ELISA Duoset kits (R&D Systems) according to the manufacturer's instructions. The section below (section 3.2.2.1.2) describes in depth the protocol used for these investigations.

3.2.2.1.2 Duoset ELISA methodology

The levels OPG, OPN, and Dkk-1 as well as IL-17 and FGF-β were quantified by the ELISA Duoset Development Kits (R&D Systems). These kits contained 1 vial each of specific capture and detection antibodies, 2 vials of the standard and a vial of Streptavidin-HRP. All the reagents were brought to room temperature and mixed thoroughly prior to experimental use.

The capture antibody and biotinylated detection antibody were reconstituted from powder to the desired concentration and then diluted to the working concentration with filtered phosphate buffered saline (PBS) (Sigma) and 1X reagent diluent (R&D Systems) respectively. The ELISA plate consists of 96 wells which were pre-coated with the specific working concentration of the capture antibody the previous day and incubated at room temperature over night to allow adherence to the plate. The following day, the plate was then aspirated and washed 3 times with 0.05% Tween 20 (R&D Systems) in PBS wash buffer. The wells were then blocked with either 300µl of 1X reagent diluent (DY995-R&D systems) or 1% Bovine serum albumin (BSA) (Sigma) for 1 hour at room temperature to prevent non-specific antibody cross-reactive binding. Patient samples, either serum or synovial fluid were diluted with 1X reagent diluent at the desired dilution with an addition of 3ug/ml Heteroblock (Omega) final concentration for 1 hour at room temperature. The

mediator standard was reconstituted to the required concentration and subsequently used to prepare a 7 point standard curve using 2 fold serial dilutions in 1X reagent diluent.

The ELISA plate was aspirated and washed three times with wash buffer. Next, 100µl of both the prepared standards in duplicate and samples, as well as a blank control were added into their respective wells. These samples were incubated for two hours at room temperature to allow binding to the capture antibody. The plate was washed three times to remove unbound samples. A 100µl of biotinylated detection antibody was added to each well and further incubated for 2 hours at room temperature. After the removal of the detection antibody, Streptavidin-HRP was diluted 1:200 in 1X reagent diluent and a 100µl was added into each of the wells. The ELISA plate was then placed away from direct light and incubated for 20 minutes at room temperature. The wash process was repeated and a 100µl of substrate solution consisting of a 1:1 mixture of colour Reagent A (H_2O_2) (R&D Systems) and Reagent B (Tetramethylbenzidine) (R&D Systems) was subsequently added to the wells. This mixture was left to incubate at room temperature for 20 minutes avoiding direct light to allow blue colour development. Lastly 50µl of "stop solution" (2N H_2SO_4), stored at 4°C was added to the wells containing the substrate solution to stop colour development. This was observed by the blue colour changing into yellow.

The plate was read immediately on a microplate reader (Titertek) with 2 filters, one at 450nm the other at 575nm. The use of dual filters allow for wavelength correction for any optical imperfections in the plate which can alter the results. The optical density (OD) was then read and a standard curve and quantification of the mediator level was automatically generated from the Titertek software and recorded.

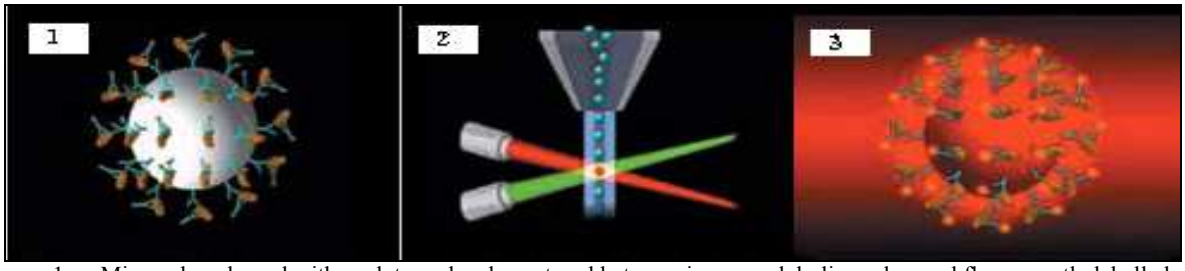
3.2.2.1.3 Quantikine ELISA methodology for the measurement of Endostatin

This kit contained a microplate pre-coated with a monoclonal antibody specific to endostatin. The plate was pre-wetted with 100µl of assay diluent to each well. Patient samples were diluted with 1X calibrator diluent at the desired dilution along with an addition of 3ug/ml Heteroblock (Omega) final concentration for 1 hour at room temperature. The endostatin standard was reconstituted to the required concentration and subsequently used to prepare a 7 point standard curve using 2 fold serial dilutions in 1X calibrator diluent. Fifty microlitres of prepared standards, blank control and samples were added to the wells. The plate was then incubated for 2 hours at room temperature on a horizontal orbital microplate shaker at 500rpm to allow binding of endostatin. The plate was then washed and aspirated 4 times using the supplied wash buffer. The addition of 200µl of endostatin conjugate, a horseradish peroxidase enzyme-linked monoclonal endostatin specific mouse antibody was added. This was incubated again at room temperature for two hours on the orbital shaker to allow the specific binding. The wash procedure was performed again to remove any unbound antibody-enzyme-reagent. Then 200µl of substrate solution (1:1 mixture of Colour Reagent A and B) was pipetted into each well and incubated for 30 minutes at room temperature avoiding direct light. During this incubation a blue colour develops in proportion to the amount of endostatin bound in the initial step of the assay. The colour development is subsequently stopped with 50µl of stop solution (2N sulphuric acid). The colour intensity or absorbance was determined by measuring optical density (OD). This was achieved by the use of a microplate reader (Titertek) set at 450nm and then at read again at 575nm for wavelength correction. The Titertek programme automatically generated the standard curve and endostatin concentration results which were subsequently recorded.

3.2.2.2 The Multiplex bead based X-multianalyte profiling (-MAP) principle

A sophisticated piece of technology called the Luminex (Bio-Plex™ 200 X-map suspension array System, Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, United Kingdom) is used to measure numerous analytes in parallel. The multiplex system has the ability to measure up to 100 analytes in a single assay. The Multiplex is based on a 2 way system incorporating the sandwich ELISA method and the fluorescence activated cell sorting principle (FACS) Flow Cytometry principle. The microparticle beads contain 2 fluorescent dyes in variable intensities to create a distinct bead set for each analyte. Molecules bind to the specific antibodies attached to the bead surface which are then subsequently tagged by fluorescent labelled reporter protein. The beads are passed individually through a two laser column in the Luminex machine, and their fluorescent biochemical reactions are measured and reported. The red laser reads the beads' unique 2 dye fluorescent emission to determine its unique analyte bead identity. The green laser then measures the reporter label to determine fluorescence quantity. The fluorescence activity within a sample is directly related to the concentration of the specific analyte in the sample. The diagram below (Figure 3.2.2.2) shows in brief the process that occurs within the Luminex machine.

Figure 3.2.2.2: The bead reading system of the Luminex machine



1. Microsphere bound with analyte molecule captured between immunoglobulin probes and fluorescently labelled reporter tags.
2. Microparticle beads within a fluidic stream pass through the red and green lasers.
3. Molecular tags & microsphere are both excited & the fluorescent intensity is identified & measured.

This rapid method for mediator level quantification was performed on numerous mediators on both serum and synovial fluid samples. All the multiplex assays were performed according to the specific manufacturers kits' instructions. Although each multiplex assay system varies in its reagent concentrations, the principle of the assay between the systems are the same and are described below.

The filter microplate was first pre-wetted with 100µl either wash buffer or assay buffer depending on the protocol. This buffer was then removed completely through the filter attached at the bottom of the plate by a vacuum manifold system (5 Hg). Analyte specific antibodies are pre-coated onto the microparticle beads. This is done by the manufacturer, as each bead has a unique identification which is determined by the 2 colour dye of the bead. The beads are diluted to the required concentration depending on the particular multiplex assay and 50µl was pipetted into each the wells. Next 50µl of prepared samples, a blank control and a 7 point standard was then added to the respective wells. This was then incubated at room temperature on a horizontal orbital plate shaker at 500rpm for two hours. This allowed the mediators in the samples and standards to bind to the specific analyte antibody coated microparticle beads. Un-bound standards and samples were removed by washing 3 times with 100µl of wash buffer using the vacuum manifold between each step. The biotinylated detection antibodies specific to the analytes of interest was diluted to the desired concentration and either 25µl or 50µl was pipetted into each well depending on the particular assay. This was then incubated on the horizontal orbital plate shaker for 30 minutes or 1 hour at room temperature, depending on the multiplex assay. The wash step procedure was performed again after the removal of the detection antibodies. Next, 50µl of Streptavidin-Phycoerythrin (PE) was added and incubated for either 30/20/10 minutes depending on the specific protocol at room temperature on the

orbital shaker out of direct light. The plate was washed a final three times through the vacuum manifold and given a final incubation on the orbital plate shaker, covered from the light for two minutes in assay buffer to resuspend the microparticle beads.

The Bio-Plex 200 equipment was calibrated to each of the specific multiplex assay protocols and the plate was immediately read in the Bio-Plex 200 after bead resuspension to determine the phycoerythrin fluorescence levels. The fluorescence level is directly proportional to the amount of specific analyte. Therefore analyte concentration levels are quantified by fluorescence level. The quantification of each of the specific analytes in the multiplex was automatically generated by the Luminex software including their relative standard curves. The software also allowed the ability to remove outlier standard results to create an optimal standard curve and the mediator quantitative levels were then recorded.

With the range of mediators investigated, a number of different dilution factors were required for serum and synovial fluid sample preparation. The 27-plex multiplex required a 1:4 dilution of both serum and synovial fluid samples as stated by the protocol. MMP-1, -2,-3,-8 and -12 were all performed with serum samples at a 1:10 dilution, while synovial fluid used a 1:1000 dilution factor. Serum samples for MMP-9 investigation required a dilution of 1:100. A 1:10,000 dilution was required for MMP-3 investigation into synovial fluid samples, while a 1:50 dilution was used for both serum and synovial fluid investigating TIMPs.

The ELISA assays for endostatin and OPN were performed using a 1:50 dilution for serum samples. Synovial fluid for measurement of OPN was diluted to 1:400 or 1:800 if the sample was above the recordable threshold at 1:50 dilution. Serum samples were diluted to

a 1:10 dilution in Dkk-1 ELISAs and synovial fluid at 1:5. OPG ELISAs required serum samples to be diluted 1:10 and a dilution factor of 1:50 for synovial fluid. FGF-β and IL-17 ELISAs were predominantly performed with serums at a 1:5 dilution factor. However a number of samples were above the recordable threshold and therefore had to be repeated at a higher dilutions ranging from 1:10, 1:20 and 1:40.

Investigation of endostatin was performed on a total of 81 patients from the baseline RA cohort. A total of 86 baseline RA patients were assayed by a polystyrene bead based 27-plex multiplex assay while the paired RA cohort was assayed with a magnetic bead based 27-plex multiplex kit. All other mediator assays were performed on all patients available.

Both ELISAs and multiplex assays were performed with patient samples in duplicate in majority of the assays. However some samples were performed in singlets due to limited well space.

The possibility of cross-reactive interference from competitive Rheumatoid Factor (RF) autoantibody was considered in these assays since both the ELISA and multiplex bead arrays are based on antibody binding systems. In order to block cross-linking by RF, patient samples in sample diluent were pre-incubated with a compound called Heteroblock (Omega Biologicals Incorporated, Bozeman, Montana, USA) at a final concentration of 3µg/ml for 1 hour prior to addition to the plate wells.

3.2.3 Statistical software and data analysis

All data was analysed using Number Cruncher Statistical Software package for Windows (NCSS 2000, NCSS Statistical Software, Kaysville, Utah, USA). A probability value of ≤ 0.05 was used to determine significance level. Various techniques from NCSS including multivariate variable selection, discriminant stepwise-selection and regression, multiple regression, forward logistic regression, one way analysis of variance (ANOVA), Two-sample and paired T-tests, principal component analysis (PCA), correlation matrices, Chi-square, odds ratio and receiver operating characteristic (ROC) analysis were used. Results were also adjusted for confounding variables such as disease duration, smoking, autoantibody presence and DMARD treatment where appropriate. Furthermore, parametric and non-parametric analyses were used depending on the distribution of the data when appropriate. Analysis of normal distribution incorporated 7 tests (Shapiro-Wilk W, Anderson-Darling, Martinez-Iglewicz, Kolmogrov-Smirnov, D' Agostion -skewness, -Kurtosis and -Omnibus test) as well as a frequency distribution histogram and normal probability plot to determine normality. In addition, the Variance Ratio and the Modified Levene equal variance test were also used when appropriate for determining equal or unequal variance.

Testing against a variable (dependent) repeatedly (multiple comparison testing) increases the likelihood of identifying a significant result purely by chance when there actually is none (type 1 error) with each additional comparison, ie. false positive rate. Adjustment for this type of error in one-way ANOVA where comparison is with 3 or more groups was the regular z-value test of the Kruskal-Wallis multiple comparison analysis for non-parametric data, whereas Scheffes's multiple comparison test was used for normally distributed data.

Other multiple comparison analyses may be considered significant after the Bonferroni adjustment for multiple testing e.g. with a p-value $\leq$0.01 for up to 5 comparison tests, $\leq$0.005 for up to 10 comparisons and $\leq$0.0001 for up to 50 comparisons. Setting a stricter p-value threshold reduces the risk of type 1 errors, however increases the risk of type 2 errors, that is failing to identify a significant result when there actually is one ie. false negative rate. Consequently, as the risk of type 2 errors rise, statistical power in turn decreases as it is 1 minus type 2 error probability. Adjustment for multiple comparisons in other analyses were generally not undertaken as they were largely exploratory in nature. Statistical power is the probability of identifying a significant result when there actually is one ie. detecting true significance. Typical power values are set to 80%, which means an 80% chance (eg. 8 out of 10 times) or greater of finding a significant result when there is one. With a reduction in the power of the analysis, the conclusion of a result as statistically significant becomes more difficult, regardless of whether it's true or false. An increase of the p-value or the number of samples can increase the power of a test. Without knowing the likely effect size and nature of response for each biomarker in these studies it was difficult to pre-determine the sample size necessary to achieve power of 80% or more. Many of the analyses were largely exploratory in nature, although the numbers used were consistent with published studies of a similar type. The results of the current study may be used to better inform future studies in their design and estimation of sample sizes to achieve sufficient power.

3.2.3.1 Principal Component Analysis biomarker profiling

PCA reduces the dimensionality of a large number of variables to a smaller set of independent components based on their data correlation pattern and the maximum number

of iterations. These component group profiles then can also be investigated for their association with disease measures and features with the various techniques mentioned above.

PCA, although exceptionally useful in reducing the number of variables, is still limited by the number of variables it can include in respect to the number of patient samples. As such, PCA was initially performed on all the mediators collectively and subsequently particular mediators were selected to be included in the final PCA analysis based on their maximum variability score.

3.2.3.2 Genesis hierarchical clustering biomarker profiling

Genesis 1.7.2 software (Institute for Genomics and Bioinformatics, Graz University of Technology, Alexander Sturn) was used to generate heat maps of multiple continuous variable measurements for each patient in a single display. This allows for visual representation of the mediator levels in patients as well as illustrating all the disease measure variables.

The Genesis programme uses a two-way hierarchical clustering method which enables groups of variables with similar expression levels to be clustered together, as well as grouping together patient samples with similar expression patterns. This can then create phenotype profiles which can be included in statistical analyses.

The range of results obtained from the multiplex and ELISA assays were extremely wide between the various mediators. As a consequence, using the in-house function of the

software Log2 transformation of the raw results was performed, and then subsequently normalized to get bring the values within range of one another. A Heat Map utilizes a colour key which displays a gradual scale of intensity (-2.0 to +2.0 score) to reflect the levels below and above the standard deviation. The increasing intensities of yellow and orange are associated with highest expression levels of mediators, while increasing intensity of blue is associated with lower levels of mediators relative to the mean. Missing or unrecorded values, or those below the limit of detection are indicated by the grey boxes. Dendrogram branch lengths and distances between nodes illustrate the extent of similarities.

Hierarchical clustering and Principal Component Analysis were both used in this study to profile mediators. Hierarchical clustering, although not a statistical tool, does generate an overall image which provides information on mediator levels in different groups of patients, and how these may relate to disease phenotype. PCA profiling in contrast, is a statistical tool that identifies the most important mediators and determines distinct profiles. Using both hierarchical clustering analysis and principal component analysis in this study maximizes the ability to identify prominent mediators which would theoretically be good targets for disease intervention. This type of analysis help to elucidate which mediators are more likely to be associated with greater disease activity and severity, as well as contributing insights into the biological mechanisms.

A summary explaining all the statistical analyses utilized and the rationale of why they were used for each cohort section is presented in a flow chart (Appendix figure 3.2.3).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 BIO

MARKER PROFILING IN EARLY AND ESTABLISHED RA

Patients with early and established RA were investigated to determine whether any specific biomarker profiles may explain worse disease in the beginning and later stages of RA. Such investigations can give insights into elucidating the pathological process and may give clues into the molecular pathways involved in the disease process as well as the progression of disease.

4.1.1 Relationship of RA features and characteristics

Initial comparative analyses of RA disease characteristics and features were performed for general assessment of the patients investigated in this study. RF and anti-CCP autoantibodies have been well documented to be associated with RA. Chi-square analysis revealed a significant association between RF and anti-CCP presence (p=0.0003), where nearly 90% of patients positive for RF were also positive for anti-CCP, and were over 6x more likely to be anti-CCP positive than RF negative patients (Table 4.1.1a). Interestingly, over 50% of patients were negative for RF but were positive for anti-CCP, whereas 11% of RF positive patients were negative for anti-CCP. This adds to the notion that anti-CCP develops first in RA disease and further demonstrates that it may be a better prognostic and diagnostic tool than RF (Greiner *et al.*, 2005, van Gaalen *et al.*, 2005, Nishimura *et al.*, 2007).

Table 4.1.1a: Association between RF and anti-CCP

	Anti-CCP n (%)		Odds Ratio (95% CI)
	Negative	Positive	
RF negative	19 (46%)	22 (54%)	Referent
RF positive	5 (11%)	40 (89%)	6.38 (2.17-18.74)

χ^2= 13.23 (df=1), p= 0.0003

Analysis also revealed that patients negative for RF were twice as likely to present with an effusion of a knee joint than RF positive patients, although this was not significant (10/41 (24%) v 6/45 (13%), OR=2.02, 95% CI 0.68-5.99, p=0.26). This was similarly found with anti-CCP negative patients who were also more likely to present with a knee effusion than anti-CCP positive patients (6/24 (25%) v 10/62 (16%), OR=1.75, 95% CI 0.57-5.34, p=0.36), which was also not significant. No significant association was found between erosive disease and patients positive for RF either (27/40 (67%) v 23/36 (64%), OR=1.17, 95% CI 0.45-2.97, p=0.81). However a significant association was found with anti-CCP (p=0.01). Patients with anti-CCP were over 3x more likely to have erosive disease than patients negative for anti-CCP (Table 4.1.1b), which is consistent with previous studies which have shown anti-CCP has a stronger relationship to erosions than RF (Visser *et al.*, 2002, Shankar *et al.*, 2006, Agrawal *et al.*, 2007).

Table 4.1.1b: Association between anti-CCP and erosive disease

	Erosions n (%)		Odds Ratio (95% CI)
	Negative	Positive	
Anti-CCP negative	12 (57%)	9 (43%)	Referent
Anti-CCP positive	14 (25.5%)	41 (74.5%)	3.76 (1.34-10.59)

χ^2= 6.78 (df=1), p=0.01

Autoantibodies have been shown to be present in individuals years before the actual onset of RA disease and increase the risk of early RA development (Rantapää-Dahlqvist *et al.*, 2003). Patients who were positive for anti-CCP were more likely to have early disease onset than anti-CCP negative patients (17/61 (28%) v 1/24 (4.2%), OR=6.16, 95% CI 1.08-35.13, p=0.01), which is in line with previous studies (Diaz *et al.*, 2011). The presence of

RF however was not associated with early onset (p=0.60). This further contributes to the notion that anti-CCP is a risk factor for early RA development and also a better prognostic and diagnostic tool than RF (Vossenaar and van Venrooij, 2004, Niewold *et al.*, 2007, Diaz *et al.*, 2011).

No significant association was found between synovial effusions and erosions (8/13 (61.5%) v 42/63 (66.7%), OR=0.78, 95% CI 0.23-2.57, p=0.75), although this could be attributed to the relatively small numbers of patients with both synovial effusions and erosions available for comparison.

4.1.2 EARLY RA

RA patients with early disease (disease duration of ≤2 years) were investigated with respective baseline (first clinical admittance) clinical, laboratory and patient characteristic data. Such investigations can give insights into the development of disease and the pathological process in early disease before its progression into established disease, which may provide valuable clues to the pathways by which RA develops.

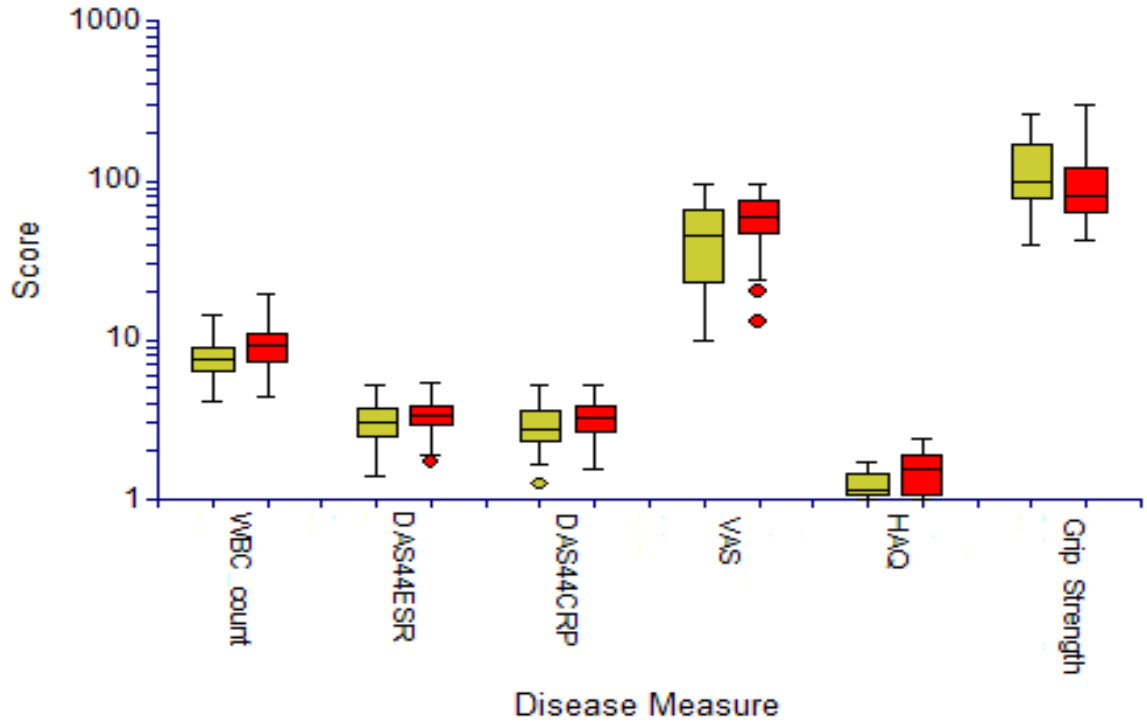
4.1.2.1.1 Relationship of RA disease features and patient characteristics with disease activity and severity in early RA

Initial analyses of baseline disease activity and severity measures with RA patient characteristics and clinical features were performed for general assessment of the patients investigated in this cohort. RF and anti-CCP have been well documented to be associated with more severe RA. Comparative analyses between positive and negative patients of both RF and anti-CCP revealed no significant differences in the level of disease activity and severity with either autoantibody (data not shown). This lack of difference may be because these particular patients are too early in their stage of RA development with ~60% (59/86) of patients having a disease duration of 6 months or less. Thus clear differences in severity may not have yet developed between positive and negative RF patients.

4.1.2.1.2 Influence of cigarette smoking

Cigarette smoking has been documented to be associated with worse RA disease (Saag *et al.*, 1997, Wolfe, 2000, Söderlin *et al.*, 2011), which was similarly demonstrated in this early RA patient cohort. Patients who had ever smoked demonstrated a significantly higher WBC count (p=0.03), DAS44ESR (p=0.04), DAS44CRP (p=0.02), VAS (p=0.02) and HAQ score (p=0.01) plus a significantly lower grip strength (p=0.04) (Figure 4.1.2a). However 74% (20/27) of non-smokers are women, which could explain the lower grip strength. No other disease measure was found to be significantly different in patients who have ever smoked (n=59) than those who had never smoked (n=27).

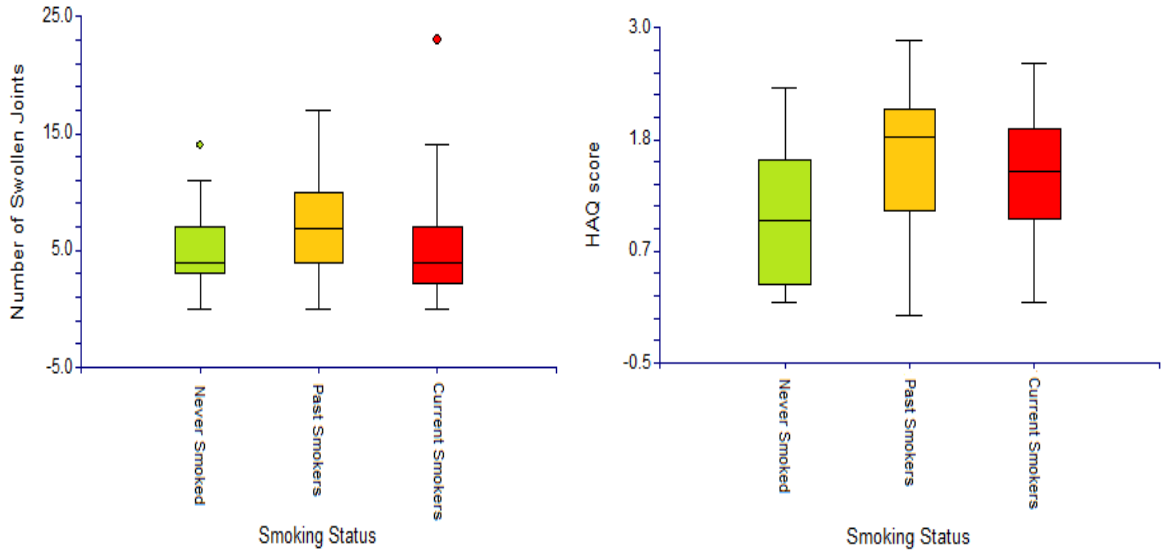
Figure 4.1.2a: Association of cigarette smoking with disease activity and severity



Green= never smoked, Red= ever smoked

Further analyses of the active smoking status of RA patients revealed however that the majority of disease activity and severity measures were not significantly different between never smokers, past smokers and current smokers, except for the number of swollen joints (p=0.04) and the HAQ score (p=0.02) (Figure 4.1.2b and c respectively). Past smokers were shown to have a significantly higher number of swollen joints than current smokers and never smokers, as well as higher HAQ scores compared to current smokers and never smokers, although this was only significant for the latter.

Figures 4.1.2b and c: Comparison of the number of swollen joints and the HAQ according to patient smoking status



The higher level of severity found in past smokers could possibly be attributed to the frequency and the amount of time patients have smoked (pack year history). However further analysis revealed that current smokers rather than past smokers (as well as never smokers) had the greatest number of smoking pack year history (30.0 (14.5-33.75) v 15.0 (8.75-40) v 0 (0-0), p=<0.0001). Patient gender also did not explain these results as there was a similar frequency of both male and female patients amongst past smokers (49% and

51% respectively). Duration of RA disease also could not explain these results, as no significant difference was found between never, past and current smokers (p=0.51), although past smokers were found to be older than current smokers (mean age 58 and 53 respectively). Differences in ages may have had a negative impact on measures of disease activity and severity as patients could have ceased smoking for other health related reasons (e.g. cardiovascular disease). In addition, the possibility of an immunosuppressive effect of current smoking may also contribute to the reduction of severity compared with past smokers (Geng *et al.*, 1996, McCue *et al.*, 2000, Ouyang *et al.*, 2000, Singh *et al.*, 2000). Interestingly, chi-square analysis also revealed RA patients who had never smoked were more likely to present with a synovial effusion than ever smokers, however this was not significant (7/27 (26%) v 9/59 (15.3%), OR=1.94, 95% CI 0.65-5.76, p=0.25). No significant association was found between smoking and erosive disease (35/51 (68%) v 15/25 (60%), OR=1.45, 95% CI 0.55-3.87, p=0.60). Patients who had ever smoked were shown to be more likely to be positive for RF than patients who have never smoked (33/59 (56%) v 12/27 (44%), OR=1.56, 95% CI 0.63-3.86, p=0.36), which is consistent with previous studies (Heliovaara *et al.*, 1993, Mattey *et al.*, 2002a, Morgan *et al.*, 2009). Furthermore, RF was also found to be more likely in current smokers than in past smokers (Table 4.1.2d) which is also in line with past studies (Wolfe, 2000, Mattey *et al.*, 2002a, Mattey *et al.*, 2002c, Westhoff *et al.*, 2008). Neither of these relationships were found to be significant, this may be due to the relatively small number of patients.

Table 4.1.2d: Association between smoking status and RF

Smoking status	RF negative n (%)	RF positive n (%)	Odds Ratio (95% CI)
Never smoked	15 (56%)	12 (44%)	Referent
Past smoker	18 (51%)	17 (49%)	1.17 (0.43-3.16)
Current smoker	8 (33%)	16 (67%)	2.40 (0.79-7.32)

χ^2=2.84 (df=2), p=0.24

In addition, categorisation by pack year history showed patients who had smoked more than 30 pack years were nearly 4x more likely to be RF positive (Table 4.1.2e). Furthermore, a significant increasing trend between smoking and the presence of RF (p=0.04), suggests a time-dose quantitative relationship between RF and frequency and duration of smoking (Wolfe, 2000, Stolt *et al.*, 2003). These findings show that current, frequent, long term smokers are more likely to be RF positive.

Table 4.1.2e: Association between pack year category and RF

Stratified pack year	RF negative n (%)	RF positive n (%)	Odds Ratio (95% CI)
0	15 (58%)	11 (42%)	Referent
0.1-15	12 (57%)	9 (43%)	1.02 (0.32-3.19)
16-30	5 (50%)	5 (50%)	1.34 (0.33-5.50)
>30	4 (25%)	12 (75%)	3.74 (0.99-14.01)

χ^2=5.03 (df=3), p-(trend)=0.04

Patients who ever smoked were also found more likely to be positive for anti-CCP positive than anti-CCP negative (45/59 (76%) v 17/27 (63%), OR=1.88, 95% CI 0.71-5.00, p=0.21) which is in line with other studies (Linn-Rasker *et al.*, 2006). This however did not reach significance, which may be due to the relatively small number of patients available. Furthermore, the presence of anti-CCP was more likely in current smokers than in past smokers, although this was not significantly associated (p=0.12) (Table 4.1.2f), suggesting that recent exposure to cigarette smoke may increase anti-CCP production.

Table 4.1.2f: Association between smoking status and anti-CCP

Smoking status	Anti-CCP negative n (%)	Anti-CCP positive n (%)	OR (95% CI)
Never smoked	10 (37%)	17 (63%)	Referent
Past smoker	11 (31%)	24 (69%)	1.27 (0.45-3.60)
Current smoker	3 (12.5%)	21 (87.5%)	3.68 (0.94-14.42)

χ^2=4.16 (df=2), p=0.12

A significant distribution between the categories of pack year history with the presence of anti-CCP was found (p=0.02) where the likelihood of anti-CCP increased with the general increasing trend of pack years (Table 4.1.2g).

Table 4.1.2g: Association between pack year category and anti-CCP

Stratified pack year	Anti-CCP negative n (%)	Anti-CCP positive n (%)	Odds Ratio (95% CI)
0	9 (35%)	17 (65%)	Referent
0.1-15	10 (48%)	11 (52%)	0.73 (0.25-2.15)
16-30	0 (0%)	10 (100%)	12.30 (0.66-228.2)
>30	2 (12.5%)	14 (87.5%)	3.53 (0.77-15.99)

χ^2=10.18 (df=3), p-(trend)=0.3

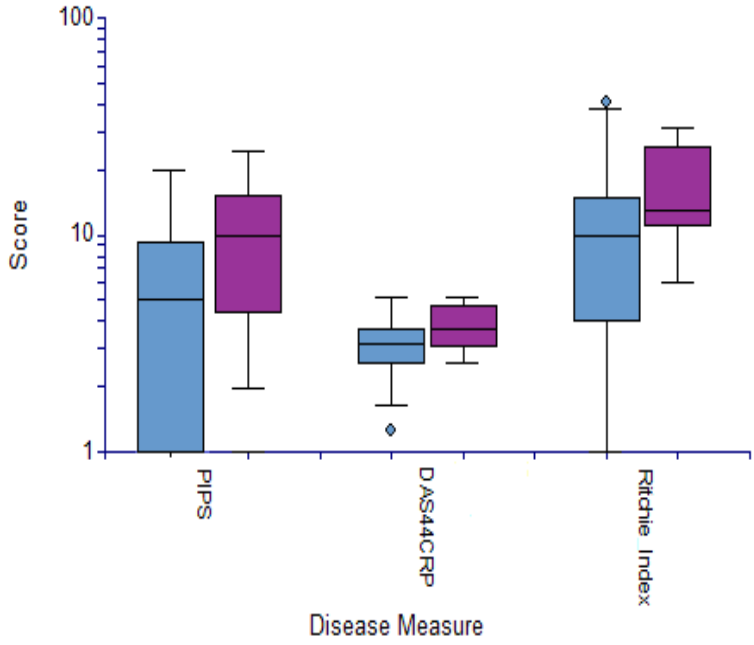
Cigarette smoking is commonly known to increase the risk of early RA development (Heliovaara *et al.*, 1993, Silman *et al.*, 1996, Symmons *et al.*, 1997, Costenbader *et al.*, 2006), where the age of onset may be different between smokers and non-smokers (Hutchinson *et al.*, 2001). However, analysis revealed that in patients categorised as having early onset (≤40 years) a significantly smaller proportion of patients (8/58) were smokers than non-smokers (10/27) (14 v 37%, OR=0.28, 95% CI 0.10-0.80, p=0.02).

4.1.2.1.3 Influence of DMARD treatment

The majority of RA patients were receiving NSAIDs (60%) at time of first attendance to clinic. Only a small group were receiving DMARDs (14%), which had been administered prior to their initial attendance. Patients receiving DMARD therapy had a significantly higher PIPS (p=0.02), DAS44CRP (p=0.03) and Ritchie Index score (p=0.04) (Figure 4.1.2h). Those patients on DMARDs were likely to have presented early with more active disease, hence the requirement of the administration of DMARDs to help alleviate disease.

No other disease measure was found to be significant, which could be due to the small number of patients on DMARDS to show any clear differences.

Figure 4.1.2h: Association of DMARDs with the PIPS, DAS44CRP and the Ritchie Index



Blue=DMARD negative, Purple=DMARD positive

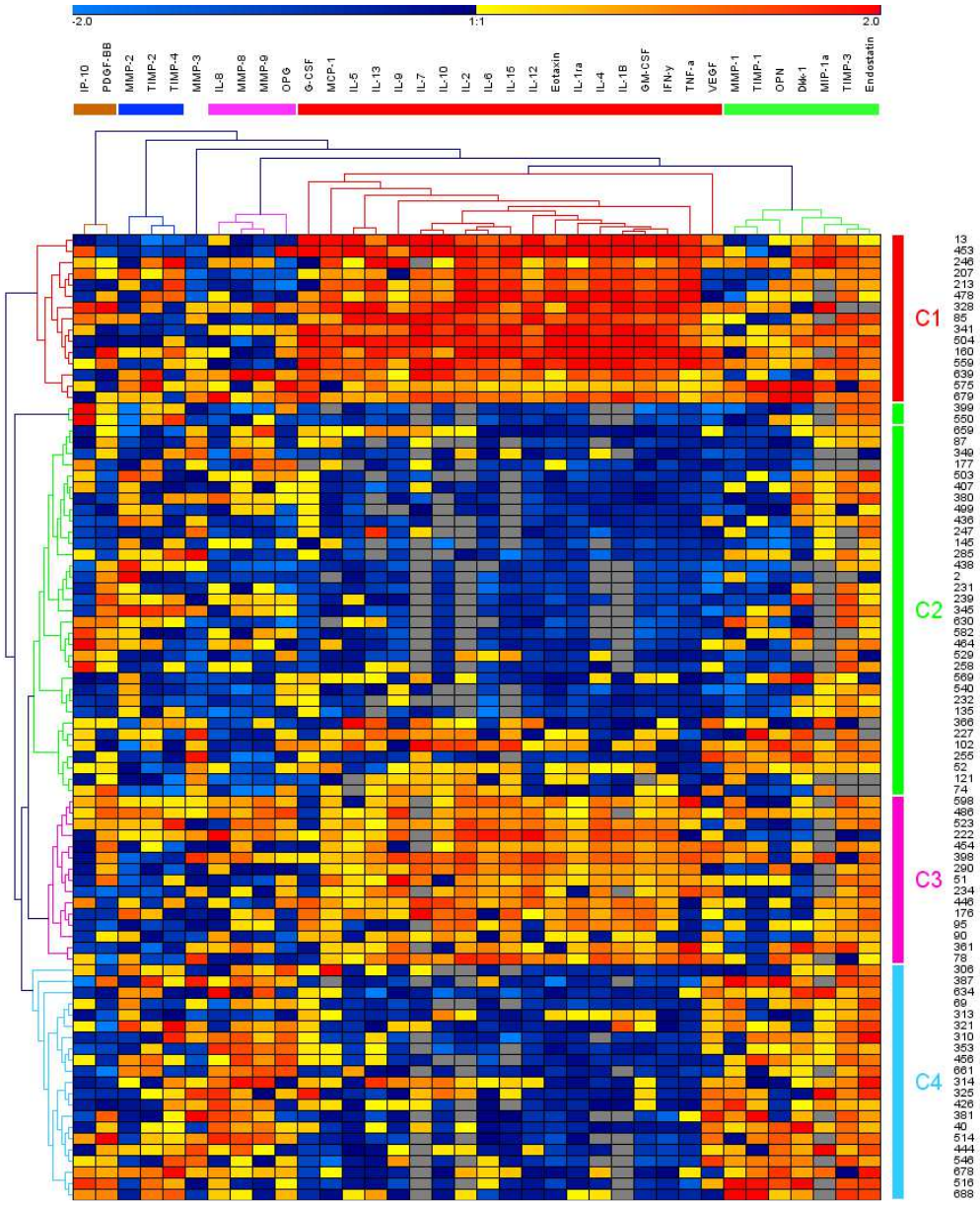
4.1.2.2 BIOMARKER PROFILING IN EARLY RA

A total of 36 mediators were investigated in this cohort of early RA patients. RANTES was excluded from analyses due to the majority of results being greater than the maximum threshold of detection. In addition, the majority of patient results for IL-17, FGF-β and MMP-12 were found to be below the minimum detection level and thus also excluded. Furthermore, the standard curves obtained with MIP-1β were considered to be unacceptable, and thus these results were also not included in analyses.

4.1.2.2.1.1 Early RA Hierarchical clustering biomarker profiling

Genesis hierarchical clustering analysis was used to identify RA patient groups based on their mediator expression. A heat map displaying the relative expression level of each mediator for every RA patient was generated (Figure 4.1.2i). After 2-dimensional hierarchical clustering, 4 distinct patient clusters denoted as C1, C2, C3 and C4 based on their mediator expression pattern similarities were identified, which further demonstrates that RA is an immunologically heterogeneous disease and may have various pathological routes.

Figure 4.1.2i: Heat map of the relative mediator expression in early RA patients



y-axis=patient identification number, x-axis=mediators

Clusters C1 and C3 displayed similar patterns of relatively high mediator expression. In contrast, clusters C2 and C4 displayed similar patterns of relatively low mediator expression, apart from some of the MMPs, TIMPs and endostatin, which suggests the possibility of immunological subtypes of varying grades of immune activity. The heat map showed the biggest differences between the high and low mediator expression clusters (C1

and C3 versus C2 and C4) were of the interleukins, as well as a number of other specific mediators (eotaxin, G-CSF, GM-CSF, MCP-1, IFN-γ, TNF-α and VEGF). This was confirmed with statistical comparisons ($p=\leq 0.05$) (data not shown), although IL-8 was additionally found to be significantly higher in the high mediator expression clusters (31.46 (24-50.7) v 24.72 (12.7-41.6), p=0.03). This 20 mediator profile of interleukins, eosonophil and monocyte chemoattractants, granulocyte/macrophage growth factors, angiogenesis factor and pro-inflammatory mediators could largely explain the difference between these potential immunological subtypes. Discriminant stepwise selection and forward logistic regression analysis of this profile revealed that IL-6 alone (p=0.0001, model p=<0.0001, model r-squared=0.46), distinguished the high from the low mediator expression clusters in particular.

4.1.2.2.1.2 Association with early RA disease activity and severity

Chi-square analysis significantly demonstrated that the C1 and C3 patient clusters also had the highest frequencies of patients positive for RF, whereas the C2 cluster showed the lowest frequency of RF positive patients followed by cluster C4 (Table 4.1.2j). No significant difference was found between patient clusters and the distribution of anti-CCP autoantibody. However, similar to that of RF seropositivity, C1 and C3 clusters had the highest frequencies of anti-CCP positive patients (Table 4.1.2j). This suggests that the 20 interleukin-prominent biomarker profile expressed in the high mediator expression clusters and particularly pro-inflammatory mediator IL-6 may be important in regard to the generation of autoantibodies as Th2 interleukin production have roles in stimulating antibody production (Janeway Jr *et al.*, 2005). The difference of mediator expression

pattern and seropositivity between the relatively high and low expression levels could suggest the possibility of a disease subtype.

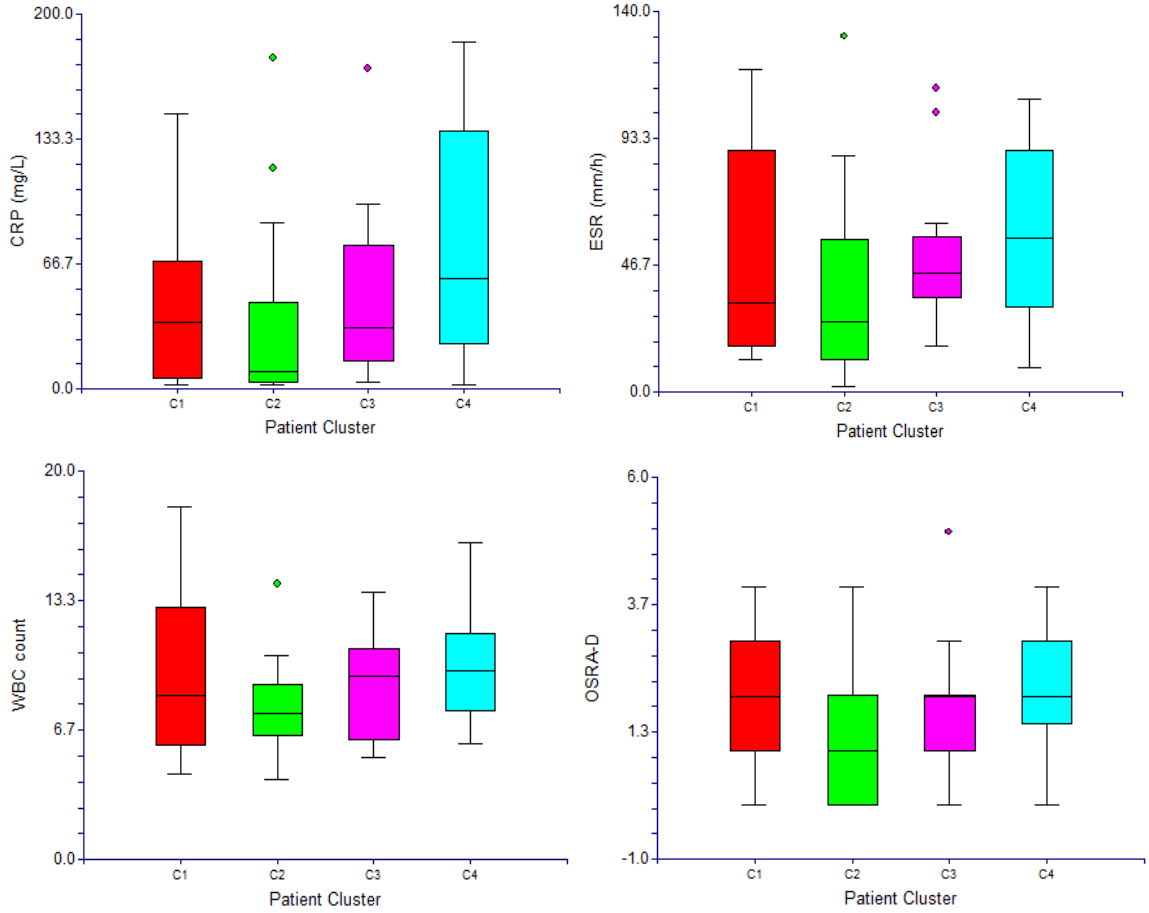
Table 4.1.2j: Association between patient clusters and autoantibodies

Patient Cluster	RF negative n (%)	RF positive n (%)	Anti-CCP negative n (%)	Anti-CCP positive n (%)
C1	2 (13.3%)	13 (86.7%)	3 (20%)	12 (80%)
C2	24 (68.6%)	11 (31.4%)	13 (37%)	22 (63%)
C3	3 (20%)	12 (80%)	1 (6.7%)	14 (93.3%)
C4	12 (57%)	9 (43%)	7 (33%)	14 (67%)
	χ^2=18.57 (df=3), p=0.0003		χ^2=5.62 (df=3), p=0.13	

No significant difference was found between patient clusters and the distribution of synovial effusions (p=0.74) or erosions (p=0.72).

Comparison of disease activity and severity measures revealed that only the OSRA-D (p=0.05) plus indices of peripheral inflammation (CRP p=0.02, ESR p=0.01, WBC count p=0.02) were significantly different between patient clusters (Figure 4.1.2k). No other disease measure was found to be significantly different. Differences were mainly between patients of the C4 and the C2 cluster, with the C4 cluster demonstrating highest scores despite both displaying relatively low mediator expression patterns.

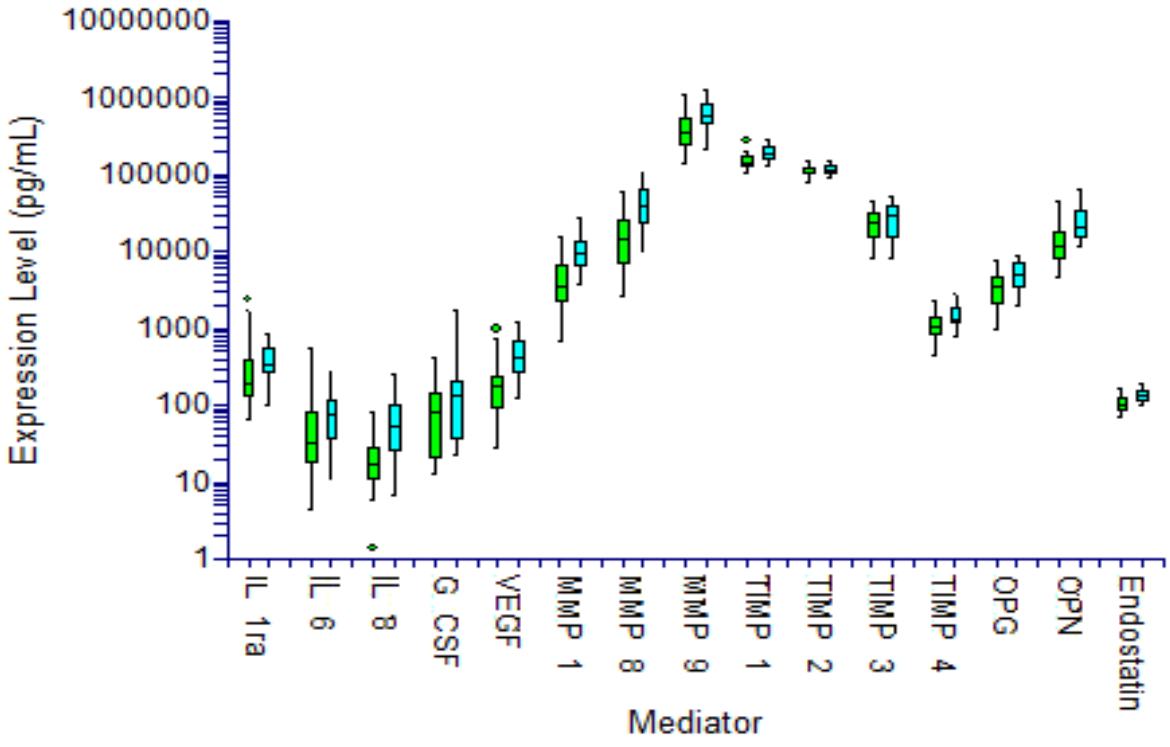
Figures 4.1.2k: Comparison between patient clusters and disease measures in early RA



As clusters 2 (n=35) and 4 (n=21) showed the largest differences in terms of disease measures despite demonstrating relatively similar mediator expression patterns, comparisons were performed to determine expression differences between the 2 clusters. Mediators IL-1ra (p=0.005), IL-6 (p=0.01), IL-8 (p=<0.0001), G-CSF (=0.03), VEGF (p=<0.0001), MMP-1 (p=0.0001), -8 (p=<0.0001), -9 (p=0.0002), TIMP-1 (p=0.0004), -2 (p=0.04), -3 (p=0.02), -4 (p=0.007), OPG (p=0.001), OPN (p=0.0002) and endostatin (p=<0.0001) were found to be significantly higher in the C4 patient cluster compared to cluster C2 (Figure 4.1.2l). This 15 mediator profile consists of pro-inflammatory cytokines, chemokines, angiogenesis and cell growth factors plus matrix remodelling mediators including ECM degrading enzymes and therefore could largely account for the worse

disease activity and severity found in the C4 cluster (Lawlor *et al.*, 2004, Nishimoto, 2006, Knudsen *et al.*, 2008, Yoo *et al.*, 2008). However, IL-1ra an inhibitor of IL-1 and OPG an osteoclastogenesis inhibitor (bone growth stimulator) and a key regulator of immune responses (Lacey *et al.*, 1998, Schiff, 2000, Feige, 2001, Cutolo, 2004), may be elevated to compensate for increased inflammation. Discriminant stepwise selection and forward logistic regression analysis of this profile revealed that a profile of high IL-8, MMP-1, MMP-8 and OPG expression in particular distinguished the C4 cluster from the C2 cluster (model p=<0.0001, model r-squared=0.54). Although the individual mediators were not separately significant (all mediators p=≥0.057), their cumulative contribution as a profile is significant. This suggests that raised expression of these mediators collectively may contribute to worse disease activity and severity.

Figure 4.1.2l Comparison of mediators between patient clusters C2 and C4



Green=C2 cluster, Blue=C4 cluster

Interestingly, the distribution of early disease onset was significantly different between the patient clusters. Cluster C2 was shown to have the highest frequency of early onset disease whereas the C4 cluster demonstrated the lowest frequency of early onset disease (Table 4.1.2m). This suggests that the elevated 15 mediator profile identified in the C4 patient cluster and in particular raised IL-8, MMP-1, MMP-8 and OPG expression may also be important in regard to delayed RA development.

Table 4.1.2m: Association between patient clusters and early RA onset

Patient Cluster	Early Onset negative n (%)	Early Onset positive n (%)
C1	13 (86.7%)	2 (13.3%)
C2	22 (63%)	13 (37%)
C3	12 (85.7%)	2 (14.3%)
C4	20 (95%)	1 (5%)

χ^2=9.68 (df=3), p=0.02

4.1.2.2.1.3 Association with cigarette smoking

Cigarette smoking has been well documented to be associated with severe RA and worse disease outcome. Chi-square analysis revealed the distribution of smokers was significantly different between patient clusters (Table 4.1.2n). The C2 patient cluster had the lowest frequency of smokers compared to the other clusters, whereas clusters C1, C3 and C4 all had similarly high frequencies of smokers. This suggests that the elevated 15 and 20 mediator profiles identified in these clusters may both be upregulated in smokers. This could also account for the low frequency of smokers in the C2 cluster with its relatively low mediator expression pattern. Interestingly, the C4 cluster demonstrated the highest frequency of smokers despite displaying a relatively low mediator expression pattern. This may be in part due to the elevated expression of immuno-regulatory mediators IL-1ra and OPG identified in this cluster. In addition, cigarette smoke has been

documented to have immunosuppressive properties which may also partially explain the pattern of low mediator expression in the C4 cluster (McCue *et al.*, 2000, Ouyang *et al.*, 2000, Oltmanns *et al.*, 2005, Lee *et al.*, 2007).

Table 4.1.2n: Association between patient clusters and smoking

Patient Cluster	Never smoked n (%)	Ever smoked n (%)
C1	4 (26.7%)	11 (73.3%)
C2	17 (48.6%)	18 (51.4%)
C3	3 (20%)	12 (80%)
C4	3 (14.3%)	18 (85.7%)

χ^2=8.70 (df=3), p=0.03

The smoking status of patients was also shown to be significantly different between patient clusters (p=0.04), where the C2 patient cluster had the lowest frequency of current smokers compared to the remaining clusters (Table 4.1.2o). This suggests that recent exposure to cigarette smoke may upregulate the expression of both the 15 and 20 mediator profiles in these clusters. Interestingly, the C4 cluster had the highest frequencies of past smokers which may partly account for the relatively low mediator expression pattern found in this cluster.

Table 4.1.2o: Association between patient clusters and smoking status

Patient Cluster	Never smoked n (%)	Past smokers n (%)	Current smokers n (%)
C1	4 (26.7%)	4 (26.7%)	7 (46.7%)
C2	17 (48.6%)	13 (37.1%)	5 (14.3%)
C3	3 (20%)	6 (40%)	6 (40%)
C4	3 (14.3%)	12 (57.1%)	6 (28.6%)

χ^2=13.21 (df=6), p=0.04

Subsequent analysis with pack year history stratified by smoking intensity between patient clusters revealed no significant differences in the distribution between patient clusters. However, the C2 cluster was found to have the lowest frequency of patients that smoked more than 15 pack years, whereas clusters C1, C3 and C4 displayed similarly high frequencies (Table 4.1.2p). This indicates that frequent exposure and large quantities of cigarette smoke may upregulate the expression of both the 15 and 20 mediator profiles.

Table 4.1.2p: Association between patient clusters and stratified pack year history

Patient Cluster	Never smoked n (%)	0.1-15 n (%)	16-30 n (%)	≥31 n (%)
C1	4 (30.8%)	1 (7.7%)	3 (23.1%)	5 (38.5%)
C2	17 (51.5%)	10 (30.3%)	3 (9.1%)	3 (9.1%)
C3	3 (25%)	3 (25%)	2 (16.7%)	4 (33.3%)
C4	2 (13.3%)	7 (46.7%)	2 (13.3%)	4 (26.7%)

χ^2=15.05 (df=9), p=0.09

Chi-square analysis was also performed between patient clusters and current DMARD treatment, however no significant difference in the distribution of DMARD patients was found between the clusters (p=0.84). This may be possibly explained by the small number of patients on DMARDs.

4.1.2.2.1.4 Early RA Hierarchical clustering results summary

These results indicate that cigarette smoking may upregulate the expression of multiple mediators, especially in response to recent exposure. The upregulation of mediators, especially interleukins and MMPs, therefore could contribute to the generation of autoantibodies and influence the level of disease activity and severity found in RA smokers. This particular mediator profile-disease activity and severity relationship may therefore account for the relatively low expression of mediators in non-smokers and thus

explain the better level of RA disease found in patients of the C2 cluster. As cigarette smoke has been documented to have immunosuppressive properties (McCue *et al.*, 2000, Ouyang *et al.*, 2000, Oltmanns *et al.*, 2005, Lee *et al.*, 2007), as well as potentially upregulate immuno-regualtory mediators (IL-1ra and OPG) this may account for the delayed onset of disease found in RA smokers. These results also illustrate that smoking may suppress or promote expression of certain mediators, and that cessation of smoking may produce a different mediator expression profile compared with that of current smokers, which in turn may influence disease severity in a unique manner.

4.1.2.2.2.1 Early RA Principal Component Analysis biomarker profiling

Due to the large number of mediators investigated in this study, principal component analysis (PCA) was employed to reduce the large number (dimensionality) of interrelated (multicollinearity) variables. An exploratory PCA of all 36 mediators was initially performed that selected a set of 17 specific mediators which provided the maximum variance (Table 4.1.2q).

Table 4.1.2q: Mediators selected for Principal Component Analysis

Selected PCA Baseline Biomarkers				
IL-1β	IL-8	TNF-α	MMP-3	OPN
IL-2	G-CSF	VEGF	MMP-8	
IL-4	GM-CSF	MMP-1	MMP-9	
IL-6	IP-10	MMP-2	Dkk-1	

Principal component analysis identified 6 principal component (PC) biomarker profiles generated from correlation patterns of the 17 selected mediators which were then used for analysis. Table 4.1.2r displays the 6 PC generated biomarker profiles and their modified relative factor loadings after varimax rotation. Positive and negative factor loadings relate to high and low expression level respectively.

Table 4.1.2r: PC profiles of 17 biomarkers and their relative factor loadings

PC 1		PC 2		PC 3		PC 4		PC 5		PC 6	
IL-2	0.91	MMP-9	0.90	VEGF	0.77	G-CSF	0.95	IP-10	0.89	Dkk-1	0.81
TNF-α	0.91	MMP-8	0.88	MMP-2	-0.72	IL-1β	0.82	MMP-2	-0.46	OPN	0.73
IL-6	0.90	IL-8	0.71	MMP-3	0.63	GM-CSF	0.79			MMP-1	0.62
IL-4	0.89										
GM-CSF	0.57										
IL-1β	0.51										

- negative factor loading

4.1.2.2.2.2 Association with early RA disease activity and severity

Regression analyses revealed various PC profiles were associated with a number of disease activity and severity measures, particularly that of the PC 3 and 6 profiles which were highly associated with the majority of RA disease measures (Table 4.1.2s). The PC 3 profile which consisted of high VEGF and MMP-3 expression, but a low level of MMP-2 was strongly associated with a large number of pain and inflammatory measures. The PC 6 profile which incorporated mediators Dkk-1, OPN and MMP-1 was shown also to be strongly associated with measures of inflammation. These matrix remodelling mediators including ECM degrading enzymes and an angiogenesis stimulator have multiple roles including chemotaxis, apoptosis, cell regulation (proliferation, migration, adhesion), inflammation etc. and may explain the worse disease activity and severity found in RA patients (Reinholt *et al.*, 1990, Yamanaka *et al.*, 2000, McCawley and Matrisian, 2001, Green *et al.*, 2003, Stamenkovic, 2003, Clavel *et al.*, 2007, Diarra *et al.*, 2007, Glass and Karsenty, 2007, Nagasaka *et al.*, 2008, Yoo *et al.*, 2008, Ueland *et al.*, 2009).

The PC 3 profile was also shown to be associated with various measures, particularly relating to the joint; the number of swollen joints, synovial effusion, the OSRA-D and the HAQ. This profile may account for the higher degree of inflammation which is normally associated with synovial effusions and swollen joints (Reinholt *et al.*, 1990, Yamanaka *et al.*, 2000, McCawley and Matrisian, 2001, Green *et al.*, 2003, Stamenkovic, 2003, Clavel *et al.*, 2007, Diarra *et al.*, 2007, Glass and Karsenty, 2007, Nagasaka *et al.*, 2008, Yoo *et al.*, 2008, Ueland *et al.*, 2009). No other PC profile was found to be associated with any other measures for function or joint damage including erosions and the Larsen hand score. This may be because early RA patients may not show sufficiently large differences in joint

damage in early stages of the disease process to be able to link them to particular mediators.

Furthermore, the PC 1 profile consisting of Th2 related interleukins IL-1β, IL-2, IL-4, IL-6 (Janeway Jr *et al.*, 2005), plus GM-CSF and TNF-α was found to be positively associated with RF autoantibodies alone. No profile was identified to be associated with anti-CCP.

Table 4.1.2s: Association between PC profiles and disease measures

Disease Measure	Variable	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	Model p-value	Model r-squared
Inflammation	CRP			<0.0001			<0.0001	<0.0001	0.60
	ESR	0.02		<0.0001			<0.0001	<0.0001	0.40
	SJ >5 (+/-)			0.03				0.02	0.06
	WBC		<0.0001				0.01	<0.0001	0.50
	Platelets (+/-)			0.004			0.004	<0.0001	0.20
	Anaemia (+/-)			0.02			0.01	0.001	0.15
	Synovial effusion (+/-)			0.004				0.002	0.10
	PIPS >5 (+/-)							NS	
	CGDA	0.008		0.001			0.01	0.0003	0.20
	OSRA-A			0.0006			0.02	0.0001	0.18
	SI	0.03	0.02	0.001			0.003	0.0002	0.23
	DAS44CRP			0.003				0.003	0.10
	DAS44ESR			0.001				0.001	0.90
Autoantibody	RF (+/-)	0.04						0.008	0.07
	Anti-CCP (+/-)							NS	
Pain	VAS			0.004				0.004	0.09
	TJ >9 (+/-)							NS	
	RI >10 (+/-)							NS	
Damage	Erosions (+/-)							NS	
	OSRA-D			0.001				0.002	0.11
	Larsen Hands score >28 (+/-)							NS	
Function	EMS >60 (+/-)							NS	
	HAQ			0.001		0.04		0.002	0.14
	Grip strength <88.5 (+/-)							NS	
Other	Ever Smoked (+/-)		0.006		0.13		0.02	0.0001	0.20

N.B Non-parametric variables were dichotomised using the median value as the cut-off level, (-) negative association, NS=Not Significant.

As PC profiles PC 3 and PC 6 were both found to be associated with the majority of inflammatory measures, further investigation was performed with mediators from both profiles as separate independent variables to determine which mediators specifically within these profiles were most strongly associated with inflammation (Table 4.1.2t). Regression analyses revealed MMP-3 to be the most strongly associated independent mediator with the majority of inflammatory disease measures, with OPN following closely behind.

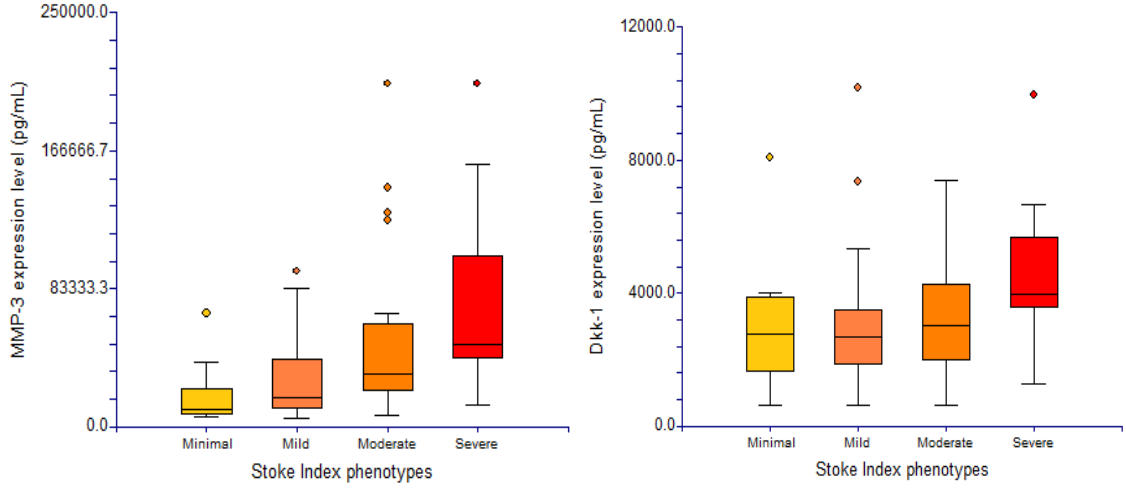
Table 4.1.2t: Association between PC 3 and PC 6 derived mediators with measures of inflammation

Inflammatory Disease Measure	PC 3				PC 6		Model p-value	Model r-squared
Variable	VEGF	MMP-2	MMP-3	Dkk-1	OPN	MMP-1		
CRP	0.004		<0.0001		0.0006	0.004	<0.0001	0.67
ESR	0.001		0.002		0.002		<0.0001	0.39
Platelet (+/-)					0.03	0.0002	<0.0001	0.21
Anaemia (+/-)			0.0007			0.03	<0.0001	0.27
CGDA >47 (+/-)		0.03 (-)	0.003				<0.0001	0.20
OSRA-A			0.004		0.02		0.0003	0.18
SI			<0.0001	0.007			<0.0001	0.27

N.B Non-parametric variables were dichotomised using the median value as the cut-off level, (-) negative association.

MMP-3 and OPN were also found to be associated with overall RA disease activity measure of the OSRA-A (Table 4.1.2t). In addition, MMP-3 and Dkk-1 were shown to be associated with another overall disease activity measure of the Stoke Index (Table 4.1.2t). Comparisons between Stoke Index disease activity categories revealed significant differences of expression between the phenotypes for both MMP-3 and Dkk-1 (p=<0.0001 and p=0.005 respectively), with a significant increasing trend of worse disease (p-(trend) <0.0001 and p-(trend)=0.001 respectively) (Figures 4.1.2u).

Figures 4.1.2u: Association of Stoke Index categories with MMP-3 and Dkk-1 expression



The PC 3 profile was also shown to be associated with additional overall disease activity measures of the DAS44CRP and DAS44ESR (Table 4.1.2s). Regression analyses were therefore also performed on the PC 3 mediators as separate independent variables to determine which mediators were most strongly associated with overall DAS activity. Analysis revealed MMP-3 to be associated with both DAS scores (p=0.001 and p=0.02 respectively), with VEGF associating also with the DAS44ESR (p=0.02), although this was lost after adjusting for smoking. Subsequent comparisons of these mediators between DAS44 phenotypes revealed MMP-3 expression to be significantly different between the phenotypes in both the DAS44ESR and DAS44CRP (p=0.006 and p=0.002 respectively), with a significant increasing trend of worse disease activity in both DAS scores (p-(trend)=0.001 and p-(trend)=<0.0001 respectively) (Figures 4.1.2v). This was similarly demonstrated with VEGF expression and the DAS44ESR (p=0.03, p-(trend)=0.005), although this seemed to be dependent on patient smokers (Figure 4.1.2w).

Figures 4.1.2v: Association of MMP-3 expression with DAS44CRP and DAS44ESR phenotypes

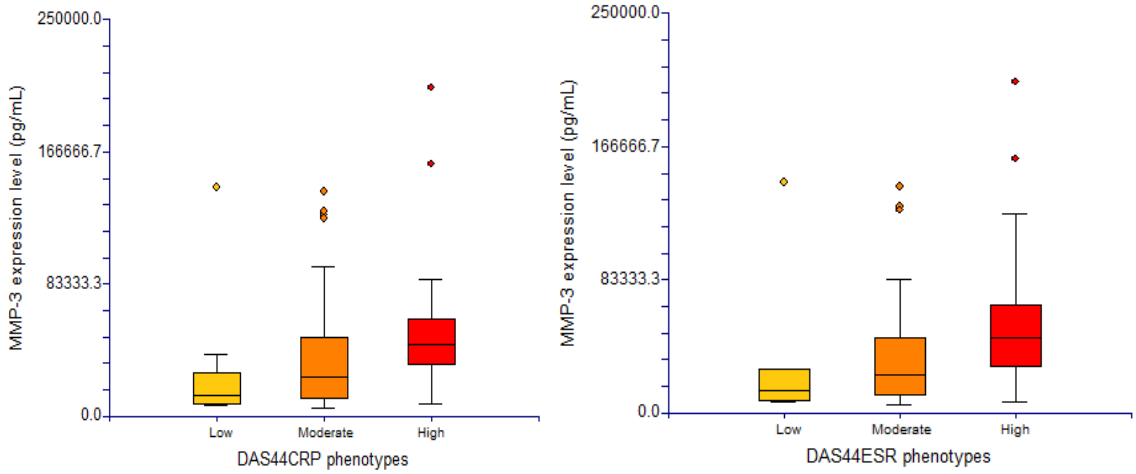
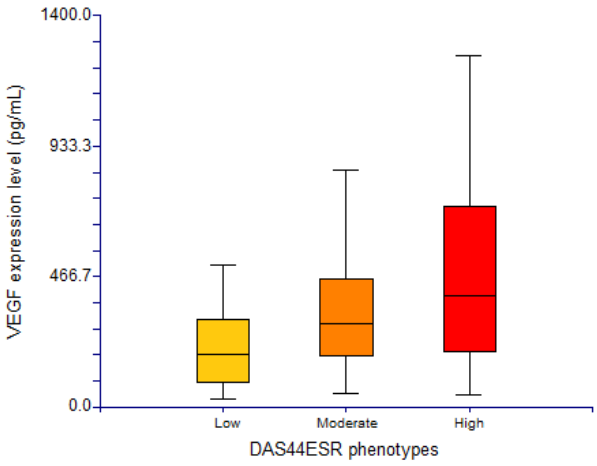


Figure 4.1.2w: Association of VEGF expression with DAS44ESR phenotypes



4.1.2.2.2.3 Association with cigarette smoking and DMARD treatment

Profiles PC 2 (IL-8, MMP-8 and MMP-9) and PC 6 (Dkk-1, OPN and MMP-1) were found to be associated with cigarette smoking with PC 4 contributing to its overall significance (model p=0.0001, model r-squared=0.20) (Table 4.1.2s). The PC 6 profile was associated with DMARD treatment (p=0.01, model p=0.005, model r-squared=0.10). This suggests that both smoking and DMARD treatment may upregulate the expression of the mediators in this profile. No PC profile at baseline was found to be associated with early onset.

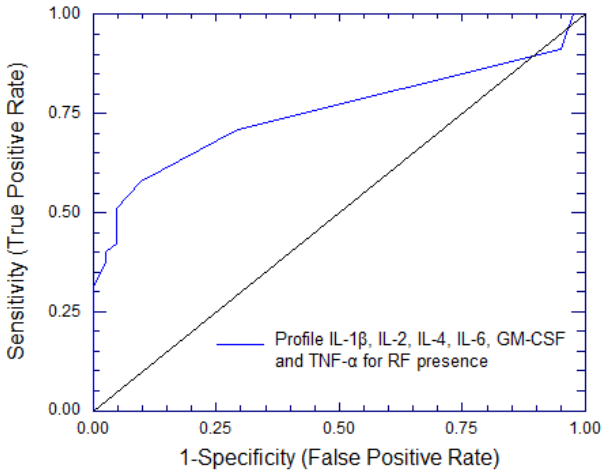
4.1.2.2.2.4 Early RA Principal Component Analysis results summary

These results suggest that cigarette smoking may upregulate various mediators that play a major role in neutrophil activation, angiogenesis and matrix remodelling. This may account for the high level of inflammation and worse overall disease activity found in early RA, as well as joint severity associated with this profile. Upregulation of these mediators may also account for the association with DMARD treatment, since patients presenting with higher levels of inflammation would have higher requirement for therapeutic intervention (Saag *et al.*, 1997, Másdóttir *et al.*, 2000, Wolfe, 2000, Mattey *et al.*, 2002c, Finckh *et al.*, 2007, Westhoff *et al.*, 2008).

4.1.2.2.3 Composite Biomarker Profiles

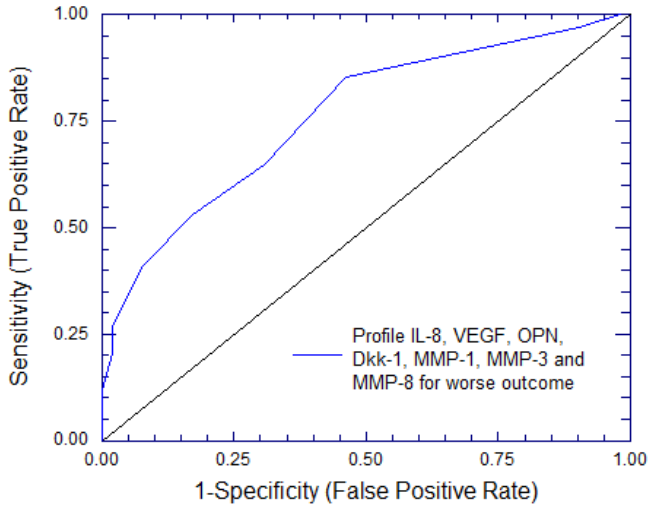
Using both hierarchical clustering and principal component analysis a biomarker profile of IL-1β, IL-2, IL-4, IL-6, GM-CSF and TNF-α was derived which was likely to be associated with RF. ROC analysis of this composite mediator profile revealed it to be "fairly" accurate in identifying patients positive for RF (AUC 0.74, 95% CI 0.62-0.84, SE 0.05, p=<0.0001) (Figure 4.1.2x).

Figure 4.1.2x: Accuracy of RF identified biomarker profile



Hierarchical clustering and principal component analysis identified 2 distinct biomarker profiles that were predominantly associated with worse disease activity and severity in these RA patients. A composite of these 2 profiles therefore could create a profile that may maximally account for worse RA disease. ROC analysis of this profile (IL-8, VEGF, OPN, Dkk-1, MMP-1, MMP-3 and MMP-8) showed it to be "fair" in identifying a worse disease activity phenotype, defined by severe DAS (score >3.7) or high Stoke Index (score 12-17) disease activity scores (AUC 0.76, 95% CI 0.65-0.86, SE 0.05, p=<0.0001) (Figure 4.1.2y).

Figure 4.1.2y: Accuracy of worse disease activity phenotype identified biomarker profile



No biomarker profile from either analysis method was found to be associated with erosive disease, however PCA did reveal a biomarker profile associated with multiple joint specific disease measures (high VEGF, MMP-3 and low MMP-2 expression). ROC analysis of this profile was subsequently performed, but it "failed" to accurately identify the presence of erosions in early RA (AUC 0.48).

4.1.2.2.4 Early RA Overall Summary

This cohort of RA patients demonstrated biomarker profiles that were predominantly associated with markers of inflammation rather than outcome disease measures although a few biomarker profiles of interest from both analysis methods were derived.

A biomarker profile of growth factors, pro-inflammatory cytokines and numerous interleukins was identified to be associated with RF production. This profile is consistent with previous studies where multiple interleukins and the same specific mediators were strongly associated with autoantibody presence (Hitchon *et al.*, 2004, Alex *et al.*, 2007,

Hueber *et al.*, 2007, Jørgensen *et al.*, 2008, Kokkonen *et al.*, 2010, Meyer *et al.*, 2010, Chandra *et al.*, 2011, Hodkinson *et al.*, 2011). Various interleukins may be involved in the generation of RF autoantibody, although IL-6 seems particularly important since it was identified in both hierarchical clustering and principal component analysis. This profile may also have the potential to predict a more severe phenotype in established RA as autoantibodies are associated with worse disease and poorer outcome (Reilly *et al.*, 1990, Myllykangas-Luosuj~irvi *et al.*, 1995, Másdóttir *et al.*, 2000, Wolfe, 2000, Mattey *et al.*, 2001b, Vencovsky´ *et al.*, 2003, Greiner *et al.*, 2005, Lindqvist *et al.*, 2005, Sihvonen *et al.*, 2005, Mewar *et al.*, 2006, Mattey *et al.*, 2007, Turesson *et al.*, 2007).

A distinct matrix remodelling biomarker profile including mediators associated with chemoattraction and angiogenesis was associated with worse disease activity and severity. This profile is unique when compared with previous profiling studies. However, individual mediators of this profile in conjunction with other mediators have been similarly found associated with greater disease activity in previous studies (Moniem and Mohammad, 2003, Kuryliszyn-Moskal *et al.*, 2006, Angelo and Kurzrock, 2007, Ateş *et al.*, 2007, Clavel *et al.*, 2007, Rioja *et al.*, 2008, Sennels *et al.*, 2008, Bazzichi *et al.*, 2009, Gaoya *et al.*, 2009, Meyer *et al.*, 2010, Ozgonenel *et al.*, 2010). This profile therefore has the potential to become a novel assessment measure to complement current RA disease activity measures.

Cigarette smoking may upregulate IL-6, IL-8, MMP-1, MMP-8, MMP-9, Dkk-1, OPN and OPG. Both hierarchical clustering and principal component analysis identified IL-8, MMP-1 and MMP-8 which is consistent with previous studies where IL-8 and MMPs were upregulated in healthy non-RA smokers (Nakamuraa *et al.*, 1998, Wang *et al.*, 2000,

Knuutinen *et al.*, 2002, Nordskog *et al.*, 2003, Raitio *et al.*, 2005, Karimi *et al.*, 2006). This further demonstrates that smoking can alter the expression of mediators and thus influence immune activity. Mediators of neutrophil activation and matrix remodelling are particularly prominent in this profile, which suggests a major role for these molecules in the association between smoking and RA.

This study demonstrates the importance of various chemoattractants, angiogenesis factors, growth factors, pro-inflammatory cytokines and matrix remodelling mediators in the pathological process of early RA, and identifies mediators which are likely to be involved in molecular pathways of the disease process during its development. Cytokines commonly accepted to be associated with RA development (e.g. IL-β, IFN-γ and TNF-α) did not provide any more information about disease activity in early RA than many of the other mediators. These findings highlight the heterogeneity of RA pathology and indicate that various markers of immune dysfunction influence the development of RA, which illustrates the complex network of mediators involved in disease. Some of these biomarkers could become novel targets for disease intervention, where specific targeting may help to lower inflammation and damage leading to reduced disease activity and severity.

4.1.3 BIOMARKER PROFILING FOR RA PROGNOSIS

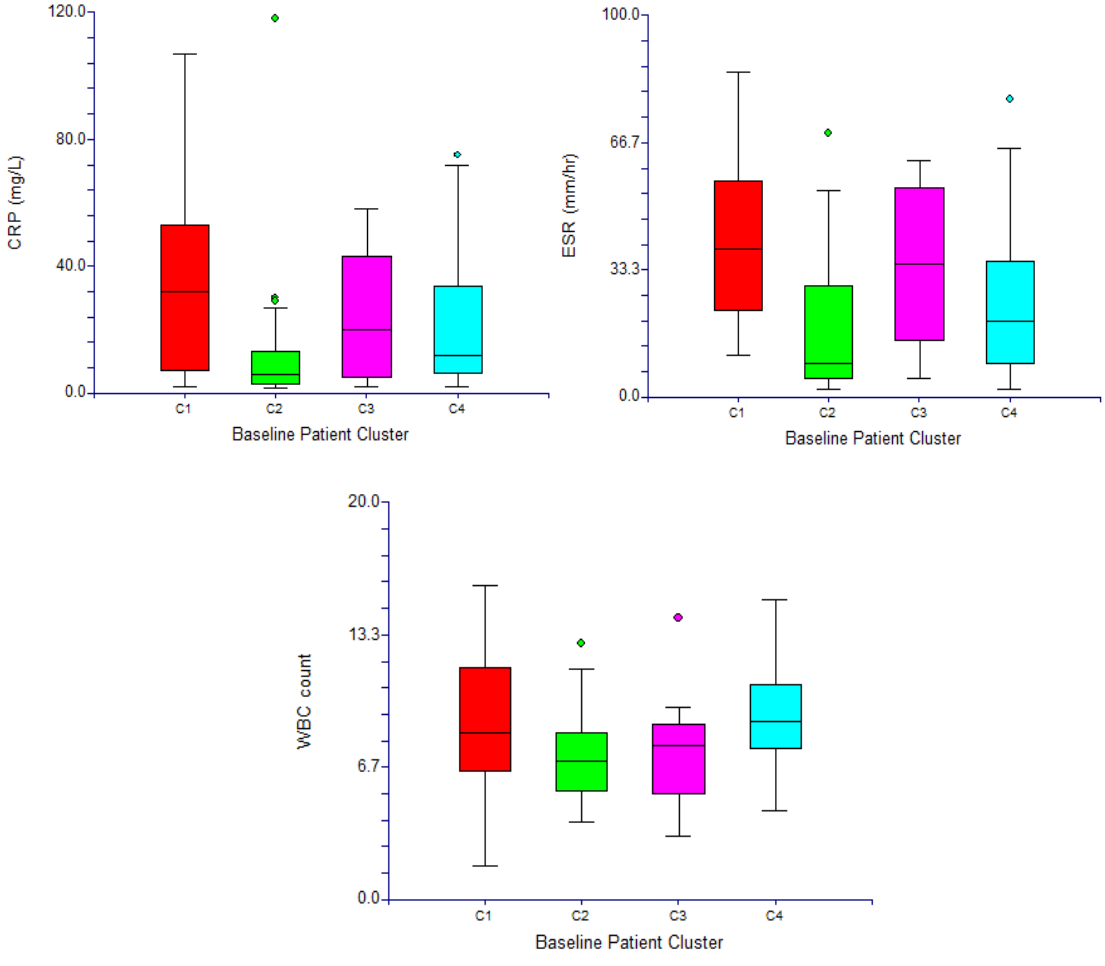
RA is a long-term disease, therefore identifying biomarkers that predict its likely course and outcome would greatly aid in the management of the disease, and become a useful tool especially for newly diagnosed early stage RA patients. Such investigations can also give clues into the pathological process of RA and how it may develop. Biomarker clusters and PC profiles identified at baseline from hierarchical clustering and the PCA method respectively were analysed with measures of disease activity and severity at 5 year follow-up to determine whether any specific profiles could predict disease outcomes in established RA.

4.1.3.1.1 Hierarchical clustering prognostic biomarker profiling

Analysis of baseline patient clusters with measures of disease at 5 year follow-up revealed the same few indices of peripheral inflammation (ESR, CRP and WBC count) to be significantly different between patient clusters as in early RA. No other measures of inflammation, pain, damage, or function at 5 year follow-up were found to be significantly different between baseline clusters. The C2 baseline patient cluster demonstrated the lowest levels of peripheral inflammation compared to the other clusters (Figure 4.1.3a). This suggests that high expression of the 15 biomarker profile especially that of IL-8, MMP-1 and MMP-8 identified from the C2 cluster at baseline may also be important in regard to peripheral inflammation in established disease as well as in early disease. Interestingly, the baseline C4 cluster no longer displayed the highest levels of peripheral inflammation compared to the other clusters at 5 year follow-up. This was now displayed by clusters C1 and C3 instead. This could suggest that the upregulated profile of

predominantly interleukins particularly that of IL-6 identified in the C1 and C3 clusters may account for the increased level of peripheral inflammation found in these clusters. This could also be due to the influence of the high frequencies of autoantibodies present in these patient clusters, as autoantibodies are documented to be strongly associated with worse RA disease (Mewar *et al.*, 2006, Agrawal *et al.*, 2007). Cigarette smoking may also in part explain this relationship as it also has been well documented to be strongly associated with worse disease severity (Saag *et al.*, 1997, Wolfe, 2000, Finckh *et al.*, 2007, Söderlin *et al.*, 2011), as these patient clusters also had high frequencies of patients who have ever and currently smoke.

Figure 4.1.3a: Comparison of baseline patient clusters and 5 year follow-up peripheral inflammation disease measures



4.1.3.1.2 Principal component analysis prognostic biomarker profiling

Analysis of PC biomarker profiles identified at baseline with measures of disease at 5 year follow-up revealed only a few associations of significance (Table 4.1.3b). PC profiles 1 and 2 consisting of IL-1β, IL-2, IL-4, IL-6, TNF-α, GM-CSF and IL-8, MMP -8 and -9 respectively were found to be associated with the ESR, WBC count and the number of platelets. This relationship with indices of peripheral inflammation was similarly shown in early RA and suggests that these mediators may also be important in inflammation in established disease.

Table 4.1.3b: Association between baseline PC biomarker profiles and 5 year follow-up disease measures

Disease Measure	Variable	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	Model p-value	Model r-squared
Inflammation	ESR >21 (+/-)	0.05			0.06			0.001	0.14
	WBC		0.0002					0.00032	0.14
	Platelets (+/-)	0.03						0.01	0.06
Damage	OSRA-D		0.03	0.0001	0.03(-)			<0.0001	0.23
Function	HAQ >1.5 (+/-)			0.02	0.10(-)			0.007	0.10

N.B Non-parametric variables were dichotomised using the median value as the cut-off level, (-) negative association, NS=Not Significant.

Analyses also revealed PC profiles 2, 3 and 4 were associated with both the OSRA-D and the HAQ, with PC 4 demonstrating an inverse relationship. The latter association suggests a possible protective effect of the PC4 profile (IL-1β, G-CSF and GM-CSF) in RA, which has also been demonstrated in a number of studies (Hartung *et al.*, 1995, Hartung *et al.*, 1997, Boneberg *et al.*, 1999, Fadok *et al.*, 2001, Boneberg and Hartung, 2002, Jinushi *et*

al., 2007, Martins *et al.*, 2011). Although these mediators have been associated with exacerbation of RA (Eastgate *et al.*, 1988, Xu *et al.*, 1989, Fiehn *et al.*, 1992), an animal study revealed that exogenous G-CSF prevented adjuvant-arthritis in rats (Brendolan *et al.*, 2003), where it can switch immune activity to a Th2 humoral response and promote immunological tolerance (Rutella *et al.*, 2005). Thus further investigation is required on the pleiotropic activity of these growth factors in RA and their ability to potentially reduce joint damage.

Further investigation of mediators from profiles PC 2, 3 and 4 as separate independent variables were performed to determine which were most strongly associated with joint damage and function at 5 year follow-up. An independent profile of elevated MMP-8 ($p=0.03$) and reduced MMP-2 ($p=0.003(-)$) expression were found to be most strongly associated with the OSRA-D at 5 year follow-up (model $p=0.0004$, model r-squared=0.15). Whereas, MMP-3 was the only mediator that was independently associated with the HAQ ($p=0.02$). MMP-3 and MMP-8 are known contributors to RA pathology and joint damage as they are ECM degrading enzymes with multiple pro-inflammatory activities (Yamanaka *et al.*, 2000, Green *et al.*, 2003), whereas MMP-2 although an ECM degrading enzyme has been shown to have pleiotropic functions with anti-inflammatory roles (McQuibban *et al.*, 2000, McQuibban *et al.*, 2002). This could partly explain the worse joint damage and functional outcome in established stage of disease as found in patients at 5 year follow-up. However ROC analysis of this profile demonstrated that these biomarkers were poor (AUC <0.6) at predicting a worse outcome at 5 year follow-up for both erosive disease and worse disease activity characterised by a severe DAS44 (>3.7 score) phenotype or a high Stoke Index phenotype (score 12-17). This biomarker profile therefore would not be useful as a prognosis marker for established disease.

4.1.3.2 Prognostic biomarker profiling for disease remission

Disease activity remission is a favourable aim to be achieved in RA patients, although a "low" disease activity phenotype (DAS44 >1.6-2.4 score) is also a desirable status for patients to achieve. The association of baseline biomarker profiles with better disease activity at 5 year follow-up was investigated to identify any biomarker profiles that could potentially predict better RA disease. The minimal disease phenotype of the Stoke Index (score 1-3) was also investigated, as it provides another indication of favourable outcome in RA. Baseline patient clusters C1 and C2 were found to have the highest frequency of disease activity favourable outcomes compared to the other clusters, however chi-square analyses revealed no significant differences between the clusters ($p=\geq 0.58$). This could be due to the small numbers of remission and low disease phenotype patients within each cluster (data not shown).

No baseline PC profiles were found to be associated with remission, low or minimal disease activity phenotypes of the DAS44CRP, DAS44ESR or Stoke Index at 5 year follow-up. Alternatively, the 17 mediators discerned as important biomarkers from the exploratory principal component analysis prior to their reduction into smaller components were used for analysis as independent variables. Logistic regression analyses revealed only a few baseline mediators from this 17 mediator selection were significantly associated with that of a favourable outcome at 5 year follow-up. In particular MMP-2 was shown to be positively associated with a minimal phenotype of the Stoke Index (p=0.04, model p=0.03, model r-squared=0.05) as well as the low phenotype of the DAS44ESR (p=0.05, model p=0.004, model r-squared=0.11). This was independently associated except after adjusting for DMARD treatment, where MMP-2 lost significance with the low DAS44ESR

phenotype. This suggests that MMP-2 may be elevated in response to DMARD treatment, especially as the majority of RA patients were receiving DMARD treatment (91%) at the time of follow-up. In addition, IL-2 an anti-inflammatory and regulatory cytokine was also significantly associated with a low DAS44ESR phenotype (p=0.02). No biomarker profile was found to be associated with a low phenotype of the DAS44CRP or the remission status of this disease activity measure. Discriminant regression however revealed a biomarker profile of IL-8 alone to be independently associated with remission status of the DAS44ESR (p=0.03, model p=0.03). This suggests that this chemotactic mediator may have a possible anti-inflammatory role in more established RA, as found in some studies where it inhibits the adhesion of leukocytes, especially neutrophils, to activated endothelial cells and thus possess immunosuppressive activities (Wheeler *et al.*, 1988, Gimbrone *et al.*, 1989, Hechtman *et al.*, 1991, Nourshargh *et al.*, 1992). Further investigation is warranted to determine the true relationship of IL-8 with more favourable outcomes, if it indeed has anti-inflammatory properties in addition to its commonly accepted pro-inflammatory role (Vjeroslava Slavić1 *et al.*, 2005). ROC analysis demonstrated that this small profile of MMP-2, IL-2 and IL-8 "poorly" (AUC 0.67) predicted a favourable outcome as characterised by either a minimal Stoke Index (score 1-3), low (>1.6-2.4 score) or remission (score $\leq$1.6) phenotype of the DAS44 at 5 year follow-up. This biomarker profile therefore would not be useful as a prognosis marker for established disease.

4.1.3.3 Summary

These analyses could not identify any biomarkers that would be effective in predicting outcome in established disease, however a profile was found that may be important in the development and progression of RA disease. Hierarchical clustering and PCA biomarker profiling both identified similar baseline mediators of elevated interleukins, a chemoattractant, a cell growth factor and pro-inflammatory cytokines plus and MMPs which were associated with inflammation within the peripheral circulation in established disease. This type of relationship was similarly demonstrated at both early and established stages of disease and therefore could potentially become an inflammatory marker for use in long term targeting for a lower disease activity over a sustained period of time.

4.1.4 ESTABLISHED RA

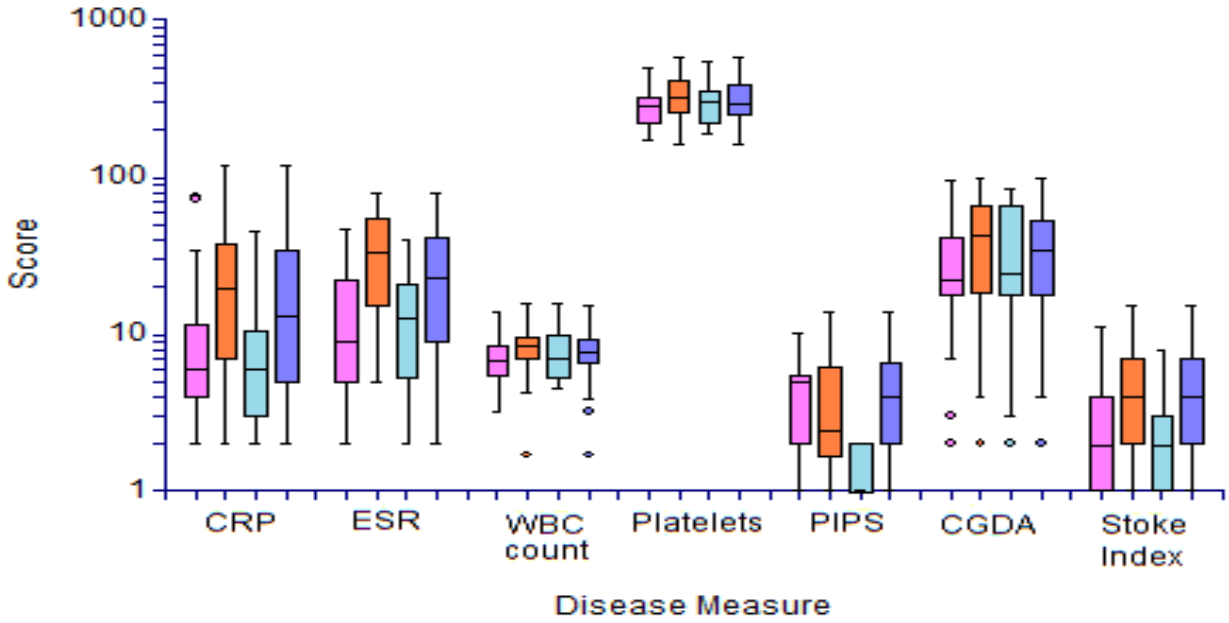
RA patients with established disease (disease duration of >5 years) were investigated with respective 5 year clinical, laboratory and patient characteristic data. The same cohort of patients from the early RA investigation was used. A total of 77 patients out of the original 86 at baseline were investigated, as the sera from 9 patients were not available at 5 year follow-up.

4.1.4.1.1 Relationship of RA disease features and patient characteristics with disease activity and severity in established RA

Initial analyses of 5 year follow-up disease activity and severity measures with RA patient characteristics and clinical features were performed for general assessment of the patients investigated in this cohort. Autoantibodies have been well documented to be associated with worse disease in RA. Comparisons between positive and negative patients for both RF and anti-CCP with measures of disease activity and severity were performed. Analyses revealed RF and anti-CCP positive patients both had significantly higher CRP (p=0.0004, p=0.01), ESR (p=0.0005, p=0.02) and Stoke Index (p=0.003, p=0.04) respectively than negative patients. The PIPS was also shown to be significantly higher in patients positive for anti-CCP (1 (0-4.75) v 0 (0-1), p=0.02), whereas patients with RF had a significantly higher WBC count (p=0.01), number of platelets (p=0.008) and CGDA score (p=0.02) (Figure 4.1.4a). Furthermore, an increasing trend with the additional presence of autoantibodies of none, one or both in the CRP (p-trend=<0.0001), ESR (p-trend=<0.0001) and the Stoke Index (p-trend=0.001) was also demonstrated, illustrating a cumulative effect on disease activity and severity. These findings are in line with previous studies that

autoantibodies are associated with more severe RA (Másdóttir *et al.*, 2000, Agrawal *et al.*, 2007).

Figure 4.1.4a: Association of autoantibodies with disease activity and severity at 5 year follow-up



Pink=RF negative, Orange=RF positive, Light blue=anti-CCP negative, Dark blue=anti-CCP positive

4.1.4.1.2 Influence of cigarette smoking

Cigarette smoking has been well documented to be associated with worse RA disease, which was similarly demonstrated in this established RA patient cohort (Saag *et al.*, 1997, Wolfe, 2000, Söderlin *et al.*, 2011). Analyses revealed the majority of disease measures were significantly higher in patients who had ever smoked than in never smokers (Table 4.1.4b). The frequency of patients who had ever and never smoked did not alter by the time of 5 year follow-up, however two patients who had previously smoked became current smokers, while 6 patients became past smokers by having ceased smoking for more than 6

months. All other patients' smoking status remained the same. Comparison of disease measures by smoking status revealed that past smokers had a significantly higher CRP, VAS, HAQ, OSRA-A, OSRA-D and overall Stoke Index and DAS44ESR disease activity (Table 4.1.4b). As patient smoking status did not alter greatly by the time of 5 year follow-up, this relationship of worse disease in past smokers could not be accounted for by the frequency or duration of cigarettes smoked (pack year history), nor by their age, gender or disease duration.

Table 4.1.4b: Association of cigarette smoking with disease activity and severity at 5 year follow-up

Disease Measure	Variable	Never smoked (n=26)	Past smokers (n=34)	Current smokers (n=17)	p-value†	Ever smoked (n=51)	p-value††
Inflammation	CRP	4.5 (3-14)	12 (6-35.5)	10 (5.5-3)	0.03	11.5 (6-34.5)	0.008
	WBC	6.6 (5.2-8.5)	8.3 (6.5-9.5)	8.2 (7-9.4)	0.14	8.3 (6.7-9.5)	0.05
	OSRA-A	2.00 ±1.97	3.41 ±2.14	3.11 ±2.47	0.04	3 (1-5)	0.01
	SI	2 (1-5)	4 (2-7)	3 (2-6)	0.04	3 (2-7)	0.01
	DAS44CRP	2.30 ±0.97	2.93 ±1.09	2.74 ±0.99	0.07	2.86 ±1.05	0.03
	DAS44ESR	2.35 ±1.00	3.03 ±1.04	2.88 ±1.04	0.04	2.98 ±1.03	0.01
Pain	VAS	24 (2.7-53.5)	51 (33.7-67.7)	40 (17.5-80)	0.002	50 (32-73)	0.001
	TJ	3 (0.7-7.2)	6.5 (2-11.2)	7 (2-11.5)	0.07	7 (2-11)	0.02
	RI	3 (0.7-10.2)	8.5 (3-15.7)	9 (2-16)	0.09	9 (2-15)	0.03
Damage	OSRA-D	1 (0-3)	2 (1-3.2)	2 (0.5-3.5)	0.05	2 (1-3)	0.01
Function	HAQ	0.87 ±0.85	1.74 ±0.81	1.47 ±0.83	0.0006	1.65 ±0.82	0.0002

The Mean ±SD for parametric data, the median (lower and upper interquartile range (IQR)) for non-parametric data. † One-way ANOVA or Kruskal-Wallis test as appropriate. †† Two-sample T-test/Aspin Welch or Mann-Whitney U/Kolmogorov test as appropriate for equal/unequal variance.

4.1.4.1.3 Influence of DMARD treatment

By the time of 5 year follow-up, the majority of RA patients were receiving DMARD treatment (91%) compared to the 14% of patients at baseline. A significant difference was found only in the PIPS between DMARD positive and negative patients. Patients receiving DMARD treatment demonstrated a significantly higher PIPS than patients negative for DMARD treatment (0 (0-0) v 0 (0-3), p=0.02). No other significant differences were found in any of the other disease measures.

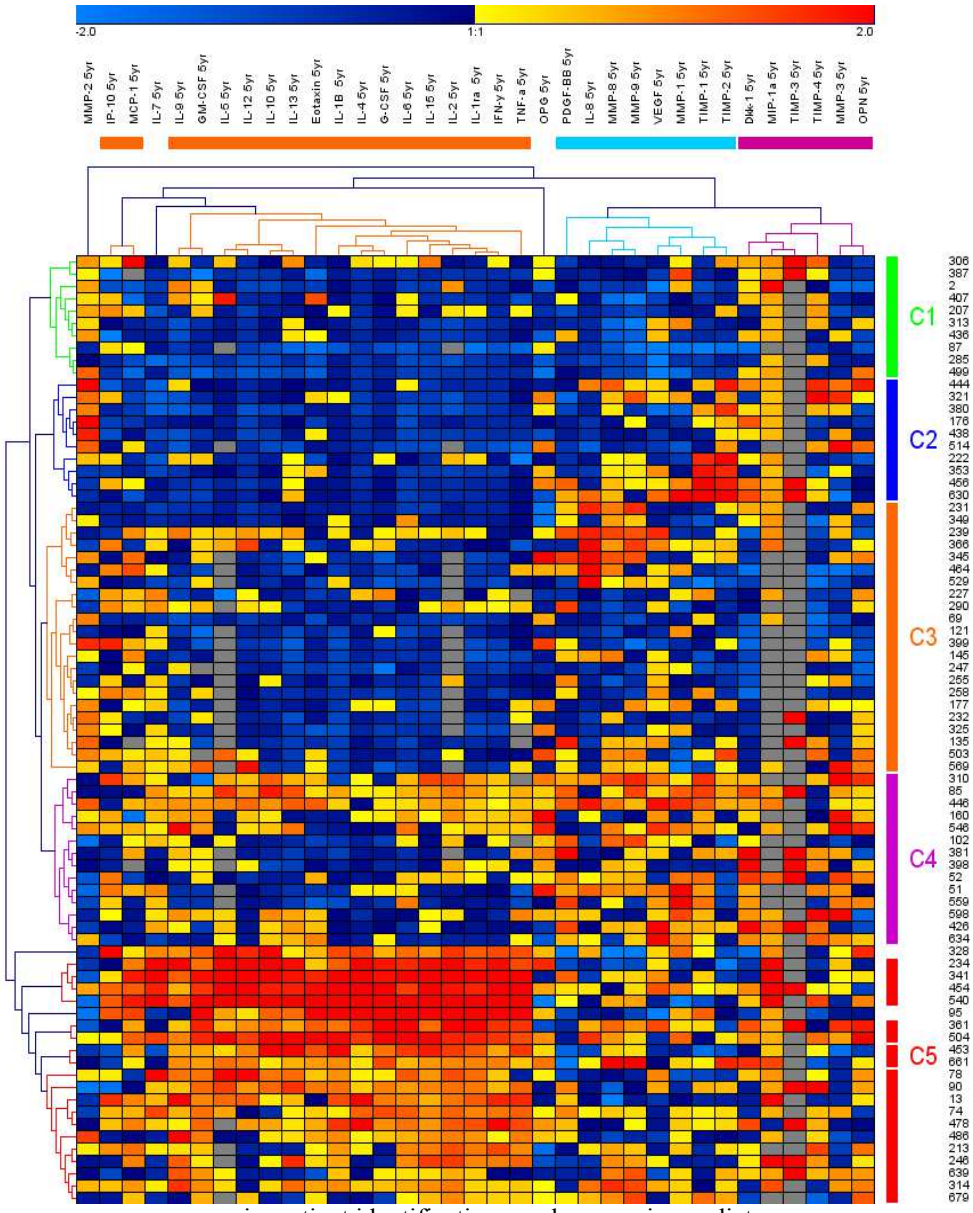
4.1.4.2 BIOMARKER PROFILING IN ESTABLISHED RA

A total of 35 mediators were investigated in this cohort of established RA patients. RANTES, IL-17, FGF-β, MIP-1β and MMP-12 were not included in these analyses as they were excluded at baseline. Endostatin was excluded from these analyses as it was not considered a mediator of high variability in principal component analysis at baseline, and thus was not investigated in 5 year follow-up patient serum.

4.1.4.2.1.1 Established RA Hierarchical clustering biomarker profiling

Hierarchical clustering analysis as described earlier (section 4.1.2.2.1.1) was similarly used and identified 5 distinct patient clusters denonted as C1, C2, C3, C4 and C5, further demonstrating that RA in an immunologically heterogenous disease and may have various pathological routes.

Figure 4.1.4c: Heat map of the relative mediator expression in established RA patients



y-axis=patient identification number, x-axis=mediators

Clusters C1, C2 and C3 displayed similar patterns of relatively low mediator expression. In contrast, cluster 5 displayed a relatively high mediator expression pattern particularly that of interleukins. It should be noted that the C5 patient cluster is not in the traditional sense a cluster as it separated into numerous smaller clusters. However, for the purposes of this investigation these were combined together due to their similar expression levels in comparison to the other patient clusters. The C4 cluster displayed a moderate expression

pattern of the investigated mediators. This heat map demonstrates the varying grades of immune activity and the possibility of immunological subtypes. The heat map showed the biggest differences between the high and low mediator expression clusters (C5 versus C1, C2 and C3) were that of the interleukins as well as a number of other specific mediators (IP-10, eotaxin, G-CSF, GM-CSF, MCP-1, IFN-γ, TNF-α and MMP-2). This was confirmed for the most part with statistical comparisons ($p=\leq 0.05$), although IP-10 was not found to be significantly different between these specific clusters (p=0.23). However, MIP-1α (14.8 (13-62.2) v 11.62 (0-14.04), p=0.002), OPN (18000 (13075-30825) v 12475 (8175-20100), p=0.008) and MMP-9 (590184.3 (433641.7-749302.3) v 465523.4 (332157.5-624322.9), p=0.04) were also found to be significantly higher in the C5 cluster. This 22 mediator profile could largely explain the difference between these potential immunological subtypes (Gruber *et al.*, 1996, Isomäki and Punnonen, 1997, Mohammed *et al.*, 2003, Stamenkovic, 2003, Lawlor *et al.*, 2004). Discriminant stepwise selection and forward logistic regression analysis of this profile revealed IL-6 alone (p=0.003) with contribution of IL-9 (p=0.08) in particular distinguished the high from the low mediator expression clusters (model p=<0.0001, model r-squared=0.52).

4.1.4.2.1.2 Association with established RA disease activity and severity

Chi-square analysis significantly demonstrated that patient cluster C5 had the highest frequencies of patients positive for RF followed by cluster C4, whereas clusters C1 and C3 displayed the lowest frequency of RF positive patients (Table 4.1.4d). The C2 cluster showed half RF positive and RF negative patients. No significant difference was found in the distribution of anti-CCP between patient clusters, although similar to that of RF seropositivity, clusters C5 and C4 had the highest frequencies of anti-CCP positive patients

(Table 4.1.4d). This suggests that the 22 interleukin-prominent mediator profile expressed in the high mediator expression cluster, and particularly pro-inflammatory Th2 humoral mediators IL-6 and IL-9, may be important in regard to the production of autoantibodies in established disease (Janeway Jr *et al.*, 2005).

Table 4.1.4d: Association between patient clusters and autoantibodies

Patient Cluster	RF negative n (%)	RF positive n (%)	Anti-CCP negative n (%)	Anti-CCP positive n (%)
C1	8 (80%)	2 (20%)	4 (40%)	6 (60%)
C2	5 (50%)	5 (50%)	3 (30%)	7 (70%)
C3	15 (68%)	7 (32%)	8 (36.4%)	14 (63.6%)
C4	5 (35.7%)	9 (64.3%)	3 (21.4%)	11 (78.6%)
C5	4 (19%)	17 (81%)	3 (14.3%)	18 (85.7%)
	χ^2=15.6 (df=4), p=0.003		χ^2=3.79 (df=4), p=0.43	

Chi-square analysis revealed no significant difference in the distribution of synovial effusions between patient clusters in established disease (p=0.37). Analysis of erosive disease revealed however a near significant difference between patient clusters (p=0.06) (Table 4.1.4e). The C5 patient cluster had the highest frequencies of patients with erosive disease. This suggests that the 22 mediator profile identified in the high C5 mediator expression cluster may also be important in regard to erosions in established disease (Gruber *et al.*, 1996, Isomäki and Punnonen, 1997, Mohammed *et al.*, 2003, Stamenkovic, 2003, Lawlor *et al.*, 2004). This profile consists of interleukins, eosonophil and monocyte chemoattractants, granulocyte/macrophage growth factors, matrix remodelling mediators and pro-inflammatory mediators and may explain for the high frequency of patients with erosive disease in this cluster (Xu *et al.*, 1989, Koch *et al.*, 1992, Seki *et al.*, 1995, Garcia-Zepeda *et al.*, 1996, Gruber *et al.*, 1996, McQuibban *et al.*, 2000, McQuibban *et al.*, 2002, Lawlor *et al.*, 2004, Janeway Jr *et al.*, 2005, Xu *et al.*, 2005, Nishimoto, 2006, Li and Rostami, 2010). Interestingly, clusters C1, C3 and C4 demonstrated relatively similar high

frequencies of patients positive for erosions (62-69%), whereas the C2 cluster had the lowest frequency of erosive patients (30%), despite clusters C1 and C3 having a similar low mediator expression pattern to the C2 cluster. Subsequent comparisons between these clusters revealed Dkk-1 (4752+2798.2 v 2238.43+1260.3, p=0.02), TIMP-1 (246897.6±100191.4 v 156825±28221.45, p=0.02) and TIMP-2 (166288.3+30961.1 v 121143+13473.44, p=0.001) to be significantly higher in the C2 cluster in comparison to clusters C1 and C3, and distinct from the C5 cluster and may be important in protecting bones from erosions. This profile incorporates inhibitors of ECM tissue degrading enzymes which regulate bone destruction and could account for the lower frequency of patients with erosive disease in this cluster (Brew *et al.*, 2000, Mannello and Gazzanelli, 2001, Baker *et al.*, 2002, Clark *et al.*, 2008, Murphy *et al.*, 2009).

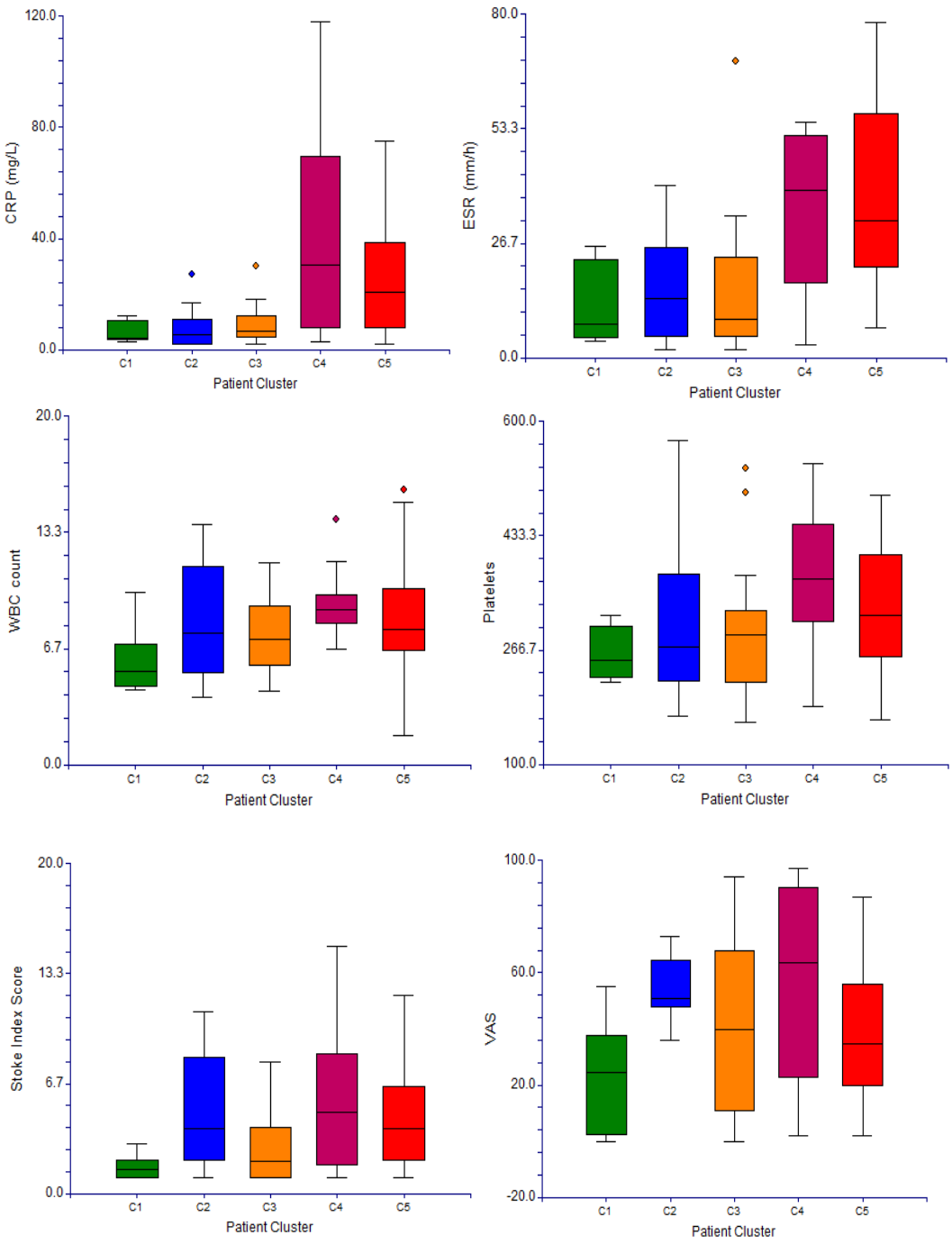
Table 4.1.4e: Association between patient biomarker clusters and presence of erosions

Patient Cluster	Erosive negative n (%)	Erosive positive n (%)
C1	3 (37.5%)	5 (62.5%)
C2	7 (70%)	3 (30%)
C3	6 (31.6%)	13 (68.4%)
C4	4 (31%)	9 (69%)
C5	3 (16%)	16 (84%)

χ^2=8.80 (df=4), p=0.06

Comparison of disease activity and severity measures revealed that the VAS (p=0.03), the Stoke Index (p=0.006) and peripheral inflammation measures (CRP p=0.0001, ESR p=0.0002, WBC count p=0.04, platelets p=0.01) were significantly different between patient clusters (Figure 4.1.4f). No other measures of disease were shown to be significantly different. Differences were mainly between patients of the C4 and the C1 cluster, with cluster C4 demonstrating the highest scores.

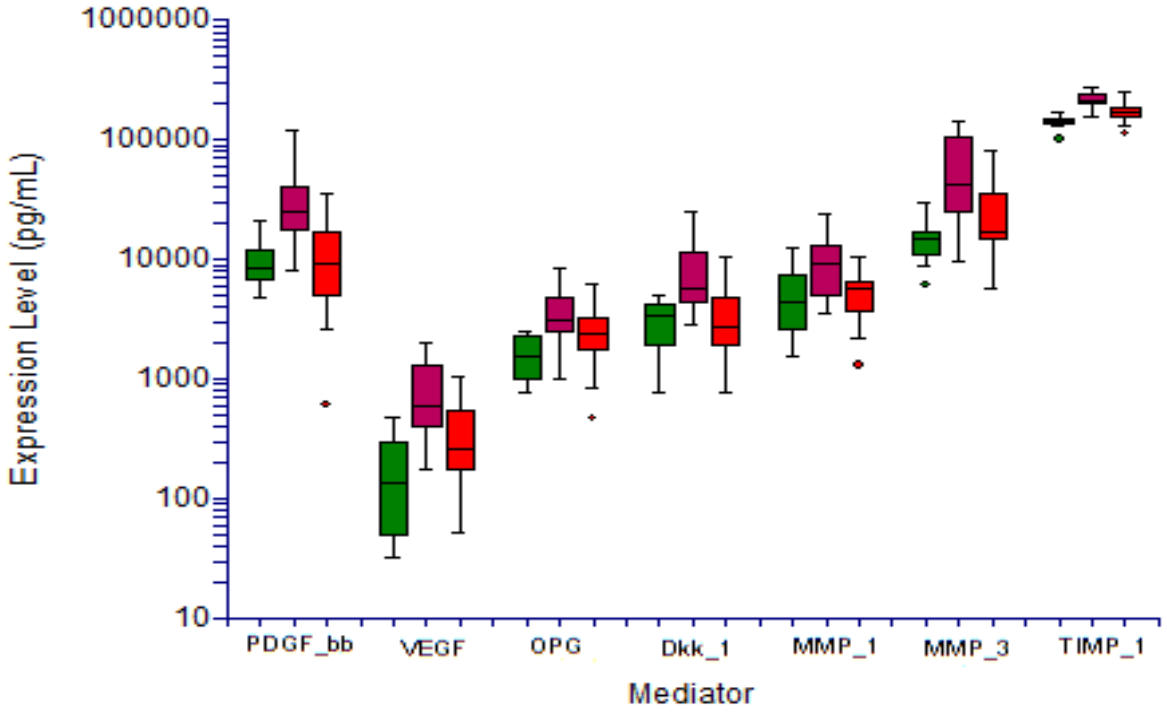
Figure 4.1.4f: Comparison between patient clusters with disease measures in established RA



Although cluster C4 demonstrated the highest scores of disease activity and severity, it displayed a moderate mediator expression pattern in comparison to the C5 cluster. Thus mediator comparisons against the C4 cluster were performed to determine which were

uniquely raised in this cluster. Analyses revealed a profile of 7 mediators in the C4 cluster which were significantly different from the C5 and C1 clusters. These were PDGF-bb (p=0.0006, p=0.0006), VEGF (p=0.01, p=0.002), OPG (p=0.04, p=0.005), Dkk-1 (p=0.003, p=0.001), MMP-1 (p=0.02, p=0.04), MMP-3 (p=0.01, p=0.001) and TIMP-1 (p=0.0006, p=<0.0001) (compared with C5 and C1 respectively, Figure 4.1.4g). This profile consisting of angiogenesis and cell growth factors plus matrix remodelling mediators including ECM degrading enzymes therefore could largely account for the worse disease activity and severity found in the C4 cluster (Paleolog, 2002, Alvarez *et al.*, 2006, Kuryliszyn-Moskal *et al.*, 2006, Yoo *et al.*, 2008). Interestingly, OPG a mediator responsible for bone growth and mineral deposition plus TIMP-1 a regulator for ECM degrading enzymes were also found to be significantly raised in cluster C4. These regulatory mediators may be upregulated as a compensatory response to the greater level of disease activity and inflammation.

Figure 4.1.4g: Comparison of 7 biomarker profile between C1, C4 and C5 patient clusters



Green=C1 cluster, Purple=C4 cluster, Red=C5 cluster

The Stoke Index score was found to be significantly different between patients clusters, however clusters demonstrated a score of no more than a median of 5 indicating that each patient cluster were of a mild or minimal overall disease activity phenotype. Chi-square analysis of Stoke Index phenotype categories showed a significant difference in distribution between patient clusters and demonstrated that patients of the C1 and C3 clusters had the highest frequencies of a minimal phenotype followed by the C2 cluster (Table 4.1.4h). This suggests that low expression of the 22 mediator profile identified in the high mediator expression cluster may also be important in regard to less overall disease activity and severity in established disease (Gruber *et al.*, 1996, Isomäki and Punnonen, 1997, Mohammed *et al.*, 2003, Stamenkovic, 2003, Lawlor *et al.*, 2004). No significant difference in the distribution of DAS44CRP (p=0.88) or the DAS44ESR (p=0.33) disease activity phenotypes was found, nor was there any difference in remission status.

Table 4.1.4h: Association of stoke index phenotypes with patient clusters

5 Year Follow-up	Stoke Index Phenotype			
Patient Biomarker Clusters	Minimal n (%)	Mild n (%)	Moderate n (%)	Severe n (%)
C1	10 (100%)	0 (0%)	0 (0%)	0 (0%)
C2	5 (50%)	2 (20%)	3 (30%)	0 (0%)
C3	16 (72.7%)	5 (22.7%)	1 (4.5%)	0 (0%)
C4	4 (28.6%)	6 (42.9%)	3 (21.4%)	1 (7.1%)
C5	8 (38.1%)	9 (42.9%)	3 (14.3%)	1 (4.8%)

χ^2=21.71 (df=12), p=0.04

4.1.4.2.1.3 Association with cigarette smoking

Cigarette smoking has been well documented to be associated with severe RA and worse disease outcome. Chi-square analysis revealed the distribution of smokers was significantly different between patient clusters, where cluster C5 was found to have the highest frequencies of smokers (Table 4.1.4i). This suggests that the elevated 22 mediator profile identified in this high mediator expression cluster may be upregulated in smokers. Interestingly, the C2 cluster demonstrated a similar frequency of smokers, despite this cluster displaying a relatively low mediator expression pattern. This suggests that raised expression of Dkk-1, TIMP-1 and TIMP-2 identified in this C2 cluster may also be important in regard to smoking in this set of patients. Furthermore, cigarette smoke has been documented to have immunosuppressive properties which may partly explain the pattern of low mediator expression in this cluster (McCue *et al.*, 2000, Ouyang *et al.*, 2000, Oltmanns *et al.*, 2005, Lee *et al.*, 2007).

Table 4.1.4i: Association between patient clusters and smoking

Patient Cluster	Never smoked n (%)	Ever smoked n (%)
C1	7 (70%)	3 (30%)
C2	2 (20%)	8 (80%)
C3	9 (41%)	13 (59%)
C4	5 (35.7%)	9 (64.3%)
C5	3 (14.3%)	18 (85.7%)

χ^2=10.80 (df=4), p=0.03

The smoking status of patients was also found to be significantly different between patient clusters in established RA (p=0.003), where cluster C5 demonstrated the highest frequency of current smokers (Table 4.1.4j). This suggests that recent exposure to cigarette smoke may upregulate expression of the 22 mediator profile. The C2 cluster however was found

to have the highest frequency of past smokers which may partly account for the relatively low mediator expression pattern displayed by this cluster. Furthermore, cigarette smoke has been documented to have immunosuppressive properties (McCue *et al.*, 2000, Ouyang *et al.*, 2000, Oltmanns *et al.*, 2005, Lee *et al.*, 2007), patients who no longer smoke therefore may express mediators without any inhibition. This may account for the raised Dkk-1 and TIMPs identified in this cluster of past smokers. TIMPs are natural MMP inhibitors and thus have roles in regulating inflammation which could also contribute to the low expression of mediators in this cluster (Brew *et al.*, 2000, Mannello and Gazzanelli, 2001, Baker *et al.*, 2002, Clark *et al.*, 2008, Murphy *et al.*, 2009).

Table 4.1.4j: Association of 5 year smoking status with patient clusters

	Smoking Status		
Patient Cluster	Non-smoker n (%)	Past smoker n (%)	Current smoker n (%)
C1	7 (70%)	2 (20%)	1 (10%)
C2	2 (20%)	7 (70%)	1 (10%)
C3	9 (41%)	11 (50%)	2 (9%)
C4	5 (35.7%)	7 (50%)	2 (14.3%)
C5	3 (14.3%)	7 (33.3%)	11 (52.4%)

χ^2=23.0 (df=8), p=0.003

Chi-square analysis did not reveal a significant association between patient clusters and pack year stratification (p=0.10), indicating that the difference between C2 and C5 patients was not due to intensity and length of time patients smoked.

Chi-square analysis between patient clusters and those receiving DMARD treatment at time of 5 year follow-up also did not demonstrate a significant difference in distribution between the patients groups (p=0.40).

4.1.4.2.1.4 Established RA Hierarchical clustering results summary

These results indicate that a mediator profile of multiple interleukins and various other mediators may be upregulated in response to smoking, especially after recent exposure. This upregulation of various mediators therefore could contribute to the increase of autoantibody production and bone erosion found in RA smokers as well as influence the level of disease activity and severity. This particular mediator profile-disease activity and severity relationship may therefore account for the relatively low expression of mediators in non-smokers and thus explain the better level of RA disease found in patients of the C1 cluster. These results also illustrate that smoking may suppress or promote expression of certain mediators, and that cessation of smoking may produce a different mediator expression profile compared with that of current smokers, which in turn may influence disease severity in a unique manner.

4.1.4.2.2.1 Established RA Principal Component Analysis biomarker profiling

As at baseline, an exploratory PCA of all 36 mediators at 5 year follow-up was initially performed. This selected a specific set of 17 mediators which provided the maximum variance and were incorporated into the principal component analysis (Table 4.1.4k).

Table 4.1.4k: Mediators selected for Principal Component Analysis

Five Year Follow-up PCA Biomarkers				
IL-1ra	TNF-α	VEGF	MMP-1	MMP-9
IL-2	G-MCSF	OPN	MMP-2	
IL-6	IP-10	OPG	MMP-3	
IL-8	PDGF-ββ	Dkk-1	MMP-8	

Principal component analysis identified 5 PC biomarker profiles generated from the correlation patterns of the 17 mediators selected which were then used for analysis. Table 4.1.4l displays the 5 PC generated biomarker profiles and their modified relative factor loadings after variamax rotation. Positive and negative factor loading relate to high and low expression level respectively.

Table 4.1.4l: PC profiles of 17 biomarkers and their relative factor loadings

PC 1		PC 2		PC 3		PC 4		PC 5	
IL-1ra	0.96	MMP-8	0.86	PDGF-bb	0.70	MMP-3	0.75	IP-10	-0.77
IL-2	0.94	MMP-9	0.82	MMP-1	0.67	OPG	0.60	Dkk-1	0.50
GM-CSF	0.91	IL-8	0.67	VEGF	0.62	OPN	0.51	OPG	-0.45
IL-6	0.90	OPN	0.41	Dkk-1	0.56	MMP-2	0.43		
TNF-α	0.87			MMP-2	-0.44				

- negative factor loading

4.1.4.2.2.2 Association with established RA disease activity and severity

Regression analyses revealed only a few significant associations of PC profiles with measures of disease (Table 4.1.4m). Predominantly, the PC 4 profile consisting of OPG, OPN, MMP-3 and MMP-2 expression was found to be independently associated with the greatest number of disease activity and severity measures, especially inflammatory indices, the VAS for pain and various joint measures including the number of swollen joints and the OSRA-D. These mediators are well known for ECM remodelling and have roles in inflammation which would account for the association with systemic inflammation and for patients experiencing worse pain (Xu *et al.*, 2005, Ateş *et al.*, 2007, Bazzichi *et al.*, 2009, Gaoya *et al.*, 2009). In contrast, OPG has been shown to have roles in bone growth, mineral deposition and immune regulation and could be elevated as a response to compensate for worse disease severity (Lacey *et al.*, 1998, Feige, 2001, Schoppet *et al.*, 2002). However, after adjusting for confounding factors disease duration, ever smoking

and DMARD treatment, PC 4 was no longer associated with the OSRA-D, suggesting that smokers present with worse severity, and thus have a higher need for DMARD treatment (Saag *et al.*, 1997, Másdóttir *et al.*, 2000, Wolfe, 2000, Mattey *et al.*, 2002c, Finckh *et al.*, 2007, Westhoff *et al.*, 2008).

Furthermore this PC 4 profile was also found to be associated with patients who have ever smoked, which suggests that smoking may promote expression of certain mediators such as upregulation of OPG, OPN, MMP-2 and MMP-3. In addition, cigarette smoke may also have an anti-inflammatory protective role in RA as OPG, a mediator responsible for bone growth, mineral deposition and immune regulation was also elevated. However this profile lost its significance when adjusted for DMARD treatment at 5 year follow-up. Intriguingly, erosions and synovial effusions were found to be negatively associated with PC 3 and PC 4 respectively. Further investigation is required to determine the true premise of this relationship.

Table 4.1.4m: Association between PC biomarker profiles with disease measures

Disease Measure	Variable	PC 1	PC 2	PC 3	PC 4	PC 5	Model p-value	Model r-squared
Inflammation	CRP (+/-)	0.04			0.02		0.0001	0.20
	ESR >21 (+/-)	0.009			0.06		<0.0001	0.24
	SJ >3 (+/-)				0.05		0.04	0.05
	WBC		0.002		0.0003	0.0006	<0.0001	0.31
	Platelets			<0.0001			<0.0001	0.21
	Anaemia (+/-)		0.03		0.001		0.0002	0.20
	Synovial effusion (+/-)				0.02(-)	0.02	0.005*	0.12
	PIPS >0 (+/-)	0.11					0.04*	0.05
	CGDA >32 (+/-)						NS	
	OSRA-A >3 (+/-)				0.01		0.005	0.09
	SI >3 (+/-)	0.03			0.002		<0.0001	0.26
	DAS44CRP >2.60 (+/-)						NS	
	DAS44ESR >2.69 (+/-)						NS	
Autoantibody	RF (+/-)						NS	
	Anti-CCP (+/-)	0.10					0.01	0.08
Pain	VAS >47 (+/-)				0.004		0.0008	0.13
	TJ >5 (+/-)						NS	
	RI >8 (+/-)						NS	
Damage	Erosions (+/-)			0.05(-)			0.04	0.06
	OSRA-D				0.03		0.03	0.05
	Larsen Hand score >28 (+/-)						NS	
Function	EMS >30 (+/-)		0.02				0.009	0.08
	HAQ >1.5 (+/-)						NS	
	Grip strength >107 (+/-)						NS	
Other	Ever Smoked (+/-)				0.05		0.03	0.05

N.B Non-parametric variables were dichotomised using the median value as the cut-off level, * Forward logistic regression only as discriminant stepwise selection unable to perform, (-) negative association, NS=Not Significant.

The Stoke Index for overall disease activity was also shown to be strongly associated with the PC 4 profile as well as the PC 1 profile which consists of granulocyte-macrophage growth factors and pro-inflammatory mediators (IL-1ra, IL-2, IL-6, GM-CSF and TNF-α) (Table 4.1.4m). Regression analyses were subsequently performed between PC profiles and the Stoke Index minimal disease phenotype (score 1-3). Analysis revealed profiles PC 1 and PC 4 to be negatively associated with minimal disease activity (Table 4.1.4n). Furthermore, analyses were subsequently performed between PC profiles and better activity phenotypes of both the DAS44 -CRP and -ESR measures (remission (<1.6) and low (1.6-2.4)) (Table 4.1.4n). The PC 4 profile was similarly revealed to be negatively associated with a low DAS44CRP. Reduced mediator expression levels in this profile therefore may account for a more favourable disease activity. Interestingly, the PC 1 profile demonstrated a positive association with a low DAS44ESR. This profile includes mediators which have anti-inflammatory activities including IL-1ra which is a well characterised inhibitor of the pro-inflammatory cytokine IL-1β, and IL-2 an immune response regulatory cytokine which could explain its association with less severe disease (Cutolo, 2004, Janeway Jr *et al.*, 2005). Furthermore, PC 3 alone was found to be associated with remission status of the DAS44ESR. This profile predominantly consists of pro-inflammatory mediators and ECM degrading enzymes, plus MMP-2 which was negatively loaded within this profile and thus did not explain for being affiliated with remission in RA patients (McQuibban *et al.*, 2000, McQuibban *et al.*, 2002).

Table 4.1.4n: Association between PC profiles with low/minimal/remission overall disease activity phenotypes

Disease Activity Phenotype	PC 1	PC 2	PC 3	PC 4	PC 5	Model p-value	Model r-squared
Stoke Index Minimal (+/-)	0.03(-)			0.002(-)		<0.0001	0.26
DAS44CRP Low (+/-)				0.06(-)		0.04	0.05
DAS44CRP Remission (+/-)						NS	
DAS44ESR Low (+/-)	0.02					0.003	0.10
DAS44ESR Remission (+/-)			0.04			0.04	0.05

(-) negative association, NS=Not significant

4.1.4.2.2.3 Established RA Principal Component Analysis biomarker profiling results summary

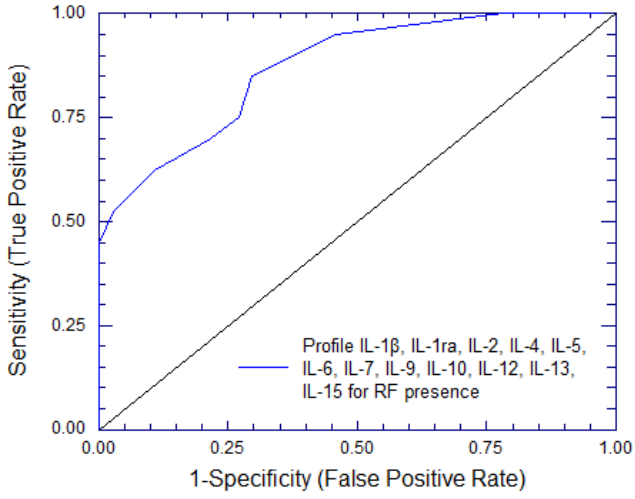
These results suggest that cigarette smoking may upregulate OPN, MMP-2 and MMP-3 expression, which could explain why these mediators were also shown to be associated with worse disease activity and severity measures in established RA. A low expression of these mediators plus elevated expression of anti-inflammatory activity IL-1ra, IL-2 and OPG would therefore be associated with better RA disease. This was demonstrated by the negative association of this mediator profile with favourable disease activity phenotypes (minimal/low) of the Stoke Index and DAS44CRP scores.

4.1.4.3 Composite Biomarker Profiles

Hierarchical clustering alone identified a biomarker profile of elevated IL-6, IL-9 plus low expression of TIMP-1 and TIMP-2 was derived which was likely to be associated with erosive disease. ROC analyses however of this profile revealed that both AUCs were "poor" at identifying erosive RA patients (AUC 0.61 and 0.60 respectively).

Principal component analysis could not identify a biomarker profile associated with RF or anti-CCP, however hierarchical clustering identified numerous interleukins and specific mediators (growth factors, chemoattractants and pro-inflammatory cytokines) which were associated with RF. Interleukin -6 and IL-9 were particularly shown to be associated with seropositivity, however ROC analysis revealed it to "poorly" identify RF positive patients (AUC 0.70). Consequently, ROC analysis of all investigated interleukins identified this profile to be "good" at accurately identifying patients positive for RF (AUC 0.87, 95% CI 0.81-0.95, SE 0.036, p=<0.0001) (Figure 4.1.4o) and indicates that multiple interleukins in collaboration are associated with RF production.

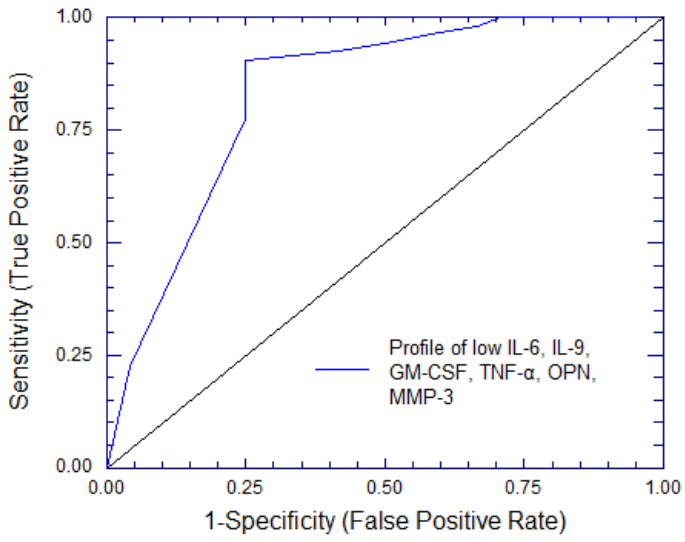
Figure 4.1.4o: Accuracy of RF identified biomarker profile



A biomarker profile incorporating PDGF-bb, VEGF, Dkk-1, MMP-1, MMP-3 and OPN which was likely to be associated with worse disease activity and severity was also derived from both the hierarchical clustering and principal component analysis. ROC analysis of this biomarker profile however showed it to "poorly" identify worse disease (defined by either a severe DAS44-CRP, -ESR (score >3.7) or high Stoke Index (score 12-17) phenotype) (AUC 0.67).

In contrast, a biomarker profile of low IL-6, IL-9, GM-CSF, TNF-α, OPN, MMP-3 expression which was likely to be associated with a better disease outcome was derived from both hierarchical clustering and principal component analysis. ROC analysis revealed this profile to be "good" at accurately identifying favourable disease activity (defined by either a minimal Stoke Index (score 1-3), low (>1.6-2.4 score) or remission (score ≤1.6) DAS44 phenotype (AUC 0.83, CI 95% 0.70-0.94, SE 0.05, p=<0.0001) (Figure 4.1.4p).

Figure 4.1.4p: Accuracy of disease activity and severity composited biomarker profile for favourable disease activity



4.1.4.4 Established RA Overall Summary

This cohort of RA patients, although considered to be at an established stage of disease, demonstrated biomarker profiles that were found to be associated with only a few measures of disease activity and severity. These were mainly markers of inflammation, rather than outcome disease measures although a few biomarker profiles of interest from both analysis methods were derived.

A biomarker profile of growth factors, chemoattractants, pro-inflammatory cytokines and especially numerous interleukins was associated with RF. This profile is in line with previous studies where multiple interleukins and the same specific mediators were strongly associated with autoantibody presence (Hitchon *et al.*, 2004, Alex *et al.*, 2007, Hueber *et al.*, 2007, Jørgensen *et al.*, 2008, Kokkonen *et al.*, 2010, Meyer *et al.*, 2010, Chandra *et al.*, 2011, Hodkinson *et al.*, 2011).

These results identified a biomarker profile of low IL-6, IL-9, GM-CSF, TNF-α, OPN, MMP-3 expression which was associated with a better level of disease activity. This profile is unique when compared with previous profiling studies, although individually these mediators have been previously described with pro-inflammatory roles in RA. Thus a reduction in their expression would likely be associated with reduced RA disease activity and severity, as well as potentially a new assessment measure for disease activity.

Cigarette smoking may upregulate IL-6, IL-9, OPG, OPN and MMP-3 which is consistent with previous profiling studies that smoking can alter expression of a variety of MMPs and cytokines and thus influence immune activity (Nakamuraa *et al.*, 1998, Knuutinen *et al.*,

2002, Nordskog *et al.*, 2003, Raitio *et al.*, 2005). This profile provides further insight into the mechanisms involved in the effects of cigarette smoking in RA.

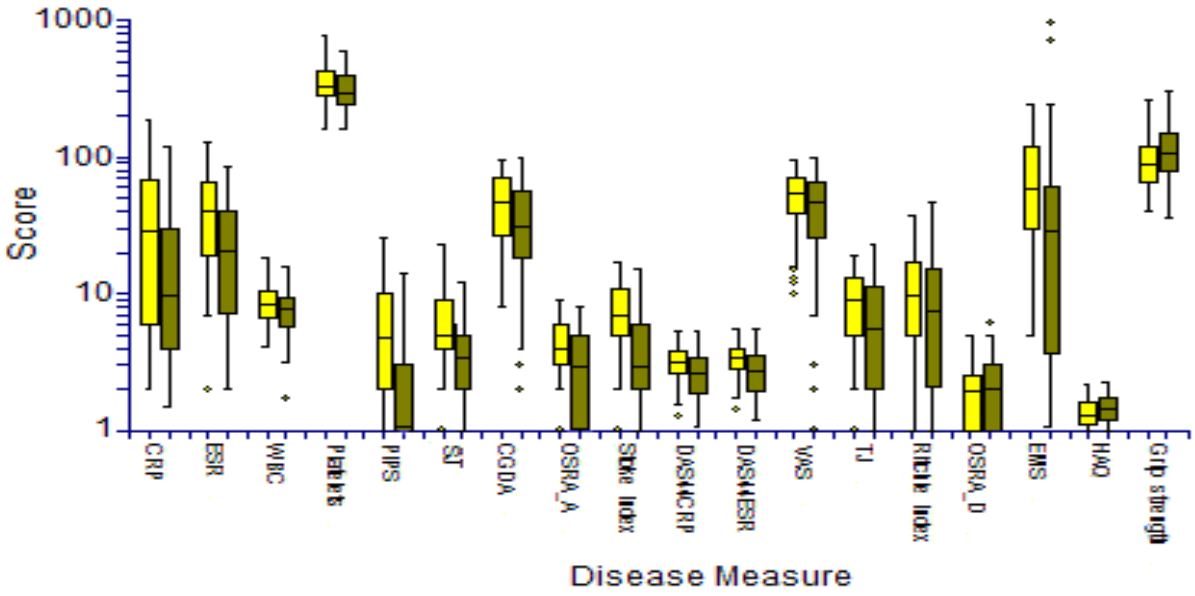
This study further demonstrates the importance of various interleukins, growth factors, matrix remodelling mediators and pro-inflammatory cytokines in the pathological process of established RA, and identifies mediators which are likely to be involved in molecular pathways of the disease process. Commonly accepted mediators of RA pathology (IL-β, IFN-γ and TNF-α) again did not provide any more information about disease activity in established disease than many of the other serum biomarkers. These findings further demonstrate the heterogeneity and the complex network of mediators involved in RA pathology and suggests that these markers of immune dysfunction could become novel targets for disease intervention.

4.1.5 BIOMARKER PROFILING RA DISEASE PROGRESSION

This study investigated biomarker profiling in both early and established RA in the same cohort of patients, which allowed the investigation of mediator expression changes with progression of the disease. Such investigations can determine whether any biomarker profiles may explain the change in activity and/or severity over the 5 year period and give possible insights into the disease process as the disease progresses. This type of investigation can further help elucidate the pathological process in RA and may give clues of the molecular pathways involved in disease progression.

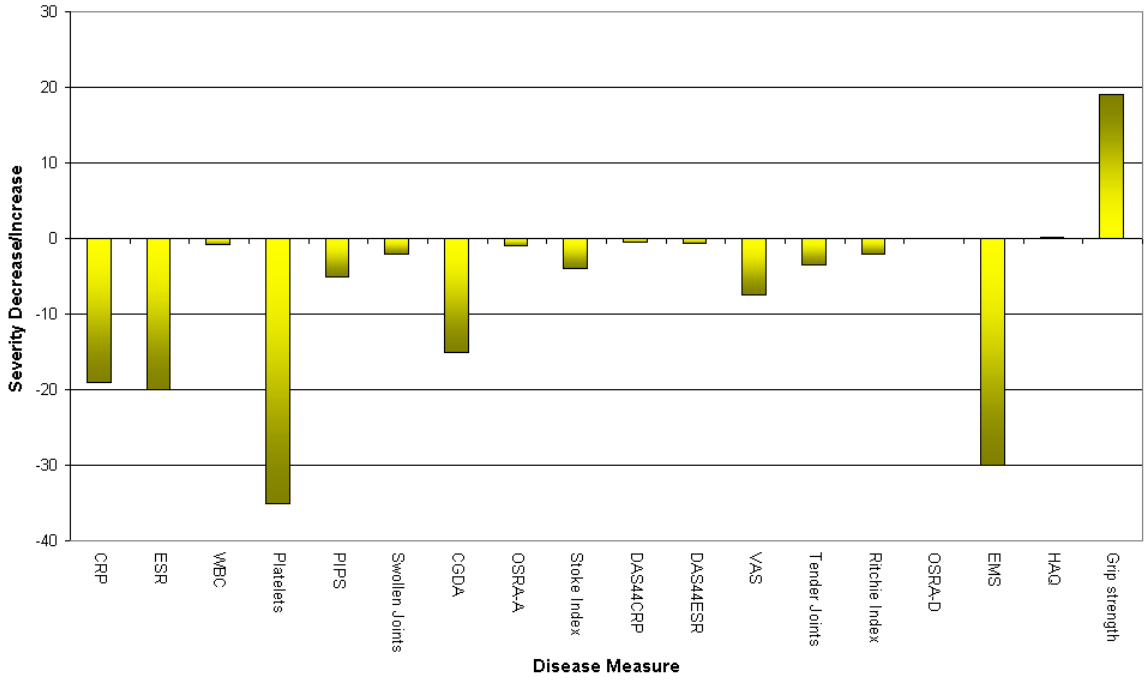
Comparison of disease measures between early and established RA revealed a significant decrease in the majority of indices by the time of 5 year follow-up ($p=\leq 0.05$) (Figure 4.1.5a and b). In particular, the level of disease activity was shown to be reduced whereas disease severity did not generally change during the 5 year period as no significant difference was found in the OSRA-D ($p=0.50$) or HAQ ($p=0.76$). However a significant improvement in function was shown in the EMS score and grip strength. The data demonstrate that these RA patients generally had a lower level of active disease at 5 year follow-up than when they first attended the clinic but only a slight or no improvement in severity measures.

Figure 4.1.5a: Comparison of disease activity and severity between early and established RA



Yellow=Early RA, Green=Established RA

Figure 4.15b: Change in disease activity and severity from early to established RA



The expression of mediators also changed between baseline and 5 year follow-up, where half of the mediators either increased or decreased during the 5 year period (Figure 4.1.5c). Half of these mediators were found to be significantly different between baseline and 5 year follow-up. In particular IL-4, IL-5, IL-6, IL-8, G-CSF, MCP-1, OPG and TIMP-3 were found to be significantly lower at 5 year follow-up ($p=\leq 0.03$), whereas IL-1β, IL-7, IL-10, IL-13, eotaxin, IP-10, MIP-1α, MMP-2 and TIMP-2 were shown to be significantly higher at 5 year follow-up ($p=\leq 0.01$) (Figure 4.1.5d).

Figure 4.1.5c: Changes in mediator expression from baseline to 5 year follow-up

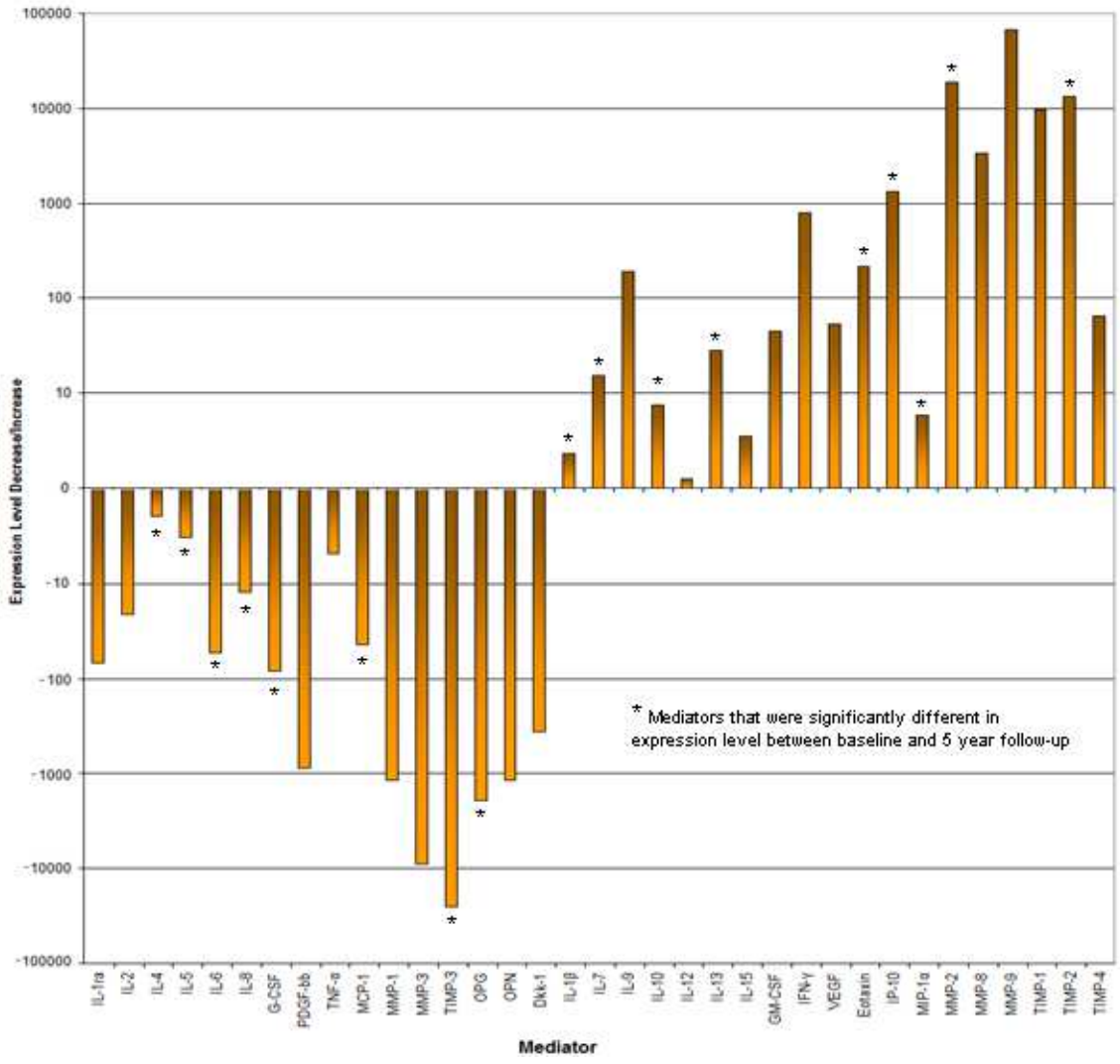
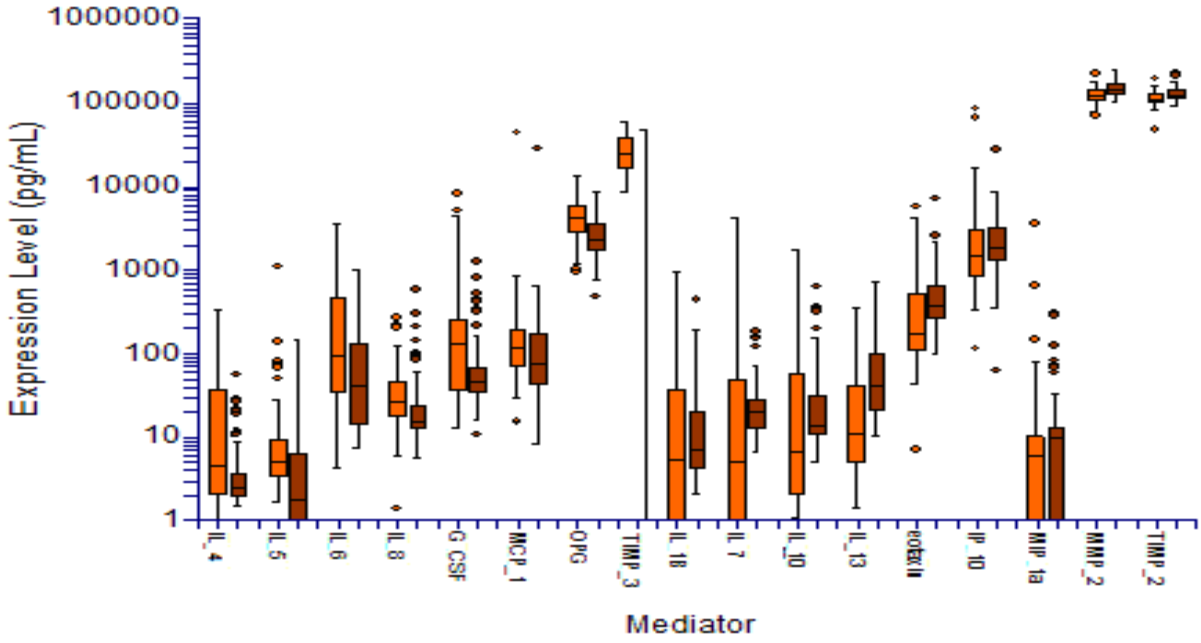


Figure 4.1.5d: Comparison of mediator expression between baseline and 5 year follow-up



Orange=Baseline, Brown=5 year follow-up

4.1.5.1 Association of mediator expression changes and changes in disease activity and severity

Changes in mediator expression and their relationship to disease activity and severity changes were analysed to determine their association with progression of RA. Various mediator expression changes were associated with changes in disease measures, in particular a decrease in IL-8, PDGF-bb, OPN, MMP-1 and MMP-3 were found to be the most associated with decreased disease activity as well as a decrease in EMS (Table 4.1.5e). PDGF-bb, OPN and MMP-1 were associated with the greatest number of disease measures, with MMP-1 predominantly associated with measures of peripheral inflammation whereas OPN was associated with measures of overall disease activity (CGDA, OSRA-A). PDGF-bb however was associated with a range of inflammatory

disease activity measures (WBC, PIPS, OSRA-A) as well as the VAS. In contrast, eotaxin and TIMP-4 were negatively associated with decreased disease activity (CGDA, OSRA-A, DAS44ESR, VAS, tender joints, Ritchie Index) indicating that elevated expression would result in an increase of overall disease activity, joint tenderness and pain. These mediators are known inflammatory chemoattractants, growth factors and matrix remodelling mediators respectively, so a reduction in their expression would likely be associated with a less active disease which may explain the lower level of disease activity displayed (Baggiolini and Clark-Lewis, 1992, Garcia-Zepeda *et al.*, 1996, Clark and Parker, 2003, Mohammed *et al.*, 2003, Stamenkovic, 2003, Vjeroslava Slavić1 *et al.*, 2005, Xu *et al.*, 2005, Alvarez *et al.*, 2006, Holmbeck and Szabova, 2006, Bazzichi *et al.*, 2009, Gaoya *et al.*, 2009). Interestingly TIMP-4 demonstrated an association with greater disease activity, although it is characterized as an inhibitor of ECM degrading enzymes (Brew *et al.*, 2000, Mannello and Gazzanelli, 2001, Clark *et al.*, 2008, Melendez-Zajgla *et al.*, 2008). This could be due to this mediator being upregulated as a means of regulating increased inflammatory activity. These relationships were largely independent of confounding factors, although reduced WBC counts was no longer associated with reduced OPN after adjusting for DMARD treatment (91% of patients positive).

Autoantibodies, erosions, the Larsen hand score and synovial effusions were recorded only once in this cohort, and therefore change over time was not recorded.

Table 4.1.5e: Association of mediator expression changes with disease measure changes

	Inflammation										Pain		Damage	Function	
Mediator	CRP >0	ESR	WBC	Platelets >0	PIPS >0	SJ	CGDA	OSRA -A	Stoke Index	DAS 44 ESR >0	VAS	TJ >0	RI >0	OSRA -D	EMS >0	HAQ >0
IL-1β																
IL-1ra			0.001(-)				0.02									
IL-2																
IL-4																
IL-5																
IL-6									0.004							
IL-7											0.01					
IL-8		0.05							0.05						0.04	
IL-9														0.01(-)		
IL-10			<0.0001 (-)						0.0004							
IL-12									0.003(-)							
IL-13																
IL-15							0.03									
Eotaxin			0.01				0.01(-)			0.04 (-)				0.008(-)		
G-CSF									0.0006 (-)							
GM-CSF														<0.0001		
IFN-γ			<0.0001													
IP-10						0.005										
MCP-1																
MIP-1α																0.15(-)
PDGF-bb			0.04		0.02			0.01			0.0005					
TNF-α			<0.0001 (-)													

Table 4.1.5e: Association of mediator expression changes with disease measure changes continued

Mediator	CRP >0	ESR	WBC	Platelets >0	PIPS >0	SJ	CGDA	OSRA -A	Stoke Index	DAS 44 ESR >0	VAS	TJ >0	RI >0	OSRA -D	EMS >0	HAQ >0
VEGF											0.01(-)					
MMP-1	0.002	<0.0001		0.0002					<0.0001							0.03
MMP-2	0.02(-)	0.01(-)														
MMP-3		0.004						0.004			0.0006					
MMP-8																
MMP-9		0.02(-)	0.0006													
OPG																
OPN			0.04				0.01	0.007	0.04(-)					0.03		
Dkk-1																
TIMP-1																
TIMP-2																
TIMP-3									0.02							
TIMP-4			0.02					0.03(-)			0.03(-)	0.01 (-)	0.03 (-)			
Model p-value	<0.0001	<0.0001	<0.0001	<0.0001	0.006	0.005	0.005	0.0001	<0.0001	0.02*	0.0005	0.007	0.02	0.0003	0.006	0.005*
Model r-squared	0.25	0.51	0.60	0.35	0.10	0.10	0.20	0.27	0.46	0.07	0.26	0.08	0.07	0.25	0.10	0.13

>0 an improvement, (-) negative association. * Forward logistic regression only as discriminant stepwise selection unable to perform. N.B DAS44CRP and Grip strength were not significant and thus not included in table.

4.1.5.2 Association of mediator changes with cigarette smoking and DMARD treatment

Regression analysis of mediator expression changes in patients who had ever smoked revealed a positive association with an increase in TIMP-2 level (p=0.01). In addition, a decrease in TIMP-3 level was found to be negatively associated with ever having smoked (p=0.005(-)). An increase in MMP-9 level was also found to be negatively associated with ever having smoked (p=0.052(-)), and this contributed to the overall model (model p=0.001, model r-squared=0.17). This indicates that cigarette smoking may upregulate the expression of TIMP-2 and TIMP-3 as well as possibly down-regulating MMP-9. This suggests that smoking may promote or suppress the expression of certain mediators which has been similarly demonstrated in previous studies (Knuutinen *et al.*, 2002, Nordskog *et al.*, 2003, Raitio *et al.*, 2005). In contrast to this study, another study found MMP-9 to be upregulated in smokers (Nakamuraa *et al.*, 1998). This provides some insight into the mechanisms involved in the effects of cigarette smoking in RA.

Little difference in the changes of mediator expression was shown in patients treated with or without DMARDs, although the small number of patients not receiving DMARDs had increased expression of IP-10 (1388.5 (423-3094.6) v 481 (-745.4-1334.115), p=0.05), TIMP-1 (33695 (22502.6-45149.4) v 8943.96 (-14897.4-34908.3), p=0.04) and TIMP-2 (30024.1 (20972.5-31394.14) v 13476.7 (-470.46-22568), p=0.01) compared to those receiving treatment. However, the numbers of patients not receiving DMARDs was small, so the significance of these results is unclear.

4.1.5.3 Summary

This cohort of RA patients displayed a less active disease by the time of 5 year follow-up, with no overall improvement in disease severity. The improvement in the level of disease activity was shown to be associated with a biomarker profile of reduced eotaxin, IL-8, PDGF-bb, OPN, MMP-1, MMP-3. This particular profile is unique to this study, although specific mediators of this profile including MMP-1 and MMP-3 have been identified to be associated with joint damage progression (Yamanaka *et al.*, 2000, Constantin *et al.*, 2002, Green *et al.*, 2003, Tchetverikov *et al.*, 2003, Ateş *et al.*, 2007, Mamehara *et al.*, 2010). In contrast to this study, eotaxin has been found to be associated with less radiographic progression, although this was only 12 months after diagnosis of early RA which may account for this discrepancy (Syversen *et al.*, 2008). The reduction of these chemoattractants, growth factors and matrix remodelling mediators appears to be independent of any effect of cigarette smoking and DMARD treatment. These mediators therefore could potentially become novel targets for inhibition of disease activity as well as the potential to be clinical markers for long term disease assessment.

4.2 COMPARISON OF BIOMARKERS IN THE PERIPHERAL CIRCULATION AND THE SYNOVIAL FLUID IN RA

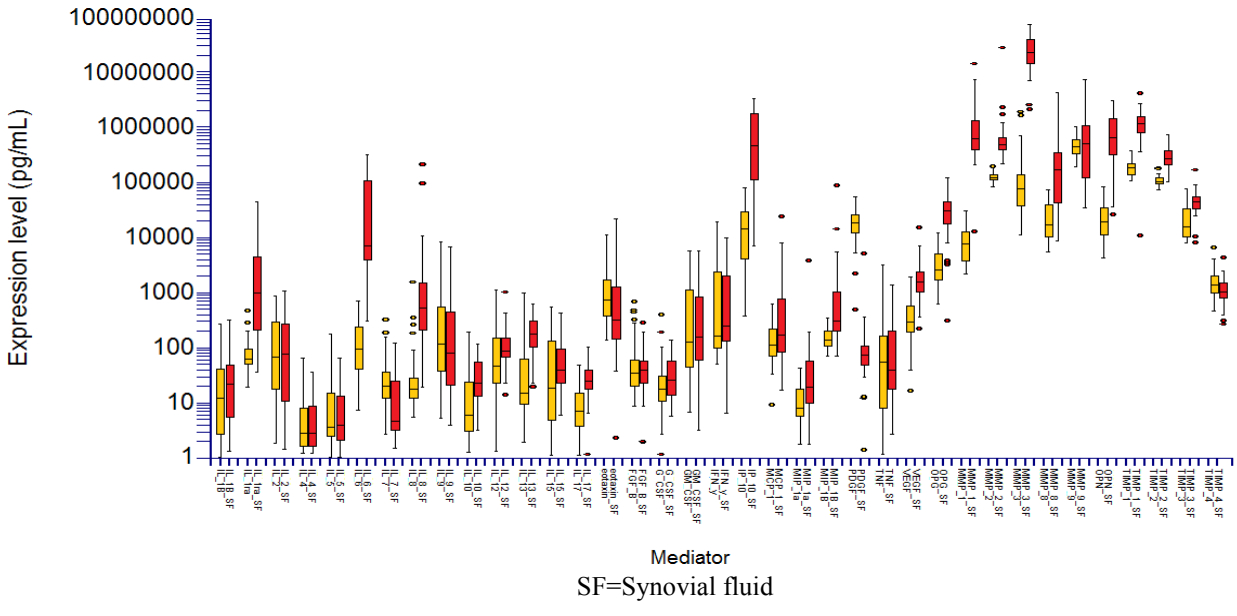
RA biomarker profiling has been commonly investigated in the serum due to its ease of procurement, despite the main site of inflammation occurring at the synovial joint. The peripheral circulation may not necessarily reflect the same level of immune activity as within the joint. Thus, RA biomarker profiling of paired serum and joint synovial fluid collected at the same time were investigated for comparison between the 2 environments. This type of investigation can also determine whether any biomarker profiles from either location are particularly associated with disease activity and severity which may provide further clues into the disease process. In addition, this investigation can also determine whether any biomarker profiles from either environment are associated with joint damage. This can elucidate the respective systemic and articular contribution, since the active inflamed joint recruits mediators from the peripheral circulation. These type of investigations can help further understand the pathological process of RA as well as give clues to the molecular pathways involved and insights into the systemic nature of RA.

Thirty-nine mediators in total were investigated in both the serum and the synovial fluid with the exclusion of MMP-12 and endostatin as they were excluded from the first RA cohort. Within the sera, the level of RANTES was usually greater than the maximum threshold of detection and thus was not included in analyses. However levels were within the detection range in the synovial fluid and so were still included in specific analyses. Dkk-1 was barely detected within the synovial fluid, and so was also excluded from analyses involving synovial fluid but was included in serum analyses. It should be noted that RA patients of this cohort were extremely varied in terms of disease duration (0.16-36

yrs, median 1.5 years, mean 6.63 years), and thus patient disease duration was taken into account in analyses wherever possible.

Initial comparisons of mediator expression in the serum and the synovial fluid were performed to investigate immunological activity within the 2 environments. In line with previous studies, paired t-tests revealed that the majority of mediators were significantly higher in the synovial fluid than in the serum (Figure 4.2a) (Steiner *et al.*, 1999, Tchetverikov *et al.*, 2004, van den Ham *et al.*, 2009, Moura *et al.*, 2010). A few mediators (IL-2 p=0.008, IL-7 p=<0.0001, IL-9 p=0.01, PDGF-bb p=<0.0001, eotaxin p=<0.0001, and TIMP-4 p=<0.0001) however were found to be significantly higher in the serum (Figure 4.2a). In contrast, no significant differences were shown for IL-4, IL-5, FGF-β, G-CSF, GM-CSF, IFN-γ and TNF-α between the 2 sites. This demonstrates that immune activity in general is greater at the site of inflammation (the active joint), although the separate sites do display some similarity in immune activity.

Figure 4.2a: Comparison of mediator expression between the peripheral circulation and synovial joint



Correlation analyses demonstrated a relationship between the 2 environments as a significant positive correlation was found in the majority of mediators (excluding IL-1ra, IL-6, MIP-1β, PDGF-ββ, OPG, MMP-1, MMP-2, TIMP-1 and TIMP-2) (Appendix figure 4.2b). Interestingly, MMP-2 and OPG were found to be negatively correlated, although this did not reach significance (Spearman's Rank r-correlation -0.18 and -0.02, p=0.24 and 0.84 respectively) (Appendix figure 4.2b). This suggests that the peripheral circulation may to an extent reflect the joint immunologically.

4.2.1 Comparison of synovial joint and peripheral circulation biomarker profiles for disease activity and severity

Combined biomarker profiling of the serum and the synovial fluid was investigated to determine whether any mediators from a specific environment are associated with disease activity and severity. Regression analyses revealed a biomarker profile of high IL-13, IL-17 and MMP-8 plus low IL-5, IL-12 and IP-10 expression from the synovial fluid was associated with disease activity and severity, whereas a profile of high IL-13, IL-15, IP-10, MIP-1α and TIMP-4 plus low PDGF-bb and MMP-2 from the serum was shown to be associated with RA activity and severity (Table 4.2c). This illustrates that no specific environment is closer in reflecting disease activity than the other, in fact both sites show associations with disease activity. However, these biomarker profiles are distinct from each other, indicating that specific mediators from both environments are associated with disease activity and severity. Only IL-13 (an inducer of the Th2 humoral response) and IP-10 from both environments were found to be associated with disease activity (Wynn, 2003), although interestingly IP-10 demonstrated a negative association in the synovial fluid. This could suggest a possible anti-inflammatory role in RA as this chemokine also

has pleiotropic activities which include inhibiting angiogenesis (Angiolillo *et al.*, 1995, Neville *et al.*, 1997, Yates-Binder *et al.*, 2012).

Table 4.2c: Combined serum and synovial fluid biomarker profiling for disease activity and severity

Mediator	CGDA >56 (+/-)	OSRA-A >5 (+/-)	SI >9 (+/-)	DAS44ESR >3.6 (+/-)	DAS44CRP >3.53 (+/-)	HAQ >1.5 (+/-)
IL-5						
IL-5 SF	0.05(-)					
IL-12						
IL-12 SF				0.05(-)		
IL-13				0.04		
IL-13 SF						0.01
IL-15	0.01					
IL-15 SF						
IL-17						
IL-17 SF	0.02					
IP-10				0.002	0.006	
IP-10 SF					0.02(-)	
PDGF-bb						0.03(-)
PDGF-bb SF						
MIP-1α		0.04				
MIP-1α SF						
MMP-2					0.03(-)	
MMP-2 SF						
MMP-8						
MMP-8 SF				0.06		
TIMP-4	0.06					
TIMP-4 SF						
Model p-value	<0.0001	0.02	NS	<0.0001	<0.0001	<0.0001
Model r-squared	0.36	0.10		0.40	0.34	0.32

NS=Not significant, (-)=negative association. N.B All analyses were performed with discriminant stepwise selection followed by forward logistic regression of selected variables.

Intriguingly, a single mediator, IL-2 an inducer of the Th2 humoral response (p=0.003) from the serum was found to be associated with RF (n=19/44 (43%)) (model p=<0.0001), whereas IP-10 (p=0.008) from the sera and TIMP-3 (p=0.006) from the synovial fluid were positively associated with anti-CCP positivity (n=35/52 (67%)) (model p=<0.0001). However, one study identified IL-2 as stimulating the production of antibodies, but not the production of RF autoantibodies (Callaghan *et al.*, 1993). Further investigation of these

autoantibody biomarker profiles in both the serum and the synovial fluid therefore is needed to determine the influence of both environments on autoantibody production.

4.2.2 Comparison of synovial joint and peripheral circulation biomarker profiles for joint damage and disability

Combined biomarker profiling of the serum and the synovial fluid was investigated to determine whether any mediators from either environment are associated with damage and destruction of the joint. Regression analyses revealed that mediators from both the peripheral circulation and the synovial joint were associated with joint disease activity and severity (SF profile: IL-2, IL-13 IL-15, IL-17 and G-CSF, serum profile: IP-10, Dkk-1 and MMP-2) (Table 4.2d). However, the synovial joint environment seems to positively reflect joint disease severity more than that of the serum, especially since MMP-2 and Dkk-1 from the serum demonstrated a negative association to joint disease activity and severity. In addition, these environment specific profiles are distinct from one another, since no mediator was found to be commonly associated in both environments. This suggests that specific mediators from both the synovial joint and the peripheral circulation are associated with joint damage and severity. Intriguingly, serum IP-10 was found to be positively associated with more joint disease indices than any other mediator from either environment. This is consistent with previous studies where an association with increased damage in the joint was shown in animal experimental arthritis, and therefore could be a potential target for RA treatment (Salomon *et al.*, 2002, Kwak *et al.*, 2008).

Table 4.2d: Combined serum and synovial fluid biomarker profiling for joint disease activity and severity

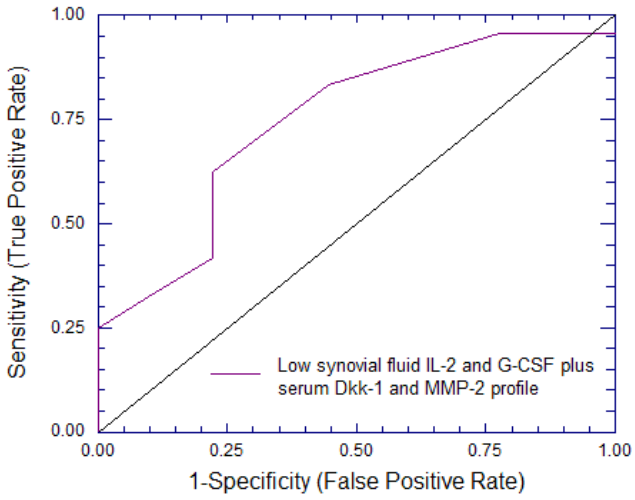
	Inflammation		Pain		Damage			Function	
Mediator	SJ >5 (+/-)	PIPS >3 (+/-)	TJ >7 (+/-)	RI >11 (+/-)	OSRA-D >2.5 (+/-)	Larsen Hand score >50 (+/-)	Erosion (+/-)	EMS >60 (+/-)	Grip strength >98 (+/-)
IL-2									
IL-2 SF									0.06
IL-13									
IL-13 SF				0.009					
IL-15									
IL-15 SF									0.008(-)
IL-17									
IL-17 SF									0.02(-)
G-CSF									
G-CSF SF									0.03
IP-10	0.01		0.002	0.004					
IP-10 SF									
Dkk-1						0.01(-)			
Dkk-1 SF	X	X	X	X	X	X	X	X	X
MMP-2				0.01(-)					
MMP-2 SF									
Model p-value	0.003	NS	<0.0001*	<0.0001	NS	0.0001	NS	NS	<0.0001
Model r-squared	0.14		NA	0.35		0.41			0.45

SF=Synovial Fluid, X=excluded mediator, NS=Not Significant, NA=Not Applicable, (-)=negative association. N.B All analyses were performed with discriminant stepwise selection followed by forward logistic regression of selected variables. *Discriminant regression alternatively used as logistic regression unable to perform.

ROC analysis of this biomarker profile of positively associated IL-13, IL-15, IL-17 from the synovial fluid and sera IP-10 were analysed with the presence of erosions (n=28/37 (75.7%)) but "failed" in accurately identifying patients with erosive disease (AUC 0.59). In contrast, ROC analysis of negatively associated IL-2 and G-CSF from the synovial fluid and Dkk-1 and MMP-2 from the serum was "fairly" accurate (AUC 0.74, 95% CI 0.55-0.93, SE 0.1, p=0.007) in identifying the presence of erosions (Figure 4.2e). This biomarker profile is unique to that of previous biomarker profiling studies and could potentially provide new strategies for disease intervention since IL-2 is a known immuno-regulatory mediator, and G-CSF and MMP-2 have been shown to have anti-inflammatory

activities (Hartung *et al.*, 1995, Boneberg *et al.*, 1999, McQuibban *et al.*, 2000, Boneberg and Hartung, 2002, McQuibban *et al.*, 2002, Martins *et al.*, 2011). Interestingly, Dkk-1 was negatively associated with joint severity despite being characterised as a matrix remodelling mediator (Diarra *et al.*, 2007). ROC analysis excluding Dkk-1 in the biomarker profile demonstrated "poorer" accuracy in identifying erosive disease (AUC 0.68). Further investigation of Dkk-1 in both the serum and synovial fluid is therefore required to determine its relative effects on joint damage.

Figure 4.2e: Accuracy of erosive disease identified biomarker profile



4.2.3 Association with cigarette smoking

Biomarker profiling and the influence of cigarette smoking in both the peripheral circulation and synovial fluid were investigated to determine whether cigarette smoking is associated with any effects on immune activity in either environment. As regression analyses could not be performed using patients who had ever smoked due to the limited range of patients, current smokers (n=14/49 (28%)) and past smokers (n=10/40 (25%)) were investigated. TIMP-3 alone from the sera was shown to be positively associated with

current smoking (p=0.02, model p=0.01, model r-squared=0.11), although no mediator from the sera was found to be associated with previous smoking. Interestingly, IL-10 alone from the synovial fluid was shown to be associated with current smoking as well as with past smoking (p=0.03, model p=0.01, model r-squared=0.12 and p=0.02, model p=0.01, model r-squared= 0.14 respectively). However, this mediator was found to be negatively associated with current smoking whereas it was shown to be positively associated with past smoking. This suggests that recent exposure to cigarette smoke may upregulate TIMP-3 in the peripheral circulation but downregulate IL-10 expression in the joint, which may be upregulated after smoking cessation. This demonstrates that smoking may promote or suppress expression of different mediators as other studies have described with various mediators (Madretsma *et al.*, 1996a, Madretsma *et al.*, 1996b, Ouyang *et al.*, 2000, Lee *et al.*, 2007, Choi *et al.*, 2008). Although these results indicate that smoking is able to influence immune activity distinctly within the 2 environments, only a small number of mediators were identified which were affected by smoking. This could be due to the limited number and range of patients available for analysis. This profile is distinct from that of the biomarker profile identified in the early RA cohort of IL-6, IL-8, MMP-1, MMP-8, MMP-9, Dkk-1, OPN and OPG. Further investigation therefore would be useful to understand the role and mechanisms of cigarette smoking in RA in both the peripheral circulation and the synovial fluid.

4.2.4 Association with DMARD treatment

Biomarker profiling and the influence of DMARD treatment in both the peripheral circulation and synovial fluid were investigated to determine whether drug treatment is associated with any effects on immune activity at either environment. Analysis revealed

IP-10 alone from the sera was found to be significantly associated with DMARD treatment (p=0.05, model p=0.003, model r-squared=0.20). No mediator from the synovial fluid was found to be associated with drug treatment. This suggests that DMARDs may upregulate an angiogenesis inhibitor in the peripheral circulation (Angiolillo *et al.*, 1995, Yates-Binder *et al.*, 2012). Although these results indicate that DMARDs are able to influence immune activity differently between the 2 environments, only a small number of mediators once again were identified which were affected by drug treatment. This could be due to the limited number and range of patients available and therefore additional investigation is needed. This profile is different to that of the biomarker profile identified in the early RA cohort which consisted of Dkk-1, OPN and MMP-1, although this may be upregulated by cigarette smoking and its association with DMARDs is one of response to worse disease associated with smoking. This also may be due to the fact that within this cohort, (n=23/51 (45%)) of patients were receiving DMARD treatment whereas only 14% of patients were receiving treatment in early RA.

4.2.5 Summary

The level of inflammatory mediators was generally higher in the synovial fluid than the peripheral circulation reflecting a greater immune activity in the environment of the synovial joint which is in line with previous studies (Steiner *et al.*, 1999, Tchetverikov *et al.*, 2004, van den Ham *et al.*, 2009, Moura *et al.*, 2010). For these particular mediators the synovial joint is likely to be the primary site of production, although a significant correlation between the levels of many of the mediators in the 2 environments reflects a close relationship between these sites (Tchetverikov *et al.*, 2004). However in some cases the level of particular mediators is higher in the peripheral circulation than the joint and is

more likely to reflect systemic production of these mediators (Moura *et al.*, 2010). The association of particular disease characteristics with specific mediators at one and/or both sites is an indication of the systemic nature of RA. Thus both environments are important in the RA pathological process with mediators from both the peripheral circulation and synovial fluid involved in the disease process contributing to overall disease activity and severity, although mediators from the synovial fluid are those of most importance for joint damage and severity.

4.3 RA BIOMARKER PROFILING IN COMPARISON WITH OTHER ARTHRITIDES

Biomarker profiling of RA (using early disease patients from the first cohort) in comparison with other arthritic diseases (psoriatic arthritis (PsA), reactive arthritis (ReA) and osteoarthritis (OA)) was carried out to determine if differences in biomarker levels could distinguish between the separate arthritides. Such investigations will allow for better understanding of the pathology of the different arthritides and may give clues to the different pathways involved in the various arthritides. A total of 35 mediators were investigated in ReA, PsA and OA patient sera (n=80) for comparison with RA. RANTES, IL-17, FGF-β, MIP-1β, MMP-12 and endostatin were not included in the analyses as they were excluded from analysis in the first RA cohort.

Initial comparisons of disease activity and severity between the different arthritides revealed significant differences in the majority of disease measures except for the WBC count and the VAS (Appendix Table 4.3a). Patients with RA demonstrated worse activity and severity compared to patients with other arthritic diseases, which reflects the characteristic inflammatory and destructive type of disease pathology in RA. ReA patients closely followed RA in terms of worse activity and severity, with OA patients displaying the lowest level of disease activity and severity overall. This is not surprising since OA is characterised as a non-inflammatory arthritis (Appendix Table 4.3a). It should be noted that many disease measure indices were not recorded or present in the other arthritides, such as nodules, erosions, Larsen hand score, RF, anti-CCP, and thus were not included. In addition, disease measures specific to particular arthritides (e.g. WOMAC in OA, PASI in PsA, DAS44, Stoke Index, OSRA-D, Ritchie Index and PIPS in RA) were not included

and only general measures of disease were analysed. As such, damage measure indices were excluded.

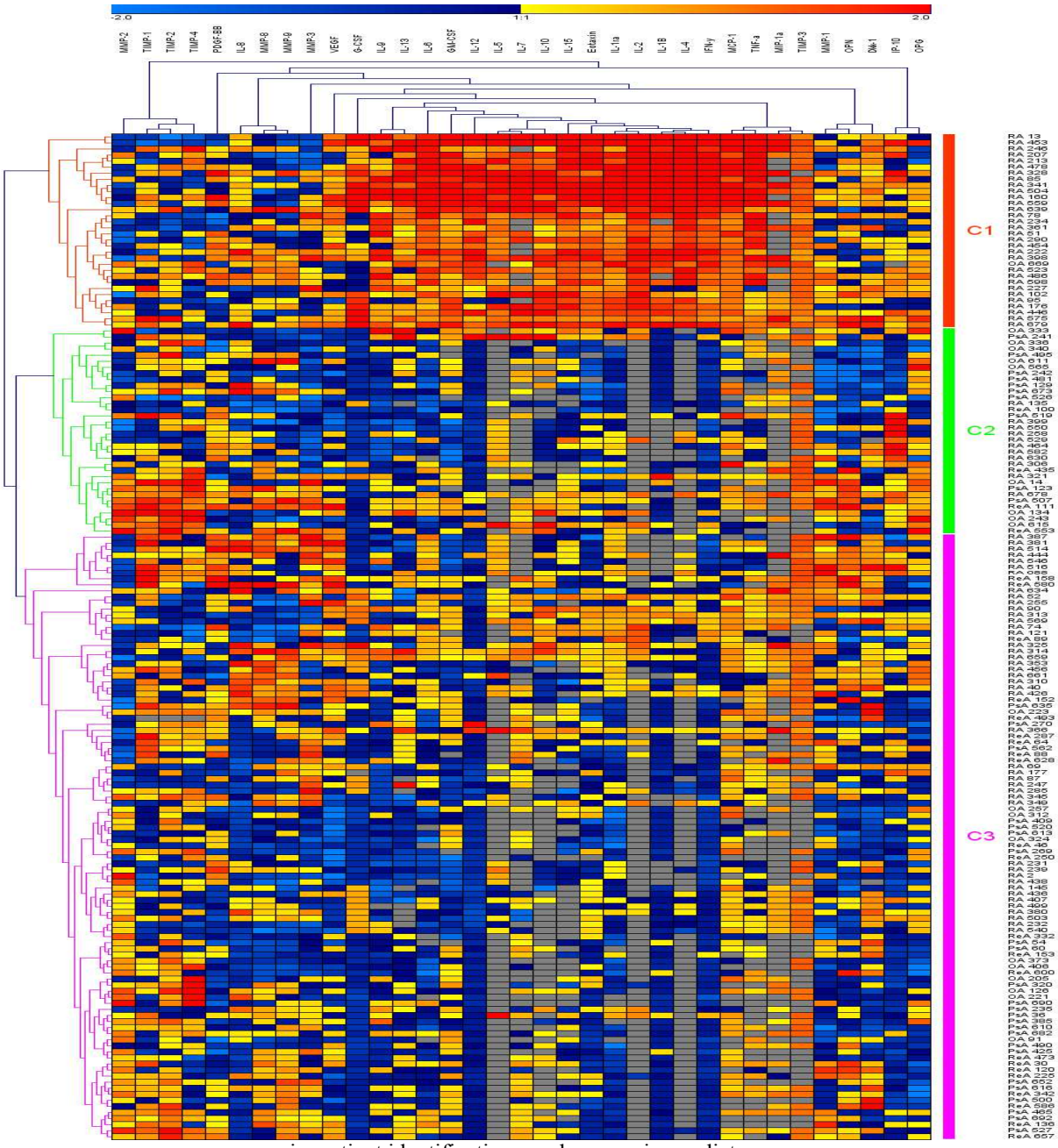
Comparison of mediator expression levels between the different arthritides also demonstrated RA patients to have the highest expression of mediators, characteristic of a more immunologically active type of arthritis ($p=\leq 0.05$, Appendix figure 4.3b) (Stabler *et al.*, 2004, Alex *et al.*, 2007, Hueber *et al.*, 2007). ReA patients closely followed RA with elevated expression of numerous mediators, while PsA and OA patients demonstrated the lowest levels of mediators. Interestingly, MMP-2 ($p=0.0001$), TIMP-1 ($p=0.04$), TIMP-2 ($p=<0.0001$) and TIMP-4 ($p=0.003$) were found to be significantly lower in RA patients in comparison to the other arthritides, particularly that of OA and PsA patients. These particular mediators have well known anti-inflammatory and regulatory activities and therefore it is not surprising that their expression is limited in RA (McQuibban *et al.*, 2000, Hoegy *et al.*, 2001, Mannello and Gazzanelli, 2001, Itoh *et al.*, 2002, McQuibban *et al.*, 2002).

4.3.1 Arthritides differentiation

Genesis hierarchical clustering analysis was used to determine if any biomarker profiles could be identified to distinguish between the different arthritides. A heat map was generated displaying the relative expression level of each mediator for every individual arthritic patient (Figure 4.3c). After 2-dimensional unsupervised hierarchical clustering, 3 distinct patient clusters denoted as C1, C2 and C3 based on their mediator expression pattern similarities were identified. No clear separations between the arthritides were revealed, as each cluster had a mixture of each of the different arthritides. A degree of

separation was found between a proportion of RA patients and the other arthritides. Thirty-six percent of RA patients grouped together in a distinct cluster (C1, n=32) of extremely high expression of half of the investigated mediators incorporating all of the interleukins and other specific mediators (eotaxin, IFN-γ, G-CSF, GM-CSF, TNF-α and MCP-1) with only 1 misclassified OA patient. Despite the C1 cluster being composed completely of RA patients bar one, it doesn't create a useful biomarker profile in terms of aiding differential diagnosis as it did not include the remaining 64% of RA patients. Elevated expression of numerous mediators is also suggestive of RA and is in line with previous studies (Alex *et al.*, 2007, van den Ham *et al.*, 2009), and therefore this cluster could indicate a RA subtype that is more immunologically aggressive.

Figure 4.3c: Heat map of the relative mediator expression in all arthritic patients

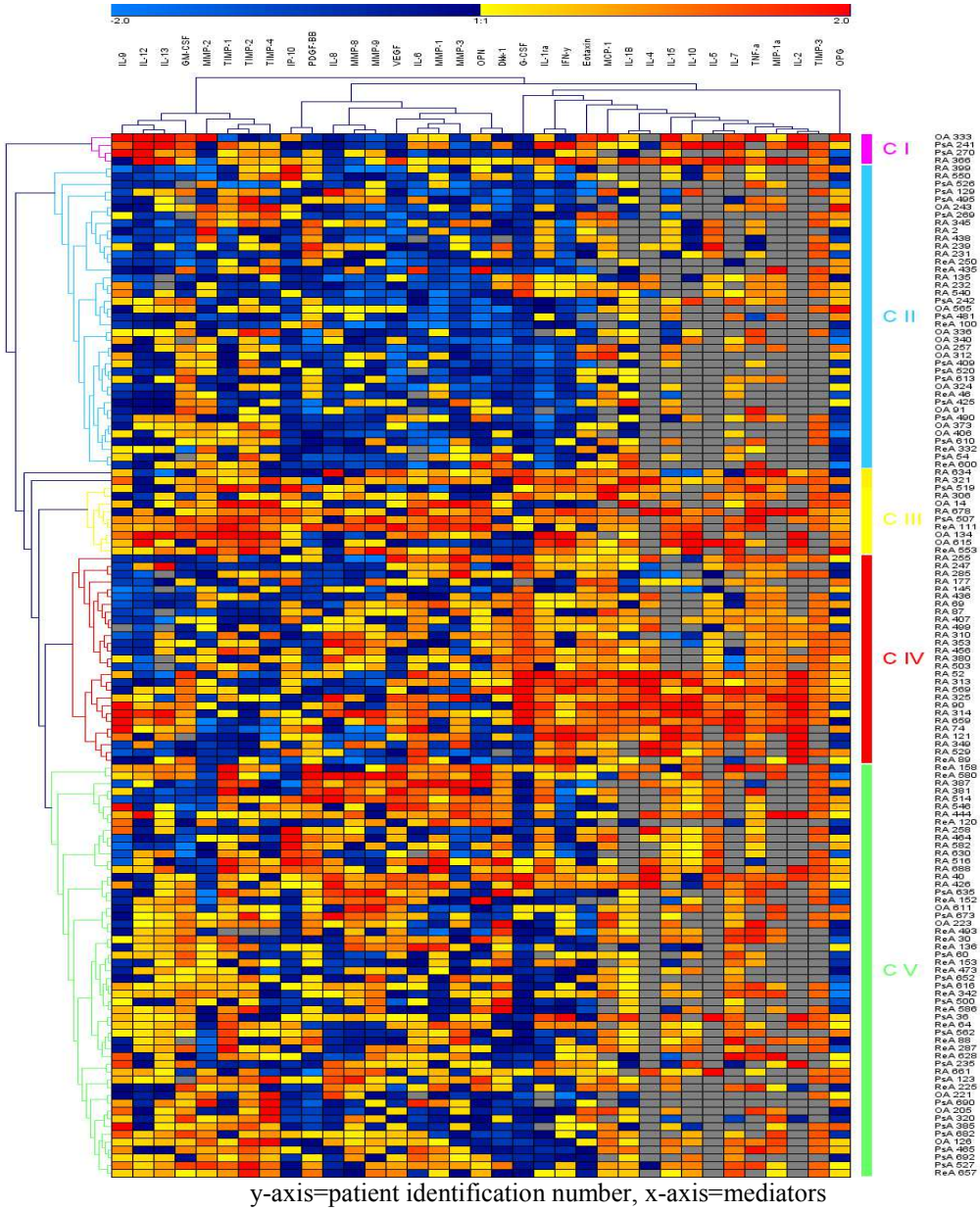


y-axis=patient identification number, x-axis=mediators

As extremely elevated mediators can skew clustering analysis in relation to the rest of the mediators, another unsupervised hierarchical clustering analysis on all the arthritides patients excluding the high mediator expression C1 RA patient cluster (plus the misclassified OA patient) was performed (Figure 4.3d). This can determine if any biomarker profiles are able to differentiate between the arthritides in patients who are

phenotypically more immunologically similar. Similar to the previous hierarchical clustering analysis performed, each identified cluster denoted as C I, C II, C III and C IV incorporated a mixture of each of the arthritides revealing no distinct separation between the different arthritic diseases. However, one cluster (C IV) grouped just under half of RA patients (47%) with only 1 misclassified ReA patient.

Figure 4.3d: Heat map of the relative mediator expression in all arthritic patients excluding the immunologically aggressive RA subtype



y-axis=patient identification number, x-axis=mediators

Comparison of mediator expressions between the C IV cluster and the remaining clusters collectively were subsequently analysed to determine a biomarker profile which could potentially identify a large proportion of RA patients. The C IV RA prominent cluster demonstrated significantly lower IL-9, IL-12, IL-13, IL-15, PDGF-bb, MMP-2, TIMP-1, -2 and -4 as well as significantly higher IL-1ra, IL-2, IL-4, IL-5, IL-6, eotaxin, G-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, TNF-α and Dkk-1 in comparison to the remaining clusters ($p=\leq 0.05$, data not shown). This 22 mediator profile could largely explain the distinction of the RA prominent cluster from the remaining patient clusters. Discriminant stepwise selection and forward logistic regression analysis of this profile revealed a biomarker profile of high IL-2 ($p=0.0003$) and G-CSF ($p=<0.0001$) expression plus low IL-9 ($p=0.01(-)$) and TIMP-4 ($p=0.02(-)$) which particularly distinguished the C IV cluster (model $p=<0.0001$, model r-squared=0.37). This cluster is different from the C1 RA prominent cluster as it displays a low expression of IL-9, IL-12, IL-13, and IL-15 which could suggest a different pathway in the development of RA pathology.

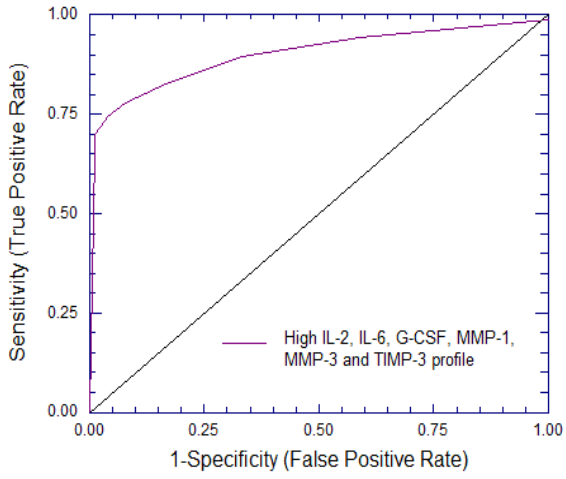
As there was no clear separation between OA, PsA and ReA in either hierarchical clustering analysis, a separate unsupervised hierarchical clustering analysis of OA, PsA and ReA excluding RA patients was additionally performed (in case all RA patients skew arthritides differentiation) (Appendix figure 4.3e). This also failed to adequately differentiate the arthritides into separate clusters. There is the possibility that the specific mediators responsible for OA, PsA and ReA pathology were not investigated in this study and therefore could not be distinguished. The diverse patterns of mediator expression displayed on the heat maps not only demonstrate the heterogeneous immunological activity between arthritides but also the immunogenic heterogeneity between individuals in each of the arthritides as well as RA.

Regression analyses were also performed to gain a statistical approach for identifying arthritis distinguishing biomarker profiles. Analysis between RA patients and the other arthritides grouped together revealed a unique 6 biomarker profile of high IL-6 (p=0.05), MMP-1 (p=0.03), MMP-3 (p=0.02) and TIMP-3 (p=<0.0001) plus low TIMP-1 (p=<0.0001(-)) and TIMP-4 (p=0.001(-)) expression which distinguished patients with RA (model p=<0.0001, model r-squared=0.36). Similar analyses were performed comparing each individual type of arthritis (ReA, PsA and OA) with the other arthritides grouped together. A small biomarker profile of elevated OPN (p=<0.0001) and low TIMP-3 (p=0.003(-)) expression distinguished ReA patients from all other arthritides (model p=<0.0001, model r-squared=0.16). Another small biomarker profile of low VEGF (p=0.01(-)) and high TIMP-2 (p=0.02) expression distinguished patients with PsA from all other arthritides (model p=0.0003, model r-squared=0.11). Patients with OA demonstrated a profile of elevated TIMP-4 (p=<0.0001) plus low MMP-1 (p=0.05(-)) and TIMP-3 (p=0.05(-)) expression which distinguished OA patients from the remaining arthritides (model p=<0.0001, model r-squared=0.17). The overall results demonstrate the important association of matrix remodelling mediators with a more inflammatory type of arthritis whereas ECM degrading enzyme inhibitors are associated with less/non-inflammatory types of arthritis. Interestingly, OA and RA distinguishing profiles display similar biomarker signatures containing MMP-1, TIMP-3 and TIMP-4 but the level of expression of these mediators is reversed in the two diseases.

Using both hierarchical clustering and regression analysis and excluding the extremely elevated mediators identified in the RA prominent cluster (C1), a composite biomarker profile of high IL-2, IL-6, G-CSF, MMP-1, MMP-3 and TIMP-3 plus low TIMP-1 and TIMP-4 was collated. ROC analysis of the elevated mediator profile revealed it to be

"excellent" in accurately identifying RA disease (AUC 0.91, 95% CI 0.86-0.96, SE 0.024, p=<0.0001) (Figure 4.3f). These mediators in combination with low expression of TIMP-1 and -4 could potentially become a specific profile to assist in RA diagnosis as well as have important roles in RA pathology. This biomarker profile in addition to a general elevated expression of numerous mediators therefore may be able to potentially identify a large proportion of RA patients.

Figure 4.3f: Accuracy of RA distinguishing composited biomarker profile



4.3.2 Disease activity and severity biomarker profiling between the different arthritides

As RA is characterised as a severe and destructive type of arthritis, analyses of mediators with disease measures between the arthritides were performed to determine whether any biomarker profiles may explain the more damaging arthritis in comparison to the other arthritides. As regression analyses could not be adequately performed due to the limited number of the other arthritic patients, Spearman's Rank correlation analysis was performed to compare with RA PCA data from the early cohort.

Numerous mediators were found to be significantly associated with various disease activity and severity measures in both ReA and RA patients. In contrast a relatively minimal number of mediators correlated with indices in OA patients and only a moderate number in PsA patients (Table 4.3g, h and i). This illustrates that numerous mediators are involved in RA pathology which reflects its characterisation as an inflammatory, immunologically active severe disease, whereas OA seems to have fewer inflammatory mediators involved in its pathology which reflects its characterisation as a non-inflammatory arthritis.

Table 4.3g: Correlations between mediators and disease measure indices in OA patients

	Inflammation						Pain		Function		
Mediator	ESR	CRP	Platelets	WBC	CGDA	SJ	VAS	TJ	EMS	HAQ	Grip strength
IL-6	0.54/0.02										
IL-9	0.61/0.006										
IL-13					0.47/0.04			0.44/0.04			
eotaxin											(-)0.55/0.02
IP-10	0.57/0.01								(-)0.63/0.002		
MCP-1							(-)0.51/0.01				
MIP-1α								0.44/0.04			(-)0.51/0.03
PDGF-bb		0.48/0.04									
VEGF		0.58/0.01		0.56/0.01	0.62/0.005	0.44/0.05		0.47/0.03			
MMP-8		0.58/0.01					(-)0.46/0.03				
MMP-9		0.53/0.02		0.47/0.03							
TIMP-1			0.59/0.007								
TIMP-2			0.57/0.01						(-)0.47/0.03		
TIMP-4			0.47/0.04							0.49/0.02	
OPG										0.54/0.01	

Values: Spearman's Rank *r* correlation/p-value. (-) negative relationship.

Table 4.3.h: Correlations between mediators and disease measure indices in PsA patients

	Inflammation					Pain			Function		
Mediator	ESR	CRP	Platelets	WBC	CGDA	SJ	VAS	TJ	EMS	HAQ	Grip strength
IL-1β					0.36/0.03						
IL-5			0.35/0.03					0.36/0.03			
IL-6	0.54/0.0009	0.64/0.00005	0.39/0.02	0.59/0.0001		0.40/0.01				0.37/0.03	
IL-7			0.36/0.03	0.34/0.04	0.43/0.01						
IL-9					0.42/0.01						
IL-10									0.36/0.03		
IL-13		0.35/0.04									
IL-15			0.45/0.006							0.39/0.02	
IP-10						0.36/0.03					
MCP-1					0.38/0.02						
PDGF-bb			0.42/0.01								
MMP-1				0.38/0.03			0.39/0.01				
MMP-2		0.39/0.02							(-)0.35/0.03		
MMP-3		0.41/0.01			0.36/0.04						0.39/0.03
MMP-8	0.44/0.009	0.36/0.03	0.40/0.01								
TIMP-1		0.40/0.01	0.34/0.04	0.44/0.007						0.41/0.01	
TIMP-2						0.34/0.04					
TIMP-4										0.35/0.04	
Dkk-1				0.37/0.03							
OPG							0.53/0.001		0.66/<0.0001	0.66/<0.0001	

Values: Spearman's Rank *r* correlation/p-value. (-) negative relationship.

Table 4.3i: Correlations between mediators and disease measure indices in ReA patients

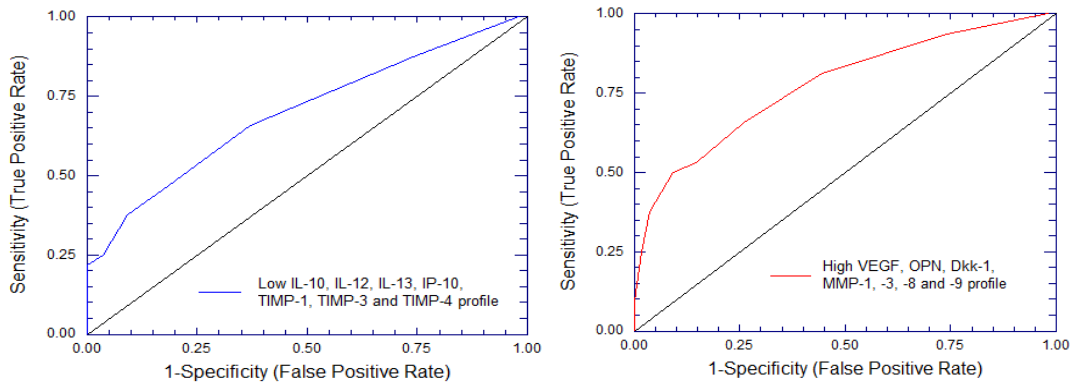
	Inflammation						Pain			Function	
Mediator	ESR	CRP	Platelets	WBC	CGDA	SJ	VAS	TJ	EMS	HAQ	Grip strength
IL-1β											0.55/0.004
IL-6	0.72/0.00002	0.85/<0.0001	0.61/0.0007	0.56/0.002		0.48/0.01					
IL-7							(-)0.42/0.03				
IL-8				0.63/0.004		0.46/0.01					
IL-10	0.40/0.04	0.39/0.04		0.42/0.03							0.44/0.02
IL-12	0.61/0.0008	0.54/0.005	0.48/0.01	0.62/0.0006		0.46/0.01					
IL-13	0.57/0.001	0.50/0.009	0.39/0.04	0.47/0.01							
IL-15				0.39/0.04							0.47/0.01
eotaxin											0.39/0.04
IFN-γ							(-)0.40/0.042		(-)0.40/0.04		0.49/0.01
IP-10	0.54/0.003	0.43/0.03	0.44/0.02		0.44/0.02	0.39/0.04		0.39/0.04			
MCP-1											0.51/0.008
VEGF	0.64/0.0003	0.54/0.005	0.52/0.006	0.50/0.008		0.45/0.02					
MMP-1	0.39/0.04	0.55/0.003	0.41/0.03								
MMP-3	0.49/0.009	0.44/0.02	0.48/0.01	0.39/0.04							
MMP-8	0.60/0.001	0.54/0.004		0.69/<0.0001		0.60/0.001				0.43/0.02	
MMP-9	0.55/0.003	0.43/0.02		0.56/0.002		0.46/0.01					
TIMP-1	0.69/0.0001	0.60/0.001	0.74/<0.0001	0.63/0.0005		0.42/0.03					
TIMP-3	0.61/0.0009	0.46/0.02	0.62/0.0008	0.52/0.006		0.52/0.007					
TIMP-4			0.40/0.04	0.39/0.04							
OPN	0.55/0.003	0.59/0.001	0.40/0.03	0.46/0.01		0.44/0.02					
OPG							(-)0.39/0.04				

Values: Spearman's Rank *r* correlation/p-value. (-) negative relationship.

Mediator profiles were found to be distinctly different between the individual arthritides for each of the disease measures, illustrating the varying pathological processes between arthritides (Tables 4.3g, h and i). ReA patients interestingly demonstrated approximately half of investigated mediators (IL-6, IL-10, IL-12, IL-13, IP-10, VEGF, OPN plus all the MMPs and TIMPs barring MMP-2 and TIMP-2) to be particularly correlated with peripheral inflammatory measures (ESR, CRP, swollen joints, platelet and WBC count), as well as the number of swollen joints, VAS and the HAQ to a degree (Table 4.3i). This profile, particularly the MMPs, OPN and VEGF similarly resembles a PCA derived profile of peripheral inflammation identified in early RA patients (early RA cohort section 4.1.3). These 2 arthritides seem to have some of the same pathological processes in common and therefore could suggest that ReA and RA may have similar disease developmental pathways. These mediators of ECM degrading enzymes, bone remodelling and angiogenesis factors also have roles in immune function (inflammation, chemotaxis, apoptosis regulation, effector cell and cytokine stimulation, etc.) (Mohammed *et al.*, 2003, Xu *et al.*, 2005, Clavel *et al.*, 2007, Van Lint and Libert, 2007, Wang and Denhardt, 2008, Yoo *et al.*, 2008, Candelario-Jalil *et al.*, 2009), which in conjunction with the cytokines incorporated in the profile could explain the higher level of peripheral inflammation seen in both ReA and RA patients (Appendix table 4.3a). Although these disease activity indices are shown to be quite comparable between RA and ReA patients (Appendix table 4.3a), RA patients still displayed the highest levels of peripheral inflammation. This could be attributed partly to Dkk-1 an indirect inducer of bone remodelling (Diarra *et al.*, 2007, Ueland *et al.*, 2009), which was uniquely associated to these indices in RA compared to the other arthritides (early RA cohort section 4.1.3). Furthermore, ReA patients and to a certain extent PsA and OA patients, showed significant correlations of IL-10, IL-12, IL-13, IP-10 and TIMPs with peripheral inflammation which were not reflected in RA patients

(Table 4.3g, h and i) (IL-10, IL-12, IL-13 and TIMPs were not included in the final PCA analysis of early RA patients due to their limited variance influence). These interleukins and IP-10 have been found to have pleiotropic actions with anti-inflammatory roles and angiogenesis inhibition (Angiolillo *et al.*, 1995, de Vries, 1995, Marie *et al.*, 1996, Xing *et al.*, 1998, Chang and Radbruch, 2007, Yates-Binder *et al.*, 2012), In addition, TIMPs are well known ECM degrading enzyme inhibitors as well as angiogenesis inhibitors and both suppress as well as induce apoptosis (Brew *et al.*, 2000, Mannello and Gazzanelli, 2001). Such counteractive activities from these mediators may explain the reduced level of peripheral inflammation in ReA patients and the remaining arthritides in comparison to RA patients (Appendix table 4.3a). ROC analysis of this biomarker profile (low IL-10, IL-12, IL-13, IP-10, TIMP-1, -3 and -4 plus high VEGF, OPN, Dkk-1, MMP-1, -3, -8 and -9 expression) revealed both the low and high profiles to be 'fairly' accurate in identifying worse RA disease (defined by either a severe DAS44-CRP, -ESR (score >3.7) or high Stoke Index (score 12-17) phenotype) (AUC 0.70, 95% CI 0.59-0.83, SE 0.06, p=0.0002, AUC 0.77, 95% CI 0.68-0.89, SE 0.05, p=<0.0001 respectively) (Figures 4.3j). As both these individual profiles were fairly accurate in identifying worse disease, their combination could potentially be useful in identifying severe RA.

Figures 4.3.j: Accuracy of low and high biomarker expression biomarker profile for worse disease activity



4.3.3 Summary

The specific biomarker profile that distinguished RA from other arthritides identified in this study is a unique signature compared to that of previous biomarker profiling studies. However, individual mediators within the profile have been identified in profiles from previous studies. Raised IL-2 and IL-6 were similarly found in studies which distinguished RA disease from PsA and OA respectively (Schlaak *et al.*, 1996, Partsch *et al.*, 1997, Raza *et al.*, 2005, Moura *et al.*, 2010). Therefore this biomarker profile could potentially assist in the diagnosis of undifferentiated arthritis where patients have an unconfirmed type of arthritis and would usually have to wait until symptoms further develop to make an accurate determination.

Past studies have usually investigated RA in comparison with other arthritides in attempt to aid in the diagnosis of RA rather than discerning worse activity and severity. Thus the biomarker profile identified in this study is especially unique in comparison to other arthritides. This biomarker profile incorporates well characterised mediators prominently involved in RA inflammation and worse disease (Mohammed *et al.*, 2003, Clavel *et al.*, 2007, Diarra *et al.*, 2007, Van Lint and Libert, 2007, Wang and Denhardt, 2008, Yoo *et al.*, 2008, Ueland *et al.*, 2009), and therefore has the potential to become be a novel assessment measure to complement current RA disease activity measures.

The mediators identified in this investigation may be important in the pathways involved in the development of RA disease as well as the pathological process, and therefore could become potential targets for therapeutic intervention, such as MMPs which were prominently identified or possibly through the use of their natural inhibitors (TIMPs) as a novel treatment approach.

4.4 Conclusion

The purpose of this research was to investigate the relationship between markers of immune dysfunction and RA. This study identified numerous interleukins, matrix remodelling mediators and pro-inflammatory biomarkers including chemoattractants, angiogenesis and cell growth factors as being involved in the pathological process, including its development and progression, and enabled its differentiation from other arthritides. These multiple biomarkers may be important in the molecular pathways of the complex cellular-mediator network involved in RA. The study illustrated and further contributed to the notion that RA is an extremely heterogeneous disease involving multiple mediators, with various possible routes of pathology. Many of the biomarkers identified in this study were shown to be associated with disease activity and severity, both systemically and from within the joint. In addition, cigarette smoking influenced biomarker expression in both environments and was associated with a biomarker profile which in turn was associated with worse disease. Thus, smoking could potentially alter immune regulation and stimulate RA development and progression.

Therapies targeted specifically at some of these biomarkers have already been developed and have proved to be important in the treatment of RA. However, this study highlights the heterogeneity of the disease process and indicates that other markers of immune dysfunction may have the potential to become novel targets for therapeutic intervention as well as creating alternative approaches to treatments. This has already been preliminary demonstrated with specific biomarkers identified in this study, where VEGF gene knock-out and OPN deficient, arthritis animal experimental models displayed reduced arthritis and were protected from joint destruction (Yumoto *et al.*, 2002, Brendolan *et al.*, 2003,

Mould *et al.*, 2003). Such studies could ultimately lead to development of strategies for manipulating immune function, leading to remission or reduction in the severity of RA. Furthermore, profiles of markers of immune dysfunction may also have the potential to become useful clinical tools as new assessment measures for the disease process as well as aid in the diagnosis of arthritides.

4.5 Limitations of the investigations

In this study, some of the data were incomplete for individual patients i.e. data on the presence of erosions, autoantibodies, smoking and disease measures. In some cases therefore, this decreased the number of patients available for analyses and limited statistical significance.

Within the first cohort, 9 patients were lost to 5 year follow-up. These 9 patients were therefore removed in comparative analysis of baseline and 5 year follow-up. The number of patients was also limited within the 2nd and 3rd cohorts of this study, and thus restricted statistical analyses. In particular, principal component analysis could not be performed to create specific PC profiles of important biomarkers. These were therefore analysed as individual markers. Furthermore, due to insufficient numbers in each of the different arthritic patient groups of the 3rd cohort, regression analyses of biomarkers with disease measures could not be performed. Alternative analyses had to be employed, and thus possible confounding factors could not be taken into consideration. It is evident that sufficient numbers of patients are required for the creation of profiles, and allowance needs to be made for patient loss and other factors when investigating multiple biomarkers using the new methodology.

Another limitation of this study was found when comparing patients between baseline and 5 year follow-up RA within the first cohort. Information was unavailable at what time point during the 5 year period patients changed their status from past to current smoking. However, it should be noted that this occurred in only 3 patients, and therefore would not dramatically alter the results in this cohort. Such information should be recorded for future studies of this nature. This type of limitation was also found with DMARD treatment, where it was not known exactly when patients started receiving DMARDs. Furthermore, it can take up to 6 months for DMARDs to have any effect, while some patients do not respond at all to this treatment. Therefore, it should be recorded at what time point patients started receiving DMARD treatment, and whether they responded to treatment.

Paired serum and synovial fluid samples of RA patients were procured at the same time as their joint flare. As a synovial effusion can occur at any time, the disease duration for each patient therefore varied considerably in the second cohort. The median disease duration of this cohort is 1.5 years, though the actual range of this cohort was from 0.16-36 years. Thus this cohort consisted of both early and established RA patients. Analysis was adjusted for disease duration wherever possible. However, this could only be performed in regression analyses, while other analyses, such as t-tests, could not incorporate this confounding factor.

Two methods for measuring mediators were used in this project: bead based multiplex assays and ELISAs. However, comparing the modern multiplex array technology with traditional ELISAs, revealed a high variability between the methodologies, as IL-17 and FGF-β levels were significantly different between the two approaches. Although these particular mediators were excluded in analysis, it does give cause for concern to the

validity of this new bead array technology system. Differences between the two methods have been previously identified (duPont *et al.*, 2005, Elshal and McCoy Jr., 2006), and although a significant correlation was demonstrated in measured mediators, the actual concentration levels differ considerably. This may be due to the different monoclonal antibodies, diluents, reagents and buffers used between the different assays, as this was also demonstrated between different kits of ELISAs (duPont *et al.*, 2005, Elshal and McCoy Jr., 2006). Limiting material variability between the assays could therefore reduce the difference of concentration levels. Furthermore, an upgrade to magnetic beads in the Luminex system from the original but now discontinued polystyrene bead system, shows significant differences between the 27 plex data. It should be pointed out that investigation between the paired samples was performed by the use of magnetic beads, while the other 27 plex assays were performed by the traditional polystyrene bead system. As a consequence, we have kept these two bead approaches separate in the analysis. Although, there are overlapping samples between the paired RA cohort and the early first cohort RA data set, these were then performed with both polystyrene and magnetic bead assays and analysed appropriately.

4.6 Further suggested work

A repeat of this study using additional patients plus complete database information would allow for larger detailed patient cohorts and thus provide more valid and confident findings. In particular it would be most valuable to repeat those investigations that were close to significance or suggested possible associations.

Possible future work could include the investigation of paired RA serum and synovial fluid samples in separate cohorts of patients with early or established disease. A comparison between early and late stages of disease at different time points could give useful clues as to how the disease progresses within the joint, and which biomarkers are responsible for bone damage and persist throughout the progression of the disease.

Another aspect for possible further investigation would be the relationship between markers of immune dysfunction in the synovial fluid and the disease process in OA, ReA and PsA patients, in comparison with RA synovial fluid biomarker profiles. This could provide unique insights into the pathological process at the primary site of attack (i.e. the joint) in the different forms of arthritis.

Further understanding of the mediator-cellular networks in RA may identify potential new molecular targets, and thus enable development of strategies for manipulation of immune function. This may include altering cell function by targeting immune regulatory molecules. A greater understanding of the multiple mediators involved in the inflammatory response may provide the opportunity to develop novel therapies for RA, as well as other arthritic diseases and autoimmune disorders.

CHAPTER 5

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CHAPTER 6

APPENDIX

Appendix figure 3.1.2: The patient smoking questionnaire

Hospital number

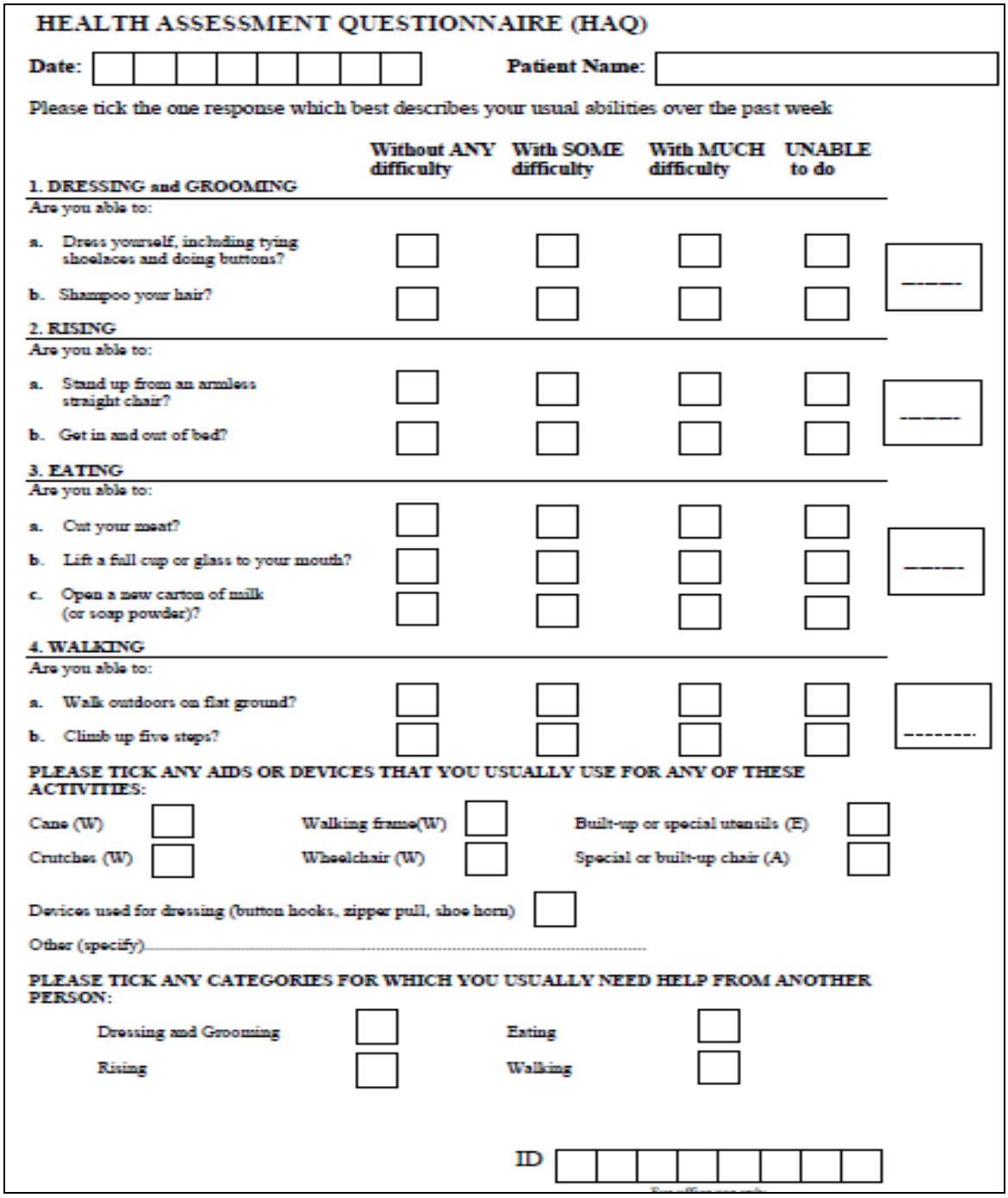
SMOKING QUESTIONNAIRE

Surname	First name	Age
Gender	Occupation	

1. Do you currently smoke cigarettes?	Yes	No
2. If not, have you ever smoked?	Yes	No
3. If you answered yes to question 1 or 2, at what age did you start smoking?		
4. How many cigarettes per day?		
5. If you answered yes to question 2, at what age did you stop smoking?		
6. Does your partner currently smoke?	Yes	No
7. If not, have they ever smoked?	Yes	No
8. Have you ever worked in a smokey atmosphere (e.g. pub)?	Yes	No
9. If yes to question 8, for how long?		

Thank you for your time.

Appendix figure 3.1.3.4: The Health Assessment Questionnaire



HEALTH ASSESSMENT QUESTIONNAIRE (HAQ)

Date: ☐☐☐☐☐☐☐☐ **Patient Name:** ☐

Please tick the one response which best describes your usual abilities over the past week

	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	UNABLE to do	
1. DRESSING and GROOMING					
Are you able to:					
a. Dress yourself, including tying shoelaces and doing buttons?	☐	☐	☐	☐	_____
b. Shampoo your hair?	☐	☐	☐	☐	
2. RISING					
Are you able to:					
a. Stand up from an armless straight chair?	☐	☐	☐	☐	_____
b. Get in and out of bed?	☐	☐	☐	☐	
3. EATING					
Are you able to:					
a. Cut your meat?	☐	☐	☐	☐	_____
b. Lift a full cup or glass to your mouth?	☐	☐	☐	☐	
c. Open a new carton of milk (or soap powder)?	☐	☐	☐	☐	
4. WALKING					
Are you able to:					
a. Walk outdoors on flat ground?	☐	☐	☐	☐	_____
b. Climb up five steps?	☐	☐	☐	☐	

PLEASE TICK ANY AIDS OR DEVICES THAT YOU USUALLY USE FOR ANY OF THESE ACTIVITIES:

Cane (W) ☐ Walking frame(W) ☐ Built-up or special utensils (E) ☐

Crutches (W) ☐ Wheelchair (W) ☐ Special or built-up chair (A) ☐

Devices used for dressing (button hooks, zipper pull, shoe horn) ☐

Other (specify).....

PLEASE TICK ANY CATEGORIES FOR WHICH YOU USUALLY NEED HELP FROM ANOTHER PERSON:

Dressing and Grooming ☐ Eating ☐

Rising ☐ Walking ☐

ID ☐☐☐☐☐☐☐☐

Please tick the one response which best describes your usual abilities over the past week

	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	UNABLE to do	
5. HYGIENE					
Are you able to:					
a. Wash and dry your entire body?	☐	☐	☐	☐	____
b. Take a bath?	☐	☐	☐	☐	
c. Get on and off the toilet?	☐	☐	☐	☐	
6. REACH					
Are you able to:					
a. Reach and get down a 5 lb object (e.g. a bag of potatoes) from just above your head?	☐	☐	☐	☐	____
b. Bend down to pick up clothing off the floor?	☐	☐	☐	☐	
7. GRIP					
Are you able to:					
a. Open car doors?	☐	☐	☐	☐	____
b. Open jars which have been previously opened?	☐	☐	☐	☐	
c. Turn taps on and off?	☐	☐	☐	☐	
8. ACTIVITIES					
Are you able to:					
a. Run errands and shop?	☐	☐	☐	☐	____
b. Get in and out of a car?	☐	☐	☐	☐	
c. Do chores such as vacuuming, housework or light gardening?	☐	☐	☐	☐	

PLEASE TICK ANY AIDS OR DEVICES THAT YOU USUALLY USE FOR ANY OF THESE ACTIVITIES:

Raised toilet seat (H) ☐ Bath seat (H) ☐ Bath rail (H) ☐

Long handled appliances for reach (R) ☐

Jar opener (for jars previously opened) (G) ☐

Other (specify) _____

PLEASE TICK ANY CATEGORIES FOR WHICH YOU USUALLY NEED HELP FROM ANOTHER PERSON:

Hygiene ☐ Gripping and opening things ☐

Reach ☐ Errands and housework ☐

ID ☐☐☐☐☐☐☐☐

For office use only

HAQ	
0	0.0
1	0.125
2	0.250
3	0.375
4	0.500
5	0.625
6	0.750
7	0.875
8	1.00
9	1.125
10	1.250
11	1.375
12	1.500
13	1.625
14	1.750
15	1.875
16	2.000
17	2.125
18	2.250
19	2.375
20	2.500
21	2.625
22	2.750
23	2.875
24	3.000

Appendix figure 3.2.3: Flow chart summarizing the rationale and the statistical analyses used in each cohort.

Flow Chart: 4.1 Biomarker profiling in early and established RA

Initial analyses were primarily performed for general assessment of the cohort.

↓

Chi-square analysis and odds ratios between disease features (ie. early onset, autoantibodies, erosions and synovial effusion) were performed to determine potentially associated distributions and its presentation likelihood.

4.1.2 Early RA

4.1.2.1.1 Relationship of RA disease features and patient characteristics with disease activity and severity in early RA

Preliminary assessment of the cohort was performed with RA features, patient characteristics and disease measures to determine their influence on disease activity and severity.

↓

Two sample T-tests of disease measures were performed with autoantibodies, cigarette smoking and DMARD treatment to determine their association to the level of disease activity and severity.

↓

Additional analyses of cigarette smoking with patients' active smoking status and disease measures were performed by one-way ANOVA to compare the level of disease activity and severity to current and past smoking.

↓

Further analysis of cigarette smoking (including patients' active smoking status and stratified pack year history) with disease features were performed by chi-square analysis and odds ratio to determine potentially associated distributions and its presentation likelihood.

4.1.2.2 Biomarker profiling in early RA

4.1.2.2.1.1 Early RA Hierarchical clustering biomarker profiling

Hierarchical clustering analysis (HCA) of mediators was performed to determine if mediator expression patterns could potentially identify biomarker profiles.

↓

Two sample T-tests between the high & low HCA identified mediator expression patient groups and mediators were performed to identify potentially associated biomarkers.

↓

Subsequently, discriminant analysis for initial biomarker selection followed by logistic regression of selected biomarkers were performed to identify a potentially associated biomarker profile.

4.1.2.2.1.2 Association with early RA disease activity and severity

Chi-square analysis of the HCA identified groups and disease features were performed to determine potentially associated distributions.

↓

One-way ANOVA of baseline disease measures between the HCA identified groups were performed to compare the level of disease activity and severity.

↓

Two sample T-tests of mediators between HCA patient groups identified with the largest difference in disease activity and severity were performed to determine potentially associated biomarkers.

↓

Discriminant analysis for initial biomarker selection followed by logistic regression of selected biomarkers were subsequently performed to identify a potentially associated biomarker profile.

4.1.2.2.1.3 Association with cigarette smoking

Chi-square analysis of the HCA identified groups and cigarette smokers (including ever smokers, patients' active smoking status and stratified pack year history) were performed to determine potentially associated distributions.

↓

Chi-square analysis of the HCA identified groups and DMARD treatment was also performed to determine potentially associated distributions.

4.1.2.2.2.1 Early RA Principal Component Analysis (PCA) biomarker profiling

An exploratory PCA of all mediators was performed to select specific mediators that provided the maximum variance and reduced the dimensionality of interrelated mediators.

↓

A second PCA was performed using selected mediators to identify specific PC biomarker profiles based on their correlation patterns.

4.1.2.2.2.2 Association with early RA disease activity and severity

Multiple and logistic regression analyses (parametric and non-parametric data respectively) of PC biomarker profiles with disease measures and features plus patient characteristics (ie. cigarette smoking and DMARD treatment) were performed after initial PC biomarker selection with multivariate and discriminant selection (parametric and non-parametric respectively) to identify potentially associated biomarker profiles.

↓

Similar regression analyses were performed on highly associated PC biomarker profiles as separate independent variables to identify potentially associated specific mediator biomarker profiles.

↓

Since specific biomarkers were associated with overall disease activity measures (ie. DAS44CRP/ESR and Stoke Index), comparisons between disease activity phenotype categories by one-way ANOVA were performed to determine disease activity trends.

4.1.2.2.3 Composite biomarker profiles

ROC analyses of composite biomarker profiles collated from both the HCA and PCA biomarker profiling methods were performed to determine its potential for accurately identifying RA features, worse disease activity and erosive disease.

4.1.3 Biomarker profiling for RA prognosis

4.1.3.1.1 Hierarchical clustering prognostic biomarker profiling

One-way ANOVA between identified HCA patient groups and 5 year follow-up disease measures were performed to identify potentially associated prognostic biomarker profiles.

↓

No ROC analysis was performed since no unique biomarker profile was identified for 5 year follow-up disease severity.

4.1.3.1.2 PCA analysis prognostic biomarker profiling

Multiple and logistic regression analyses after initial discriminant or multivariate selection were performed with PC biomarker profiles and 5 year follow-up disease measures to identify potentially associated prognostic biomarker profiles.

↓

Similar regression analyses were performed on baseline PC biomarker profiles that were highly associated to 5 year follow-up disease measures as separate independent variables, to identify potentially associated mediator specific biomarker profiles.

↓

ROC analyses of the identified prognostic biomarker profile were performed to determine its potential for accurately predicting worse disease activity and erosive disease.

4.1.3.2 Prognostic biomarker profiling for disease remission

Chi-square analysis between baseline HCA identified groups and 5 year follow-up overall disease activity phenotype categories were performed to determine potentially associated distributions for predicting favourable outcomes (ie. remission, minimal and low phenotype statuses).

↓

Logistic regression of baseline PC profiles and 5 year follow-up favourable outcomes failed to identify potentially associated prognostic biomarker profiles.

↓

Discriminant analysis for initial selection of the baseline mediators identified from the exploratory PCA with 5 year follow-up favourable outcomes was alternatively performed. Logistic and discriminant regressions (when required) of selected biomarkers was performed to determine potentially associated prognostic biomarker profile.

↓

ROC analysis of the identified prognostic biomarker profile were performed to determine its potential for accurately predicting favourable outcomes.

4.1.4 Established RA

4.1.4.1.1 Relationship of RA disease features and patient characteristics with disease activity and severity in established RA

Preliminary assessment of established RA cohorts' patient characteristics, disease measures and features were similarly performed as early RA cohorts' initial assessment as previously described (Flow Chart 4.1.2.1.1) with two sample T-tests, one-way ANOVAs,

chi-square analyses and odds ratios to determine their influence on disease activity and severity.

4.1.4.2 Biomarker profiling in established RA

4.1.4.2.1.1 Established RA Hierarchical clustering biomarker profiling

HCA biomarker profiling as previously described (Flow Chart 4.1.2.2.1.1) with two-sample T-tests, discriminant analysis and logistic regression were similarly performed in established RA to identify potentially associated biomarker profiles.

4.1.4.2.1.2 Association with established RA disease activity and severity

Chi-square analysis of the HCA identified groups and disease features were performed to determine potentially associated distributions.

↓

Two-sample T-test was performed with HCA patient groups identified with greatest frequency of erosions to determine potentially associated erosive biomarker profile.

↓

One-way ANOVA of baseline disease measures between the HCA identified groups were performed to compare the level of disease activity and severity.

↓

Two sample T-tests of mediators between HCA patient groups identified with the largest difference in disease activity and severity were performed to determine potentially associated biomarkers.

↓

Since an overall disease activity measure was associated between HCA groups, a chi-square analysis of HCA groups and overall disease activity phenotype categories were performed to determine potentially associated distributions.

4.1.4.2.1.3 Association with cigarette smoking

Chi-square analysis of the HCA identified groups and cigarette smokers (including ever smokers, patients' active smoking status and stratified pack year history) were performed to determine potentially associated distributions.

↓

Chi-square analysis of the HCA identified groups and DMARD treatment was also performed to determine potentially associated distributions.

4.1.4.2.2.1 Established RA Principal Component Analysis (PCA) biomarker profiling

An exploratory PCA of all mediators was performed to select specific mediators that provided the maximum variance and reduced the dimensionality of interrelated mediators.

↓

A second PCA was performed using selected mediators to identify specific PC biomarker profiles based on their correlation patterns.

4.1.4.2.2.2 Association with established RA disease activity and severity

Multiple and logistic regression analyses of PC biomarker profiles with disease measures and features plus patient characteristics were performed after initial PC biomarker selection with multivariate and discriminant selection to identify potentially associated biomarker profiles.

↓

Since specific biomarkers were associated with specific overall disease activity measures, discriminant analysis and logistic regression were performed with disease activity favourable phenotypes (ie low, minimal, remission) to determine potentially associated biomarker profiles for better disease.

4.1.2.2.3 Composite biomarker profiles

ROC analyses of composite biomarker profiles collated from both the HCA and PCA biomarker profiling methods were performed to determine its potential for accurately identifying RA features, worse and favourable disease activity.

4.1.5 Biomarker profiling RA disease progression

Comparisons between early and established RA were performed to determine changes in disease and identify potentially associated biomarker profiles.

↓

Two-sample T-tests of baseline and 5 year follow-up disease measures were performed to compare the level of disease activity and severity between early and established RA.

↓

Mathematical calculations between baseline and 5 year follow-up disease measures were performed to determine the level of disease activity and severity change from early to established disease.

↓

Mathematical calculations were similarly performed with mediators to determine the change in expression level from early to established RA.

↓

Two-sample T-tests of mediators were similarly performed to compare the level of mediator expression between the 2 stages.

4.1.5.1 Association of mediator expression changes and changes in disease activity and severity

Discriminant analysis of mediator expression changes with disease measure changes for initial biomarker selection followed by logistic regression of selected biomarkers were performed to determine potential associated biomarkers.

4.1.5.2 Association of mediator changes with cigarette smoking and DMARD treatment

Discriminant analysis of mediator expression changes with ever smokers for initial biomarker selection followed by logistic regression of selected biomarker were performed to determine the potential influence of cigarette smoking on mediator changes.

↓

Discriminant analysis and logistic regression of mediator expression changes were similarly performed with DMARD treatment to determine the potential influence of DMARDs on mediator changes.

Flow Chart: 4.2 Comparison of biomarkers in the peripheral circulation and the synovial fluid in RA

Initial comparisons between the serum and the synovial fluid were preformed for preliminary assessment of the peripheral circulation and the joint.

↓

Paired T-tests of mediators were performed to compare the level of immune activity between the 2 environments.

↓

Spearman's rank correlation analyses of mediators were consequently performed to determine the relationship between the 2 sites since the joint recruits mediators from the peripheral circulation.

4.2.1 Comparison of synovial joint and peripheral circulation biomarker profiles for disease activity and severity

Analyses were performed to determine whether biomarkers from a particular environment were associated with disease activity and severity in comparison to the other site.

↓

Discriminant analysis of both serum and synovial fluid mediators together for initial biomarker selection followed by logistic regression of selected biomarkers were performed to identify associated biomarkers from a specific site.

↓

No ROC analysis was performed since no specific environment was closer in reflecting disease activity and severity than the other.

4.2.2 Comparison of synovial joint and peripheral circulation biomarker profiles for joint damage and disability

Analyses were performed to determine whether biomarkers from a particular environment were associated with joint activity and severity in comparison to the other site.

↓

Discriminant selection of both serum and synovial fluid mediators together followed by logistic regression (or discriminant regression as an alternative when required) of selected biomarkers were performed to identify associated biomarkers from a specific site.

↓

Since both environments were associated with joint damage, in particular the synovial joint, ROC analysis of the collated biomarker profile were performed to determine its potential for accurately identifying erosive disease.

4.2.3 Association with cigarette smoking and 4.2.4 Association with DMARD treatment

Analyses were performed to determine whether cigarette smoking were associated with any effects on immune activity in either environment.

↓

Discriminant selection of the serum and the synovial fluid mediators (separate analyses) followed by logistic regression of selected biomarkers in both current and past smokers were alternatively performed (ever smokers could not be performed due to limited patient range) to identify potentially associated biomarkers at both environments.

↓

Analyses were similarly performed to determine whether DMARD treatment were associated with any effects on immune activity in either environment.

↓

Discriminant selection and logistic regression of selected biomarkers in both the serum and the synovial fluid of DMARD patients were performed to identify potentially associated biomarkers at both environments.

Flow Chart: 4.3 RA biomarker profiling in comparison with other arthritides

Initial comparisons of RA and the other arthritides were preformed for preliminary assessment.

↓

One-way ANOVA's of disease measure indices were performed to compare the level of activity & severity between the arthritides.

↓

Mediators were subsequently compared by one-way ANOVA to determine potentially associated biomarkers.

4.3.1 Arthritides differentiation

Analyses were performed to identify potentially associated biomarker profiles for distinguishing RA from the other arthritides.

↓

Hierarchical clustering analysis (HCA) of mediators was performed to determine if mediator expression patterns could differentiate the arthritides.

↓

Another HCA excluding a RA subgroup with high mediator expression identified from the previous HCA was performed since extreme expression patterns can skew HCA.

↓

HCA identified a RA prominent subgroup that was analysed with two-sample T-tests with remaining patients to determine potentially associated biomarkers.

↓

Subsequently, discriminant analysis for initial biomarker selection followed by logistic regression of selected biomarkers were performed for identification of a potentially associated RA biomarker profile.

↓

A separate HCA excluding all RA patients was also performed to determine if mediator expression patterns could differentiate between the other arthritides.

↓

A statistical approach was also employed, where discriminant analysis of all mediators followed by logistic regression of selected biomarkers for each arthritis were also performed, for identification of a potentially associated biomarker profile for each of the arthritides.

↓

ROC analysis of the composite biomarker profile collated from the 2 profiling methods were performed to determine its potential for accurately distinguishing RA and as a diagnosis profile.

4.3.2 Disease activity and severity biomarker profiling between the different arthritides

Analyses were performed to identify a potential biomarker profile for worse RA in order to identify more severe and damaging type of arthritis.

↓

Since initial analyses identified no biomarker profiles for severity, Spearman's rank correlation of mediators & disease measure indices were performed on each arthritis excluding RA (regression analyses could not be performed due to small patient numbers) to identify potentially associated biomarker profiles.

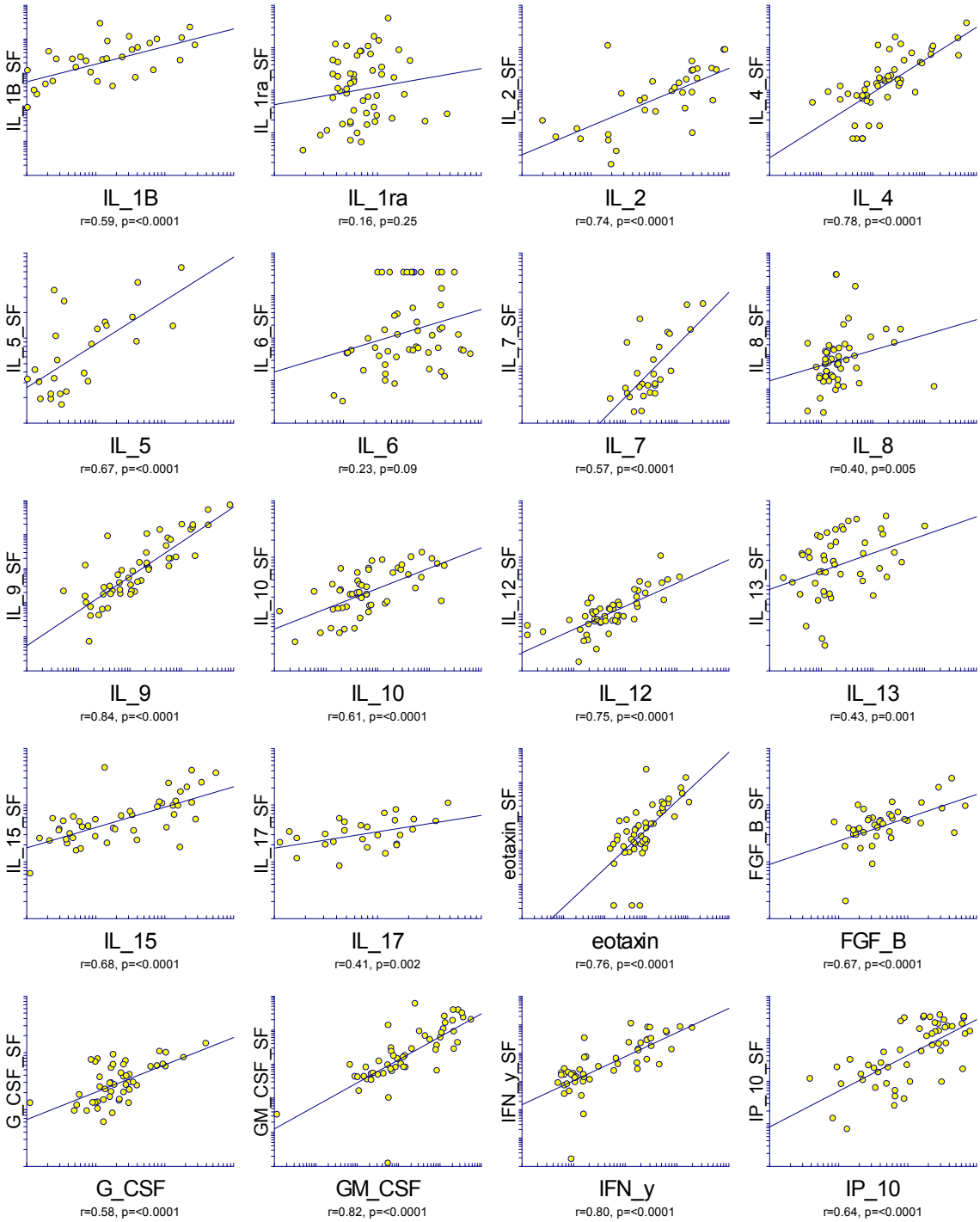
↓

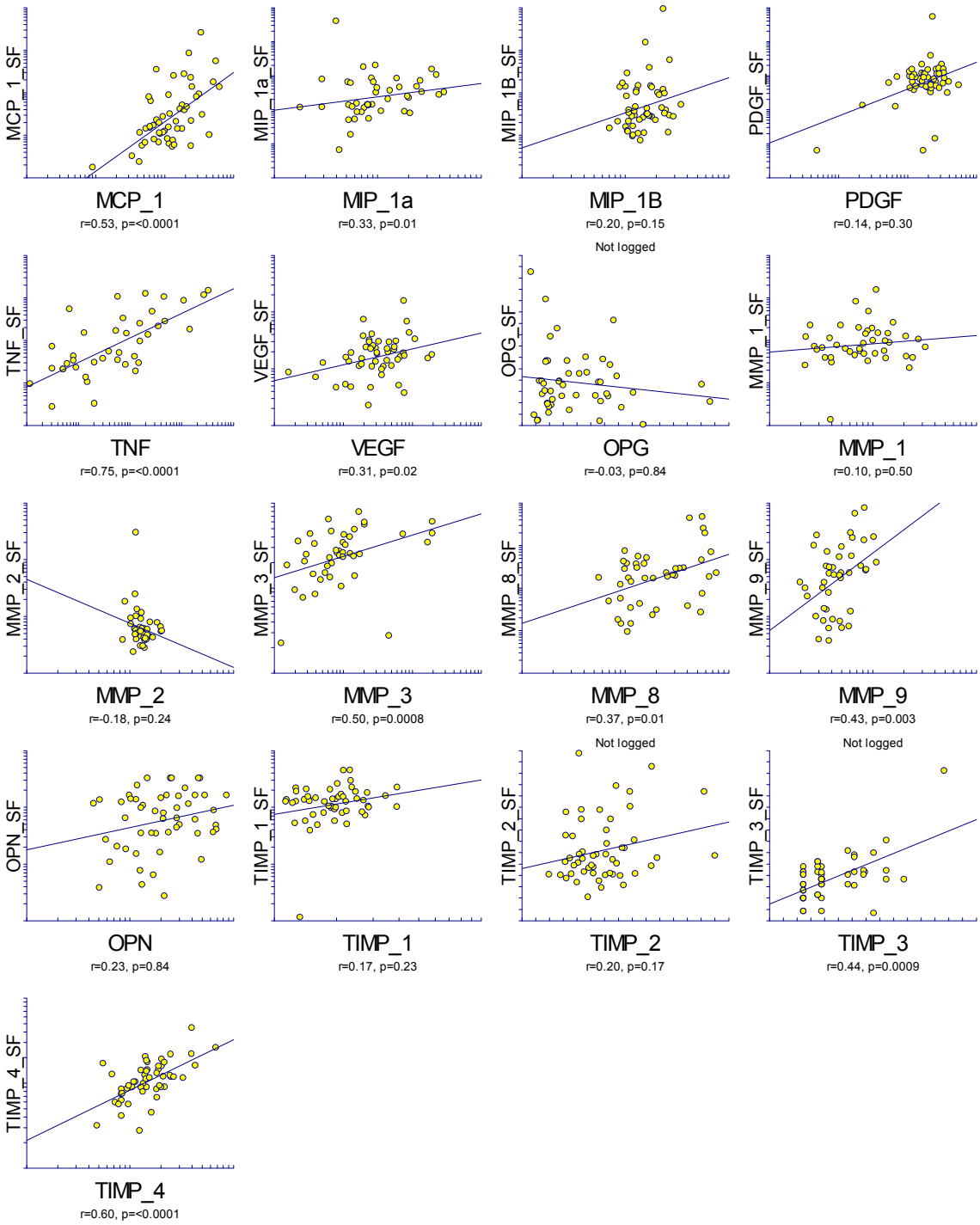
Correlated biomarker profiles and early RA PCA biomarker profiles were manually compared to identify a biomarker profile potentially associated with RA disease severity.

↓

ROC analysis of the identified RA severity biomarker profile was performed to determine its potential for accurately identifying worse disease.

Appendix figure 4.2b: Correlation matrix of mediators (logged) (excluding Dkk-1 and RANTES)



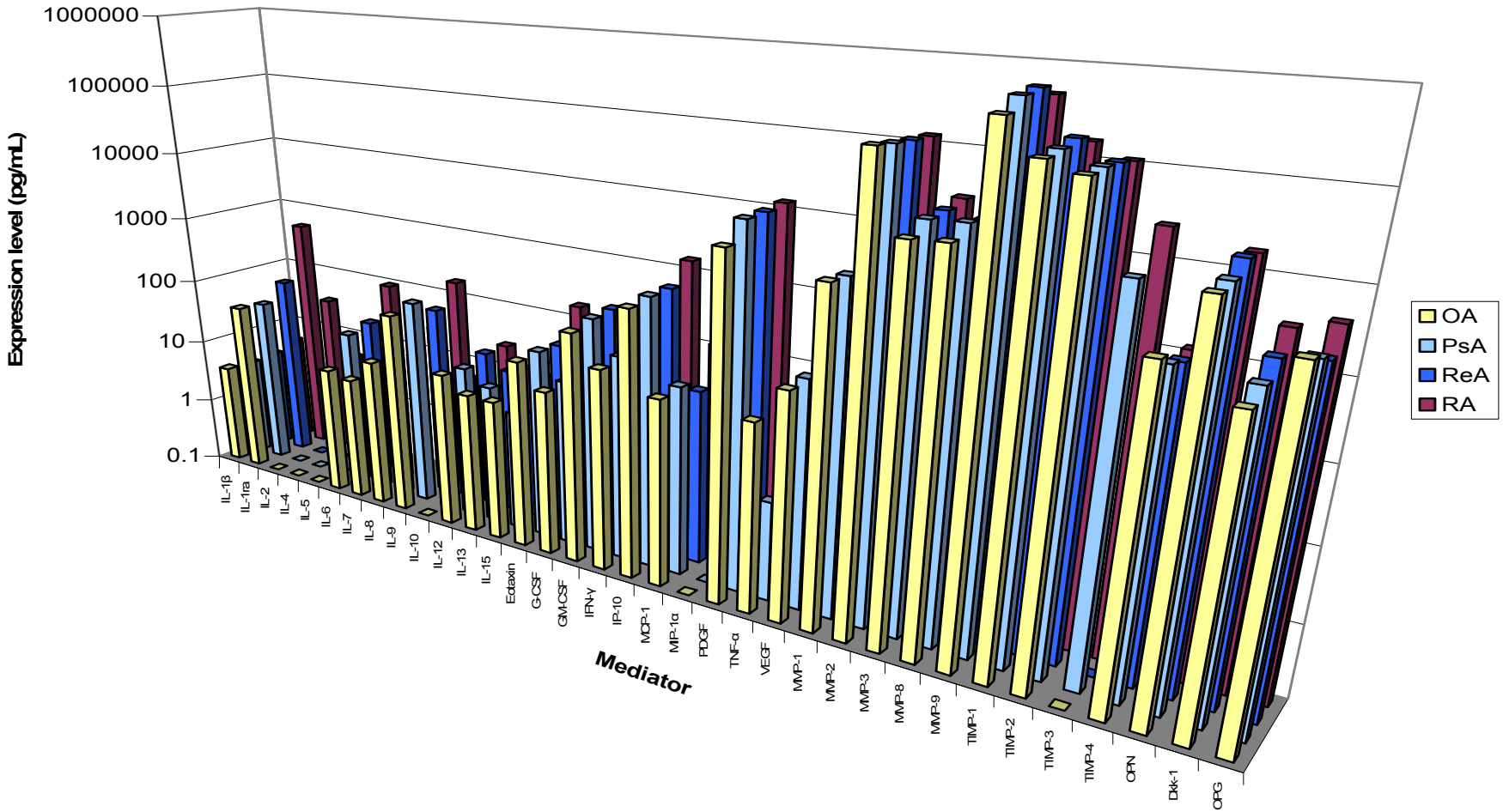

MCP_1_SF
MCP_1
r=0.53, p=<0.0001
MIP_1a_SF
MIP_1a
r=0.33, p=0.01
MIP_1B_SF
MIP_1B
r=0.20, p=0.15
PDGF_SF
PDGF
r=0.14, p=0.30
TNF_SF
TNF
r=0.75, p=<0.0001
VEGF_SF
VEGF
r=0.31, p=0.02
Not logged
OPG_SF
OPG
r=-0.03, p=0.84
MMP_1_SF
MMP_1
r=0.10, p=0.50
MMP_2_SF
MMP_2
r=-0.18, p=0.24
MMP_3_SF
MMP_3
r=0.50, p=0.0008
MMP_8_SF
MMP_8
r=0.37, p=0.01
MMP_9_SF
MMP_9
r=0.43, p=0.003
Not logged
OPN_SF
OPN
r=0.23, p=0.84
TIMP_1_SF
TIMP_1
r=0.17, p=0.23
Not logged
TIMP_2_SF
TIMP_2
r=0.20, p=0.17
Not logged
TIMP_3_SF
TIMP_3
r=0.44, p=0.0009
TIMP_4_SF
TIMP_4
r=0.60, p=<0.0001

Appendix Table 4.3a: Comparison of disease measures between the different arthritides

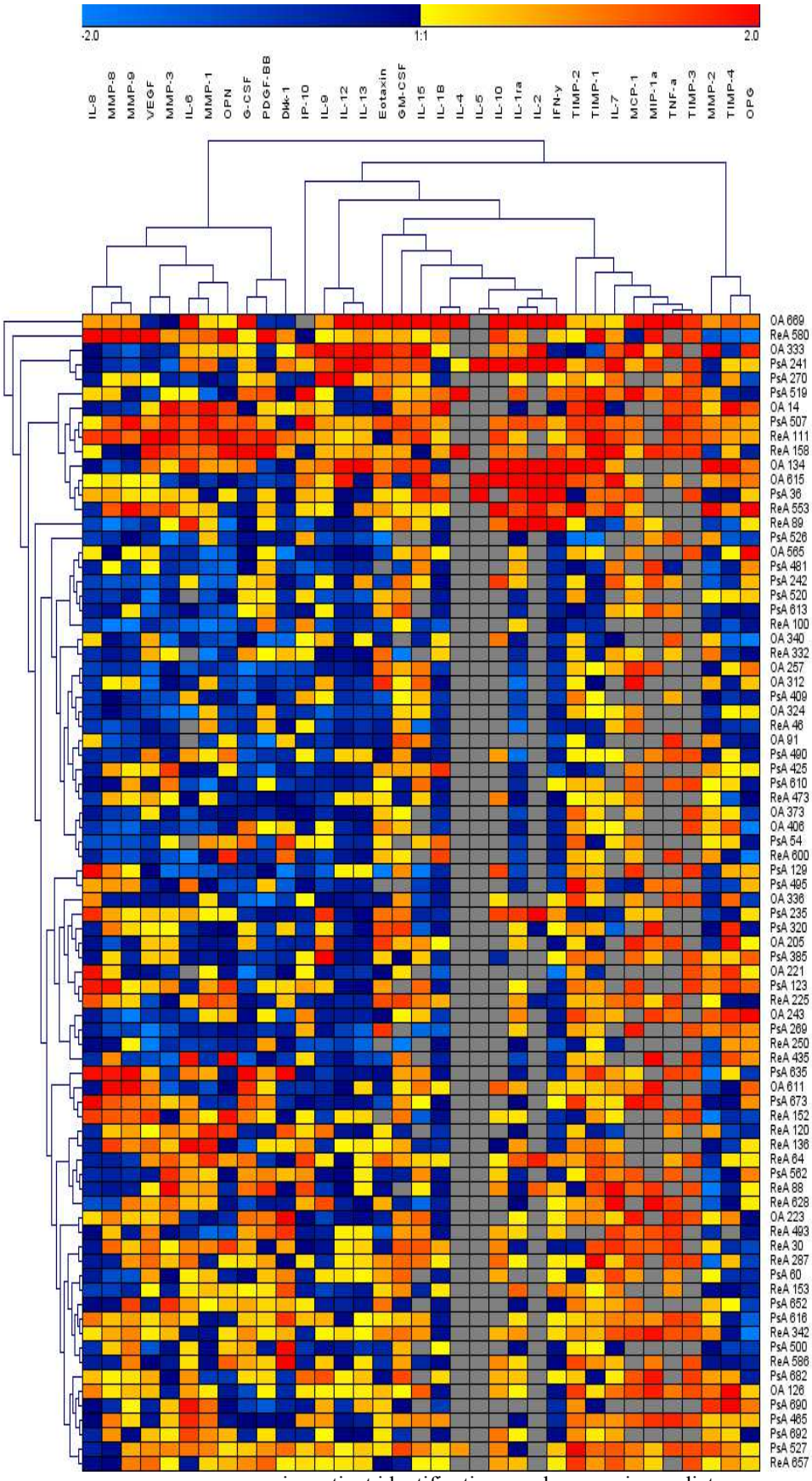
Disease Measure	Variable	OA	PsA	ReA	RA	p-value†
Inflammation	ESR	17 (7-29.7)	24 (13-37)	33.5 (7.5-71.5)	41 (19-66.7)	0.0008
	CRP	5 (2.7-11)	13 (6-25)	18 (3-110)	29 (6-69)	0.001
	Platelets	259 (244-312)	300.5 (266.7-350.2)	349 (260.2-481)	339 (286.5-429.5)	0.004
	WBC	7.1 (6.2-9.3)	8.3 (7.1-9.6)	8.75 (7.2-9.6)	8.5 (6.6-10.5)	0.25
Pain	CGDA	23.5 (8.7-50.2)	35 (21-56.5)	35 (24-53)	47 (26.7-71)	0.006
	SJ	2 (1-3)	3 (1.75-5)	2 (1-4)	5 (3-9)	<0.0001
	VAS	37.5 (13.2-62.7)	65 (33-78.2)	50.5 (31.5-74.5)	55 (39-72)	0.16
	TJ	3 (1-4.7)	6 (1.7-10)	2.5 (1.7-5.2)	9 (4.5-13)	<0.0001
Function	EMS	10 (0-75)	45 (3.7-120)	30 (0-75)	60 (30-120)	0.009
	HAQ	0.93 (0.3-1.5)	0.87 (0.3-1.5)	0.56 (0.21-1.4)	1.5 (0.7-2)	0.0004
	Grip strength	212.5 (120-289.5)	135 (89-260)	300 (232.5-300)	88.5 (65-120.2)	<0.0001

The Mean $\pm$SD for parametric data, the median (lower and upper interquartile range (IQR)) for non-parametric data. † One-way ANOVA or Kruskal-Wallis test as appropriate.

Appendix figure 4.3b: Comparison of mediator expression levels between the different arthritides



Appendix figure 4.3e: Heat map of 35 biomarkers in OA, PsA and ReA patients



y-axis=patient identification number, x-axis=mediators