Metabolomics of Inflammatory Arthritis

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Abstract

Background Rheumatoid arthritis (RA) is associated with both local and systemic inflammation which influences the function of the whole body as well as local tissues in the joints. Significant consequences of this are changes in metabolism. Hence, we hypothesised that chronic inflammation alters metabolism and that the metabolic profile of an individual patient with early inflammatory arthritis predicts the subsequent course of disease. Furthermore, we suggested that these metabolic changes would identify biomarkers of response to treatment in inflammatory arthritis and provide novel insights into disease mechanisms.

Methods Using NMR spectroscopy of serum, urine and synovial fibroblasts we derived metabolic profiles and subjected these to multi-parameter analyses to identify metabolic differences associated with inflammation.

Results We were able to predict outcome in patients with early arthritis using material derived from cultured synovial fibroblasts but were unable to do so using serum. There was a significant association between CRP levels in the patients' serum and the metabolic profile of their synovial fibroblasts and their serum. There was also a significant association between the metabolomic fingerprint of synovial fibroblasts and the fibroblasts' IL6 production. We found differences in metabolites between urine samples of RA and psoriatic arthritis (PsA) patients and were able to predict responses to anti-TNF therapy in patients with RA.

Discussion Our results demonstrate that underlying inflammatory processes drive significant changes in metabolism that can be measured in the peripheral blood, synovial fibroblasts and urine samples in patients with inflammatory arthritis.

Declaration

I have carried out all the work in this thesis myself including writing all the initial manuscripts that have been published from this thesis apart from:

1. Synovial biopsies which were done by Dr Andrew Filer and initial culture was done by a technician.
2. Measurement of glutamine levels in the urine which were done at Birmingham Childrens Hospital
3. Some of the clinical samples were collected by other clinicians including myself
4. The serum samples from group 1 of the early arthritis cohort were processed by Dr Stephen Young but analysed by me.

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Publications

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Abbreviations

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
ANOVA	Analysis of variance
Anti-CarP	Anti-carbamylated antibodies
AS	Ankylosing spondylitis
ATIC	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase
BCAA	Branched chain amino acids
CD	Crohn's disease
CHD	Coronary heart disease
CSF	Cerebrospinal fluid
CTLA-4	Cytotoxic T-lymphocyte associated 4
CU	Chronic non-infectious uveitis
DAS	Disease activity score
DATA	Differentiating the mechanism of action of anti-TNF-α agents
DMARDs	Disease-modifying anti-rheumatic drugs
D_2O	Deuterium oxide
EBV	Epstein-Barr virus
ELISAs	Enzyme-linked immune-sorbent assays
EPA	Eicosapentaenoic acid
EULAR	European League Against Rheumatism
GALGO	Genetic ALGOrithm
GSTM1	Glutathione S-transferase M1
HIF	Hypoxia-inducible factor
IBD	Inflammatory bowel disease
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
LV	Latent variable
LIU	Lens-induced uveitis
MCP	Metacarpopharyngeal
MCP-1	Monocyte chemotactic protein-1
MIP-1α	Macrophage inflammatory protein-1α
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NOAR	Norfolk Arthritis Register
OA	Osteoarthritis
PADI4	Peptidyl arginine deiminase type IV
PBS	Phosphate buffered saline
PCA	Principal component analysis
PLSDA	Partial least squared discriminate analysis
PLSR	Partial least squares regression analysis
PPM	Parts per million
PsA	Psoriatic arthritis
PTPN22	Protein tyrosine phosphate, non-receptor type 22
RA	Rheumatoid arthritis
RANKL	RANK ligand

RANTES	Regulated on activation, normal T cell expressed and secreted
RASFs	Rheumatoid arthritis synovial fibroblasts
ReA	Reactive arthritis
RF	Rheumatoid factor
SE	Shared epitope
SF	Synovial fluid
SNP	Single nucleotide polymorphism
SIL6R	Soluble IL6 receptor
STAT4	signal transducer and activator of transcription 4
TCR	T cell receptor
TCZ	Tocilizumab
TMA	Trimethylamine
TMSP	Trimethylsilyl 2,2,3,3-tetradeuteropropionic acid
TNF-α	Tumour necrosis factor-alpha
TNFAI3	TNFα-induced protein 3
UC	Ulcerative colitis
VEGF	Vascular endothelial growth factor
VIP	Variable importance of the projection

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1.0 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by swelling, tenderness and destruction of synovial joints (Smolen and Aletaha 2008). It leads to severe disability and premature mortality (Kyburz and Finckh 2013). Its aetiology is not fully understood but genetic and environmental factors both contribute (Smolen and Aletaha 2008). In addition to synovitis a systemic inflammatory response is also seen involving changes in many systems and organs.

Systemic inflammation leads to alterations in metabolism and several studies have investigated the resulting changes in low molecular weight metabolites in patients and animal models of inflammation (Bezabeh et al. 2009;Lin et al. 2009;Marchesi et al. 2007;Sinclair et al. 2010;Young et al. 2009). Nuclear magnetic resonance (NMR) has been used to identify alterations in a broad range of different metabolites simultaneously and from such studies several key metabolites have been identified, providing insights into the mechanisms of disease and also representing potential biomarkers which can be used to follow the disease and assess responses to therapy (Brindle et al. 2003;Sreekumar et al. 2009). RA has significant effects on metabolism and in this project I aimed to use NMR to identify potential biomarkers for outcome and response to therapy in inflammatory arthritis and to provide novel insights into mechanisms of disease.

1.1 Rheumatoid Arthritis

1.1.1 Classification

Careful classification of diseases is an important step in allowing more accurate and consistent identification of patient populations with clearly defined characteristics for inclusion in research studies and the first criteria for RA were proposed in 1956 by a

committee of the American Rheumatism Association (Bennett et al. 1956) These classified patients as having 'definite', 'probable' or 'possible' RA. In 1958 the same committee made revisions to improve sensitivity and specificity but the criteria were (Ropes et al. 1959) felt to be cumbersome and were never widely used. Not until 1987 were classification criteria widely accepted and these were developed by the American College of Rheumatology (ACR) (Arnett et al. 1988)(Table 1.1). Over this 30 year period several forms of arthritis which had previously been classified as RA (including many of the seronegative spondyloarthropathies) had been given separate identities necessitating the development of new RA criteria. The new criteria demonstrated 91-94% sensitivity and 89% specificity for RA when compared to control subjects who did not have RA (Arnett et al. 1988).

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posterioanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localised in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

Table 1.1 The 1987 revised criteria for the classification of rheumatoid arthritis*

*For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded.

Although the 1987 classification criteria were used widely for several decades, they were criticised for their lack of sensitivity in early RA (Harrison et al. 1998) and so this led to the development of the 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for RA (Aletaha et al. 2010) (Table 1.2). Several recent studies have assessed how accurately these identify patients with early RA in patients with newly presenting inflammatory arthritis and they have found that the 2010 criteria allowed earlier classification of RA than the 1987 criteria (Cader et al. 2011;Humphreys and Symmons 2013;van der Helm-van Mil AH and Huizinga 2012), but that they also led to over-classification of RA in patients as several patients, whose arthritis had resolved, were classified as having RA (Cader et al. 2011). Researchers have found that the sensitivity of the 2010 criteria has increased compared to the 1987 criteria but the specificity has decreased (van der Helm-van Mil AH and Huizinga 2012). This is concerning as these criteria are classification criteria used to standardise inclusion into studies rather than to make a diagnosis of RA so specificity is extremely important.

Criterion	Score
A. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low=positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Table 1.2 The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis

*For classification purposes, a patient shall be said to have definite rheumatoid arthritis with a score of ≥ 6/10

1.1.2 Aetiology

1.1.2.1 Genetic factors

Rheumatoid arthritis has a complex aetiology with genetic and environmental factors contributing to pathological events. Based on twin studies the relative contribution of genetic factors is approximately 50% for the entire syndrome of RA (MacGregor et al. 2000). There have been extensive efforts to identify the associated genes and most of these studies have focussed on linkage and candidate gene associations. More recently genome-wide association studies have been used to study RA and several other autoimmune conditions (Burton et al. 2007). The association between RA and alleles of the HLA-DRB1 locus (shared epitope) has long been recognised (Gregersen et al. 1987) and these are the strongest genetic risk factors for RA. They encode a common amino acid motif, called the shared epitope (SE), in the β chain of the MHC class II HLA-DR molecule. The

presence or absence of genetic polymorphisms within the DRB1 locus is an important susceptibility factor. The genetic association of HLA-DR with RA, together with the presence of MHC class II-expressing cells and T cells in the rheumatoid synovium, led to the hypothesis that MHC class II-dependent T-cell and B-cell activation is responsible for driving the disease process in RA.

The second most important genetic risk factor in RA is PTPN22 (protein tyrosine phosphate, non-receptor type 22), a gene encoding a protein tyrosine phosphatase that has a role in T cell and B cell signalling (Begovich et al. 2004;Burkhardt et al. 2006;Hinks et al. 2005). A single nucleotide polymorphism (SNP) that results in the substitution of arginine to tryptophan (R620W) affects its interaction with Src tyrosine kinases involved in regulation of T cell receptor (TCR) signalling. This leads to altered regulation of TCR signalling during thymic selection and may permit autoreactive T cells to escape clonal deletion, thereby predisposing to autoimmunity (Vang et al. 2005). The PTPN22 R620W polymorphism has been associated with increased IgG autoantibodies against an immunodominant collagen type II epitope in early rheumatoid arthritis (Burkhardt et al. 2006).

More recently, several other genetic loci associated with RA susceptibility have been identified by large-scale genotyping of RA patients for SNPs. Such studies have highlighted RA-associated polymorphisms in a region containing TNF receptor-TRAF-1 and C5 complement (Plenge et al. 2007a;Plenge et al. 2007b), and in the gene encoding signal transducer and activator of transcription 4 (STAT4) (Remmers et al. 2007). Four SNPs in the STAT4 gene are associated with an increased risk of RA, most significantly

rs7574865. STAT4 is an intracellular molecule that transduces signals downstream of type I interferons, IL-12 and IL-23 and regulates differentiation of Th1 and Th17 cells, and is known to be upregulated in the RA synovium. Other loci associated with disease susceptibility and severity include CTLA4 which down regulates CD28-mediated T cell co-simulation; peptidyl arginine deiminase type IV (PADI4) which catalyses post-translational modifications of arginine to citrulline; and TNFα-induced protein 3 (TNFAI3) which functions as a negative regulator of NF-κB (Eyre et al. 2012). A range of other potential genetic associations remain to be investigated.

All the genes that have been identified so far are associated with multiple autoimmune diseases including juvenile idiopathic arthritis, type 1 diabetes, coeliac disease and Graves disease. So far, no locus has been identified which is exclusively associated with RA (Barton and Worthington 2009). Although, there has been some progress in identifying the genetic factors that contribute to RA the most important genetic factors (the SE and PTNP22) seem only to predispose to ACPA (anti-citrullinated protein antibody) positive disease (Bax et al. 2011), suggesting that ACPA positive RA may be a completely different disease to ACPA negative RA. The heritability of ACPA negative RA is comparable to that of ACPA positive RA but the genes responsible for this association have not yet been identified due to the smaller population sizes studied (van der Woude et al. 2009).

Hence, it seems that although genes are important, interactions of the genotype with environmental factors are necessary to precipitate and drive the disease. The factors which

contribute to the other 50% of the aetiology include age, infections, diet, smoking and hormones which are considered in the following sections.

1.1.2.2 Environmental factors

1.1.2.2.1 Smoking

Smoking is known to increase the risk of RA with several studies showing a positive link in women. Fewer studies have been carried out in males but the risk is felt to be even greater (Sugiyama et al. 2010). A population-based case-controlled study conducted in Norfolk (NOAR) found that people who had ever smoked had an increased risk of developing RA (Symmons 2005) and a recent prospective study demonstrated an increased risk of developing RA in antibody positive patients with this risk increased if they had a high body mass index (de Hair et al. 2013).

Several studies have shown a relationship between smoking and RF positivity. In those that smoke, glutathione S-transferase M1 has been linked to increased severity of RA (Mattey et al. 2002). Glutathione S-transferase polymorphisms affect the ability to detoxify chemicals in cigarette smoke which may influence the progression of RA.

Smoking can affect metabolism in other ways. Chronic smoking causes marked oxidative stress which may be responsible for a constant inflammatory process. Oxidative stress is associated with low antioxidant levels (Baka et al. 2009) and smoke contains high amounts of free radicals. Smoking induces the depletion of the endogenous antioxidant glutathione, resulting in cell injury (Moriarty et al. 2003).

Recent data suggest that smoking is linked only to ACPA positive RA (Baka et al. 2009). ACPA positive RA and ACPA negative RA are thought to be genetically distinct where HLA-DRB1 shared epitope alleles and the RA susceptibility polymorphisms in PTPN22 are associated only with ACPA positive RA (Bowes and Barton 2008). Data suggest that smoking increases the risk of ACPA positive RA only in shared epitope positive patients (Baka et al. 2009;Mahdi et al. 2009). There seems to be a 4 way relationship between smoking, periodontitis, the shared epitope and ACPA positive RA with periodontitis being more common in patients that smoke and linked to the shared epitope (Lundberg et al. 2010) and periodontitis being more prevalent in patients who have ACPA positive RA (Dissick et al. 2010). These epidemiological and longitudinal studies have helped to produce data which can give us insights into the pathogenesis of ACPA positive RA.

Current research suggests that smoking triggers citrullination in the lungs and autoimmunity (to citrullinated self-molecules) starts in the lungs and then spreads to other areas such as the joints (Klareskog et al. 2011). The citrullination of proteins in the lung causes enhanced binding of citrullinated peptides and in genetically susceptible individuals this could lead to the production of ACPA. Inflammatory arthritis accompanied by citrullination of proteins in the joints develops later and recruitment of ACPA from the circulation leads to the formation of immune complexes. Evidence of increased citrullination has been seen in the lungs, arthritic joints, extra-articular inflammatory sites in RA, inflamed muscle and inflamed lymphoid organs (Klareskog et al. 2008). Immune complex formation leads to the production of cytokines and autoantibodies and results in chronic RA (Klareskog et al. 2008).

1.1.2.2.2 Diet

Antioxidants neutralize the actions of reactive oxygen species and hence reduce some of the effects of inflammation. The NOAR (Norfolk Arthritis Register) EPIC study found that those individuals with the greatest vitamin C intake had the lowest risk of developing polyarthritis (Pattison et al. 2004a). In contrast, high red meat intake has been shown to be associated with an increased risk of developing RA (Pattison et al. 2004b). Red meat is a rich source of iron and could promote oxidative damage since it has been shown to accumulate in the rheumatoid synovial membrane (Blake et al. 1981). Fish consumption and omega-3 fatty acids have also been found to be associated with a reduced incidence of RA and this may be due to the eicosanoids in omega-3 fatty acids competing with arachidonic acid resulting in less proinflammatory eicosanoids (Shapiro et al. 1996).

Data demonstrate that alcohol consumption has a protective effect on the development of RA, with a 40-50% reduction in risk for the highest consumers (Bergstrom et al. 2013;Kallberg et al. 2009). Further studies have shown that alcohol consumption influences the severity of RA, as measured by radiographic progression, with a trend towards reduced radiographic progression in alcohol drinkers compared to non-drinkers. In particular, male patients with RA who consume alcohol had less radiographic progression than male non-drinkers (Nissen et al. 2010). This shows that lifestyle may have a very important part to play in the aetiology of RA.

1.1.2.2.3 Infections

Several infections including Epstein-Barr virus (EBV) have been linked to RA. RA patients have higher titres of anti-EBV antibodies and impaired T-cell responses to EBV antigens compared to controls, and EBV DNA has been found in the synovium of patients

with RA (Toussirot and Roudier 2008). However, an epidemiological study using the NOAR cohort, looking at a range of infections, found no association between RA and measles, rubella, glandular fever, hepatitis or TB (Symmons et al. 1997). This study did, however, find an association between development of RA and previous blood transfusions (Symmons et al. 1997).

Periodontitis, which is a chronic inflammatory disease causing significant tooth loss, has also been linked to RA. Epidemiological and clinical studies have suggested that periodontitis and tooth loss are more prevalent in individuals with RA but the strength of this association is uncertain (Chen et al. 2013;de Pablo et al. 2009), but has been biologically linked to enhanced citrullination and so is a continuing area of research.

1.1.2.3 Biological factors

1.1.2.3.1 Age

While RA can occur at any age, there is a low incidence in men under the age of 45 and the peak age of onset in women is 45 to 54 years of age using the 1987 ACR classification criteria and 55-64 years of age using the 2010 classification criteria (Humphreys et al. 2013). The incidence is generally much higher in women than men but over the age of 75 the incidence is higher for men than women (Humphreys et al. 2013). The prevalence rises with age and is highest over the age of 75. The incidence rates are generally similar using the 1987 or the 2010 classification criteria (Humphreys et al. 2013) but men generally have much more severe disease than women (Symmons 2005) and the disease may be more severe with later onset (Van Der et al. 1991). The reasons for this association with age are the subject of much debate. For example, antioxidants are low in

the elderly (Hu et al. 2000) which may allow oxidative damage to accumulate in long lived cells such as lymphocytes. Elderly humans have dysregulated immune responses with changes in both adaptive and innate immunity. A shift in T cells to a predominantly memory phenotype may contribute to the decline in IL2 production, and an increased tendency to produce a Th2 profile of cytokines (Ely et al. 2007) which may explain the increased occurrence of autoantibody levels. An increase in the basal level of CRP in the elderly suggests a low grade inflammatory process is present (Lisko et al. 2012) which may provide an environment conducive to the occurrence of the more overt inflammation seen in diseases such as RA.

1.1.2.3.2 Hormones

RA frequently remits during pregnancy and there is an increased incidence postpartum. Current oral contraceptive pill use has a protective effect and there is an association with miscarriage and termination of pregnancy of an increased risk of developing RA (Symmons 2005). Oral contraceptive use in women seems to act early in the development of RA as it has been found to have an inverse relationship with rheumatoid factor in asymptomatic genetically susceptible women (Bhatia et al. 2007). What is still not clear is whether hormonal changes explain the gender differences seen in RA as it is well known that RA is more common in females than males. As well as the hormone variation between females and males there is also a difference in body fat composition which may explain the gender differences seen. Females have a higher body fat mass composition compared to males and high body fat mass has been shown to be linked with RA (Book et al. 2009).

1.1.2.4 Summary

Several factors have been associated with the aetiology of RA but the main genetic factors (SE and PTNP22) and the most strongly associated environmental factor (smoking) seem only to be associated with ACPA positive RA. Work involving these aetiological factors has given us insights into the pathogenesis of ACPA positive RA but there is still a lot of uncertainty regarding aetiology; in particular in the context of ACPA negative RA.

1.1.3 Pathology

The synovium is the primary site of inflammation in RA. The synovial membrane is normally composed of a single cell lining layer of macrophages and fibroblasts. The sub-lining layer is composed of relatively acellular connective tissue and blood vessels. In RA the synovial membrane becomes hyperplastic with macrophages and synovial fibroblasts infiltrating the lining layer and T cells, B cells, plasma cells and macrophages infiltrating the sub-lining layer (Bartok and Firestein 2010).

In patients with RA, the sub-lining layer becomes oedematous and vascular. The inflamed synovium forms a pannus lesion which invades the adjacent cartilage and bone promoting articular destruction by osteoclasts, chondrocytes and synovial fibroblasts (Karmakar et al. 2010).

Several types of immune cell are present in the synovium, which are very active metabolically. During chronic inflammation leucocytes are recruited in response to changes in the expression of proinflammatory adhesion molecules and chemoattractant cytokines and they accumulate within the synovium (Raza et al. 2006).

I will discuss the role of synovial fibroblasts further as these are phenotypically unusual in RA and an important cell type that I have studied in my project.

1.1.3.1 Synovial fibroblasts

Inflammation and its persistence in RA result from complex interactions between haematopoietic and stromal cells (Buckley et al. 2001;Parsonage et al. 2005) but research into the pathogenesis of the disease has traditionally neglected the role of stromal cells. Fibroblasts are primarily responsible for the synthesis and remodelling of extracellular matrix components in tissues. However, their ability to produce and respond to growth factors enables the maintenance and homeostasis of the adjacent epithelial and endothelial cells (Naylor et al. 2013). Activated RA synovial fibroblasts (RASFs) have an important role in determining the site at which inflammation occurs and the persistence of inflammation in the joint microenvironment (Takemura et al. 2001). Fibroblast activation causes survival and retention of leukocytes leading to accumulation at sites of inflammation (Iwamoto et al. 2008), hence preventing the resolution of chronic inflammation (Filer et al. 2006). Fibroblasts have an important role in altering the behaviour of lymphocytes that accumulate within chronically inflamed joints by the production of the cytokines interferon-β, interleukin 6 (IL-6) and transforming growth factor-β and the chemokines stromal cell-derived factor-1 and CXCL12 (Buckley 2003). Fibroblasts also provide an environment which inhibits T-cell apoptosis, and type I interferons produced by fibroblasts and macrophages have been identified as the principal factor responsible for prolonged T-cell survival in the rheumatoid joint (Pilling et al. 1999).

RASFs are characterised by a round, large pale nucleus with prominent nucleoli, indicating active RNA metabolism. RASFs can be isolated from synovial tissue and expanded in cell culture over several passages without undergoing contact inhibition (Lafyatis et al. 1989) which is different from other cells such as macrophages.

The mechanism underlying the expansion in numbers of RASFs in the lining layer of the synovial membrane is not fully understood. In the joint, fibroblasts have limited access to nutrients and oxygen within the poorly vascularised synovium and yet expansion of fibroblast numbers still occurs. This may be due to the migration of mesenchymal stem cells from the circulation or local expansion of a stem cell pool within the synovium. However, growth in the size of the RASFs population is most likely due to an imbalance between cell proliferation, survival and death (Bartok and Firestein 2010). The synovial environment in RA promotes survival of RASFs. RASFs are protected from apoptosis by the activity of NF-κβ. NF-κβ is highly activated in RA and provides a strong pro-survival signal (Han et al. 1998). Expression of the tumour suppressor gene p53 is induced by cell damage and leads to cell cycle arrest and if the damage is substantial p53 directs cells towards apoptosis. There is an increase in p53 expression in the rheumatoid synovial lining but a lack of apoptosis. Several mutations have been found in the p53 gene (Firestein et al. 1997) in RA synovium, potentially eliminating a key pathway that regulates cell survival which may explain the lack of apoptosis seen. However, this observation has not been widely reproduced.

Cartilage and bone destruction are the hallmarks of RA. Over a decade ago, RASFs were shown to attach to and invade normal human cartilage (Mueller-Ladner et al. 1996) and recent data suggest that RASFs are primarily responsible for cartilage damage (Bartok and Firestein 2010). In situ hybridisation studies confirm that RASFs are the source of matrix metalloproteinases and cathepsins which destroy articular cartilage and bone (Tolboom et al. 2002). RASFs are also involved in bone degradation through activation of osteoclasts and by producing RANKL (Bartok and Firestein 2010).

RASFs are the dominant cell type in the hyperplastic synovial membrane. It is possible that these cells may be a significant source of metabolites as it has been shown that administration of FGF 21 (a member of the fibroblast growth factor family) to mice, resulted in significant changes in plasma lipids and glucose (Li et al. 2009). FGF 21 has significant effects on fibroblasts and adipocytes by regulating lipolysis (Li et al. 2012), which demonstrates a potential role for RASFs to be involved in the regulation of lipid related metabolites found in the blood. Hence, RASFs are an important cell to study in the context of metabolomics.

1.1.3.2 Inflammatory mediators

Cytokines are important regulators of inflammation since some, such as TNFα, IL-6 and IL-1, promote inflammatory responses which induce cartilage degradation and promote a cell-mediated immune response (Lefebvre et al. 1990). Other cytokines such as IL-4, IL-10 and IL-13 function mainly as anti-inflammatory molecules (Isomaki and Punnonen 1997). Key biological targets that have been identified as being involved in a destructive

inflammatory reaction are COX-2, pro-inflammatory interleukins, TNFα, migration inhibition factor, interferon gamma and matrix metalloproteinases (Ivanenkov et al. 2008).

Several of the inflammatory mediators above have been shown to play a role in a broad range of inflammatory diseases. This is true of CRP, a widely used marker of systemic inflammation which is secreted by several cell types and is capable of directly activating immune cells. This suggests that CRP is an active inflammatory mediator which has systemic and local affects (Montecucco and Mach 2009).

White adipose tissue has been shown to secrete several inflammatory mediators known as adipokines or adipocytokines. They induce their effects by binding to selective transmembrane receptors. Leptin is the most studied adipocytokine and is thought to have an important role in the inflammatory process (Montecucco and Mach 2009).

1.1.4 Autoantibodies

1.1.4.1 Rheumatoid factor

RA is often referred to as an autoimmune disease due to the presence of self-reactive antibodies. Rheumatoid factor (RF), an antibody that binds to the Fc region of IgG is detectable in the serum of the majority of patients with RA (70% - 80%). However, RF is also found in up to 5% of normal individuals and in patients with other systemic diseases such as Sjogren's syndrome and many systemic infections, suggesting its presence is a general consequence of immune activation rather than a RA-specific feature. RF can be detected many years prior to clinical onset (Aho et al. 1985;Rantapaa-Dahlqvist et al. 2003) and levels of RF correlate with disease severity, with patients who are seropositive

having more aggressive disease and a worse prognosis (Symmons and Silman 2006;van et al. 1992).

1.1.4.2. Anti-citrullinated protein antibodies

Anti-citrullinated protein antibodies (ACPA) are highly specific for RA. The most frequently used assay in clinical practice is the CCP2 ELISA which captures ACPAs with a broad range of specificities (Ioan-Facsinay et al. 2011). ACPAs have been described in 41-68% of patients with early RA (Kastbom et al. 2004;Nielen et al. 2004;Raza et al. 2005a;van Gaalen et al. 2004) and in many cases ACPA emerge prior to the onset of symptoms (Berglin et al. 2004;Nielen et al. 2004). The presence of ACPA is associated with more severe joint destruction and greater disease activity, and seropositivity at diagnosis is an important predictor of a more aggressive disease course (Forslind et al. 2004;Kastbom et al. 2004;Vallbracht et al. 2004). ACPA are specific for autoantigens modified by citrullination through deimination of arginine to citrulline, a post-translational modification which increases the affinity of potential autoantigens for the binding groove of the MHC class II β-chains (Hill et al. 2003). Although ACPA are specific and predictive for RA, the role for these antibodies in the disease pathogenesis is not clear. Kuhn *et al* have demonstrated that ACPA have a directly pathogenic role in the collagen-induced mouse model of arthritis, indicating an important role for citrullinated protein antigens in driving pathological events in RA (Kuhn et al. 2006). A recent study targeting citrullinated peptides from α-enolase, vimentin, fibrinogen and collagen type II demonstrated that HLA-DRB1 SE, PTNP22 and smoking are associated with the presence of specific ACPA reactivities rather than anti-CCP levels (Lundberg et al. 2013). Current research does

suggest a role for citrullinated proteins in the presence of smoking in the pathogenesis of RA in a genetically susceptible population (Klareskog et al. 2008).

There is also evidence of immunity to other posttransationally modified antigens including homocitrullination (carbamylation) (Klareskog et al. 2014;Mydel et al. 2010;Shi et al. 2014). Carbamylation takes place at low levels in healthy individuals but at increased levels in people with RA. Anti-carbamylated fetal calf serum antibodies and anti carbamylated human fibrinogen antibodies have been identified in both ACPA positive and ACPA negative RA patients. The presence of anti-carbamylated antibodies (anti-CarP) in ACPA negative patients is associated with more severe joint damage. Anti-CarP antibodies are also found in patients with arthralgia and are associated with an increased risk of developing RA independent of ACPA (Shi et al. 2014). Anti-CarP may be a very useful tool in ACPA negative patients.

1.2 Early Rheumatoid Arthritis

1.2.1 Phases of rheumatoid arthritis

Many of the characteristic features of RA described above are derived from studies of well-established disease. However, RA appears to develop clinically in several stages, usually beginning with swelling and pain in a limited number of joints, followed by a subsequent increase in the number of affected joints and severity of symptoms, and finally resulting in destruction and deformity (Deane et al. 2010b;Gerlag et al. 2012;Schaeverbeke et al. 2012).

Even following the development of clinical symptoms, evidence suggests that during the first few months, patients with early inflammatory arthritis who subsequently develop RA have a distinct but transient synovial fluid cytokine profile that is not seen in other early arthritides and not present in established RA. Synovial fluid from patients with early RA contained elevated levels of T cell, macrophage and stromal cell related cytokines IL-2, IL-4, IL-15 and GM-CSF compared to those early arthritis patients who did not develop RA. This cytokine profile is also not present in established RA (Raza et al. 2005b). However, evidence suggests that when looking at processes within the synovium, the same changes are seen within the first year of diagnosis of RA as seen in those with longer standing disease (Tak 2001).

Synovial histology appears normal with no evidence of infiltrates in ACPA positive arthralgia patients without evidence of synovial inflammation (van de Sande et al. 2011) suggesting that the development of synovial inflammation occurs at the time of clinically apparent synovitis. However, these biopsy samples were taken from the knee which is not the most common site of involvement in early RA.

The serum of patients with very early RA (< 6 weeks duration) has a very distinct cytokine pattern which favours B cell activation and survival. The levels of APRIL and BAFF are significantly higher in patients with very early RA compared to other types of early arthritis, controls and established RA (Moura et al. 2011). Other serum cytokines such as IL-1, IL-6, IL-10, IL-12, IL-15, TNFα, GM-CSF and sTNFRII have been found to be elevated in the preclinical phases of RA but the results from several studies have been conflicting (Deane et al. 2010a;Deane et al. 2010b;Schaeverbeke et al. 2012;Sokolove et

al. 2012). This may be due to differences in the cohorts studied or due to the methodologies and cut offs of the assays used. Further research into pre clinical RA will lead to better understanding of the disease and preventive strategies for RA.

1.2.2 Importance of treating rheumatoid arthritis early

It is important to identify patients who will develop RA as early as possible as it is now known that a reduction in inflammation associated with early therapy limits the articular and extra-articular manifestations of RA (O'Dell 2002;Raza et al. 2006). It has also been shown that aggressive early treatment in these patients leads to earlier functional improvement and less radiographic damage (Goekoop-Ruiterman et al. 2005). Evidence suggests that if patients with RA are treated with DMARDs within 12 weeks of symptom onset they have less joint destruction and a greater chance of drug free remission (van der Linden et al. 2010).

1.2.3 Predicting outcome in early rheumatoid arthritis

Identifying patients that will go on to develop RA can be difficult. Algorithms have been developed to try and identify those patients with undifferentiated arthritis who will go on to develop RA. These algorithms have used a combination of clinical symptoms, joint counts and the presence of antibodies such as RF and ACPA. The Leiden group have introduced a prediction rule which includes 9 variables: sex, age, localisation of symptoms, morning stiffness, tender joint count, swollen joint count, CRP, RF and ACPA (van der Helm-van Mil et al. 2007). The positive and negative predictive values of this score were good for the high and low levels but a large proportion of patients (25%) had an intermediate score for whom it was still difficult to predict outcome. Hence, there are still

many patients that it is difficult to predict outcome for (Raza and Filer 2009) and there is a need for better methods of prediction in these patients.

1.2.4 Predicting response to treatment in rheumatoid arthritis

Treatments for RA have advanced significantly over the last decade but despite the availability of several specific therapies the process of selecting a treatment for an individual patient remains empirical. However, there has been some progress towards personalised treatment of RA.

Methotrexate is the most commonly used disease-modifying anti-rheumatic drug (DMARD) to be used in the treatment of RA and progress towards personalisation of therapy in RA has been more successful for methotrexate than for any other drug, though studies have not yielded consistent results. Several demographic and clinical factors such as male sex, young age, late-onset RA, low disease activity, RF and ACPA status, non smoker, short disease duration, mild functional impairment and low level of radiological damage have been found to correlate with higher rates of remission in RA for patients treated with methotrexate (Katchamart et al. 2010). However, a study from the NOAR register found that clinical and laboratory factors such as age, gender, age at disease onset, baseline RF and CRP were poor predictors of response to treatment with methotrexate (Hider et al. 2009). Several inflammatory cytokines have been evaluated for their potential to predict response to methotrexate therapy. It has been shown that patients who had a good response to methotrexate therapy had a significantly lower ratio of IL-1ra/IL-1 beta, cytokines which are produced by peripheral blood mononuclear cells (Seitz et al. 2003). High serum TNF levels have also been found to be negatively correlated to treatment

responses to methotrexate in patients with RA (Maillefert et al. 2010). Red blood cell methotrexate polyglutamate concentrations have also been proposed as biomarkers of response to treatment in RA (Dervieux et al. 2006) but more research is needed as studies have demonstrated conflicting results (Stamp et al. 2010).

There have been several studies looking into the genetic factors governing responses to treatment with methotrexate. The presence of the 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase (ATIC) SNP has been shown to be associated with a better response to treatment with methotrexate (Keith et al. 2012). Multivariate analysis has demonstrated that four SNPs are associated with favourable treatment responses to methotrexate treatment when these data are combined with clinical data (Keith et al. 2012). Assessment for the presence of these SNPs is not used in clinical practice and this may be due to lack of statistical power in the studies and racial variation. NMR based metabolomics has also been used to predict responses to treatment to methotrexate in RA (Wang et al. 2012). A recent study demonstrated that serum metabolites correlated with an effective response to methotrexate in patients with early RA (Wang et al. 2012).

Following the introduction of biological treatment in RA the ability to predict response to therapy is even more important due to the different modes of actions and cost of these drugs. Several clinical factors have been shown to be associated with a poor response to TNF antagonists such as smoking and a high level of disability at baseline (Hyrich et al. 2006). Lower levels of disease activity and concurrent use of DMARDs, particularly

methotrexate have been shown to be associated with a better response to TNF antagonists (Hyrich et al. 2006).

Several genetic susceptibility markers such as the shared epitope have been investigated to determine if they can predict response to biological therapy but there has been no convincing evidence (Emery and Doerner 2011). A meta-analysis assessed the association between TNF gene polymorphisms and response to TNF antagonists in RA but found no association (Potter et al. 2009). A genome-wide association approach has also been conducted to try and identify genetic factors predicting response to TNF antagonists in RA (Umicevic et al. 2013). They were unable to replicate any markers that have been previously reported but did identify eight new genetic loci, which will need validation in an independent cohort (Umicevic et al. 2013).

There have also been several proteomic studies investigating response to TNF antagonists. One study identified a combination of 24 biomarkers comprising autoantibodies and cytokines which enabled prediction to TNF antagonists (Hueber et al. 2009). Another study has identified the cytokines monocyte chemoattractant protein and epidermal growth factor to be associated with a good response to TNF antagonists (Fabre et al. 2008). However, these studies are limited by small sample size.

Baseline TNF levels have also been investigated in the plasma and the synovium to determine if they have a role in the prediction of response in TNF antagonists (Marotte and Miossec 2010). A study using plasma levels of TNF demonstrated that the clinical

response of patients with high levels of plasma TNF significantly improved with higher levels of TNF antagonists (Takeuchi et al. 2011). In general, high local and systemic levels of TNF prior to treatment with TNF antagonists have been found to be associated with a good response (Marotte and Miossec 2010).

A recent study has investigated the association between central nervous system activity and response to TNF antagonists. This study found that responders to TNF antagonists had higher baseline activation in thalamic, limbic and associative areas of the brain using MRI scanning (Rech et al. 2013). Cytokine concentrations in synovial fluid have also been analysed to determine any prediction of response to TNF antagonists. A study found that synovial fluid levels of IL-6, IL-2 and G-CSF may predict responses to TNF antagonists (Wright et al. 2012). Again, all these studies have small sample sizes.

Several studies have been conducted to try and predict responses to another biological therapy, rituximab. Studies have demonstrated that the presence of antibodies (RF) and ACPA) are associated with better responses to rituximab (Emery and Doerner 2011;Narvaez et al. 2011). A protein biomarker, the interferon signature has been shown to be related to response to rituximab therapy in RA. A study found that patients with a type I interferon low signature had much better responses to rituximab than those with a high signature (Thurlings et al. 2010).

In summary, several studies have attempted to predict response to biological treatments in RA but they have given conflicting results and are limited by small sample size so further studies are still needed.

1.3 Metabolism in inflammatory disease

1.3.1 The inflammatory process

An acute inflammatory reaction is characterised by the classic cardinal signs of inflammation: heat, redness, swelling and pain (Tracy 2006). In experimental settings the temporal relationships between oedema, accumulation of leukocytes and accumulation of monocytes and macrophages are well established. These events, in self-limited inflammatory reactions, are coupled with the release of local factors which prevent further release of leukocytes, which allows resolution (Serhan 2009). The transition from acute inflammation to chronic inflammation is widely viewed as an excess of pro-inflammatory mediators (Burger and Dayer 1995).

1.3.2 Metabolic consequences of inflammation

Many factors contribute to the complex course of inflammatory reactions. Microbiological, immunological and toxic agents can initiate the inflammatory response by activating a variety of humoral and cellular mediators. In the early phase of inflammation, excessive amounts of interleukins and lipid-mediators are released and play an important role in the pathogenesis of organ dysfunction (Heller et al. 1998). Arachidonic acid (AA) is released from membrane phospholipids during inflammatory activation and is metabolised to prostaglandins and leukotrienes. Various strategies have been used to regulate the excessive production of lipid mediators on different levels of

biochemical pathways, such as inhibition of phospholipase A2, the trigger enzyme for release of AA, blockade of cyclooxygenase and lipoxygenase pathways and the development of receptor antagonists against platelet activating factor and leukotrienes (Heller et al. 1998). Some of these agents exert protective effects in different inflammatory disorders such as septic organ failure, RA or asthma, whereas others fail to do so. Encouraging results in cardiovascular morbidity have been obtained by dietary supplementation with long chain omega-3 fatty acids like eicosapentaenoic acid (EPA) (Calder and Yaqoob 2009). In states of inflammation, EPA is released to compete with AA for enzymatic metabolism inducing the production of less inflammatory and chemotactic derivatives (Heller et al. 1998).

When investigating inflammation it is important to take into account the many facets of the inflammatory environment that have the potential to play a role in pathology. Hypoxia is known to be prevalent in inflammatory environments such as those associated with wounds, malignant tumours, bacterial infections and autoimmunity (Eltzschig and Carmeliet 2011;Murdoch et al. 2005). Increasing hypoxia in the inflammatory site is associated with poorer disease outcome such as increased macroscopic synovitis in rheumatoid arthritis (Ng et al. 2010).

Normal physiological oxygen levels are thought to range between 5-12% oxygen (compared to 21% atmospheric oxygen). However, hypoxic tissue oxygen levels in pathological environments can range from as little as 0.5% oxygen to around 2.5% oxygen (Biniecka et al. 2010). Local hypoxia develops as a result of either blood vessel occlusion by inflamed tissues, or when existing supply is insufficient for increased cellular density caused by infiltrating or proliferating inflammatory cells (Biniecka et al. 2010).

Additionally, circulating phagocytes can block blood vessels reducing blood flow into the inflammatory site (Sitkovsky and Lukashev 2005). Normal tissue structures can lend themselves to hypoxia where they are poorly perfused, such as the synovium or the cornea. Tissue alteration associated with inflammation can contribute to hypoxia by altering pressure within the blood vessels causing vessel occlusion and increasing distances between blood vessels (Jawed et al. 1997;Mapp et al. 1995).

There is considerable evidence to support the fact that the inflamed synovium is a hypoxic environment. The tumour environment is known to be hypoxic and extensive angiogenesis reveals the requirement of the tissue to have a better oxygen supply. In RA, oxygen levels in synovial fluid have been directly measured revealing lower oxygen tensions compared with synovial fluid from osteoarthritic patients and patients with traumatic joint injuries (Lund-Olesen 1970).

An elegant cellular oxygen detection system is used by cells to respond to changes in environmental oxygen. Reductions in environmental oxygen lead to the stabilisation of the transcription factor hypoxia-inducible factor (HIF) which is otherwise targeted for depletion in oxygen-rich environments. HIF consists of two subunits α and β and the stability of the α subunit is regulated in an oxygen-dependent manner. Under hypoxic conditions the HIF-α escapes degradation and dimerizes with HIF-β (Aro et al. 2012). HIF expression is therefore suggestive of hypoxic exposure, and has been detected in autoimmune diseases such as RA and multiple sclerosis (Gaber et al. 2009;Hollander et al. 2001;Lassmann 2003). HIF is known to be important in inflammatory development, for example loss of HIF-1a in macrophages is associated with impaired aggregation, motility, invasiveness and killing of bacteria (Cramer et al. 2003).

Hypoxia and HIF stabilisation has a large effect on cellular metabolism. HIF causes a preference for glycolytic metabolism over oxidative phosphorylation by inducing the expression of glycolytic enzymes. This allows ATP generation to continue in the absence of sufficient oxygen albeit at a much reduced efficiency per molecule of glucose. It also induces the upregulation of lactate dehydrogenase A therefore promoting the conversion of pyruvate to lactate (Wheaton and Chandel 2011). Lactate has been detected in many chronic inflammatory conditions such as in inflamed joints (Chang and Wei 2011;Treuhaft and MCCarty 1971) multiple sclerosis, pulmonary inflammation (Serkova et al. 2008) and is thought to play a role in wound healing (Trabold et al. 2003). Conversely, the acidosis associated with increasing lactate concentrations is thought to play a pathogenic role in cell transformation and autoantigen development in some inflammatory environments (Chang and Wei 2011). The detection of lactate in metabolomic studies of disease suggests therefore that there may be an inflammatory component to the disease being investigated which may aid future treatment and understanding. It is also generally accepted that the RA joint is hypoxic and this may cause metabolic changes which could aid our understanding of the disease pathogenesis.

Systemic inflammation causes changes in metabolism. An indication of the strong link between inflammation and metabolic processes is seen in cachexia, the loss of cellular mass associated with disease. The discovery of the involvement of TNFα in this process earnt it the name ‘cachexin’. While TNFα is now known more generally as a mediator of inflammatory responses, the ability of inflammatory cytokines to have such profound effects on cellular and metabolic processes is informative. Rheumatoid cachexia is a result of chronic inflammation and this is characterised by the loss of muscle mass and

preservation of fat mass (Evans et al. 2008). Classically cachexia is characterised by a low BMI. Muscle wasting is a common feature of RA but low BMI is uncommon as the fat mass is preserved or even increased (Summers et al. 2008). Hence, RA patients may present with either the classic low BMI cachexia (1-13% of RA population) (Munro and Capell 1997) or more frequently, the rheumatoid cachexia (10-20% of RA with controlled disease and 38% of patients with active RA) (Engvall et al. 2008;Metsios et al. 2009).

The muscle loss that occurs in rheumatoid cachexia is thought to be due to proinflammatory cytokines such as TNF, IL-1 and IL-6. TNF promotes proteolysis through the ubiquitin-proteasome pathway. There is also some evidence that cytokines may prevent an increase in muscle protein synthesis in response to feeding (anabolic resistance) (Summers et al. 2010). In rheumatoid cachexia the degree of muscle wasting is associated with the disease activity of RA (Summers et al. 2010).

Low or normal levels of free testosterone are found in men and women with RA and this may contribute to rheumatoid cachexia. Population studies have shown that obesity (particularly visceral fat accumulation) is associated with low testosterone levels. Physical activity is reduced in patients with RA, though they have a normal diet. Therefore patients with RA have a positive energy balance and tend to store fat (Metsios et al. 2008b). This may be relevant in rheumatoid cachexia (Summers et al. 2010).

Metabolism is complex and is influenced by genetics and environmental factors. Systemic inflammation causes changes in metabolism and several studies have looked at individual metabolites in patients and animal models of inflammation. We therefore need to look at

several metabolites together and the systemic analysis of metabolites known as metabolomics allows us to do this. Using this approach a number of metabolites have been identified in inflammatory diseases which have provided insights into the mechanisms of disease and are also potential biomarkers.

1.4 Metabolomics

Given the profound systemic and localised changes in metabolism provoked by inflammation and inflammatory cytokines that have been outlined above, it is not surprising that the experimental approach known as metabolomics has been used to investigate several inflammatory diseases.

1.4.1 The metabolome

The metabolome is the name given to the complete set of small molecule chemicals found within a biological sample. The term metabolome has arisen as a combination of the words "metabolite" and "chromosome". The reason for this term was to demonstrate that metabolites are indirectly encoded by genes. The term "metabolome" was first used in 1998 and was designed to match with existing biological terms referring to the complete set of genes (the genome), the complete set of proteins (the proteome) and the complete set of transcripts (the transcriptome). The study of the metabolome is called metabolomics.

Unlike proteomics and genomics we are presently unable to identify the complete set of metabolites in metabolomics as identification of metabolites is still difficult and metabolite libraries can not identify all metabolites that can presently be measured and we are presently unable to measure anything with a concentration below 5µM.

The metabolome reflects the interaction between an organism's genome and its environment. As a result, the metabolome can act as an excellent probe of its phenotype (i.e. the product of its genotype and its environment). We are aware that genetic and environmental factors are involved in the aetiology of RA, hence metabolomics may be a useful tool in investigating the aetiology of RA. The rationale behind metabolomics is that changes in a disease will cause alterations in the levels of certain metabolites. To qualify as a metabolite, or to be considered to be part of the metabolome, a small molecule must typically have a molecular weight <1500 Da eg. glycolipids, polysaccharides, short peptides and not proteins (measured by proteomics), RNA or DNA (measured by genomics).

Metabolomics has been used to identify novel biomarkers and to look into the mechanisms of disease process. Metabolomics attempts to systematically identify and quantify metabolites from biological samples using approaches including mass spectrometry (MS) or nuclear magnetic resonance (NMR).

1.4.2 Approaches to metabolomics

Metabolomics attempts to systematically identify and quantify metabolites from biological samples. The small molecules represent the end result of complex biological processes in a given cell, tissue, or organ, and thus form attractive candidates to understand disease mechanisms. Metabolites represent a diverse group of low molecular weight structures including lipids, amino acids, peptides, nucleic acids, and organic acids, which makes

comprehensive analysis a difficult analytical challenge. The recent rapid development of a variety of analytical platforms based on mass spectrometry (MS) and nuclear magnetic resonance (NMR) have enabled separation, characterization, detection, and quantification of such chemically diverse structures.

Unlike transcriptomics and proteomics, the molecular identity of metabolites cannot be deduced from genomic information. Thus, the identification and quantification of metabolites must rely on sophisticated instrumentation such as MS and NMR spectroscopy. Each of these technologies has its own unique advantages and disadvantages. Optimal selection of a particular technology depends on the specific goals of a study and is usually a compromise amongst sensitivity, selectivity, and speed.

1.4.2.1 Mass Spectrometry

MS offers a good combination of sensitivity and selectivity. Modern MS provides highly specific chemical information that is directly related to the chemical and the high sensitivity of MS allows detection and measurement of very low levels of many primary and secondary metabolites. These unique advantages make MS an important tool in metabolomics (Lei et al. 2011).

1.4.2.2 NMR spectroscopy

NMR spectroscopy was developed in the 1940s but with the evolution of higher magnetic field strengths sensitivity has improved (Nicholson and Lindon 2008). NMR is highly selective and non-destructive and is generally accepted as the gold standard in metabolite structural elucidation, but it suffers from lower sensitivity than MS. NMR is the only detection technique which does not rely on separation of the analytes, and the sample can thus be recovered for further analyses (Nicholson and Lindon 2008). A wide range of

small molecule metabolites can be measured simultaneously hence, NMR is close to being a ‘universal detector’. The main advantages of NMR are high analytical reproducibility and simplicity of sample preparation (Beckonert et al. 2007). NMR is also felt to be more reliable than MS for determining concentrations of molecules (Nicholson and Lindon 2008).

1.4.3 Metabolomics of inflammatory disease

Metabolomics can be thought of as ‘systems medicine’ as it requires a thorough clinical assessment in combination with laboratory measurements to systematically and accurately identify metabolites in a meaningful manner from small quantities of biological sample. I will now discuss metabolomics in the context of inflammatory diseases in further detail.

1.4.3.1 The Gut

Crohn’s disease (CD) is a chronic debilitating inflammatory disease of the bowel. The exact aetiology is unknown but is thought to be related to the deregulation of the immune response towards gut microflora (Strober et al. 2007). Urinary metabolite profiling has been carried out on a mouse model of CD and five key metabolic differences were identified between the CD model and controls. This suggested that there are alterations of tryptophan metabolism, fucosylation and fatty acid metabolism in CD mice and the authors concluded that fucose and xanthurenic acid could be useful markers of gut inflammation (Lin et al. 2009).

A further mouse model of inflammatory bowel disease (IBD) investigating urinary metabolites identified that there was an increase in trimethylamine (TMA) and fucose compared to controls. The increase in TMA was parallel to the progression of IBD (Murdoch et al. 2008). A mouse model of ulcerative colitis (UC) investigating serum and urinary metabolites (Schicho et al. 2010) found that both serum and urine samples were equally accurate for detecting colitis but the metabolites responsible for the differences were different for both biofluids.

Faecal extracts have also been used to study IBD (Bezabeh et al. 2009). It is sometimes difficult to distinguish CD from UC and earlier identification could aid treatment and prognostication. Metabolomic analysis of faecal extracts of patients with both inflammatory diseases showed reduced levels of butyrate, acetate, methylamine and TMA compared to control (Marchesi et al. 2007). Comparing the UC and CD samples glycerol, alanine, isoleucine, leucine, lysine and valine were present in higher quantities in CD compared to UC. Acetate was lower in CD compared to UC (Marchesi et al. 2007). Metabolic differences were more marked in CD indicating that inflammation is more extensive in CD compared to UC.

Urinary metabolites have also been used to distinguish CD and UC in humans (Williams et al. 2009). They found that specific urinary metabolites related to gut metabolism differed between CD, UC and controls. Hippurate was lowest in CD and differed significantly between CD, UC and controls. Formate levels were higher in CD than in UC or controls and 4-cresol sulphate was lower in CD than in UC or controls (Williams et al. 2009).

Hippurate has been shown to be modulated according to gut microbes and this difference is likely to reflect changes in intestinal microbes.

A recent study of IBD in humans used serum, plasma and urine samples to try and distinguish UC from CD and to distinguish IBD from controls (Schicho et al. 2012). The authors identified that all three biofluids could be used to distinguish between IBD and controls but different metabolites were more prominent in different biofluids (methanol, mannose, formate, isoleucine, urea and citrate for plasma and serum but mannitol, allantoin, xylose, carnitine, betaine and hippurate in urine). They felt that the metabolic differences were less pronounced between UC and CD.

In summary, several studies have looked at metabolomics of IBD and have demonstrated that in mice and in humans TMA is an important marker of IBD (Marchesi et al. 2007;Murdoch et al. 2008) using both urine samples or faecal extracts. Hence, TMA may be a useful biomarker for IBD. Several different biofluids have also been used in the metabolomics of IBD and though they all yield important distinguishing metabolites the metabolites are different between the biofluids suggesting that different biofluids may give us different input into the aetiology of disease.

1.4.3.2 The Eye

As a closed and immunopriveleged site, the eye provides an ideal system for metabolic analysis. Metabolic products of inflammatory infiltrates accumulate in the vitreous fluid of the eye and may be extracted during ocular corrective surgery. Metabolomics has been

used to look at vitreous humour in order to differentiate ocular inflammatory diseases (Young et al. 2009). Vitreous fluid samples were taken from patients undergoing retinal surgery and analysed using NMR. Patients had various retinal disorders including chronic non-infectious uveitis (CU), acute lens-induced uveitis (LIU), proliferative diabetic retinopathy, proliferative vitreoretinopathy, rhegmatogenous retinal detachment, candida endopthalmitis and varicella zoster virus acute retinal necrosis. The different disease groups showed clear separation using principle component analysis (PCA) and partial least squared discriminate analysis (PLSDA). The majority of the patients had LIU and CU. When looking at LIU and CU specifically there was clear separation and urea, oxaloacetate and glucose were all raised in LIU compared to CU. As urea and oxaloacetate are both involved in the urea cycle it suggests that there is more active inflammation in the LIU patients (Young et al. 2009).

NMR has also been used to determine ocular metabolism in porcine eyes (Greiner et al. 1985). Using phosphorous NMR they found phosphorous containing metabolites in aqueous and vitreous fluids (Greiner et al. 1985). In addition to quantifying metabolites, phosphorous NMR can be used to monitor the rate of metabolic change in a specific biochemical reaction and the rate of change in the concentration of a particular metabolite (Greiner et al. 1985). Phosphorous NMR provides a non-invasive method to analyse ocular tissues metabolically and detect subtle biochemical changes that precede manifestations of disease. Such detection may allow for early and more effective therapeutic intervention.

1.4.3.3 Neurological disease

Multiple sclerosis is a chronic inflammatory disease affecting the nervous system. Its aetiology is still not completely understood (Ibrahim and Gold 2005) but it is characterised by demyelination, axonal loss and breakdown of the blood-brain barrier (Trapp et al. 1999). It is a heterogeneous, relapsing and remitting disease and different treatments have been shown to work at different stages of disease (Rieckmann and Smith 2001), so it is important to identify biomarkers that enable identification of different phases.

IL-1β and TNF-α have been found to be associated with a broad spectrum of neurological diseases including multiple sclerosis. Griffin *et al* investigated rat urine samples to determine whether NMR spectroscopy could detect the presence of IL-1β and TNF-α induced lesions and distinguish between the pathology caused by the cytokines (Griffin et al. 2004). They used an adenoviral vector to induce chronic endogenous expression of either IL-1β or TNF-α and found significant differences between the groups, with the IL-1β treated group showing increases in leucine, isoleucine, valine, n-butyrate and glucose whilst the TNF-α treated group showed increases of citrate, 2-oxoglutarate and succinate (Griffin et al. 2004).

NMR spectroscopy has also been used to analyse cerebrospinal fluid (CSF) in patients with multiple sclerosis and evidence suggests that there are increased CSF levels of lactate, creatinine and fructose in multiple sclerosis compared to control patients (Nicoli et al. 1996). Two additional unidentified signals were found to be elevated in multiple sclerosis and the compound responsible for both these signals has now been identified as B-hydroxyisobutyrate (Lutz et al. 2007). This is a typical partial degradation product of

branched-chain amino acids and increased B-hydroxyisobutyrate in urine samples is thought to be due to respiratory-chain deficiency leading to impaired oxidation of NADH (Chitayat et al. 1992). However the level of B-hydroxyisobutyrate in these experiments was much higher than the level found in multiple sclerosis patients. The precise role of B-hydroxyisobutyrate in multiple sclerosis needs further investigation.

Using a rat model of multiple sclerosis, metabolomics of CSF has revealed changes in metabolite levels over the course of the disease suggesting profound changes in central nervous system metabolism over the course of disease (Noga et al. 2012). The pathways that were altered included nitric oxide synthesis, energy metabolism, polyamine synthesis and levels of endogenous antioxidants.

1.4.3.4 Lung disease

Pulmonary inflammation contributes to the pathogenesis of a number of lung diseases and there is a growing need for validated experimental models that can help our understanding of disease pathogenesis and therapeutic intervention. Traditionally animal models have been used but they have their own problems in representing human disease. Genetic manipulation can greatly enhance animal models. NMR has had some application in the quantification of experimental lung injury.

Serkova et al used Magnetic Resonance Imaging and NMR to try and detect and quantify injury in mice following intratracheal administration of inflammatory cytokines (Serkova et al. 2008). Pulmonary inflammation was induced by intratracheal administration of IL-

1β and TNF-α. Lung tissue was used for the NMR metabolomics and they demonstrated that with pulmonary inflammation there was a 50% depletion of ATP and a corresponding elevation of the lactate to glucose ratio suggesting a shift to anaerobic metabolism during inflammation. These returned to control levels at 24 hours (Serkova et al. 2008) illustrating that intratracheal administration of IL-1β and TNF-α leads to profound but reversible pulmonary inflammation which is detectable by NMR.

1.4.3.5 Osteoarthritis

Osteoarthritis (OA) is a complex disease and has a multifactorial pathogenesis. It has many known risk factors such as age, sex, obesity, activity level, prior joint damage and genetic susceptibility but is not classically thought of as an inflammatory disease, though it may have an inflammatory element. There are currently no disease-modifying drugs for OA and very few in development.

Synovial fluid (SF) has been used to investigate OA via NMR. SF is widely regarded to be a good medium to study as the SF is the first place where the degradation products, enzymes and signal transduction molecules involved in OA are released from the cartilage matrix. The SF should therefore have a higher concentration of metabolites compared to blood, lymph or urine.

Damayanovich et al used SF from a canine model of OA to look at metabolic profiles using NMR (Damyanovich et al. 1999a). They compared metabolites from experimentally induced canine knee OA SF to metabolites from SF of normal canine knees and found

large increases in lactate and decreases of glucose in OA SF compared to normal SF suggesting that the intraarticular environment of an OA joint is more hypoxic and acidic than a healthy joint. They also found increased levels of pyruvate, lipoprotein associated fatty acids, glycerol and ketones in OA SF suggesting that lipolysis may be an important source of energy in OA. There were also elevated levels of N-acetylglycoproteins, acetate and acetamide in OA SF especially with progressive OA (Damyanovich et al. 1999a).

In order to try and investigate further the mechanisms underlying OA progression, Damayanovich *et al* looked at the effect of joint afferent nerve injury (Damyanovich et al. 1999b) using a bilateral canine model of OA. Paired SF samples were taken from dogs which had undergone bilateral anterior cruciate ligament transaction, unilateral knee denervation and contralateral sham nerve exposure. Increases in glycerol, hydroxybutyrate, glutamine, creatinine, acetate and N-acetyl-glycoprotein were seen in the SF from denervated compared to control knees. This suggested that the metabolite differences seen in the denervated knees was due to the aggravation of OA caused by joint denervation (Damyanovich et al. 1999b). Hydroxybutyrate is also found in SF of RA patients (Naughton et al. 1993a) suggesting that it is more of a marker of joint destruction rather than being specific for any joint disease.

Another group used guinea pigs to look at OA (Lamers et al. 2003) as they develop spontaneous progressive knee OA with features similar to human disease. The earliest histological features appear at 3 months but this progresses to extensive cartilage degeneration after 12 months. Urine samples were collected from these OA pigs and from healthy pigs at 10 and 12 months of age. They identified a metabolic fingerprint that

reflected OA changes in the pigs composing of lactic acid, malic acid, hypoxanthine and alanine suggesting their involvement in OA (Lamers et al. 2003). The presence of hypoxanthine suggests that OA may be an inflammatory disease due to the increased oxygen demand and altered purine metabolism.

Mass spectroscopy has also been used to look for novel biomarkers of knee OA (Zhai et al. 2010). They looked at serum samples of unrelated Caucasian females with and without knee OA where knee OA was defined as radiographic, medically diagnosed or total knee replacement due to primary OA. They found that the ratio of valine to histidine and the ratio of xleucine to histidine was significantly associated with knee OA in humans (Zhai et al. 2010). These ratios have potential clinical use as an OA biomarker. OA branched chain amino acids (BCAA) are raised which may drive the release of acetoacetate and 3-hydroxybutyrate and these can result from the partial oxidation of leucine. BCAA are essential amino acids and therefore cannot be synthesised within the body. An increased level of BCAA may suggest an increased rate of protein breakdown or be secondary to collagen degradation. BCAA increase production of the cytokines IL-1, IL-2, TNF and interferon (Bassit et al. 2000) which could drive the collagen degradation.

1.4.3.6 Rheumatoid arthritis

Hyaluronic acid is a major component of the proteoglycan aggregate of articular cartilage which is required for the functional integrity of extracellular matrix. In RA, SF hyaluronate is depolymerised by the action of reactive oxygen radical species (Parkes et al. 1991) and hyaluronidase activity is absent in both normal and inflamed SF. Generation of reactive oxygen species plays a principal part in synovial hypoxic reperfusion injury

(Farrell et al. 1992). This occurs as increased intra-articular pressure during exercise exceeds synovial capillary perfusion pressure leading to impaired blood flow (Mapp et al. 1995).

In 1993, the Inflammation Research Group at the London Hospital Medical College looked at the NMR profiles of RA SF and matched serum samples (Naughton et al. 1993a). The NMR profiles of SF were markedly different from their matched serum samples and there were high levels of lactate in the SF compared to the serum and low levels of glucose in the SF compared to the serum. These changes are consistent with the hypoxic status of the rheumatoid joint (Naughton et al. 1993a). All the SF samples (RA and control) had lower levels of chylomicron and very-low-density-lipoprotein associated triglycerides compared to their matched serum samples. The SF samples also had high levels of ketone bodies compared to their matched serum samples. These results suggest that the intra-articular environment has an increased utilisation of fats for energy even though it is hypoxic (Naughton et al. 1993a;Naughton et al. 1993b). They were unable to compare the control SF to the rheumatoid SF due to the low levels of SF aspirated.

Serum from mice has been used to identify a metabolite biomarker pattern associated with RA (Weljie et al. 2007). Using NMR they found that uracil, xanthine and glycine could be used to distinguish arthritic from control animals (Weljie et al. 2007). The presence of the metabolites suggests that nucleic acid metabolism may be highly affected in RA and there may be an association with oxidative stress.

More recently, a group in Denmark have looked at the plasma of patients with RA (Lauridsen et al. 2010). They found differences in the metabolic profiles between patients with RA and healthy controls and differences between patients with active RA and RA in remission. The metabolites that they identified which discriminated between the patients with active RA and healthy controls were cholesterol, lactate, acetylated glycoprotein and lipids. The lactate levels represented oxidative damage and thus indirectly reflected active inflammation. This suggests that the underlying inflammatory processes drive significant changes in metabolism that can be measured in the peripheral blood and metabolomics may prove useful as a measure of the extent of disease, potentially separating low disease activity states from patients in true remission.

A recent clinical study has used synovial fluid from patients with RA to investigate lipid profiles (Giera et al. 2012). They identified almost 70 different lipid components; some with anti-inflammatory and some with pro-resolving properties. Traditional Chinese medicine has also been used in combination with metabolomics to further categorise different types of patients with RA (van Wietmarschen et al. 2012). They investigated the clinical chemistry, metabolomics of urine and plasma samples and symptom profiles of 39 patients with RA. They combined this with traditional Chinese medicine (using Cold and Heat type methodology) and demonstrated significant biochemical differences between different subgroups of patients with RA suggesting that there are different mechanisms of disease progression and that treatments could be tailored accordingly. Metabolomic studies in RA have also illustrated that they can predict the response to a particular treatment (Wang et al. 2012). In a study of 38 patients with active RA there was a significant difference in the serum levels of certain metabolites between those who demonstrated a

clinical response to methotrexate monotherapy compared to those who did not respond (Wang et al. 2012).

1.4.3.7 Inflammatory changes in metabolites

As summarised above there is now a growing body of literature describing metabolomic changes in inflammatory diseases, both in humans and animal models. There have been few studies looking at metabolomics in RA. The studies looking at metabolomics in other inflammatory disease may help with our study in RA as there appears to be several common metabolic features of inflammation. Multiple metabolites have been identified in the other inflammatory disorders using various biofluids but there is a theme of increased energy requirement with inflammation. Studies in RA demonstrate that patients with RA have an increased energy requirement mainly due to the increased production of TNF (Metsios et al. 2008a). The studies in multiple sclerosis (Nicoli et al. 1996), OA (Damyanovich et al. 1999a) and inflammatory lung disease (Serkova et al. 2008) have found an increase in lactate and the studies in inflammatory eye disease (Young et al. 2009) and inflammatory lung disease (Serkova et al. 2008) have shown a reduction in glucose. The studies in RA point to the joint having a hypoxic environment (Naughton et al. 1993a;Naughton et al. 1993b) and an association with oxidative stress (Weljie et al. 2007). Xanthine has been shown to distinguish RA from controls in mice (Weljie et al. 2007) and this has also been shown in inflammatory bowel disease (Schicho et al. 2012).

Though there are several metabolites which have been identified in many inflammatory conditions there are unique metabolites associated with individual disease suggesting that there may be some distinctive features within the metabolic profiles of arthritis patients

which may be identifiable from a metabolomic analysis and enable us to gain insights into the aetiology and pathogenesis of RA.

1.5 Summary

RA involves a complex interaction between genes and the environment with a significant effect on systemic metabolism. As metabolism is influenced by both genetics and lifestyle analysis of metabolites may provide novel insights into the early stages of inflammatory disease.

1.6 Aims and hypothesis

The central aims for this project are:

1. To test the hypothesis that chronic synovial inflammation alters metabolism.
2. To test the hypothesis that the metabolic profile expressed by an individual patient in the earliest stage of inflammatory arthritis predicts the subsequent course of the disease. I will test whether we can use metabolomics to generate a 'fingerprint' characteristic of arthritis-dependent changes in blood and in vitro cultured synovial fibroblasts.
3. To test the relationship between disease outcome and the metabolic profile to identify novel biomarkers for RA and inflammatory disease.

2.0 Materials and methods

2.1 Samples

2.1.1 Serum

Blood was collected in vacutainer tubes containing clotting accelerator (Greiner Bio-one) at the first visit to the early arthritis clinic at Sandwell and West Birmingham Hospitals NHS Trust. Samples were then transferred to the University of Birmingham where they were centrifuged at 600g for 10 minutes, serum removed and then stored at minus 80^0C until analysis. Samples were also collected at follow up visits.

2.1.2 Urine

Mid-stream urine samples were collected at the first visit to the clinic, centrifuged as above and stored at minus 80^0C.

2.1.3 Fibroblasts

Synovial fibroblasts from the early arthritis patients and established RA (DMARD naive) were grown from the biopsy material taken using ultrasound guidance (de Sande et al. 2011) via explant culture. Tissue was collected from multiple regions within the knee, ankle or metacarpopharyngeal (MCP) joints in which there was evidence of grey scale synovitis. Ultrasound guidance was used to introduce a single port through which tissue was sampled using custom manufactured 2.0mm cutting edge forceps (knee and ankle) or a 16g core biopsy needle (MCP joint). Each assessment was carried out using multiple biopsy samples taken systematically from different locations in the joint in order to overcome synovial heterogeneity (Scire et al. 2007). Normal synovial fibroblasts were obtained from patients presenting with knee pain for arthroscopy, who had no macroscopic

or clinical evidence of inflammation. For the longstanding established RA control experiments tissue was obtained from the Royal Orthopaedic hospital and fibroblasts were cultured from these. All samples were kept on ice until they could be processed at the university. Synovial tissue was cut into 1mm sections to allow more efficient breakdown of extracellular matrix components and subsequent growth of cells and collected into 10 ml of wash buffer (RPMI, 20mMHEPES). Resulting cell suspension was washed twice and cultured in fibroblast medium. Cultures were incubated in 0.2% collagenase at 37^{o}C (Salmon et al. 1997) allowing adherent cells to become attached to the plastic. All cells at passage four were run through a flow cytometer under basic single colour staining panel for quality control to ensure that all cells were fibroblasts. Panel included fibroblast marker (CD90) for conformation plus a macrophage (CD68) and an endothelial cell marker (CD31) for exclusion.

2.2 Fibroblast cell culture

2.2.1 General culture guidelines

Fibroblast cultures were supplemented with fresh culture medium once weekly by replacing two thirds of the old culture medium with new culture medium.

2.2.1.1 Fresh complete fibroblast medium

This was composed of:

RPMI 1640 (Sigma R0883) (for full list of components see appendix)

10% Foetal calf serum

1% (100X) MEM Non-essential amino acids (Sigma M7145)

1% (100mM) Sodium Orthopyruvate (Sigma S8636)

2mM glutamine

100U/ml penicillin

100micrograms/ml streptomycin

2.2.1.2 Phosphate buffered saline

Phosphate buffered saline (PBS) was prepared from tablets purchased from Oxoid (BR14A) by dissolving 1 tablet in 100ml of water and autoclaving before use.

2.2.2 Trypsinisation of cells (splitting)

When the fibroblasts were visually confluent they were split (i.e. cells were trypsinised and reseeded into new flasks at a lower density). By convention, every time a culture is split its 'passage number' rises by 1. The reagents used were complete fibroblast medium, (10X) Trypsin-EDTA solution (Sigma T4174) and PBS.

1. A bottle of fresh complete fibroblast medium was pre-warmed to 37^{O}C.
2. (2X) trypsin-EDTA solution was prepared by diluting the stock solution 1:4 in PBS.
3. Conditioned medium was collected from the flask and kept for the new flasks.
4. The cells were washed with PBS once to remove excess medium.
5. (2X) trypsin-EDTA solution was added to the flask to cover the cells. Flasks were incubated at 37^{0}C for five minutes.
6. Microscopy was used to check that the cells had detached.
7. 8mls of fresh complete fibroblast medium were added to the flask and the cells were collected into a polypropylene tube.
8. Tube was centrifuged at 300g for 6 minutes. Supernatant was discarded.
9. Cells were resuspended in 1ml of complete fibroblast medium and cell numbers and viability assessed using a Neubauer counting chamber following staining with Trypan blue.
10. 33% conditioned medium was prepared by diluting conditioned medium1:2 in fresh complete fibroblast medium.
11. The pellet was resuspended in 33% conditioned fibroblast medium and seeded into flasks as required.

2.2.3 Cell quenching and extraction protocol:

Fibroblasts were trypsinised between passages once cells had reached confluence but when adequate number of fibroblasts for NMR experiments had been obtained metabolites were extracted using methanol, since trypsinisation has been shown to affect NMR results (Teng et al. 2009).

1. Media was aspirated (2x1ml was kept for NMR and cytokine analysis and frozen at -80°C)
2. 1x wash with 10ml PBS at room temperature and fluid aspirated
3. 1x wash with 10ml 60% methanol at -48°C (by placing the methanol on dry ice) and fluid aspirated
4. 3.5 ml -48°C methanol added and flask placed on dry ice
5. cells scraped into methanol with a cell scraper
6. cell extract transferred into two 2ml microcentrifuge tubes and snap frozen in liquid nitrogen
7. 3x freeze/thaw cycle (liquid nitrogen/dry ice)
8. Samples dried under vacuum using a Speedvac centrifugal drier overnight and stored at -80°C prior to liquid/liquid extraction

2.3 Cell extraction procedure to produce samples for NMR

Once the fibroblasts were extracted, a methanol-chloroform liquid cell extraction procedure was used to precipitate the proteins and remove lipids to leave the water soluble metabolites in the extract.

1. all four 1.5ml polypropylene microcentrifuge tubes (two from each of the two T75 flasks) in succession were re-suspended in methanol:water (600µl:540µl)** and pooled into a glass vial containing 600µl chloroform
2. glass vial was vortexed for 30seconds
3. vial left to stand on ice for 10 minutes
4. vial centrifuged at 4°C, 1500g for 10 minutes
5. vial left to stand on bench for 5 minutes
6. upper polar layer removed and placed into an polypropylene microcentrifuge tube
7. polar fraction dried in speedvac overnight and stored in -80°C prior to NMR analysis

- **300µl methanol and 300µl water were added to the 2nd polypropylene microcentrifuge tube, vortexed for 30seconds, etc. until the supernatant was transferred to the 4th polypropylene microcentrifuge tube. The supernatant was then transferred to the glass vial with 600µl chloraform. 300µl methanol and 240µl water were added to the 1st polypropylene microcentrifuge tube (in order to recover as much as possible), vortexed for 30seconds and spun for 30seconds at 16000g, transferred to 2nd polypropylene microcentrifuge tube and repeat vortexed and spun, etc. until the supernatant was transferred, vortexed and spun into the 4th polypropylene microcentrifuge tube, then pooled into the glass vial with the first supernatant and chloroform.

2.4 Sample preparation for NMR

2.4.1 Materials

3-trimethylsilyl 2,2,3,3-tetradeuteropropionic acid (TMSP), Isotec.

NMR tubes:standard series 5mm NMR tubes, Norell.

NMR tubes standard series 1.7mm, Bruker.

Filters: Nanosep 3K omega, Pall.

Deuterium oxide (D_2O) 99.9%, Aldrich.

$NaH_2PO_42H_2O$ (S/3760/53, Fisons Scientific Equipment)

Sodium azide (S8032-100G), Sigma

2.4.2 Preparation of sodium phosphate buffer

A phosphate buffer of pH 7 was prepared by mixing 0.624g $NaH_2PO_42H_2O$, 40mg sodium azide, 3.44mg TMSP and 4mls of D_2O to give a final concentration of D_2O of 10%. Final volume of 10mls was made using distilled water and pH was adjusted to pH7 using 2M NaOH.

2.4.3 Serum samples

After thawing, some serum (unfiltered) was diluted 1:1 with D_2O/H_2O containing NaCl (150mM) TMSP and sodium phosphate (20mM) pH 7. Filters were washed 6 times using warm water (38^0C) (0.5ml) and each time centrifuged at 3 000g for 15minutes to remove preservative glycerol (Tiziani et al. 2008). After thawing some sera was filtered and

centrifuged at 4^0C for 15 minutes at 10 000g. Filtered sera (50µl) were diluted with 16.7µl of buffer containing D_2O, TMSP, sodium azide and sodium phosphate.

2.4.4 Urine samples

After thawing, urine samples (1ml) were centrifuged at 16000g for 5minutes. Samples were prepared using a standard protocol that has been used in other studies of urine (Viant et al. 2007). Urine samples were buffered with phosphate buffer (100mM), made 10% with D_2O and 0.5mM with TMSP and the pH adjusted (twice over 30minutes) to pH 7.0. The samples were centrifuged at 15700g for 2 minutes and the pH of the samples was adjusted again to obtain a pH of 7-7.05. 0.6ml of sample was placed into a standard 5mm NMR tube for spectroscopy.

2.4.5 Fibroblast medium samples

Filters were washed 6 times using warm water (38^0C) (0.5ml) and each time centrifuged at 3 000g for 15minutes to remove preservative glycerol (Tiziani et al. 2008). Fibroblast medium was then filtered and centrifuged at 4^0C for 15 minutes at 10 000g. Samples (50µl) were diluted with 16.7µl of buffer containing D_2O, TMSP, sodium azide and sodium phosphate.

2.4.6 Fibroblast cells

Once the sample had been dried in the Speed-vac, 50µl of water and 16.7µl of buffer containing D_2O, TMSP, sodium azide and sodium phosphate was added to each sample.

2.5 Metabolomic analysis

One-dimensional ^{1}H spectra were acquired at 300K using a standard spin-echo pulse sequence with water suppression using excitation sculpting on a Bruker DRX 500MHz NMR spectrometer equipped with a cryoprobe or using a standard NOESY sequence on a Bruker 600MHz NMR spectrometer. Excitation sculpting makes use of pulsed field gradients and generally produces much flatter baselines (Jerschow 1999). It is useful in samples which contain large amounts of protein, which will distort the baseline. Excitation sculpting was not required on the Bruker 600 as all samples were filtered. Two-dimensional ^{1}H J-resolved (JRES) spectra were also acquired to aid metabolite identification (Ludwig and Viant 2010).

Samples were processed and data normalised with respect to the TMSP signal. Spectra were read into Prometab (Viant 2003) which is custom written software in Matlab (version 7, The Math Works, Natick, MA), and were truncated to a 0.8-10.0 ppm (parts per million) range. Spectra were segmented into 0.005 ppm (2.5Hz) chemical shift 'bins' and the spectral areas within each bin were integrated. In many metabolomics studies, NMR spectra are divided into bins of fixed width. This spectral quantification technique is used to reduce the number of variables for pattern recognition techniques and to mitigate effects from variations in peak positions. Spectra were corrected for baseline offset and then normalised to a total spectral area of unity and a generalised log transformation was applied, which stabilises the variance of the data effectively making the data more suitable for multivariate analysis (Viant 2003). Binned data were then compiled into a matrix, with each row representing an individual sample.

2.6 Statistical analyses

2.6.1 Principal components analysis (PCA)

The data bins from groups of spectra were mean centred and then assessed by PCA using the PLS_Toolbox (version 5.8) (Eigenvector Research) in Matlab (release 2009a) (Mathworks). PCA analysis identifies regions of the spectrum which allow segregation in order to identify metabolites.

PCA is a mathematical technique that yields patterns and relationships in multivariate datasets, enabling an understanding into the causes and effects behind these relationships. The basis of this method is the generation of a new coordinate system where the new variables are independent linear combinations of the original variables and also capture some features in the original data. A feature illustrates some aspect of the data, described as numerical values for each object. The fundamental basis behind PCA is the relationship between the numerical values for each object (Webb-Robertson et al. 2005).

PCA is a mathematical procedure which transforms a number of possibly correlated variables in a large multivariate dataset into a smaller number of uncorrelated variables called principal components, with little information loss. The first principal component accounts for as much of the variability in data as possible and each succeeding component accounts for as much of the remaining variability as possible. PCA simply describes the data.

2.6.2 Partial least square discriminant analysis (PLS-DA)

PLS-DA was used to perform supervised clustering of samples (Chauchard et al. 2004;De Jong 1990) and on some occasions an orthogonal signal correction was applied (OPLS-DA) to enhance the separation of groups and to achieve better models in multivariate calibration (Gavaghan et al. 2002). The PLS-DA models were cross-validated using Venetian blinds (Chauchard et al. 2004;De Jong 1990), a method which re-assigns randomly selected blocks of data to the PLS-DA model to determine the accuracy of the model in correctly assigning class membership.

2.6.3 GALGO

GALGO (Genetic ALGOrithm), a statistical package available in the statistical environment R, uses a genetic algorithm search procedure coupled to statistical modelling methods for supervised classification (Trevino and Falciani 2006). The results of GALGO analyses are presented as principle component analysis (PCA) plots where the X and Y axes represent first and second principle components providing the greatest variation between samples, and the next largest unrelated variation respectively. GALGO analysis was cross validated using K-fold cross validation where the original sample is randomly partitioned into subsamples and each observation is used for both training and validation.

GALGO, like any genetic algorithm, is based on randomly generated data whereas the fundamental principle behind PCA and PLSDA are the correlation of different components within the data. GALGO undergoes several ‘generations’ of analysis and in each generation the fitness of each individual within the data is evaluated. The individuals which fit best are then selected and the next generation of analysis begins. The algorithm

is complete when the maximum number of generations has been produced or a satisfactory fitness level has been achieved for all the individuals. It has been shown that genetic algorithms optimise the results of other models by removing irrelevant variables, which dramatically improves the classification ability of the model (Ramadan et al. 2006).

2.6.4 Partial least squares regression analysis (PLS-R)

PLS-R is a regression method that identifies which metabolites can predict a continuous variable and enables the correlation of complex metabolomic data with a relatively simple clinical parameter. This analysis yields r^2, a measure of the cross-validated goodness-of fit of the linear regression, while permutation testing (multiple analyses using random data subsets) assessed the significance of this prediction (McLeod et al. 2009).

2.6.5 Identification of metabolites

Lists of metabolites providing the greatest discrimination between groups were then identified for each technique. Using multivariate analyses, peaks with large loadings (for PCA), weightings (for PLS-DA) or regression coefficients (for PLS-R) were identified. For the PLS-DA models variable importance for projection (VIP) were calculated to produce a summary of how important each variable was in the data for predicting the classification. NMR databases (Human Metabolome Database version 2.5) and Chenomx NMR suite (Chenomx, professional version 4.0) (Weljie et al. 2006) were used to identify the metabolites (Figure 2.1). The two dimensional JRES were also used to identify metabolites using the FIMA software (Ludwig et al. 2012).

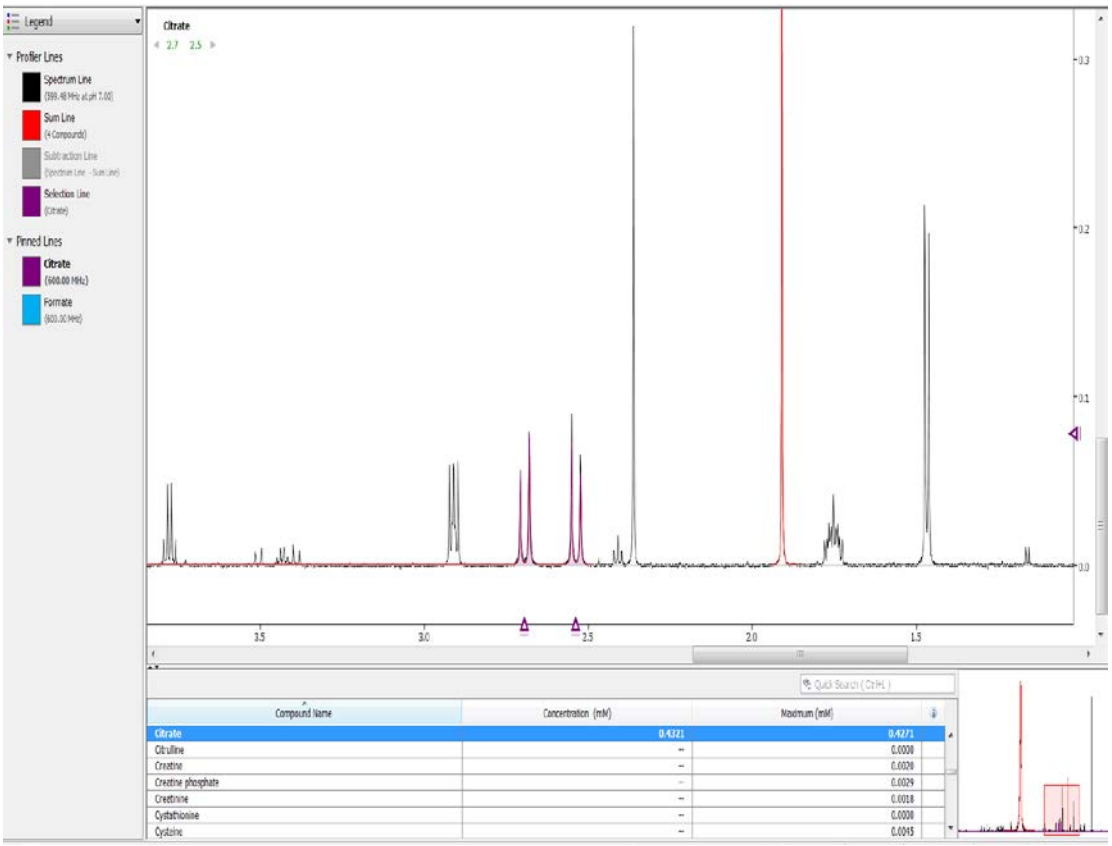


Figure 2.1 Chenomx profiler identifying metabolites in a spectrum. A spectrum is uploaded into chenomx and the chenomx profiler uses its library to identify metabolites in the spectra. The metabolite citrate is matched by the profiler as the peaks of the metabolite citrate (2.7, 2.5) match that in the spectrum.

2.7 Enzyme-linked immune-sorbent assays (ELISAs)

Levels of IL-6 in supernatants (in duplicate) of cultured fibroblasts were measured by ELISA according to the manufacturer's instructions (eBioscience: human IL-6). Plates were washed using the Bio-red Bioplex Pro plate washer. The assay was read using the Anthos htIII spectrophotometric plate reader and Stingray software. Wash buffer used was 1 x PBS, 0.05% Tween-20 and stop solution was sulphuric acid.

1. ELISA plate coated with 100µl/well of capture antibody in Coating Buffer. Plate sealed and incubated over night at 4^0C.

2. Wells aspirated and washed five times with >250µl/well of wash buffer.
3. Wells blocked with 200µl/well of Assay Dilutent. Incubated at room temperature for one hour.
4. Wells aspirated and washed five times with >250µl/well of wash buffer.
5. 100µl/well of standard and 100µl/well of sample added to the appropriate well. Plate sealed and incubated at room temperature for two hours.
6. Wells aspirated and washed five times with >250µl/well of wash buffer.
7. 100µl/well of detection antibody added. Plate sealed and incubated at room temperature for one hour.
8. Wells aspirated and washed five times with >250µl/well of wash buffer.
9. 100µl/well of the enzyme Avidin-HRP added. Plate sealed and incubated at room temperature for 30 minutes.
10. Wells aspirated and washed seven times with >250µl/well of wash buffer.
11. 100µl/well of substrate solution added to each well. Plate incubated at room temperature for 15 minutes.
12. 50µl of stop solution added to each well.
13. Plate read at 450nm.

2.8 Multiplex analysis

Supernatants (in duplicate) of cultured fibroblasts were used for the multiplex analyses. The assay was performed according to manufacturer's instructions using the following VersaMAP™ Custom Premixed Multiplex (R&D systems):

MMP-9, IL-8, IL-10, VEGF, MIP-1 alpha, IL-4, MMP-3, MMP-1, MCP-1, MMP-13, RANTES, IP-10

Plates were washed using the Bio-rad Bioplex Pro plate washer and the assay was read using the LUMINEX 100 ^{TM} System.

1. 100 µl of wash buffer added to each well. Liquid removed through the filter at the bottom of the plate using a vacuum manifold.
2. 50 µl of the Microparticle Cocktail added to each well.
3. 50 µl of standard or sample added to each well. Plate sealed and incubated at room temperature on a horizontal microplate shaker for two hours.
4. Vacuum manifold used to wash the plate by removing the liquid. 100µl of wash buffer added to each well and liquid removed again.
5. 50 µl of diluted Biotin Antibody Cocktail added to all the wells. Plate sealed and incubated at room temperature on a horizontal microplate shaker for one hour.
6. Vacuum manifold used to wash the plate by removing the liquid. 100µl of wash buffer added to each well and liquid removed again.
7. 50 µl of diluted Stretavidin-PE added to each well. Plate sealed and incubated at room temperature on a horizontal microplate shaker for 30 minutes.
8. Vacuum manifold used to wash the plate by removing the liquid. 100µl of wash buffer added to each well and liquid removed again.
9. 100µl of wash buffer added to each well to resuspend the microparticles. Plate sealed and incubated at room temperature on a horizontal microplate shaker for 2 minutes.
10. Plate read and double discriminator gates set at 7500 and 15 500.

3.0 Quality Control of the biofluids used

3.1 Blood

3.1.1 Introduction

The ready accessibility of blood from most patients means that blood cells, plasma and serum are often used in metabolomic studies. Blood is a useful biofluid in metabolomic studies as it contains a broad complement of metabolites (Zhang et al. 2010) but a lack of standardization for collection protocols may impact on the validity and reproducibility of results. Sample quality control can be an issue, particularly when samples are collected in a routine clinical setting, where variation in sample handling can significantly impact on the quality of the sample and hence the data derived from it. This has also been identified as an important issue in assessment of RNA in transcriptomic studies (van der Veen et al. 2009). Metabolites may be susceptible to variation since they may be subject to enzymatic or chemical conversion after sample collection. Subsequent storage conditions may also have an effect, for example, the number of freeze-thaw cycles the sample has gone through and the storage temperature (Teahan et al. 2006). Causes of such biases are not always obvious but can result from the type of tube in which the sample was collected and the length of time the sample was left at room temperature before being processed, cooled or frozen for storage.

A number of groups have investigated the stability of components of human plasma and serum over time but the majority of analytes targeted in these studies have been proteins, enzymes and metal ions (Boyanton and Blick 2002;Zhang et al. 1998). There are relatively few studies of the effects of variations in sample handling for metabolomics analyses. Plasma or serum metabolites are subject to constant flux as long as catalytically

active enzymes or interacting proteins are present and so removal of proteins is desirable to enhance the consistency of metabolite content prior to analysis. One disadvantage of removing proteins is that metabolites bound to proteins are removed. However, this has little impact on NMR spectra of metabolites since when these molecules are tightly bound to proteins; they would have large line widths and so are unlikely to be resolved in NMR spectra. Some small molecules with an intermediate affinity for proteins (KD μM to mM) would show altered intensities. This potential minor loss has to be balanced against the increased reproducibility of deproteinised samples and, of the various methods used to remove proteins from blood samples, ultrafiltration (using nanosep centrifugal filters) was found to be superior to precipitation (acetone extraction, acetonitrile extraction, perchloric acid precipitation and methanol/chloroform extraction) in retaining metabolite concentration and offering reproducibility (Tiziani et al. 2008).

Metabolomic studies have demonstrated good reproducibility of metabolite concentrations over variable time periods of sample handling in both plasma and serum (Yu et al. 2011). However, studies of the relative benefits of using these fluids have given conflicting results with one study showing that reproducibility over time (approximately one hour) was significantly better for plasma than for serum (Yu et al. 2011) and another study illustrating that human serum metabolite profiles were more reproducible (i.e. clustered more closely) than those from human plasma (Liu et al. 2010).

In order to minimize the artefactual variability in data derived from either serum or plasma it important to use a highly reproducible standard operating procedure for collection and preparation of blood samples for metabolomics. However, in bringing metabolomic

analyses into the mainstream of clinical practice in the routine hospital clinic, one of the least controllable variables is the time taken between the taking of the blood and storage, at low temperature, of the plasma or serum. A previous study assessed the effects of delays of up to 3 hours on the metabolites (Teahan 2006). However, in a routine clinic, delays of longer than this can occur. Furthermore Teahan et al only assessed the impact of sampling handling variables on whole serum/plasma NMR spectra, whereas in more recent studies the strong preference is to remove proteins prior to analysis.

In order to further investigate the factors which impact on the variability of metabolite analysis that might impinge on samples collected in a routine clinical environment, we have assessed the effects of time delays of up to 6 hours, on the stability of metabolite profiles derived from deproteinised serum and plasma collected from healthy donors. We have also assessed the cooling of samples on ice and an initial centrifugation to sediment cells, prior to further processing in these donors.

3.1.2 Materials and methods

3.1.2.1 Patients

3 healthy individuals (males 42, 48 and 59 years of age) were recruited from staff at the University of Birmingham. The local ethics committee approved the study and all donors gave written informed consent (see appendix). None of the subjects had a current infection and had had no infections within the previous month. They also had none of the following: inflammatory arthritis, inflammatory bowel disease, psoriasis, liver disease, inflammatory lung disease or asthma, kidney disease or multiple sclerosis.

3.1.2.2 Experimental plan

We aimed to investigate the stability of components of human plasma and serum over time ex vivo, assessing whether placing blood samples on ice immediately after collection and prior to processing and whether rapid versus delayed separation of cells from serum and plasma affected the metabolic profiles measured in serum and plasma. We also aimed to compare these different conditions for filtered and non-filtered serum and plasma. Seven tubes of serum (containing clotting accelerator (Greiner Bio-one)) and eight tubes of plasma (containing lithium heparin (Greiner Bio-one)) were taken from the 3 donors. The pathway that was followed for sample handling is shown in Figure 3.1. The addition of anticoagulants such as lithium heparin or EDTA to whole blood inhibits clot formation and reduces the loss of blood proteins and their bound substrates. It has been shown that EDTA produces strong signals for CaEDTA and MgEDTA complexes as well as free EDTA in ^{1}H NMR spectra (Nicholson et al. 1983) and so plasma was collected using lithium heparin.

The earliest post collection processing time chosen for the serum samples was 30 minutes – the length of time it takes for blood to consistently clot in the presence of clot accelerator. The other time points were chosen to reflect clinical practice. Final serum and plasma samples were stored at minus 80°C until NMR analysis. Samples were prepared as 2.4.3 and NMR spectra acquired.

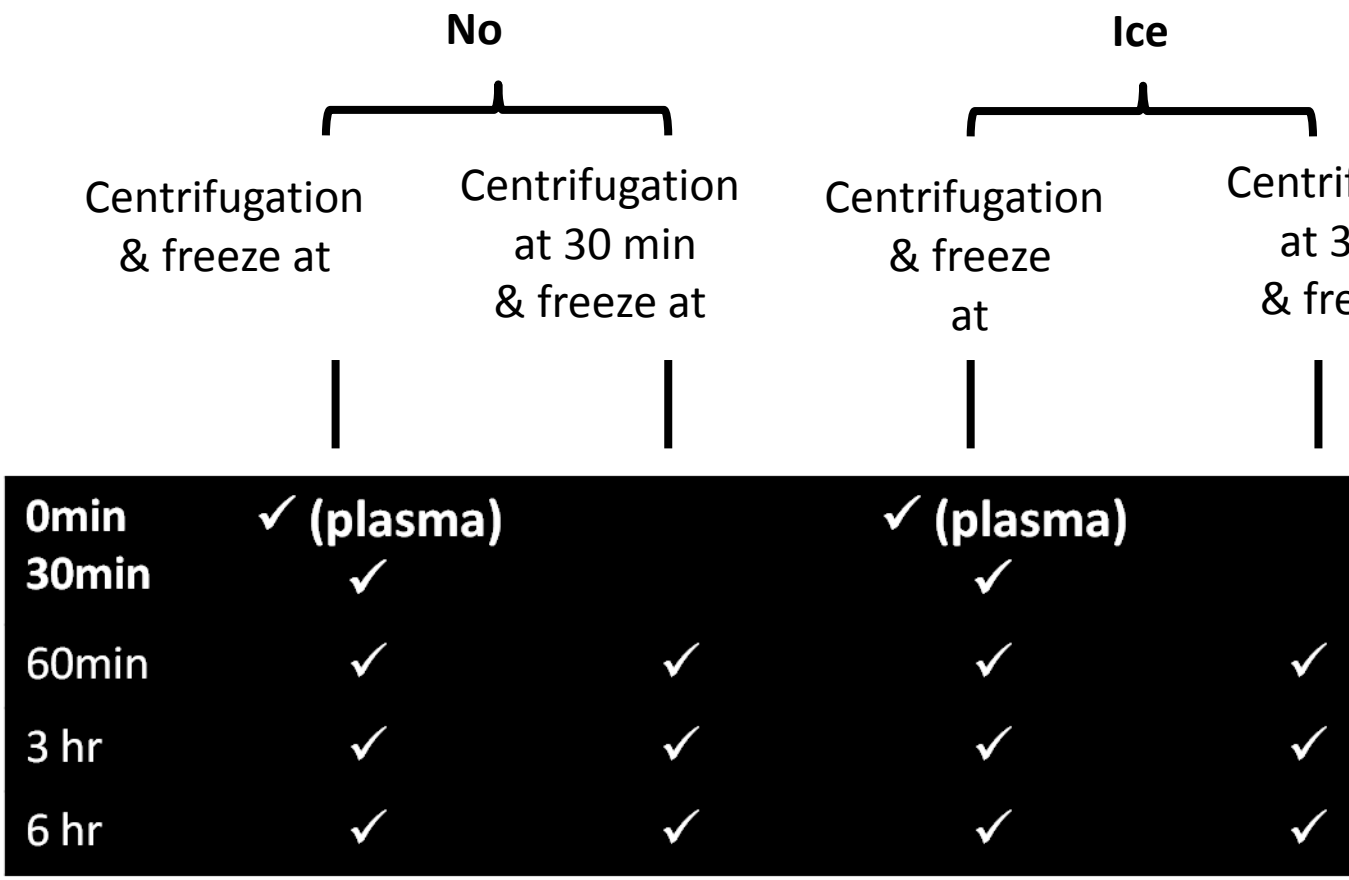


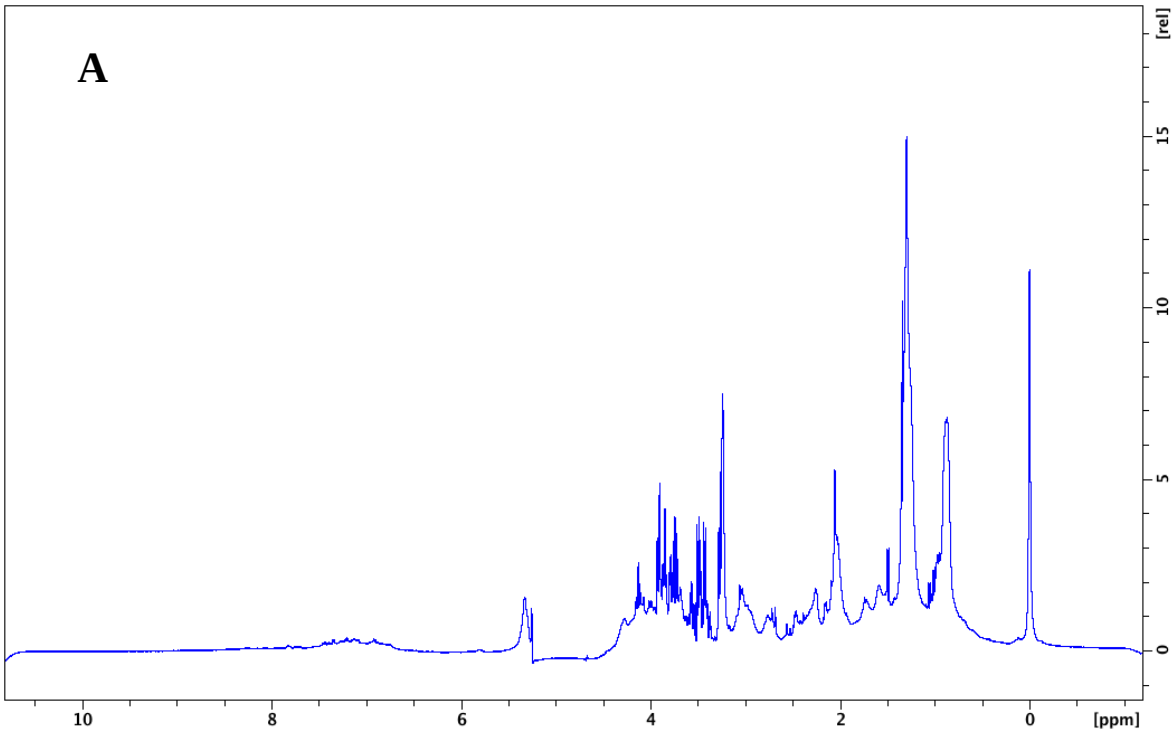
Figure 3.1: Experimental plan for investigating the effect of different sample collection methods on metabolomic analysis of blood (plasma and serum). Plasma was investigated at all time points but serum was not spun until 30minutes. Centrifugation was at 600g for 10 minutes.

3.1.3 Results

3.1.3.1 Unfiltered plasma and serum

NMR spectra of plasma and serum are shown in Figure 3.2. Here we assessed a number of sample collection and processing variables that may impact on the use of metabolomics within a clinical setting. These included time post blood collection and prior to centrifugation to separate the clot from serum and cells from plasma. When data from all the different sampling conditions used to produce the unfiltered plasma were aggregated from the 3 donors, and subjected to non-supervised analysis (Figure 3.3A), this demonstrated a clear separation of donor 2 but some overlap between donors 1 and 3

(p<0.0001 when comparing the PC1 values using one-way analysis of variance (ANOVA)). Non-supervised analysis of unfiltered serum (Figure 3.3C) produced a clear separation of all the donors regardless of the sampling conditions (p<0.0001 for the PC1 values using ANOVA). Supervised analyses (Figure 3.3B and 3.3D) enhanced the separation for unfiltered plasma and serum with good sensitivity and specificity.



B

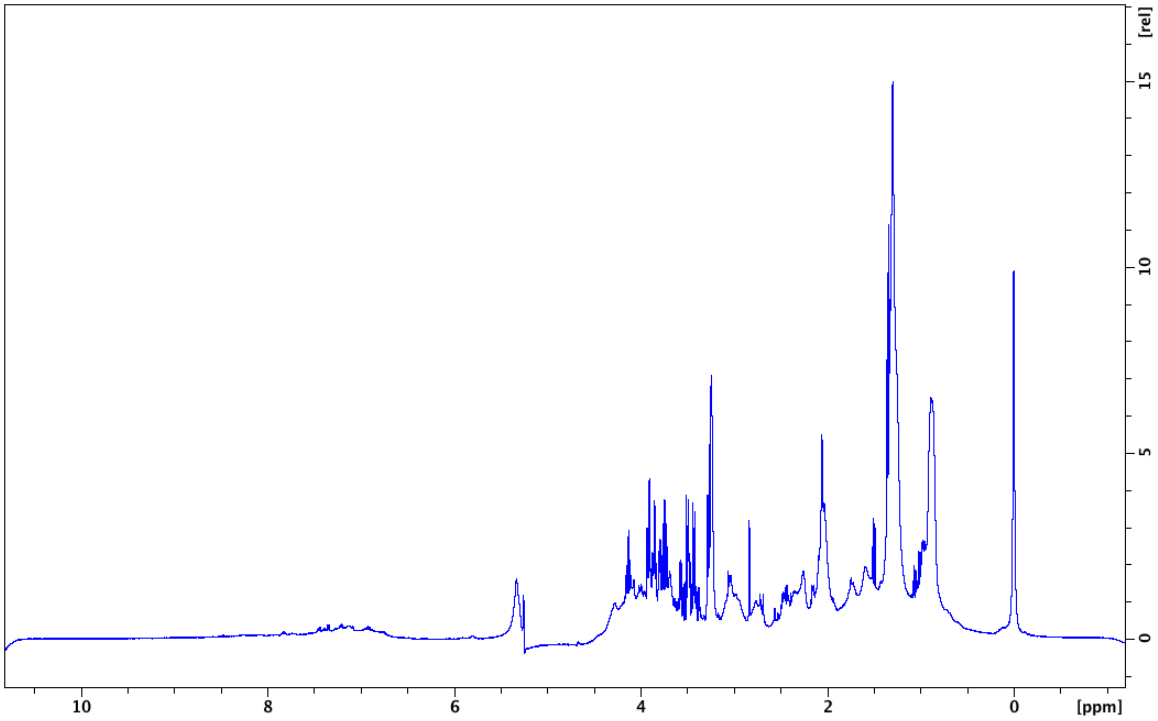


Figure 3.2: NMR spectra of plasma (A) and serum (B) from the same individual.

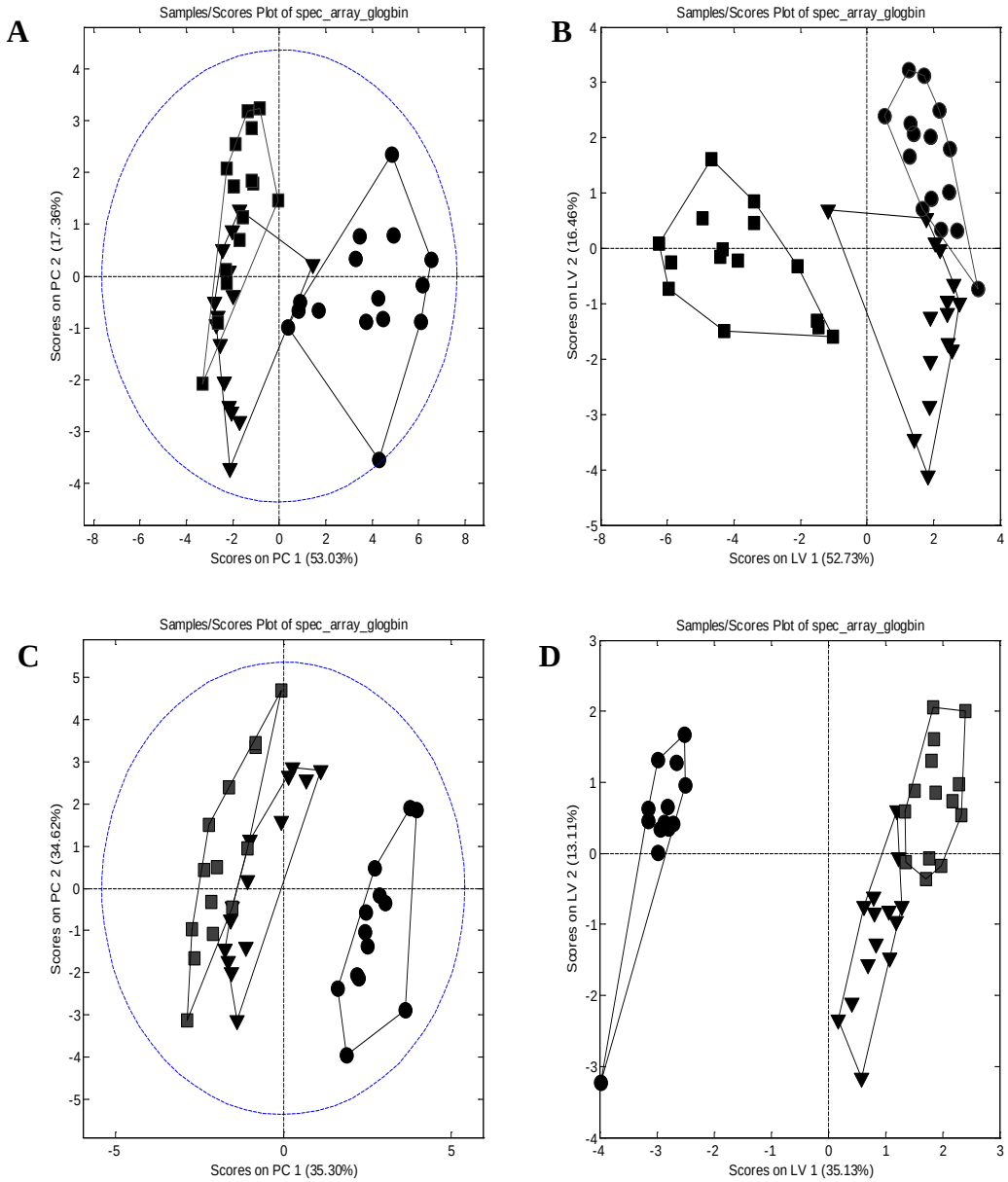


Figure 3.3: 1D 1H NMR spectra of non-filtered plasma and serum from all 3 control donors for all conditions combined (time points, ice/no ice and initial centrifugation). The values on the axis labels indicate the proportion of the variance captured by each principal component. ▼=control 1 ●=control 2 and ■=control 3. PCA of plasma samples (A) and supervised analysis (PLS-DA) of plasma samples (B) separate with a sensitivity of 100% and specificity of 96.7%. PCA of serum samples (C) and supervised analysis (PLS-DA) of serum samples separate with a sensitivity of 92.9% and specificity of 100% (D).

3.1.3.2 Filtered plasma and serum

Non-supervised analysis of filtered plasma (Figure 3.4A) demonstrated that there was separation of donors 1 and 3 but some overlap with donor 2 when combining all the sampling conditions for each donor ($p<0.0001$ for the PC1 values using ANOVA). Non-supervised analysis of filtered serum (Figure 3.4C) illustrated that there was some overlap of all the donors ($p=0.0219$ for the PC1 values using ANOVA). Supervised analyses (Figure 3.4B and 3.4D) enhanced the separation for filtered plasma and serum for all 3 donors regardless of the sampling conditions with good sensitivity and specificity.

These data demonstrate that the main variance in the metabolic profiles of collected biofluids resulted from differences between individuals and not from the effect of the different sampling handling protocols. This implies that the metabolic differences between individuals may still be detected where samples are not collected in a strictly controlled manner (e.g. differing time periods between collection and freezing of serum or plasma derived from the blood) or whether serum or plasma is collected.

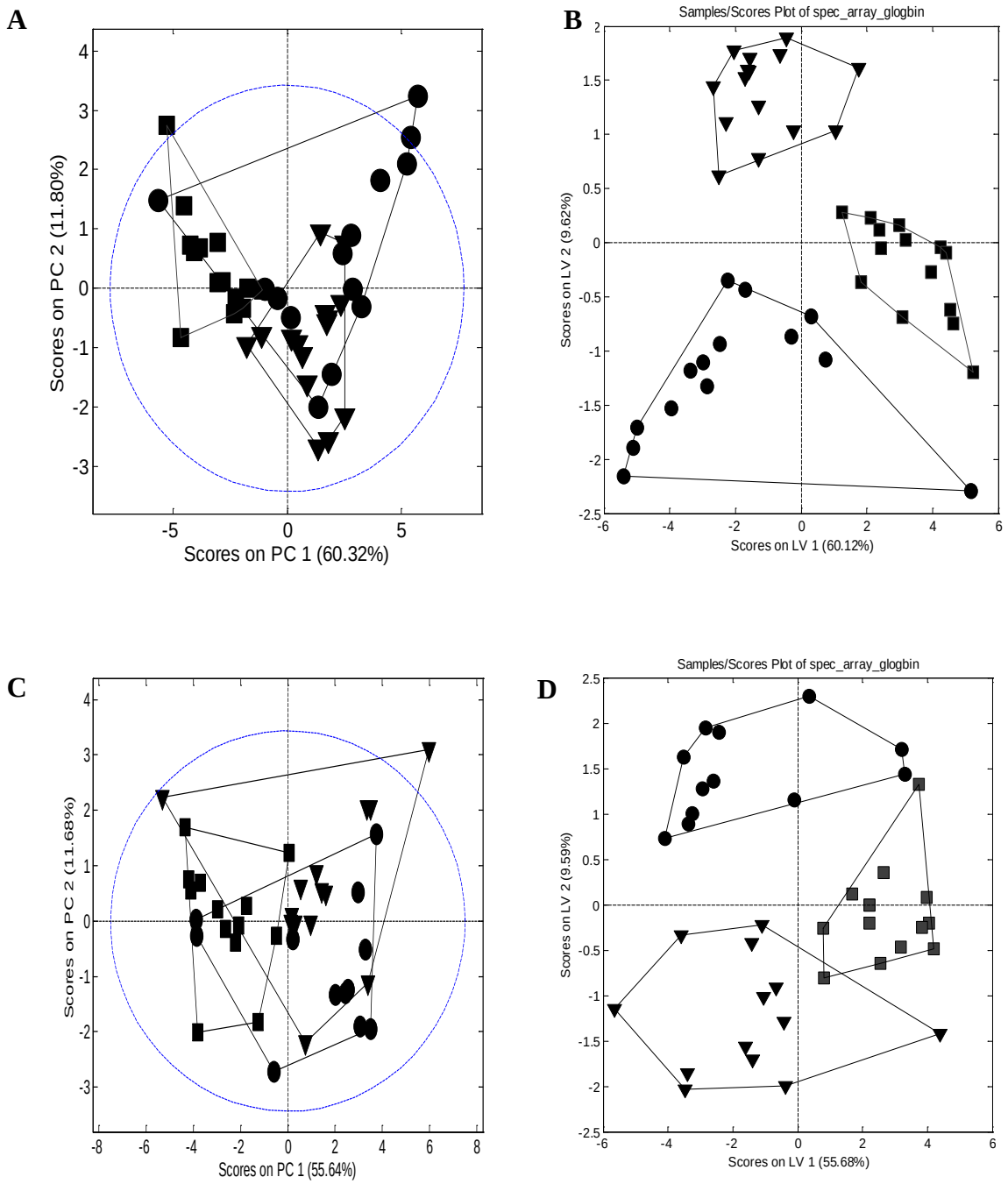


Figure 3.4: 1D 1H NMR spectra of filtered plasma and serum from all 3 control donors for all conditions combined (time points, ice/no ice and initial centrifugation). The values on the axis labels indicate the proportion of the variance captured by each principal component. ▼=control 1 ●=control 2 and ■=control 3. PCA of plasma samples (A) and supervised analysis (PLS-DA) of plasma samples (B) separate with a sensitivity of 92.9% and specificity of 93.1%. PCA of serum samples (C) and supervised analysis (PLS-DA) of serum samples (D) separate with a sensitivity of 92.3%and specificity of 96.3%.

3.1.3.3 Stability of metabolites over time

Metabolic profiles of both plasma and serum displayed good stability of metabolites and concentrations (over 6 hours) regardless of the sampling conditions. Inter-donor variability was much greater than the intra-donor variability for both filtered and non filtered blood (Figures 3.3 and 3.4). Interestingly, inter-donor variability was more pronounced for non-filtered blood than for filtered blood, perhaps deriving from the presence of lipids or lipoproteins in non-filtered blood, which may differ significantly between individuals. Using the PLSDA models for serum and plasma separately, peaks with a VIP of >1 were identified which were responsible for the separation between samples taken at baseline and those taken at 360 minutes (Table 3.1). Lactate ($p=0.0174$) and pyruvate ($p=0.0017$) were the only metabolites for which concentrations altered significantly when plasma remained in contact with cells and similarly lactate ($p=0.0417$) and pyruvate ($p=0.0278$) altered significantly when serum was in contact with cells and clot (Figure 3.5). Variation due to sample collection time was minimized by incubation of blood on ice prior to separation into serum or plasma or by an initial centrifugation to minimise cell to supernatant contact. Both approaches successfully mitigated the effects of cellular contact on lactate concentrations. In contrast, pyruvate still altered significantly in the serum on ice ($p=0.0174$) but did not alter significantly if there was an initial centrifugation (Figure 3.5).

Significant differences in metabolic profiles were found between the blood samples that had been left for different time periods prior to centrifugation and these were similar for plasma and serum.

Peaks (ppm)in plasma which had a VIP>1	Peaks (ppm)in serum which had a VIP>1
8.46	7.83
4.66	4.14
4.14	4.13
4.13	4.12
4.12	4.11
4.10	3.92
3.92	3.90
3.90	3.86
3.86	3.83
3.84	3.75
3.80	3.72
3.72	3.56
3.70	3.52
3.57	3.50
3.55	3.46
3.54	3.43
3.51	3.36
3.50	3.24
3.48	3.03
3.41	2.83
3.39	2.43
3.27	2.42
3.25	2.38
3.24	2.07
3.23	2.06
2.45	2.04
2.38	2.03
1.93	1.49
1.48	1.47
1.33	1.34
1.32	1.33
1.05	1.04
1.00	0.98
0.94	0.95

Table 3.1 Peaks in ppm from the PLSDA weightings plot of serum and plasma over time which had a Variable Importance of Projection (VIP) of >1.

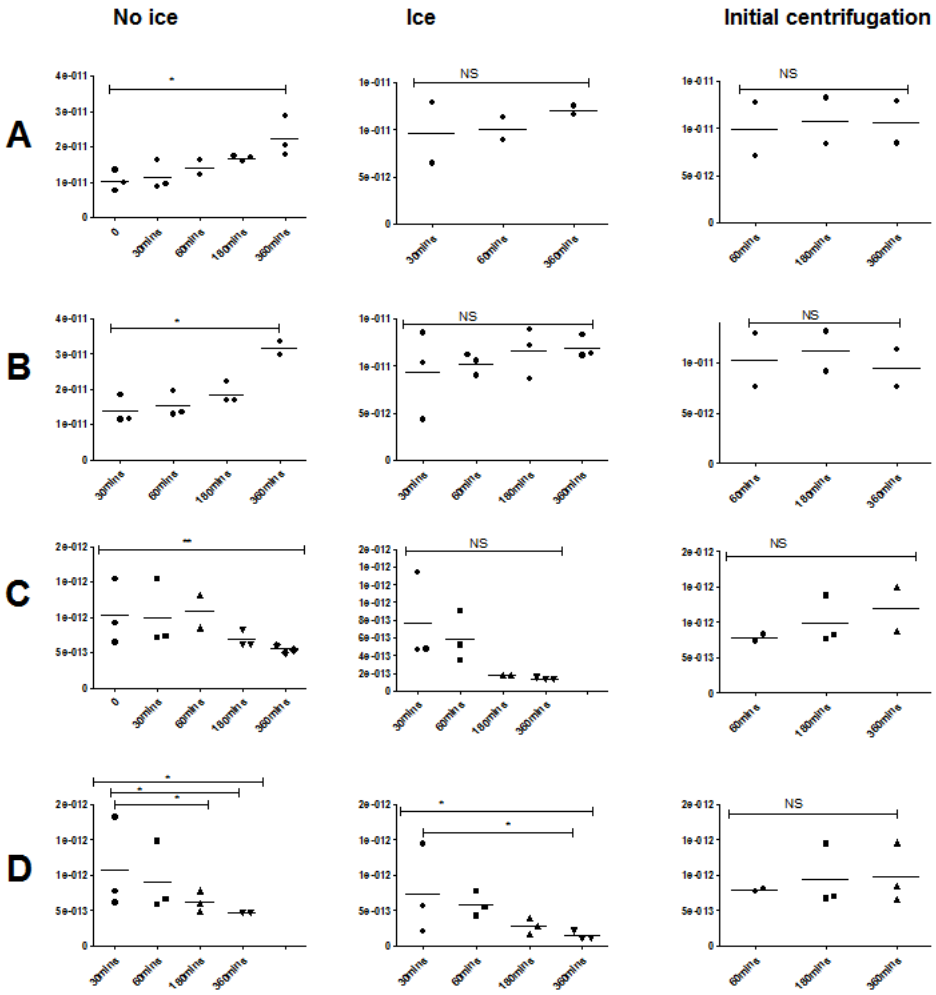


Figure 3.5: Filtered blood from control samples at different time points highlights differences in the metabolite lactate in the plasma (A) and in the serum (B) and pyruvate in the plasma (C) and in the serum (D) with time. Friedman test; p*<0.05, p**<0.01, NS=not significant.

3.1.4 Discussion

Others have studied the effect of varying sample collection times on metabolomic data (Teahan et al. 2006). Teahan et al investigated the effect of storage on ice or not, post collection and prior to further processing, for both plasma and serum and a range of time delays in sample processing. In this study we extended the time course to include a 6 hour time point to reflect the longest time delay likely to be experienced in most routine clinical settings. Here we have demonstrated a gradual increase in lactate up to 3 hours but a larger

increase after this point. We have also demonstrated that the increase in the concentration of pyruvate parallels that for lactate. Teahan et al also found variation in lactate arising from the serum-clot time, with the largest variation being with the longest delay in processing, though clotting on ice seemed to reduce this variation (Teahan et al. 2006). This was based on the analysis of whole serum and plasma, which are not ideal samples for metabolomic analysis due to the potential for protein-dependent distortion of the spectra. However, our data demonstrate in both filtered (deproteinised) and whole plasma and serum that only lactate and pyruvate change significantly and that no other confounding metabolites were revealed by the filtration process.

This study demonstrates that significant variation in levels of lactate and pyruvate may result over time following sample collection, and that this may be affected by sampling conditions for both plasma and serum (Figure 3.5). Pyruvate and lactate are intimately linked in the glycolytic pathway. This variation in pyruvate and lactate concentration may be mitigated by initial centrifugation of the sample and likely reflect the pyruvate consumption and lactate production by red blood cells (Wiback and Palsson 2002). Ideally, time prior to centrifugation should be kept to a minimum and samples should be collected on ice to reduce variation. However, if centrifugation or ice are not available in the clinical setting and sample processing cannot be performed in a timely manner; we suggest it may be important to exclude lactate and pyruvate in subsequent analyses. This work demonstrates that there is very little variation in the concentration of other metabolites and indicates that, with the above caveats, blood samples from routine clinical practice can confidently be used in metabolomic studies.

3.2 Urine

3.2.1 First void or random urine

Several studies have used urine samples for metabolomic analysis (Lin et al. 2010;Williams et al. 2009). We are aware that it is important for samples to be collected in a controlled manner to obtain consistent and accurate NMR results. We wished to ascertain the reproducibility of different forms of urine samples. A first void urine sample and a random urine sample were collected on three different occasions from eight control subjects. Triplicates of each sample were analysed by NMR.

The PCA data are shown in Figure 3.6 and the PLSDA data are shown in Figure 3.7 and 3.8. There are marked differences between the First Void and the Random urine samples of each individual control. However, it is still possible to distinguish each individual control subject very well from the other controls. The three separate urine samples for each collection type from each individual are very reproducible. For example samples 1, 2 and 3 are very similar and samples 4, 5 and 6 are very similar but 1, 2 and 3 are very different from 4, 5 and 6.

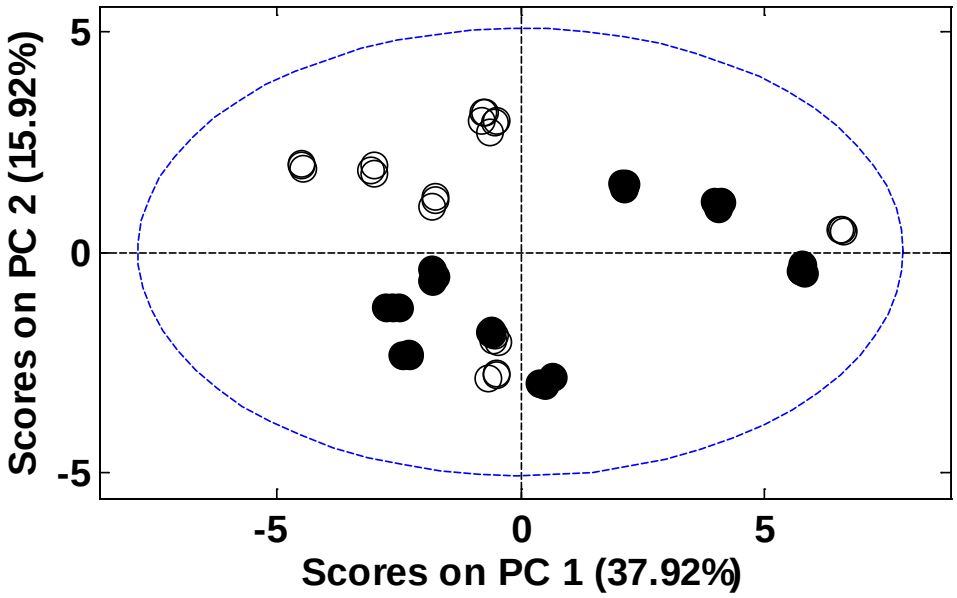


Figure3.6: 1D 1H NMR spectra of First Void (●) and Random (○) urine samples were subjected to PCA. The values on the axis labels indicate the proportion of the variance captured by each principal component. This demonstrates marked differences between first void and random urine samples.

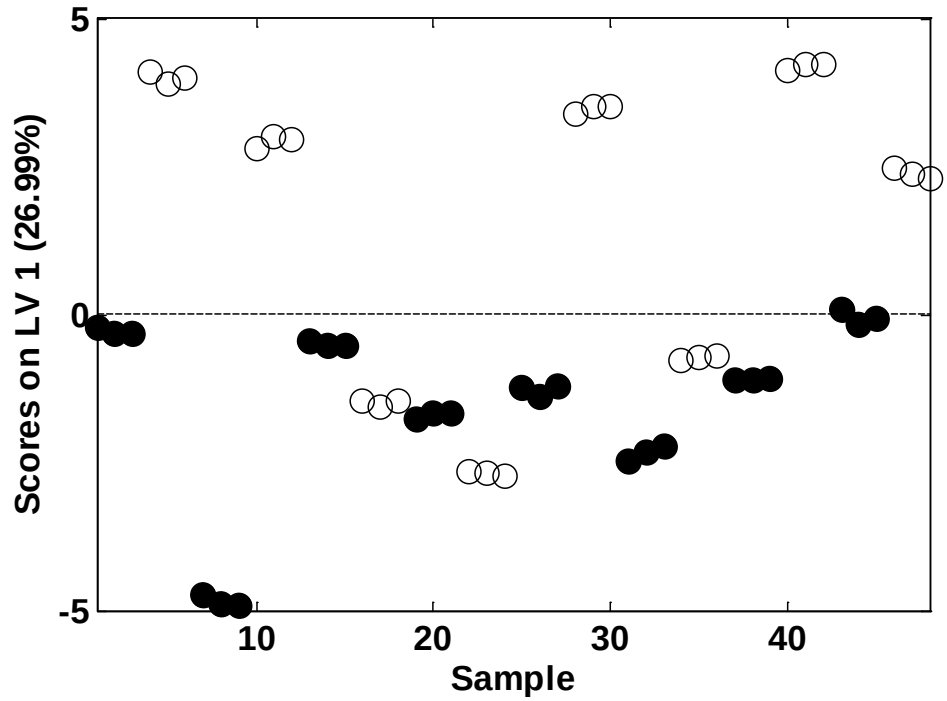


Figure 3.7: 1D 1H NMR spectra of First Void (●) and Random urine samples (○) were subjected to supervised analysis (PLS-DA). The values on the y axis label indicate the proportion of the variance captured by each principal component. The first 3 first void urine samples (●) are from the same control. The three random urine (○) samples for the same control donor follow the 3 first void (●) samples. First void urine samples are very different from random urine samples for each individual.

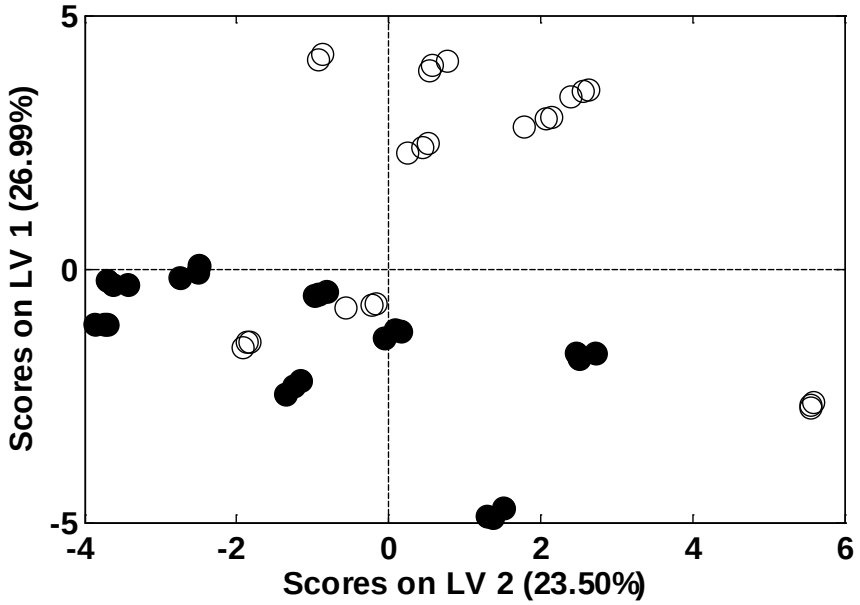


Figure 3.8: 1D 1H NMR spectra of First Void (●) and Random urine samples (○) were subjected to supervised analysis (PLS-DA). The values on the axis labels indicate the proportion of the variance captured by each latent variable. Urine samples of the same sampling type are very reproducible.

3.2.2 Discussion: urine

Urine is a very important and useful biofluid for investigating metabolites. It is easily accessible and requires little preparation for NMR as it only contains small amounts of protein so there is little interference or need for filtration. NMR analysis is strongly influenced by pH and as the pH of urine can be extremely variable samples need a higher concentration of phosphate buffer (Lindon et al. 1999;Miyataka et al. 2007). There has been no previous work using urine samples in the metabolomics of RA as most studies have used serum, plasma or synovial fluid (Lauridsen et al. 2010;Naughton et al. 1993a;Parkes et al. 1991;Weljie et al. 2007). Urine samples have been used for metabolomic studies quite widely in other inflammatory conditions including inflammatory bowel disease (Lin et al. 2009;Lin et al. 2010;Williams et al. 2009) and have produced interesting and useful findings.

We found large differences between the first void and random urine samples for each individual but the individuals could be easily identified from each other and the samples were very reproducible. Previous work looking at urine sample collection has shown that normal urine is metabolically stable for 24 hours post collection at room temperature (Maher et al. 2007). However, differences in the NMR spectra have been seen with the different methods of urine collection e.g. 24hour urine, first void urine and random urine samples. Previous work has demonstrated that the inter-donor variability is the greatest factor but the time of day that a sample is collected does play a part. Others have found that first void urine was the least reliable method of collecting urine in terms of reproducibility and that samples taken later in the afternoon were more reliable (Lenz et al. 2003;Maher et al. 2007). This is thought to be the effect of creatinine being most concentrated in the morning. Therefore, in view of reproducibility and ease of sample collection we concluded like others that the use of random urine samples was appropriate for our metabolomic studies in RA (Assfalg et al. 2008).

3.3 Number of fibroblasts required to produce a NMR spectrum

3.3.1 Introduction

No previous published work has been done using fibroblast cells in NMR based metabolomics although there is a significant body of work using NMR in cancer cells. Cancer cells grow very rapidly and the number of cells used in these experiments has been approximately 50 million (Musacchio et al. 2009). Fibroblast cells grow much slower so we wanted to define the minimum number of cells we could use to produce a NMR spectrum which could be used to potentially distinguish between fibroblasts in different metabolically active states.

3.3.2 Materials and methods

Samples were produced using 1 x 10^6 synovial fibroblast cells. A representative spectrum produced is shown in Figure 3.9.

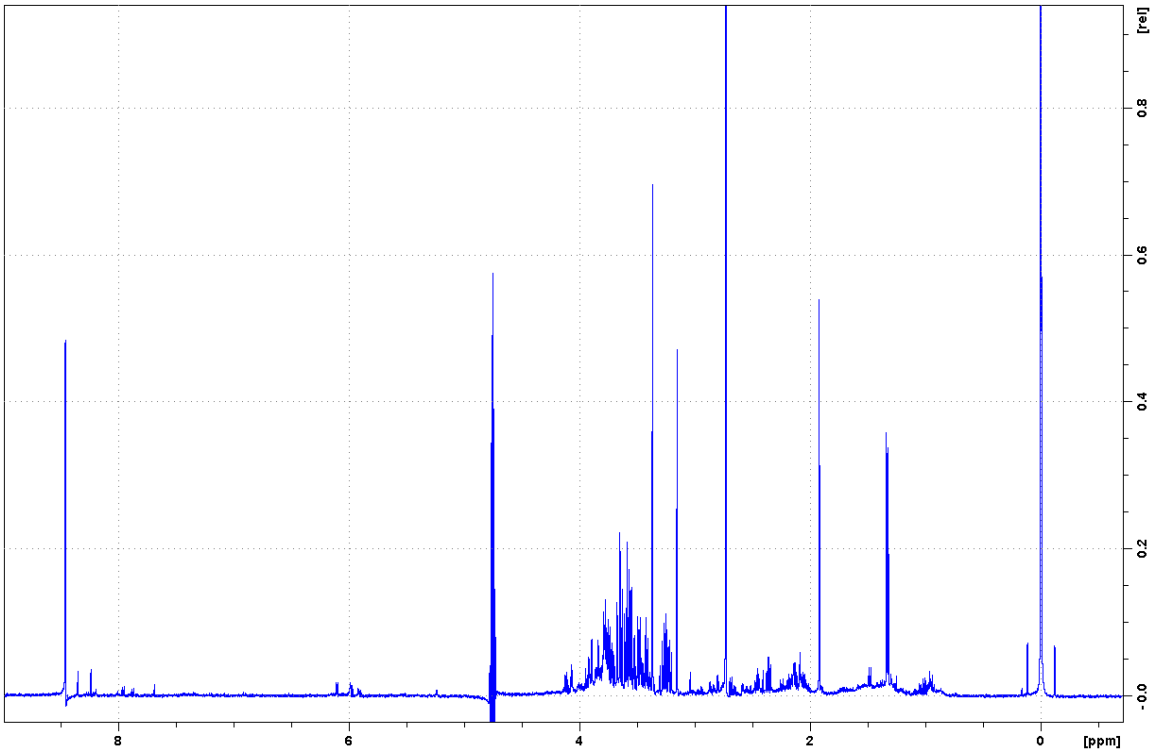


Figure 3.9: Spectrum produced using 1 x 10^6 synovial fibroblast cells demonstrating several metabolites

Extracts from one million cells produced a spectrum with good resolution (peaks in close proximity could be easily distinguished) but we wished to ascertain if spectra of fibroblasts could be used to assess potential differences between early inflammatory arthritis that resolves and early arthritis that persists. We therefore compared one, two and three T75 flasks of synovial and dermal fibroblasts (as these sites can be distinguished in terms of T cell and neutrophil accumulation) from two different patients with established RA to see if the two sites could be distinguished metabolically. Each T75 flask usually contains

between half a million and one million fibroblasts at confluence. It is well known that fibroblasts from different anatomical regions display characteristic phenotypes that are maintained after prolonged culture in vitro, suggesting that many fibroblasts have an imprinted phenotype that is stable (Buckley et al. 2001). Fibroblasts from patients with established RA were fed as per section 2.2.1. Two or three flasks were combined and the cells were extracted as per section 2.2.3 and 2.3.

3.3.3 Results

The PCA data shown in Figure 3.10 demonstrates the differences between the fibroblasts of the 2 different RA patients and the differences between synovial and dermal fibroblasts. We then overlaid the spectra for 1, 2 and 3 flasks for each cell line to identify the differences in metabolites due to the different numbers (Figure 3.11).

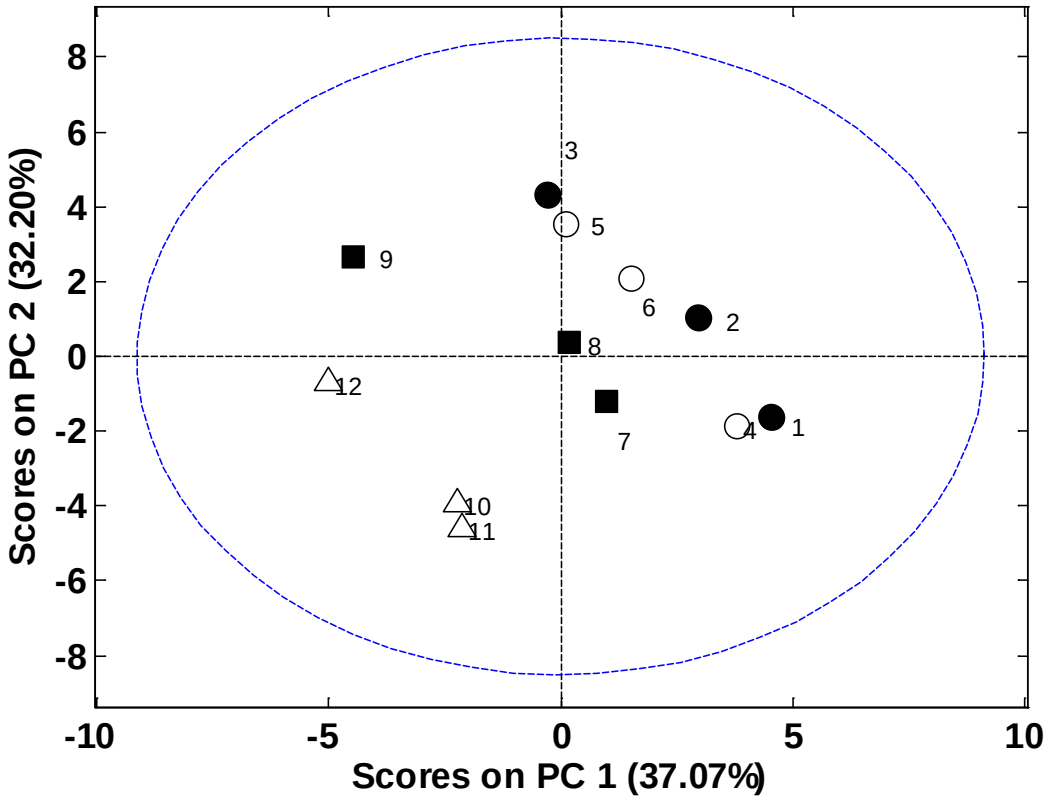
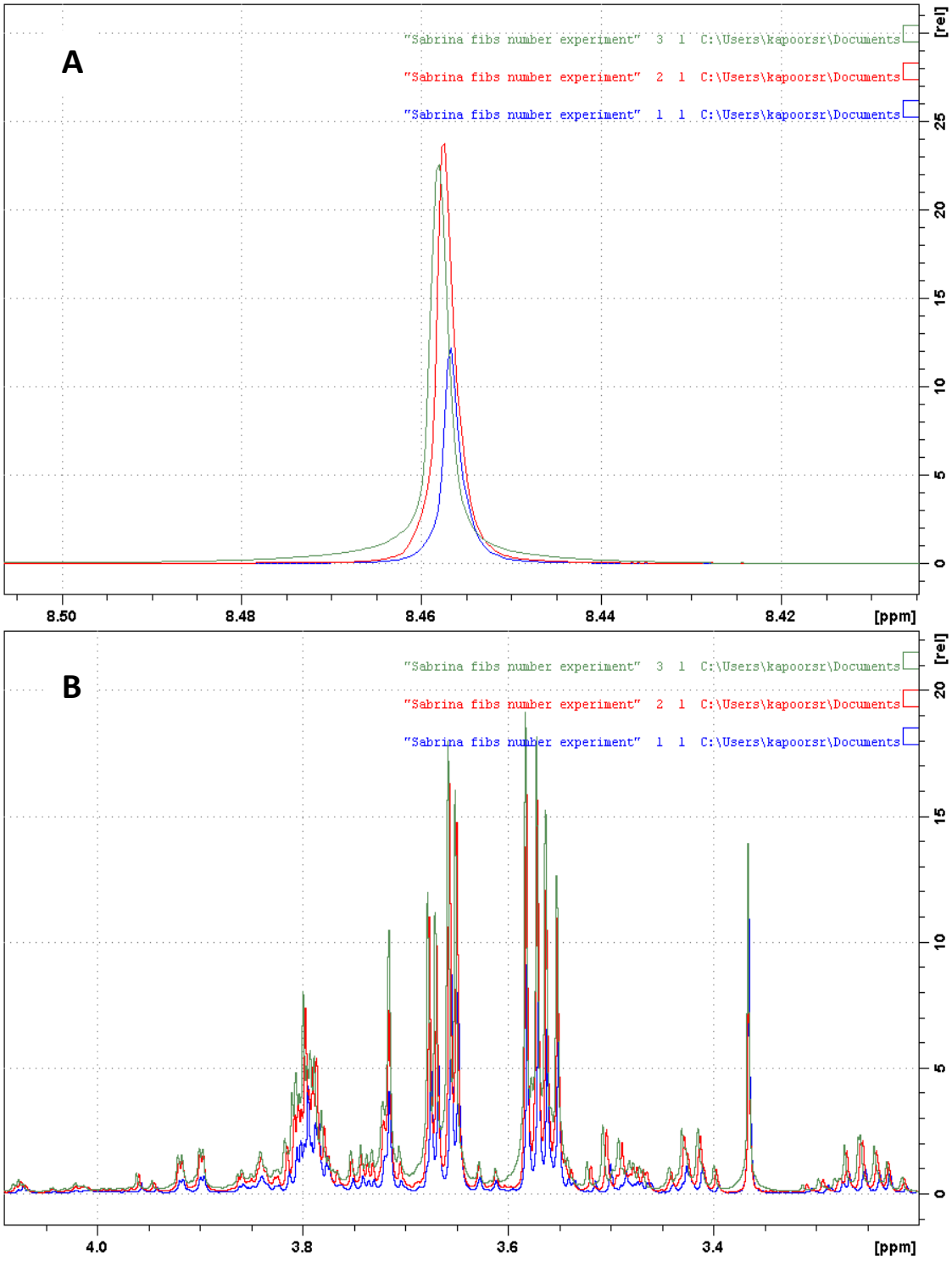


Figure 3.10: 1D 1H NMR spectra of RA synovial and dermal fibroblasts were subjected to PCA. The values on the axis labels indicate the proportion of the variance captured by each principal component. The synovial fibroblasts (●) and the dermal fibroblasts (○) represent fibroblasts from the same patient. The synovial fibroblasts (■) and the dermal fibroblasts (Δ) represent fibroblasts from the same patient. Samples 1, 4, 7 and 10 represent one T75 flask, samples 2, 6, 8 and 11 represent two T75 flasks and samples 3, 5, 9 and 12 represent three T75 flasks.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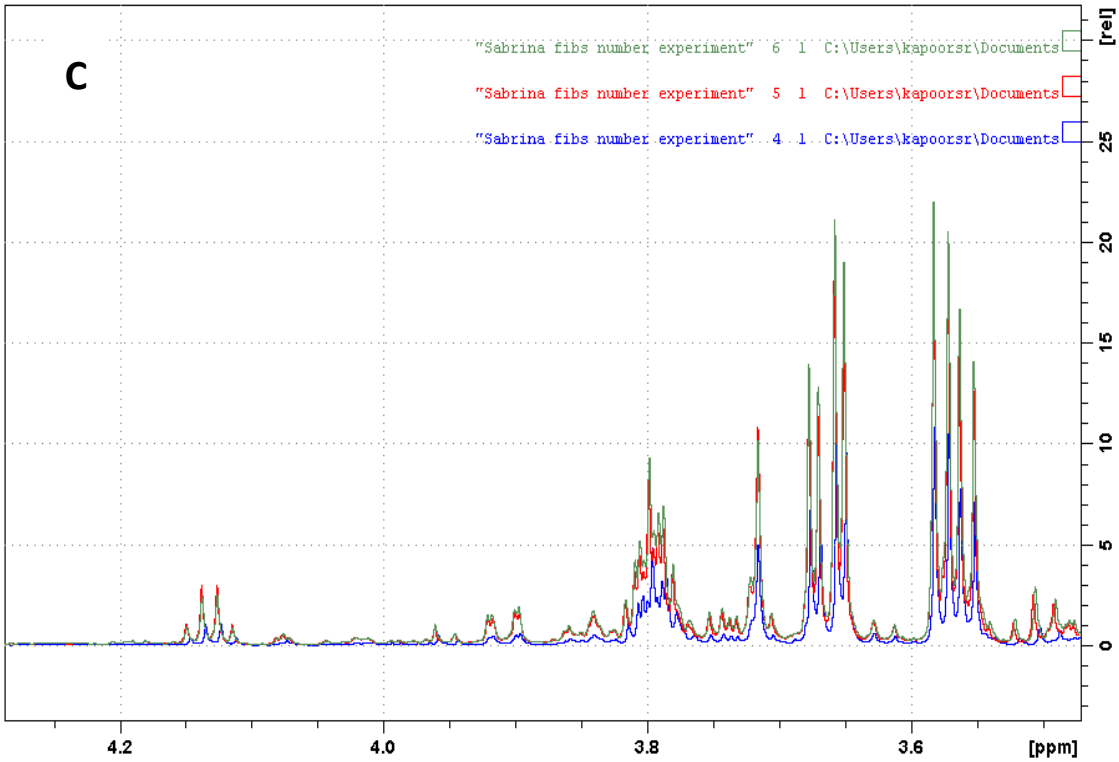
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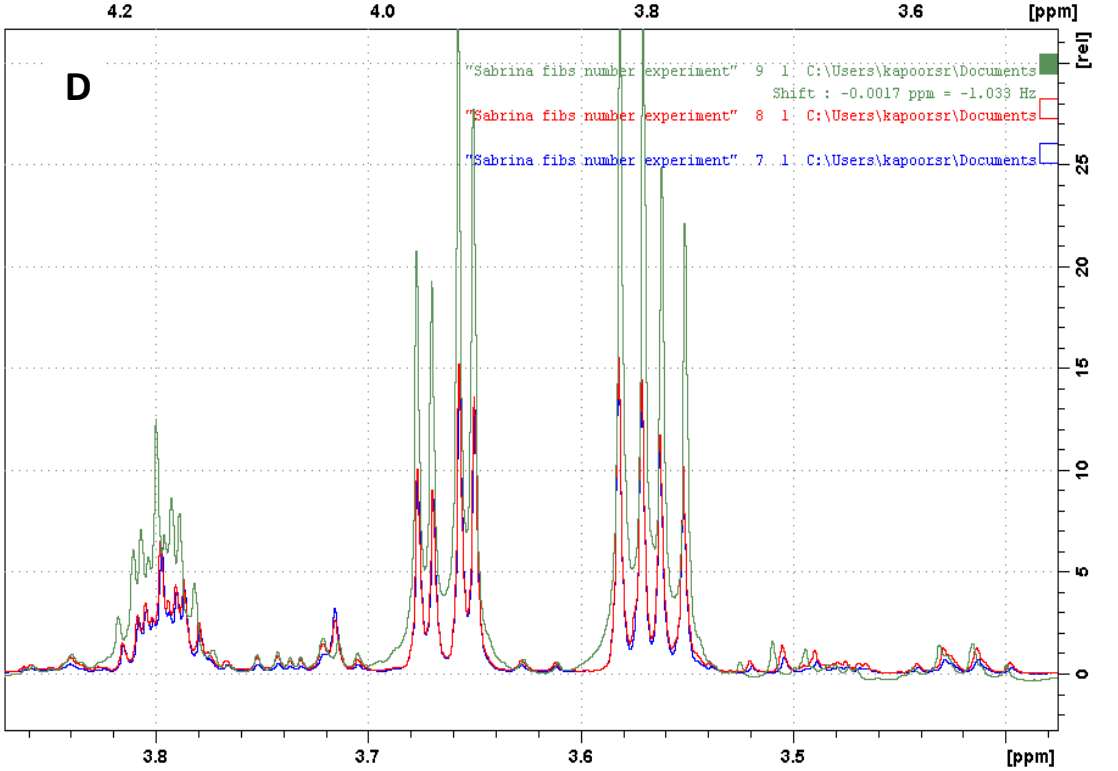
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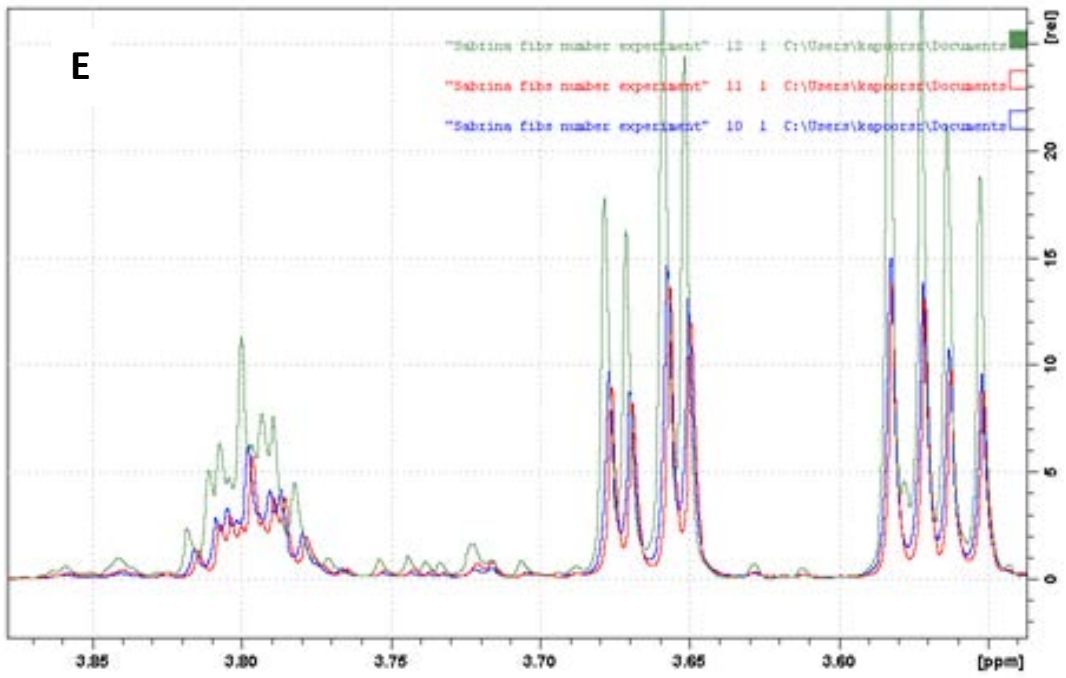


Figure 3.11 1D 1H NMR spectra from one, two and three T75 flasks of RA synovial and dermal fibroblasts from two different established RA patients.

A and B illustrate the synovial fibroblasts and C illustrates the dermal fibroblasts from the same RA patient. D illustrates the synovial fibroblasts and E illustrates the dermal fibroblasts from the other RA patient. One T75 flask is depicted in blue, two T75 flasks are depicted in red and three T75 flasks are depicted in green.

On overlying the spectra no peaks were visibly lost but the peak heights of the metabolites were much smaller for one flask compared to two or three flasks. The peak heights were similar for two and three T75 flasks particularly for synovial and dermal fibroblasts of cell line 22 (Figure 3.11 A, B and C).

3.4 Fibroblast medium

3.4.1 Introduction

Fibroblasts are grown in complete fibroblast medium. Metabolites are used by the cells from this medium and are also extruded from the cells into this medium. We envisaged that this conditioned medium may be a good source for studying the metabolic activity of fibroblasts.

The medium of cells has not been used for NMR previously but we were interested to explore whether it might give us a significant amount of useful information. When fibroblasts are split the passage number increases by one and this is done when the cells appear visually confluent which is very subjective. How confluent the cells are may affect metabolism, although it is felt that synovial fibroblasts do not undergo contact inhibition (Lafyatis et al. 1989). The number of fibroblasts present, and the quantity of metabolites extracted, may also affect metabolism, though this should not affect our results, since the analysis of the NMR data involves a normalisation of the NMR spectra to correct for differences in overall concentration.

In order to investigate the number of fibroblasts present, and the quantity of metabolites extracted, I collected fibroblast medium from cell lines at different weeks of growth. To investigate the effect of different passages on metabolic profiles I examined the fibroblast medium of a cell line and compared the different passages (P2 to P10) to see how different their NMR spectra were. Most researchers use synovial fibroblasts in the range of P2 to P5 as at higher passages there is a reduction in proliferation rate and senescence (Zimmermann et al. 2001). Though cells either differentiate or we select different

subpopulations over time, central pathological characteristics which appear to be imprinted such as IL-6 expression and invasiveness in the SCID mouse model remain present over many passages (Hirth et al. 2002;Mueller-Ladner et al. 1996). A recent study has looked at the effect of different passages in RASF and gene expression and they found that there was little change in gene expression using P2-P4 but there were alterations in gene expression in passages above this (Neumann et al. 2010). However, we were not aware if higher passages affect metabolites and we aimed to determine this by analysing our samples.

3.4.2 Materials and methods

The conditioned medium of established RA synovial fibroblasts was kept and stored during the feeding process. The samples were prepared as section 2.4.5 and were compared to normal fresh complete fibroblast medium. Conditioned medium was also taken from the same synovial fibroblasts one week after each split to represent medium from different passages. To try and standardise the process as much as possible these samples were collected from a set number of cells (10 000) one week after splitting. Fibroblast medium was also collected from cell lines at different weeks of growth after this to represent different numbers of cells. Fibroblast cultures were supplemented with fresh culture medium once weekly by replacing two thirds of the old culture medium with new culture medium. The old culture medium was collected at weekly intervals. It was not possible to count the number of cells accurately during the growth period without splitting the cells but the increase in number of cells can be seen visually by microscopy without splitting. The spectra from these samples were compared to determine the contribution of variability in cell numbers to the results of NMR experiments.

3.4.3 Results

There are clear differences between fresh fibroblast medium and conditioned medium and this difference all seems to be on PC1 (Figure 3.12).

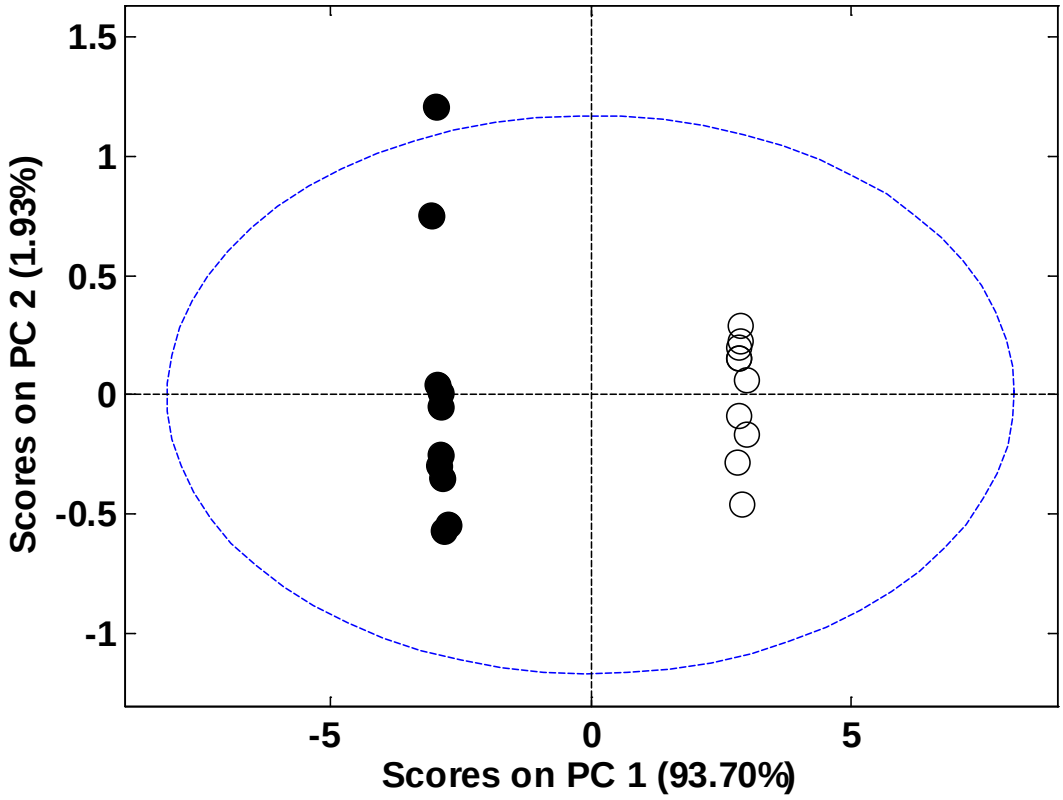


Figure 3.12: 1D 1H NMR spectra of normal (●) and Conditioned (○) fibroblast medium were subjected to PCA. The values on the axis labels indicate the proportion of the variance captured by each principal component. All the conditioned medium was taken from one established RA cell line. There are marked differences between the normal and conditioned fibroblast media.

There are clear differences between conditioned medium from synovial fibroblasts of different cell lines, regardless of passage by both PCA (Figure 3.13) and PLSDA (Figure 3.14).

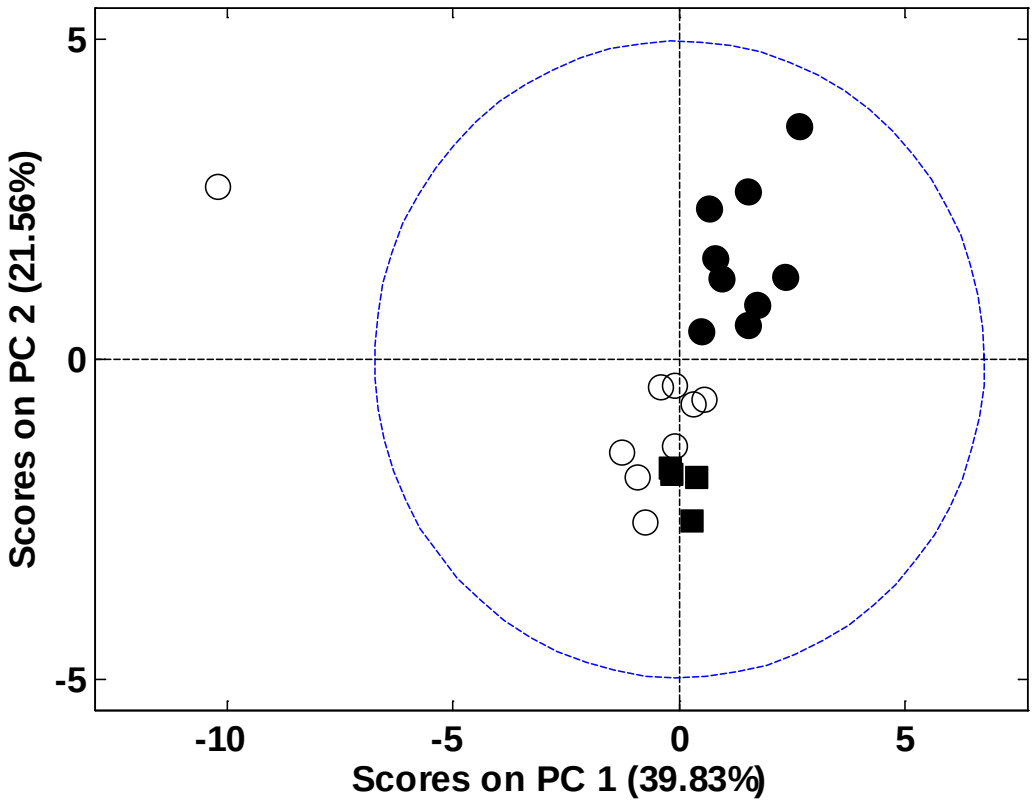


Figure 3.13 1D 1H NMR spectra of medium from fibroblasts at different passages (P2 to P10) were subjected to PCA. The values on the axis labels indicate the proportion of the variance captured by each principal component. The different symbols represent the three different cell lines. The cell lines are easily distinguishable regardless of the passage.

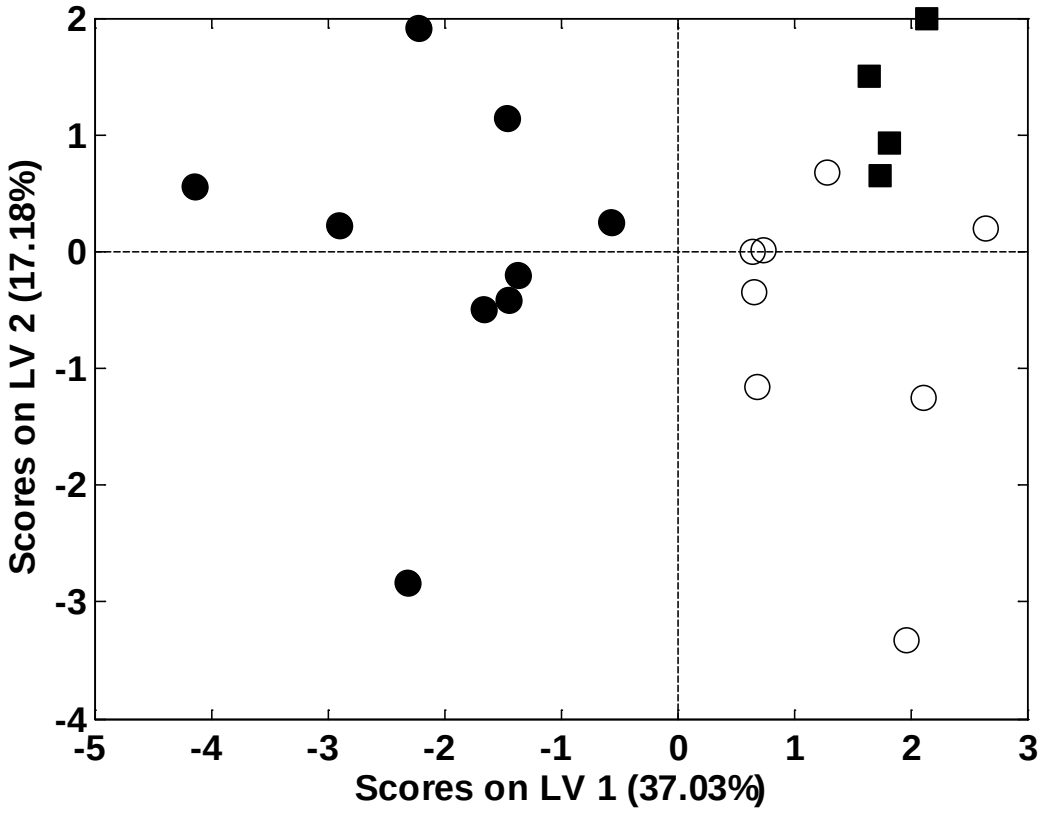


Figure 3.14 1D 1H NMR spectra of medium from fibroblasts at different passages were subjected to supervised analysis (PLS-DA). The values on the axis labels indicate the proportion of the variance captured by each principal component. The different symbols represent the different cell lines. The cell lines are easily distinguishable regardless of the passage.

There are clear differences between conditioned medium of different cell lines on both PCA (Figure 3.15) and PLSDA (Figure 3.16) regardless of the number of fibroblasts that have been growing in it.

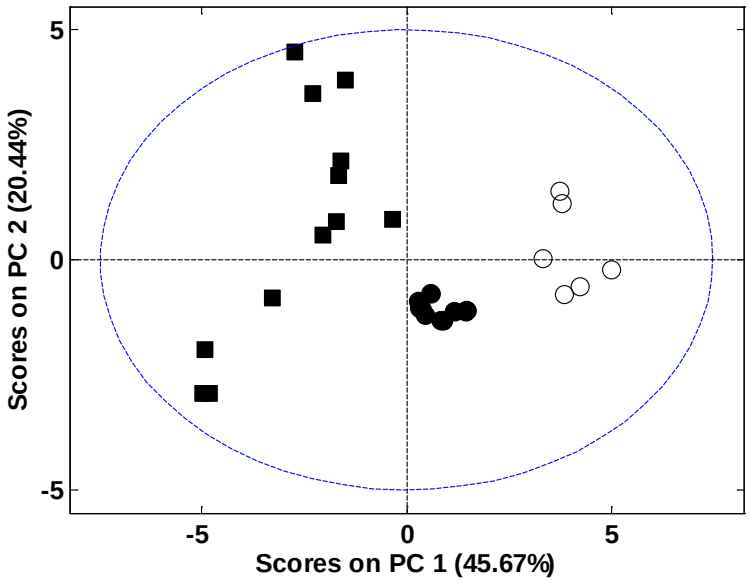


Figure 3.15 1D 1H NMR spectra of fibroblast medium containing different numbers of fibroblasts were subjected to PCA. The values on the axis labels indicate the proportion of the variance captured by each principal component. The different symbols represent the three different cell lines. The cell lines are easily distinguishable regardless of the number of fibroblasts.

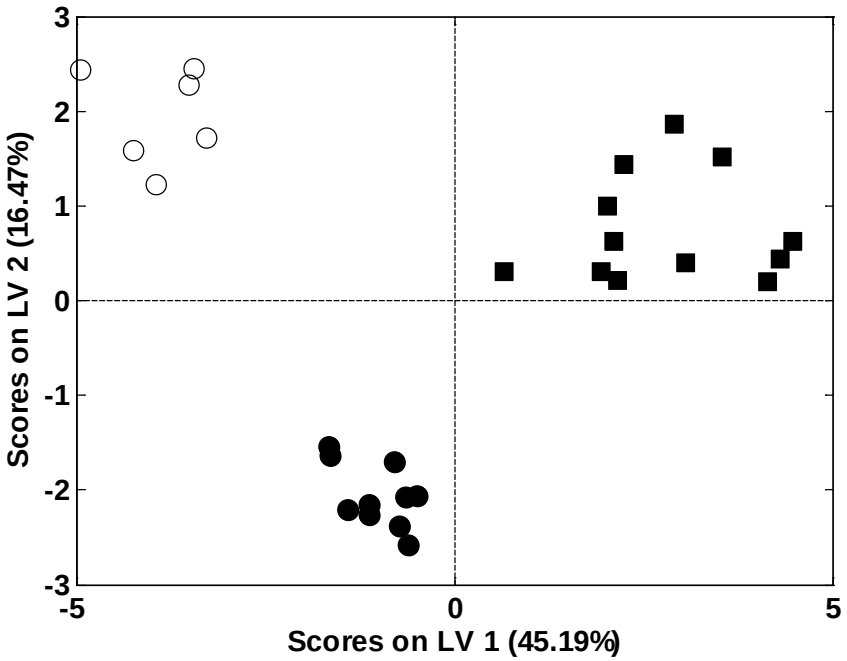


Figure 3.16 1D 1H NMR spectra of fibroblast medium containing different numbers of fibroblasts were subjected to supervised analysis (PLS-DA). The values on the axis labels indicate the proportion of the variance captured by each principal component. The different symbols represent the different cell lines. The cell lines are easily distinguishable regardless of the number of fibroblasts and PLSDA enhances this separation.

3.5 Discussion: Fibroblasts and fibroblast medium

There has been no published research using fibroblasts in NMR based metabolomics. The majority of research in NMR based metabolomics has used cancer cell lines (Teahan et al. 2011) and this has been based on large number of cells, a position which is made feasible by the fact that the cell lines are fast growing. As no previous research has used fibroblasts and they are much slower growing than cancer cells we wanted to determine the minimum number of fibroblast cells that we could use in our experiments.

The study we did using approximately 1 million cells did produce a NMR spectrum but we were unsure whether this spectrum would give us sufficient information to detect any potential differences between the fibroblasts from patients with different types of inflammatory arthritis. We compared spectra of dermal and synovial fibroblasts from two different patients as we hypothesised that there would be metabolic differences between these sites in view of the research demonstrating differences in the pattern of T cell and neutrophil accumulation between these sites (Filer et al. 2006). The PCA results demonstrate differences (Figure 3.10) between the synovial and dermal fibroblasts for one, two and three T75 flasks. We were unaware which metabolites may alter between fibroblasts from early arthritis that go on to develop persistent arthritis and fibroblasts from patients whose arthritis resolves. Hence, we compared the spectrum from one, two and three flasks of the same cell type and line (Figure 3.11) to determine if any metabolites were lost by using fewer cells. We did not identify any metabolites that were lost but we did find that the peak heights were much smaller when cells were used from one flask compared to two or three flasks. Also, the cell lines that we used for this experiment grew

quite well compared to average synovial fibroblasts as the number of cells per flask varied from 0.7 million to 1.6 million.

Our work in cell culture illustrates that dermal fibroblast grow more rapidly than synovial fibroblasts and the number of cells in a confluent flask are also greater. Hence, we hypothesised that the synovial fibroblasts from patients with early arthritis may not all grow as well as the fibroblasts in this experiment. We also speculated that the metabolic differences between fibroblasts from different sites may be greater than the differences between fibroblast from the same site in different conditions. Therefore we used two T75 flasks for all our future experiments.

The conditioned medium from fibroblasts from different cell lines could be distinguished regardless of the passage or the number of fibroblast that had been grown in it. This suggests that NMR may be able to differentiate between the metabolic profiles of different RA patients using fibroblast medium as the investigating biofluid.

3.6 Conclusions

We have conducted quality control experiments for several different biofluids which can be used in NMR based metabolomics. We have demonstrated that both filtered and unfiltered serum and plasma can be used from routine clinical practice. Blood samples should be processed in a timely manner as the metabolites lactate and pyruvate alter over time. However, there is very little variation in the concentration of other metabolites and indicates that, with the above caveats, blood samples from routine clinical practice can

confidently be used in metabolomic studies. Our work with urine samples illustrated marked differences between random urine sample and first void urine samples but good reproducibility for each collection type. Therefore, in view of reproducibility and ease of sample collection we concluded that the use of random urine samples was appropriate for our metabolomic studies in RA.

Our work with fibroblast cells comparing the number of fibroblasts grown in one, two and three T75 flasks did not demonstrate any loss of metabolites between the three sampling conditions but we did find a difference in peak height between one flask compared to two or three. Therefore we chose to use two T75 flasks for all future experiments. Our work using fibroblast media illustrated that normal fibroblast media could be easily distinguished from conditioned media and that different cell lines could be clearly identified regardless of the passage or number of fibroblasts. However, to minimise any confounding factors we planned to use media from cells with similar numbers and those that were passage matched. All this work suggests that these biofluids may give us interesting results using NMR based metabolomics.

4.0 DATA (Differentiating the mechanism of action of anti-TNF-α agents) study

4.1 Introduction

The introduction of anti-TNFα treatment has revolutionised the management of RA (Furst et al. 2011;Keystone et al. 2004;Maini et al. 1999;Spencer-Green 2000). Several agents are available within this class but response rates are imperfect: only 26-42% of patients achieve a good EULAR response within 6 months (Bazzani et al. 2009;Bennett et al. 2005;Rau 2005). Given the high cost of these therapies, and implications for disease progression in non- responders waiting for 3 to 6 months for clinical reassessment, the ability to predict treatment responses at baseline is an important goal. Several approaches have been used to attempt to predict responses to TNF antagonists but most of these are relatively weak. Clinical factors such as smoking and high levels of disability at baseline are associated with a poor response to TNF antagonists (Hyrich et al. 2006) and concurrent use of methotrexate is associated with a better response to TNF antagonists (Hyrich et al. 2006). Multiple genetic factors have been investigated for predicting responses to TNF antagonists in RA but there has been no convincing evidence (Emery and Doerner 2011;Potter et al. 2009;Umicevic et al. 2013). Proteomic studies have also been used to investigate responses to TNF antagonists but these are limited due to small sample size (Fabre et al. 2008;Hueber et al. 2009). Hence, new approaches are needed.

In addition to synovitis there are widespread systemic effects mediated by proinflammatory cytokines that impact upon metabolism. Muscle wasting is a common feature of RA and its extent is associated with RA disease activity (Summers et al. 2010) but low BMI is uncommon, as fat mass is preserved or even increased (Summers et al.

2008). The extent of the metabolic changes and the types of metabolites seen may therefore be good markers of cytokine mediated inflammatory processes in RA.

Metabolomic analysis, based on nuclear magnetic resonance (NMR) spectroscopy of biofluids, can be used to identify a broad range of metabolites simultaneously. Using this approach, the identification of several metabolites in cancer and cardiovascular disease has provided insights into disease mechanisms and has highlighted their potential as biomarkers of disease activity and response to therapy (Brindle et al. 2003;Pan et al. 2011;Sreekumar et al. 2009).

Systemic changes in many low molecular weight metabolites are reflected by their levels in urine and, indeed, metabolomic analysis of urine samples has been used in inflammatory conditions such as inflammatory bowel disease (IBD) (Murdoch et al. 2008;Schicho et al. 2010;Williams et al. 2009) to successfully distinguish different types of IBD, and to identify the presence of ongoing intestinal inflammation. Metabolomic profiles have also been shown to alter during therapy (Pan et al. 2011). Consequently, we sought to assess whether metabolomic profiles in the urine may have a role in predicting responses to TNF antagonists in patients with RA and PsA.

Psoriatic arthritis (PsA) is an inflammatory arthritis associated with psoriasis. RA and PsA are different diseases both clinically and pathologically. However, clinically they can often be difficult to distinguish as it is possible for a patient with PsA to be classified as having RA using the 1987 ACR classification criteria (Arnett et al. 1988). Pathologically

PsA is very different to RA in terms of the osteoclast and osteoblast progenitor numbers and the high levels of RANKL and osteoprotegerin in PsA (Veale et al. 2005). The cytokines also vary between the synovial tissue of RA and PsA with the interferon gamma levels being significantly higher in RA patients (Canete et al. 2000). Patients with PsA often have lower levels of inflammation, less deforming disease and less systemic involvement than patients with RA. Recent data suggests that patients with PsA may not respond to the same treatments used in RA (Kingsley et al. 2010). Hence, it is very important to predict responses to treatment in patients with RA and PsA and to distinguish between patients with RA and PsA.

4.2 Patients

Patients were part of a multicentre study (Glasgow Royal Infirmary (PsA patients only), Queen Elizabeth Hospital, Birmingham (PsA patients only), and Charing Cross Hospital, London (RA patients only) comparing responses to infliximab and etanercept. All patients were aged 18 or over. RA patients were required to fulfil 1987 ACR classification criteria (Arnett et al. 1988), to be positive for rheumatoid factor (RF) and/or anti-CCP antibodies, have a disease duration $\geq$ 6 months and a DAS28 score > 4.0. The PsA patients were required to have psoriasis at screening, $\geq$3 swollen and $\geq$3 tender peripheral joints, negativity for RF and anti-CCP antibodies and a disease duration $\geq$ 6 months. All patients had failed treatment with at least one DMARD and were treated with methotrexate at a dose of at least 7.5mg weekly, stable for at least 4 weeks prior to commencing anti-TNFα therapy. No other DMARDs were allowed within the 4 weeks prior to commencing treatment but prednisolone was allowed provided the dose remained stable and did not exceed 10mg daily.

Participants were randomised to either infliximab 3mg/kg at weeks 0, 2 and 6 and then every 8 weeks until week 46, or etanercept 25mg twice weekly for 52 weeks. Therapy was kept stable for the first 3 months. After 3 months, therapy could be changed as required, including escalation of methotrexate therapy to 25mg weekly in apparent non-responders. Clinical data, including ESR, DAS28 and HAQ scores, were collected at baseline and monthly up to week 52. A good clinical response was defined as a DAS 28$\leq$ 3.2 and a DAS 28 improvement> 1.2 after therapy (van Gestel et al. 1998) in RA. A good response in PsA was defined as an improvement in 2 factors (with at least one being a joint score) with worsening in none of the following four factors: patient and physician global assessments, tender and swollen joint scores (Clegg et al. 1996). Random urine samples were collected from the patients at baseline and at 12 weeks and were snap frozen and stored at -80^{0}C. The study was conducted in compliance with the Helsinki declaration and ethical approval was obtained from the West Glasgow Ethics Committee. All subjects gave written informed consent.

Urine samples were prepared as described in section 2.4.4 and then subjected to NMR spectroscopy. Glutamine levels were measured in the urine samples using high performance ion-exchange chromatography and xanthurenic acid levels were measured using a fluorometric method (Liu et al. 1996) which used a solid-phase extraction column.

4.3 Results

Demographic data are shown in Table 4.1.

	RA	**PsA**
Number of patients from whom baseline urine samples were available	19	21
Females	19 (100%)	10 (48%)
Median age	49.9 years (IQR 44.15-64.12)	47.84 years (IQR 35.24-58.49)
Smokers	2 (11%)	4 (19%)
Concomitant drug (Immunosuppressant)	8 (42%)	5 (24%)
Concomitant drug (NSAID)	10 (53%)	12 (57%)

Table 4.1: Table to compare the demographic details of the patients with established Rheumatoid arthritis and established Psoriatic arthritis included in the DATA study.

4.3.1 RA and PsA at baseline

To determine if systemic metabolic differences were observable between established RA and established PsA 1D NMR spectra of urine samples were acquired. After normalising and binning, these data were analysed by PCA to generate an unbiased overview of the major metabolite differences between RA and PsA patients. This is shown in Figure 4.1 and there is no clear separation of the two groups.

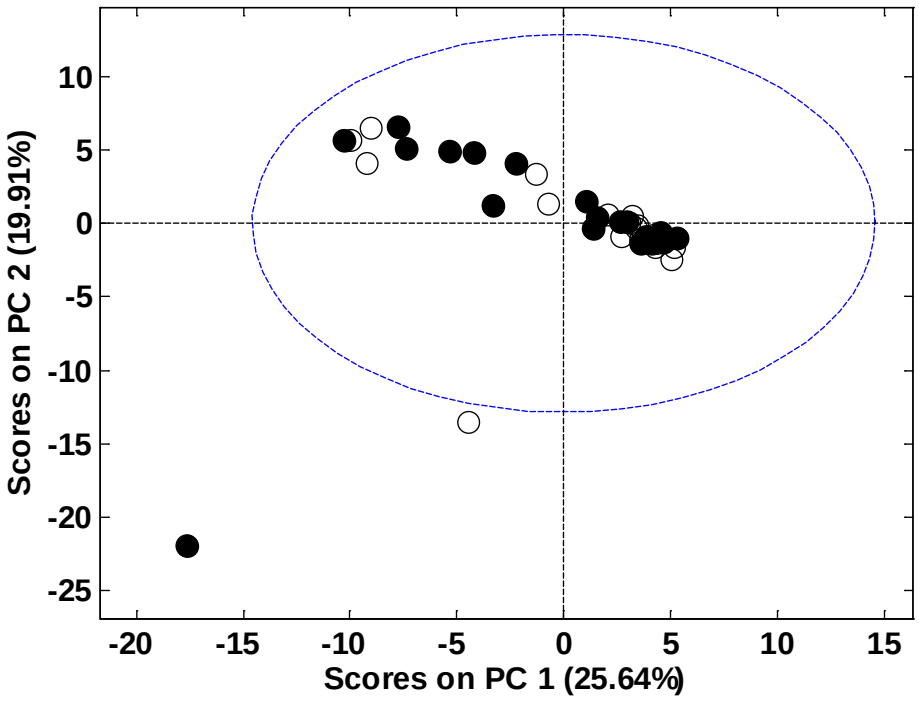


Figure 4.1: 1D 1H NMR spectra of urine samples of patients with RA (●) and PsA (○) patients were subjected to PCA. The values on the axis labels indicate the proportion of the variance captured by each principal component. There are 19 urine samples from patients with RA and 21 urine samples from patients with PsA.

4.3.1.1 PLS-DA analysis

The supervised analysis PLS-DA enhanced the separation of the two groups of samples (RA and PsA). The optimised model comprised 7 latent variables (LV) which captured 96.7% of the variance. The major contribution to the separation was made by LV1 and LV2 (Figure 4.2). This model was cross validated using Venetian blinds approach and was shown to identify samples with PsA with a sensitivity of 75% and specificity of 61% and samples with RA with a sensitivity of 61% and specificity of 75%.

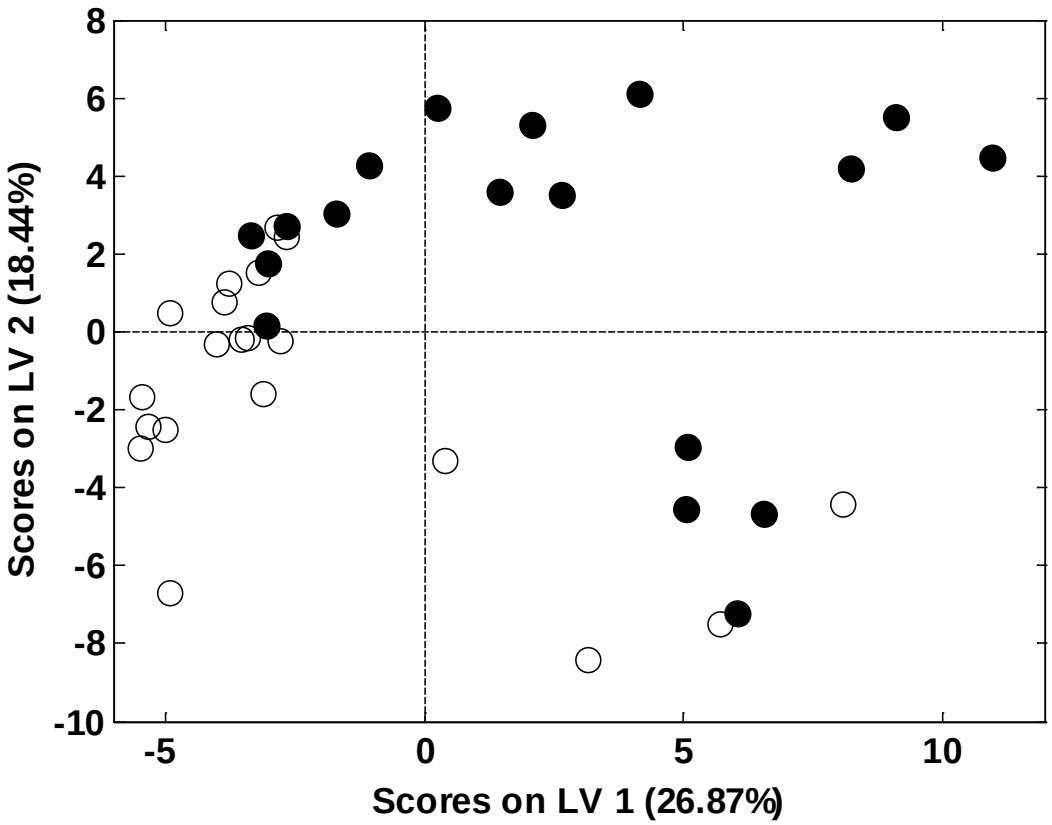


Figure 4.2: 1D 1H NMR spectra of urine samples of patients with RA and PsA were subjected to supervised analysis (PLS-DA). The values on the axis labels (LV 1 and 2) indicate the proportion of the variance captured by each principal component. There are 19 urine samples from patients with RA and 21 urine samples from patients with PsA. (sensitivity of 75% and specificity of 61%)

4.3.1.2 Metabolite identification

A weighting plot derived from the PLS-DA model is shown in figure 4.3. The weightings provide an overall picture of the discriminating features. Figure 4.4 and figure 4.5 show the prominent peaks which discriminate RA from PsA.

In RA the prominent peaks may represent 2-phenylglycine and phenylacetic acid. These are shown in Figure 4.4. In PsA the prominent peaks may represent creatinine. This is shown in Figure 4.5.

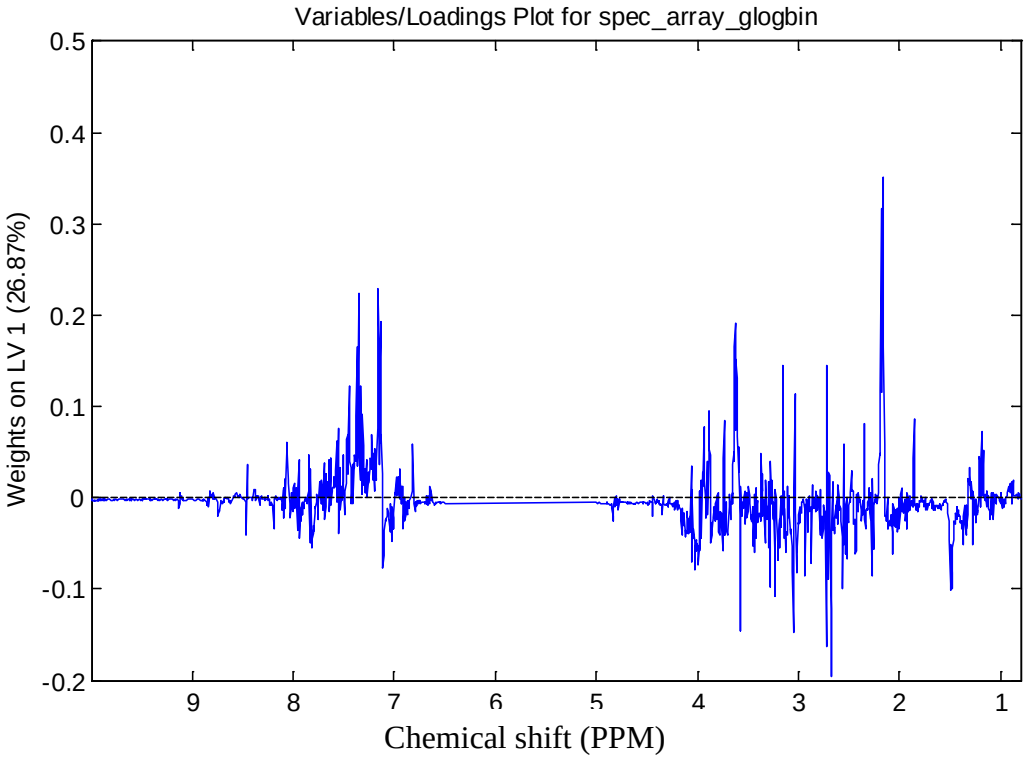


Figure 4.3: Entire weighting plot derived from PLS-DA of data from urine samples of patients with RA and PsA

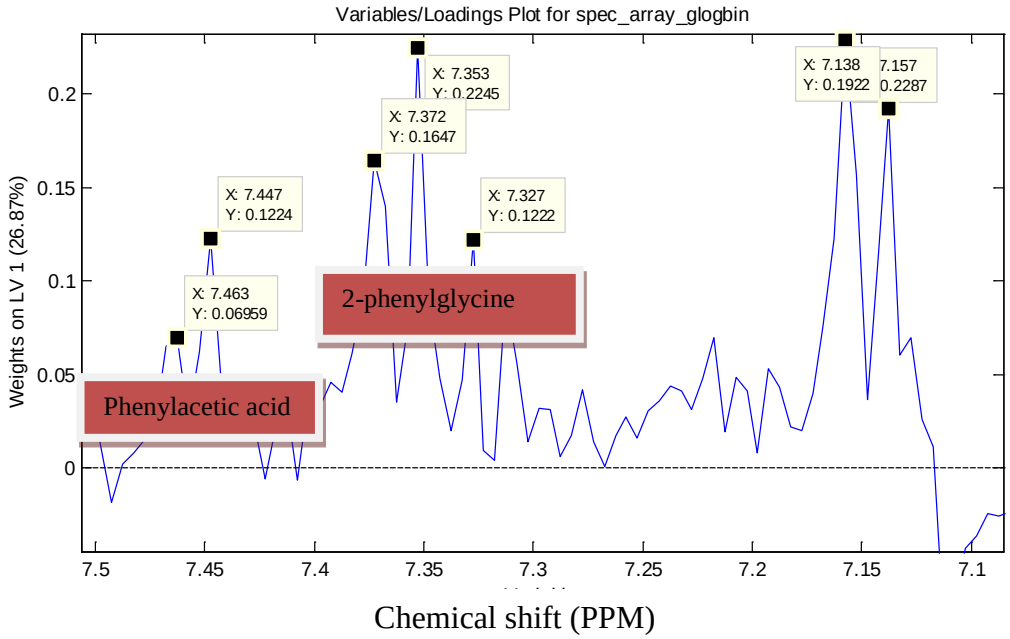


Figure 4.4: Weighting plot showing prominent peaks and their relevant ppm. The doublet represents Phenylacetic acid and the triplet represents 2-phenylglycine.

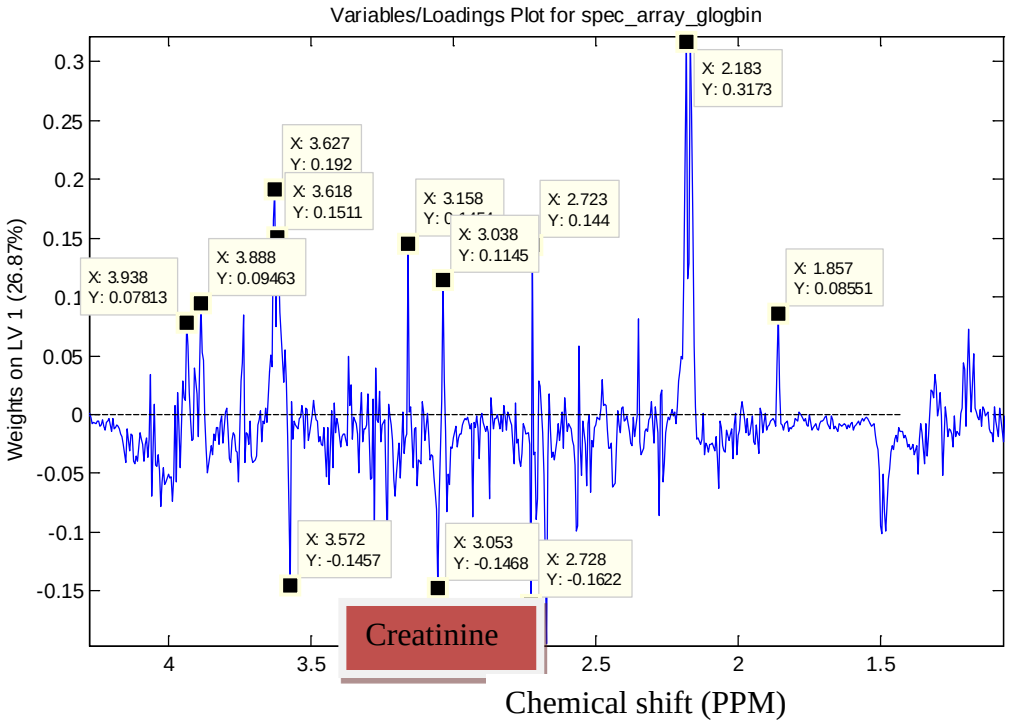


Figure 4.5: Weighting plot showing prominent peaks and their relevant ppm. The position of creatinine is shown on the weighting plot.

4.3.1.3 Relationship between CRP levels and metabolites in RA and PsA patients

My work using serum (chapter 5) has demonstrated a strong relationship between current inflammation (as tested by CRP) and the subsequent metabolic profile. We wanted to determine whether the difference we had observed between the RA and PsA samples was due to a difference in inflammation (CRP levels) or something else.

We used PLS-R (section 2.6.4) to investigate the relationship between CRP and the metabolites in RA and CRP and the metabolites in PsA separately. We found no significant association between the CRP and RA metabolites (p=0.5) and no significant association between the CRP and PsA metabolites (p=0.56) suggesting that the difference seen on the PLSDA model is not simply due to inflammation.

Using an unpaired T-test there was no significant difference between the CRP levels of the RA and the PsA patients (p=0.945).

4.3.1.4 Differences between males and females

All our patients with RA were female and 48% of the patients with PsA were female. We wished to ascertain whether these differences in gender were responsible for the changes that we were identifying between RA and PsA.

I first investigated the patients with PsA and split these into females and males to see if there were any differences in their metabolic profiles. The PCA data are shown in Figure 4.6 and the PLSDA data are shown in Figure 4.7. The PCA data show no clear differences between the females and males with PsA. The supervised analysis PLS-DA enhances the separation of the two groups of samples (males and females) and clear differences were seen. The optimised model comprised 8 latent variables (LV) which captured 99.7% of the variance. The major contribution to the separation was made by LV1. This model was cross validated using Venetian blinds approach and was shown to identify females with a sensitivity of 70% and specificity of 82% and males with a sensitivity of 91% and specificity of 50%.

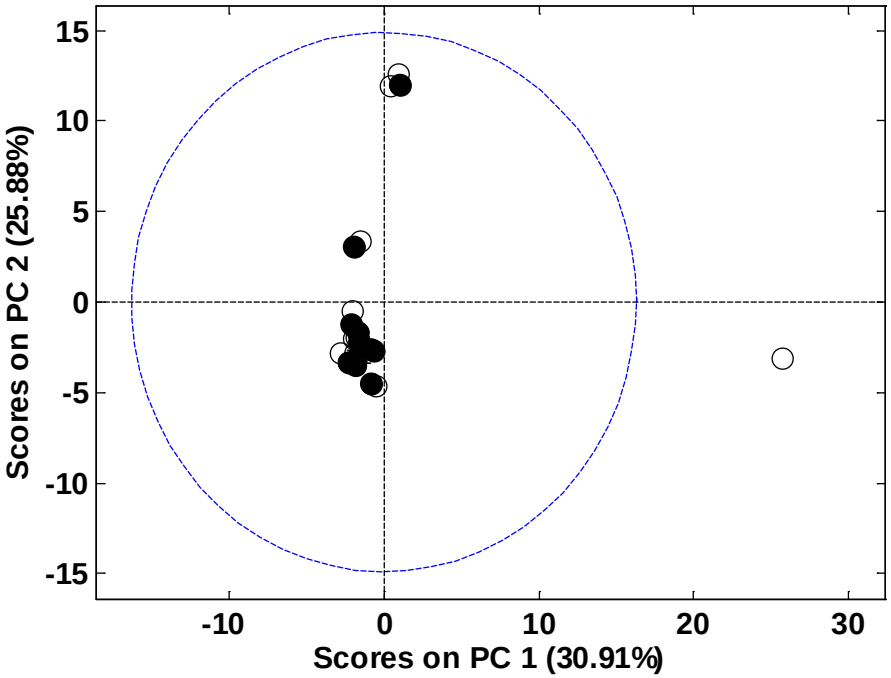


Figure 4.6: 1D 1H NMR spectra of urine samples of the male (●) and female (○) patients with PsA were subjected to PCA. The values on the axis labels indicate the proportion of the variance captured by each principal component.

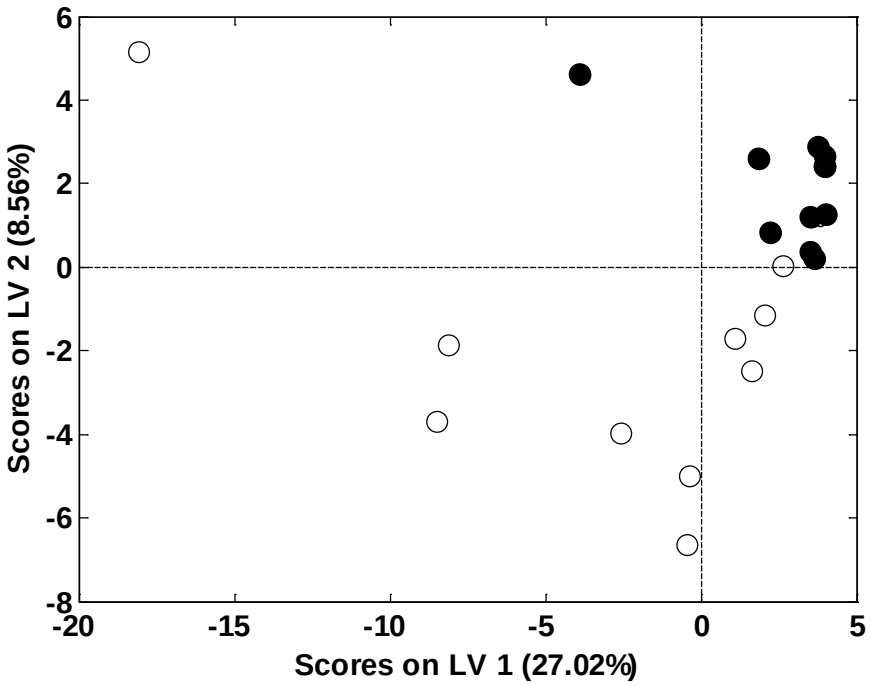


Figure 4.7: 1D 1H NMR spectra of urine samples of the male (●) and female (○) patients with PsA were subjected to supervised analysis (PLS-DA). The values on the axis labels indicate the proportion of the variance captured by each principal component.

The RA and PsA baseline data were analysed again just using the female patients in order to remove the variation created by gender. The PCA data are shown in Figure 4.8 and the PLSDA data are shown in Figure 4.9. The PCA does show some differences between the RA and PsA patients and this is enhanced by the PLSDA model. The optimised model comprised 4 latent variables (LV) which captured 69.1% of the variance. The major contribution to the separation was made by LV1. This model was cross validated using Venetian blinds approach and was shown to identify samples with RA with a sensitivity of 60% and specificity of 57.9% and samples with PsA with a sensitivity of 57.9% and specificity of 60%.

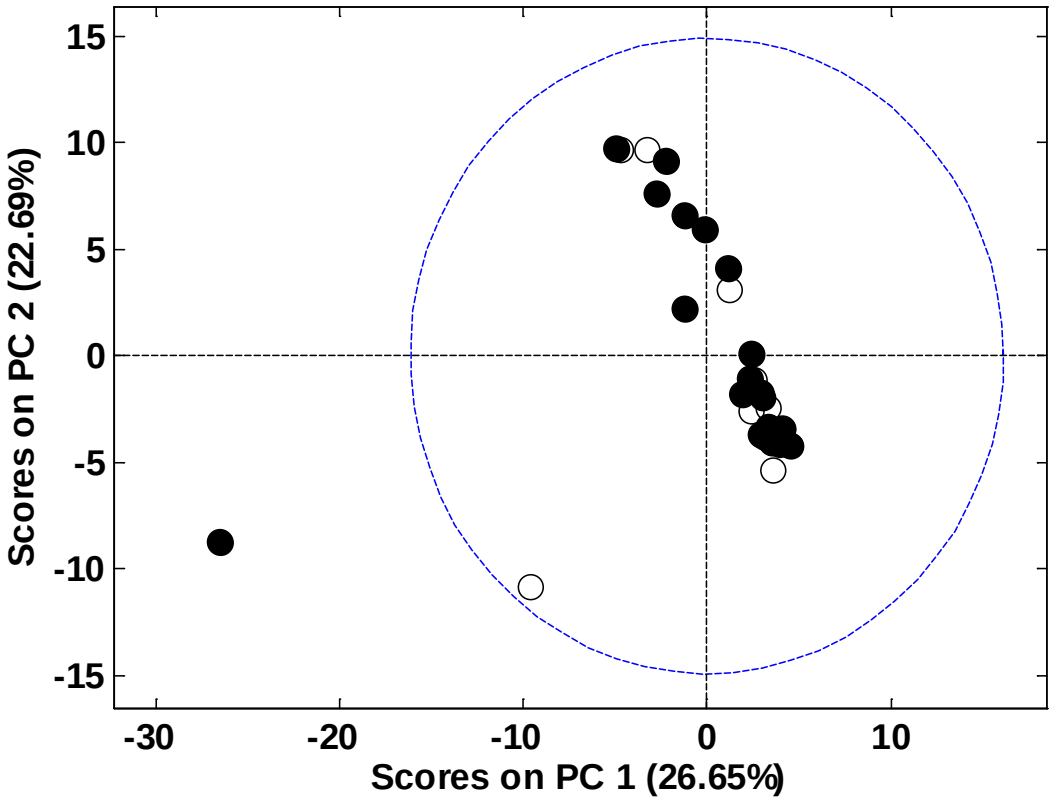


Figure 4.8: 1D 1H NMR spectra of urine samples of female patients with RA (●) and PsA (○) were subjected to PCA. The values on the axis labels indicate the proportion of the variance captured by each principal component.

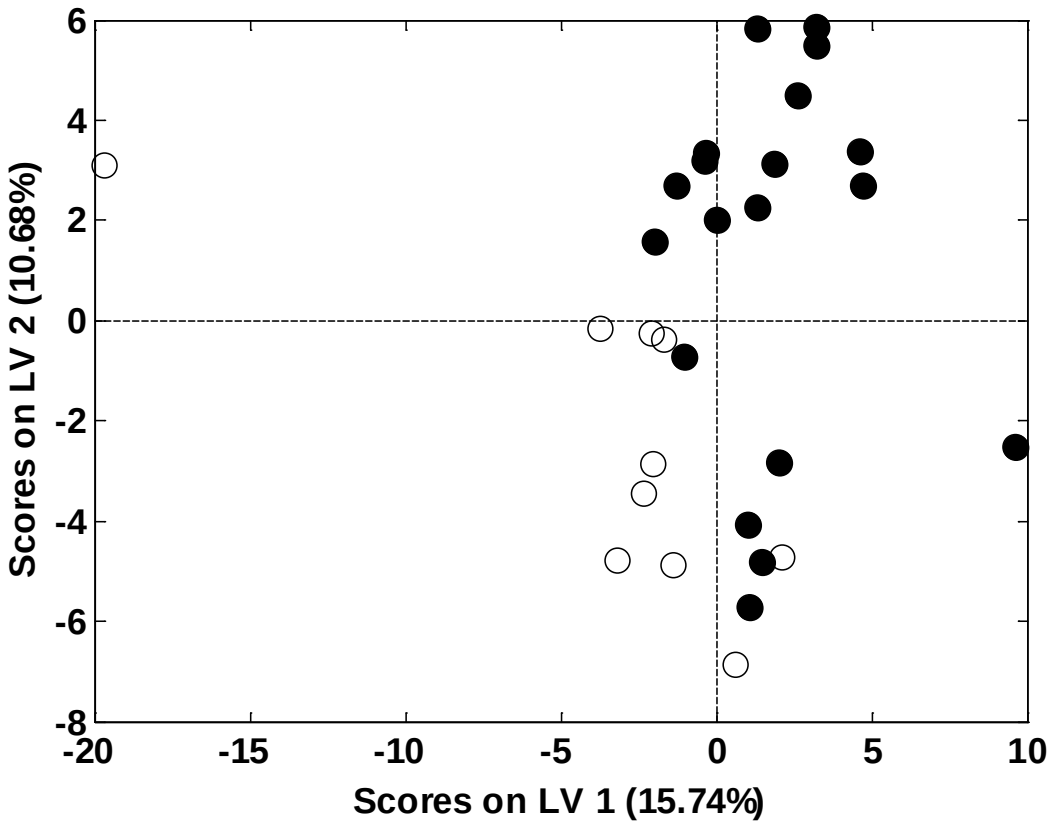


Figure 4.9: 1D 1H NMR spectra of urine samples of female patients with RA (●) and PsA (○) were subjected to supervised analysis (PLS-DA). The values on the axis labels (LV1 and LV2) indicate the proportion of the variance captured by each principal component.

Although, there are differences between the urine samples of female and male patients with PsA there are still differences between the urine samples of the RA and PsA patients when the variability of gender has been removed.

4.3.2 RA and predictors of response to anti-TNFα therapy

After 12 months of anti-TNF therapy RA patients were divided into two groups according to their response, as determined by EULAR criteria (Table 4.2). Response to anti-TNF therapy was also assessed at 3 months but only four patients had a good response (as determined by EULAR criteria) at this stage. For PsA patients only one patient did not

respond to treatment with a TNF antagonist according to the predefined response criteria. It was therefore not possible to study prediction of response in PsA.

	Good response to TNF antagonists (n=7)	**Not a good response to TNF antagonists (n=9)†**	**P value**
Age (years);mean (SD)	50.0 (13.40)	52.67 (12.83)	0.69‡
Female (number (%))	7 (100)	9 (100)	
BMI; mean (SD)	26.16 (3.75)	26.97 (5.64)	0.78‡
Prednisolone (patient number)	1	2	1‡‡
NSAIDs (patient number)	4	4	1‡‡
Baseline methotrexate dose (mg/week); mean (SD)	13.57 (4.76)	15.83 (6.12)	0.43‡
DAS28; mean (SD)	6.041 (1.06)	6.46 (0.91)	0.41‡
CRP (mg/ml); mean (SD)	21.31 (15.97)	7.02 (7.97)	0.03‡
Rheumatoid factor positive (patient number)	6	8	1‡‡
Anti CCP antibody positive (patient number)	6	8	1‡‡
Urinary albumin to creatinine ratio; mean (SD)	1.60 (2.71)	0.29 (0.45)	0.17‡

Table 4.2: Baseline characteristics of RA patients divided according to response to anti-TNF therapy at 12 months.
† "Not a good response" corresponds to moderate and poor response as determined by EULAR criteria.

‡p value calculated using unpaired t test.

‡‡p value calculated using Fishers exact test.

NMR spectra of stored baseline urine samples were acquired and analysed in order to identify differences between the two groups as follows:

Supervised PLS-DA analysis (Figure 4.10A) shows a clear distinction between patient groups segregated according to clinical response. This model comprised 1 latent variable (LV) which captured 35.5% of the variance. This model distinguished samples with or without a good response with a sensitivity of 66.7% and a specificity of 57.1%. A

weightings plot, which indicates regions of the NMR spectra which contribute to this separation (Figure 4.10B), was used to identify the discriminatory metabolites responsible for the difference in response and these are shown in Table 4.3.

The PLS-R model represents the 90 “bins” or regions of the spectra which had the greatest influence on the correlation with the change in DAS28. The GALGO model identified the bins which had the greatest influence on the separation. For the PLS-DA model the metabolites were identified from the weightings plot, which indicates regions of the NMR spectra which contribute to the separation. From the bins identified using GALGO and PLS-R the metabolites were identified for the top 20 bins. From the PLS-DA weightings plot the top 20 peaks were identified and the metabolites identified from these.

GALGO analysis was then used to reanalyse the data, firstly in order to verify the results obtained using a further supervised analysis technique, but secondly to utilise the superior modelling power of the GALGO genetic algorithm, which more effectively removes irrelevant variables. The PCA plot yielded by Galgo analysis shows a clear distinction between RA patients segregated according to clinical response (Figure 4.10C). The cross validation of this model was shown to distinguish samples from patients who would not have a good response and samples from patients who would have a good response with a greatly improved sensitivity of 88.9% and specificity of 85.7%. GALGO analysis was further used to identify the discriminatory metabolites responsible for the difference in response as shown in Table 4.3.

Finally, the relationship between baseline metabolite profiles and the change in DAS28 over 12 months was assessed using PLS-R. This analysis was repeated 100 times with and without randomisation of the NMR bin data. There was a significant association between the change in DAS28 and baseline RA metabolites ($p=0.04$). Permutation testing with 90 NMR bins included (as optimised by forward selection) demonstrated that the regression model was statistically valid ($p<0.01$).

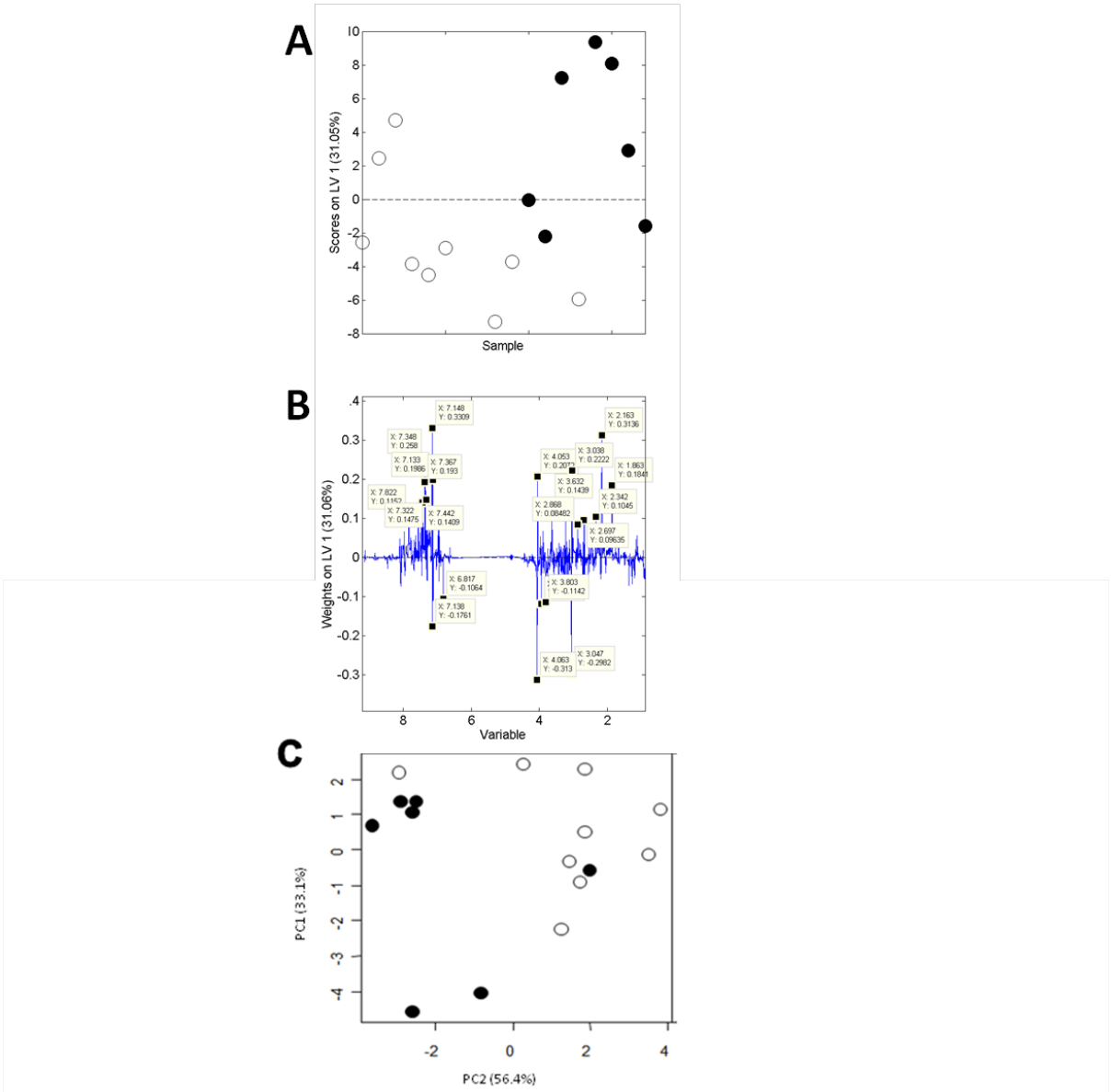


Figure 4.10:Metabolic fingerprinting distinguishes between baseline urine samples from RA patients who go on to have good response or not at 12 months.

A. 1D ^{1}H NMR spectra of baseline urine samples from RA patients who go on to have a good response (●) or not (○) to TNF antagonists at 12 months were subjected to supervised analysis (PLS-DA). The values on the axis labels indicate the proportion of the variance captured by each latent variable.

B. Weightings plot of the PLS-DA model of spectral data from baseline urine samples of the RA patients who go on to have good response or not at 12 months highlight major regions of the spectra that distinguish between the sample groups. The values on the x axis indicate chemical shift (ppm) and the values on the y axis indicate the proportion of the variance captured by each latent variable.

C. 1D ^{1}H NMR spectra of baseline urine from RA patients who go on to have a good response (●) or not (○) to TNF antagonists at 12 months were subjected to PCA using GALGO. The values on the axis labels indicate the proportion of the variance captured by each principal component.

Rank	Metabolite (chemical shift (ppm)) PLS-DA	Variable Importance of the Projection (VIP) values	Metabolite (chemical shift (ppm)) GALGO	Metabolite (chemical shift (ppm)) PLSR
1	↑**Histamine** (7.15)	91.54	Trimethylamine (2.87)	Citrate (2.67, 2.53, 2.56)
2	↑**Glutamine** (2.16, 2.34)	87.72	Thiamine (7.41)	**Phosphocreatinine** (3.04, 3.94)
3	↓**p-hydroxyphenylpyruvic acid** (4.06, 7.14, 6.82, 7.12)	**72.72**	**Histamine** (7.24, 8.01, 8.00, 7.12)	**Ethanolamine** (3.16, 3.74)
4	↓**Phosphocreatine** (3.05, 3.94)	**60.58**	**Thymine** (7.37, 7.35, 1.86)	**Creatinine** (4.05, 4.06, 3.05)
5	↑**Thymine** (7.35, 1.86)	**61.8**	**Ethanolamine** (3.17, 3.80)	**Histamine** (7.13, 3.28, 7.15)
6	↑**Creatinine** (3.04, 4.05)	**50.58**	**Phenylacetic acid** (7.29, 7.39, 7.30)	**Glutamine** (2.16)
7	↑**Xanthurenic acid** (7.37, 7.13)	**36.71**	**Glutamine** (2.12, 2.13)	**P-hydroxyphenylpyruvic acid** (6.82, 6.8225, 4.06, 7.12)
8	↑**Phenylacetic acid** (3.63, 7.44. 7.32)	**21.48**	**Xanthurenic acid** (6.96)	Dimethylamine (2.70)
9	↑**Xanthine** (7.82)	**12.68**	**Xanthine** (7.90)	**Xanthurenic acid** (7.15)
10	↓**Ethanolamine** (3.80)	7.59	Tartaric acid (4.38)	
11			3-phosphoglyceric acid (4.14)	

Table 4.3 Baseline urinary metabolites most strongly correlated with response to anti-TNF therapy using PLSR, Galgo and PLSDA models.
The top 20 bins (PLS-R and GALGO) and peaks (PLS-DA) were identified; their corresponding chemical shifts and the metabolites these represent are shown. The metabolites which are underlined have been identified by all three methods and the metabolites in bold have been identified by two different methods.

VIP values were calculated using PLS-DA.

↑ up-regulation of metabolites in the urine samples of the patients that had a good response to TNF antagonists.

↓ down- regulation of metabolites in the urine samples of the patients that had a good response to TNF antagonists.

There was a significant difference between the CRP level of those patients that responded to TNF antagonists compared to those that did not respond (p=0.03). We therefore used PLS-R to further analyse the relationship between CRP and baseline metabolites in order to investigate potential confounding variables; this did not reveal any significant association (p=0.52), suggesting that the difference we have found is independent of the inflammatory processes reflected in the CRP levels. Grouping the metabolite data into quartiles according to the CRP values also failed to separate patient groups on PCA or PLS-DA. Previous studies have shown that patients with RA have subclinical nephropathy (Niederstadt et al. 1999;Pedersen et al. 1995) and that the urinary albumin to creatinine ratio is a sensitive marker of disease activity in RA (Pedersen et al. 1995). We measured the albumin to creatinine ratio in the urine samples and there was no significant difference between the albumin to creatinine ratio of those patients that responded to TNF antagonists compared to those that did not respond (p=0.17) (Table 4.2). We also performed regression analysis for metabolic profiles at baseline against albumin to creatinine ratio and this was not significant (p= 0.31) suggesting that the relationship we have found between baseline urinary metabolic profiles and DAS28 is independent of micro-albuminuria.

4.3.3 Comparison of metabolites predicting response to therapy in RA

Metabolites that are associated with a change in DAS28 are shown in Table 4.3. The metabolites histamine, glutamine, xanthurenic acid and ethanolamine were identified by all three analytical methods. Furthermore, several metabolites were identified by at least two of the three different methods, including p-hydroxyphenylpyruvic acid, phosphocreatine,

thymine, creatinine, phenylacetic acid and xanthine. These findings cross-validate the analyses used. We were also able to identify glutamine and xanthurenic acid in the urine samples that were used for NMR analysis using ion-exchange chromatography and a fluorometric method respectively. There was a good correlation between the NMR peaks heights and the assayed levels of xanthurenic acid ($p=0.001$, $r=0.73$ using the Spearman correlation test) and a strong trend in the results for the glutamine ($p=0.07$, $r=0.46$ using the Spearman correlation test), which help validate our interpretation of the NMR data. However, the assayed levels of glutamine and xanthurenic acid were not significantly higher in the urine samples of the patients who had a good response, which suggests that while these individual metabolites contribute strongly to the discrimination, the whole set of metabolites present in the fingerprints is needed to fully separate the groups.

4.3.4 Effect of TNFα antagonists on metabolite profiles

The details of the patients on etanercept and infliximab are shown in Table 4.4. We investigated the effect of anti-TNF therapy on metabolic profiles longitudinally, comparing baseline and 12 week (during therapy) urine samples using supervised PLS-DA analysis (sensitivity 71.4% and specificity 57.1% in RA and sensitivity and specificity of 61.1% in PsA) and GALGO (sensitivity 100% and specificity 82.9% in RA and sensitivity 71.8% and specificity 69.5% in PsA). Using the weightings plot we identified that in patients with RA who responded to TNF antagonists, there were high levels of glutamine, phenylacetic acid and histamine in the baseline urine samples and higher levels of methylamine and creatinine in the urine sample post anti-TNF therapy. Similar changes in metabolites were also seen in the urine samples of the patients with PsA who responded to TNF antagonists (Figure 4.11).

	Patients on infliximab (n=18)	Patients on etanercept (n=16)	P value
Age (years); mean (SD)	48.6 (10.60)	47.46 (14.78)	0.19‡
Female (number (%))	13 (72)	13(81)	0.68‡‡
BMI; mean (SD)	28.43 (4.56)	28.36 (9.33)	0.98‡
Prednisolone (patient number)	6	3	0.63‡‡
NSAIDs (patient number)	10	9	0.99‡‡
DAS28 at baseline (RA patients only); mean (SD)	6.22 (0.95)	6.35 (1.05)	0.80‡

Table 4.4: Characteristics of all RA and PsA patients divided according to treatment.

‡p value calculated using unpaired t test.

‡‡p value calculated using Fishers exact test.

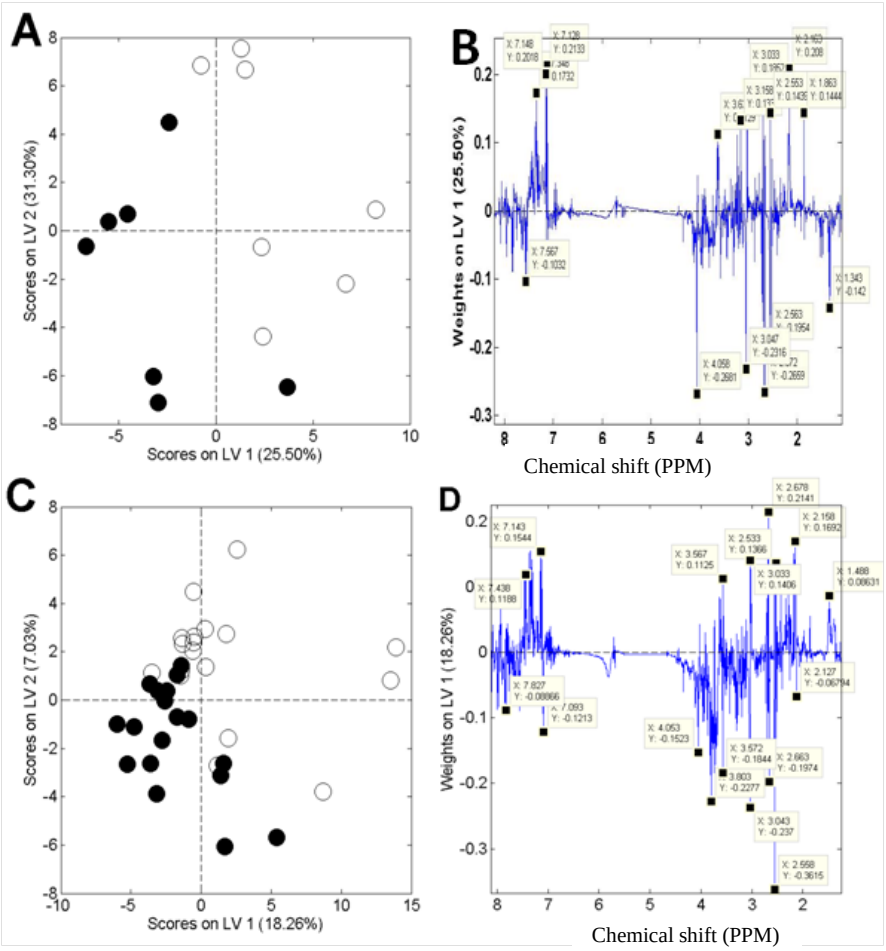


Figure 4.11 Metabolic fingerprinting enables identification of metabolites that alter post treatment with TNF antagonists in patients that have a good response.

A. 1D ^{1}H NMR spectra of urine samples from RA patients at baseline (○) and 12 weeks (●)who go on to have a good response to TNF antagonists at 12 months were subjected to supervised analysis (PLS-DA). The values on the axis labels indicate the proportion of the variance captured by each latent variable.

B. Weightings plot of the PLS-DA model of spectral data from urine samples of the patients with RA who responded to TNF antagonists highlights major regions of the spectra that distinguish between the baseline and 12 week samples. The values on the x axis indicate chemical shift (ppm) and the values on the y axis indicate the proportion of the variance captured by each latent variable.

C. 1D ^{1}H NMR spectra of urine samples from PsA patients at baseline (○) and 12 weeks (●)who go on to have a good response to TNF antagonists at 12 months were subjected to supervised analysis (PLS-DA). The values on the axis labels indicate the proportion of the variance captured by each latent variable.

D. Weightings plot of the PLS-DA model of spectral data from urine samples of the patients with PsA who responded to TNF antagonists highlights major regions of the spectra that distinguish between the baseline and 12 week samples. The values on the x axis indicate chemical shift (ppm) and the values on the y axis indicate the proportion of the variance captured by each latent variable.

Combining RA and PsA patients with a good response, we assessed which urinary metabolites changed after 12 weeks treatment with infliximab and with etanercept using supervised PLS-DA analysis (sensitivity 84.6% and specificity 55.6%) (Figure 4.12) and GALGO (sensitivity 86.2% and specificity 100%). Using the weightings plot we found that increases in hippuric acid, citrate and lactic acid were seen with infliximab treatment and increases in choline, phenylacetic acid, urea, creatine and methylamine were seen with etanercept treatment. Due to the small patient numbers, we could not investigate the effects of etanercept and infliximab in RA and PsA separately.

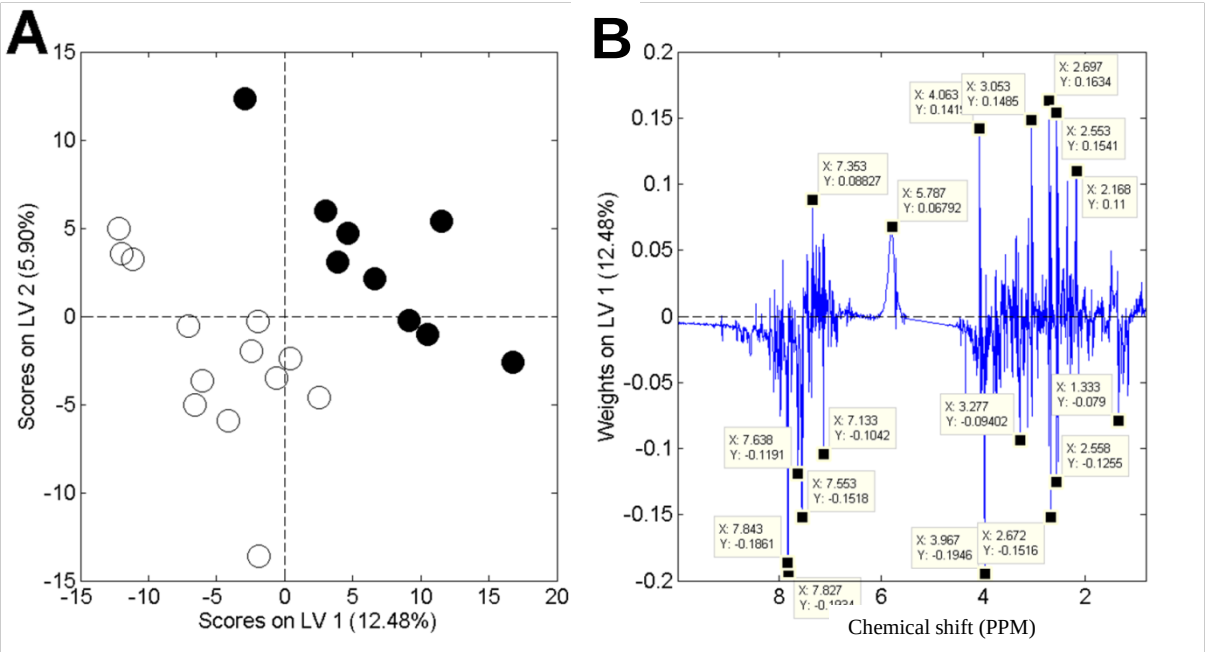


Figure 4.12 Metabolic fingerprinting of urine samples from RA and PsA patients.

A. 1D ^{1}H NMR spectra of urine samples from RA and PsA patients 12 weeks post treatment with infliximab (○) and etanercept (●)who had a good response to treatment were subjected to supervised analysis (PLS-DA). The values on the axis labels indicate the proportion of the variance captured by each latent variable.

B. Weightings plot of the PLS-DA model of spectral data from urine samples of the RA and PsA patients post treatment with infliximab and etanercept who go on to have good response at 12 months highlight major regions of the spectra that distinguish between the sample groups. The values on the x axis indicate chemical shift (ppm) and the values on the y axis indicate the proportion of the variance captured by each latent variable.

4.4 Discussion

4.4.1 RA and PsA at baseline

Our NMR data illustrate that there are differences in the urinary metabolite profiles between patients with RA and PsA. Since acquiring these data other research has shown that patients with RA and PsA can be distinguished using mass spectroscopy of blood samples (Madsen et al. 2011), thus giving us further confidence in our data. The differences we observed were not due to differing levels of inflammation since there were no significant differences between the CRP levels of the PsA and the RA patients and our regression analysis did not show any significant association between CRP and metabolites.

We have demonstrated that urine can be used to detect differences in metabolism in patients with RA and PsA. No previous work on urinary metabolomics in inflammatory arthritis has been published though there have been some analyses of serum and synovial fluid (Lauridsen et al. 2010;Madsen et al. 2011;Naughton et al. 1993b;Weljie et al. 2007). Urine has however been used to look at metabolism in inflammatory bowel disease (Williams et al. 2009) in both mouse (Lin et al. 2009;Schicho et al. 2010) and human models (Schicho et al. 2012;Williams et al. 2009). The metabolites identified vary between the different models and are not common to all inflammatory conditions and this may be due to other biofluids being used in studies of other inflammatory conditions.

There were large gender differences between the RA and PsA patients. Even when the variability of gender was removed there were still differences between the metabolic profiles of patients with RA and PsA. Our work identified several metabolites which were responsible for the separation between patients with RA and PsA. Creatinine was one of

the discriminating metabolites with higher levels in the urine samples from patients with PsA but none of the patients in our cohort had renal impairment (as defined by a raised serum creatinine) at baseline. Other discriminating metabolites were phenylglycine and phenylacetic acid with levels raised in the urine samples of the RA patients. Phenylglycine is a normal constituent of human urine and raised phenylacetic acid levels have been found in patients with end stage renal failure (Jankowski et al. 2003)and after chocolate consumption (Rios et al. 2003). They have also been postulated as a marker for depression (Davis et al. 1994). Reduced levels of phenylacetic acid have been found in the urine, plasma and cerebrospinal fluid of depressed patients (Davis et al. 1994). We have no formal data on the mood status of our patients on joining the study but we were not aware of any differences in mood between the patients with PsA and the patients with RA. Generally patients with RA are more likely to have associated depression as they usually have a more debilitating disease than those with PsA and this may explain the higher levels of phenylglycine in our RA patients. The metabolites that differentiated the urine samples of patients with PsA and RA in our study were different to the metabolites that have been found to distinguish plasma samples of patients with PsA and RA (Madsen et al. 2011). Madsen et al identified glutamine, aspartic acid, glutamic acid, succinate and histidine to be the major discriminatory metabolites but other work has illustrated that the same metabolites are not found to differentiate different disease states in different biofluids (Sreekumar et al. 2009).

4.4.2 Predictors of response to anti-TNFα therapy in RA

There were clear differences in the metabolic profiles of baseline urine samples of patients with RA who responded well to anti-TNF therapy compared with those who did not. This difference may be important as a novel predictor of responses to TNF antagonists. We

have used 3 different data analysis methods to predict response and each found that similar metabolites contributed. We have used GALGO as well as PLS-DA as it has been shown that genetic algorithms optimise the results by removing irrelevant variables and dramatically improve the classification ability of models (Ramadan et al. 2006). All three methods identified histamine, glutamine, xanthurenic acid and ethanolamine, while both PLS-DA and PLSR identified creatinine, p-hydroxyphenylpyruvic acid and phosphocreatine and both PLS-DA and GALGO identified phenylacetic acid and xanthine. Histamine, glutaminephenylacetic acid, xanthine, xanthurenic acid and creatinine were up regulated in the urine samples of the patients that had a good response to therapy whilst ethanolamine, p-hydroxyphenylpyruvic acid and phosphocreatine were down regulated.

One metabolite we identified as a strong discriminator in baseline urinary metabolites was histamine. Urinary histamine metabolites have also been suggested as a marker of disease activity in inflammatory bowel disease (Winterkamp et al. 2002) suggesting it may be a generic marker of inflammatory processes. Histamine is most obviously associated with mast cell dependent processes such as allergy, and histamine has been identified as a constituent of synovial fluid in arthritis (Buckley et al. 1997). Histological examination of synovial infiltrates in early rheumatoid arthritis has shown mast cells to be present (Tak et al. 1997), suggesting that these cells could be the source of the discriminating histamine. However, an alternative but significant route for histamine generation is via histidine degradation. Histamine arises in many tissues by the decarboxylation of histidine (Stifel and Herman 1971). It has long been known that TNF α promotes cachexia associated with chronic inflammatory disease and this cytokine is known to have direct effects in accelerating muscle breakdown leading to the release of free amino acids including histidine (Goodman 1991). Consistent with this, levels of histidine have been shown to be

considerably higher in patients with RA and systemic lupus erythematosus (Sitton et al. 1987) compared to controls. Several of the other metabolites that we have observed were also associated with catabolic processes and tissue degradation for example, glutamine, xanthurenic acid and ethanolamine, can result from tryptophan (Figure 4.13) and other amino acid degradation pathways. Tryptophan has been shown to be down-regulated in plasma of patients with ankylosing spondylitis (AS) compared with to controls (Gao et al. 2008). The release of tryptophan from its binding serum protein has been shown to correlate with improvement in disease activity in AS (Gao et al. 2008) and this may be the same in RA.

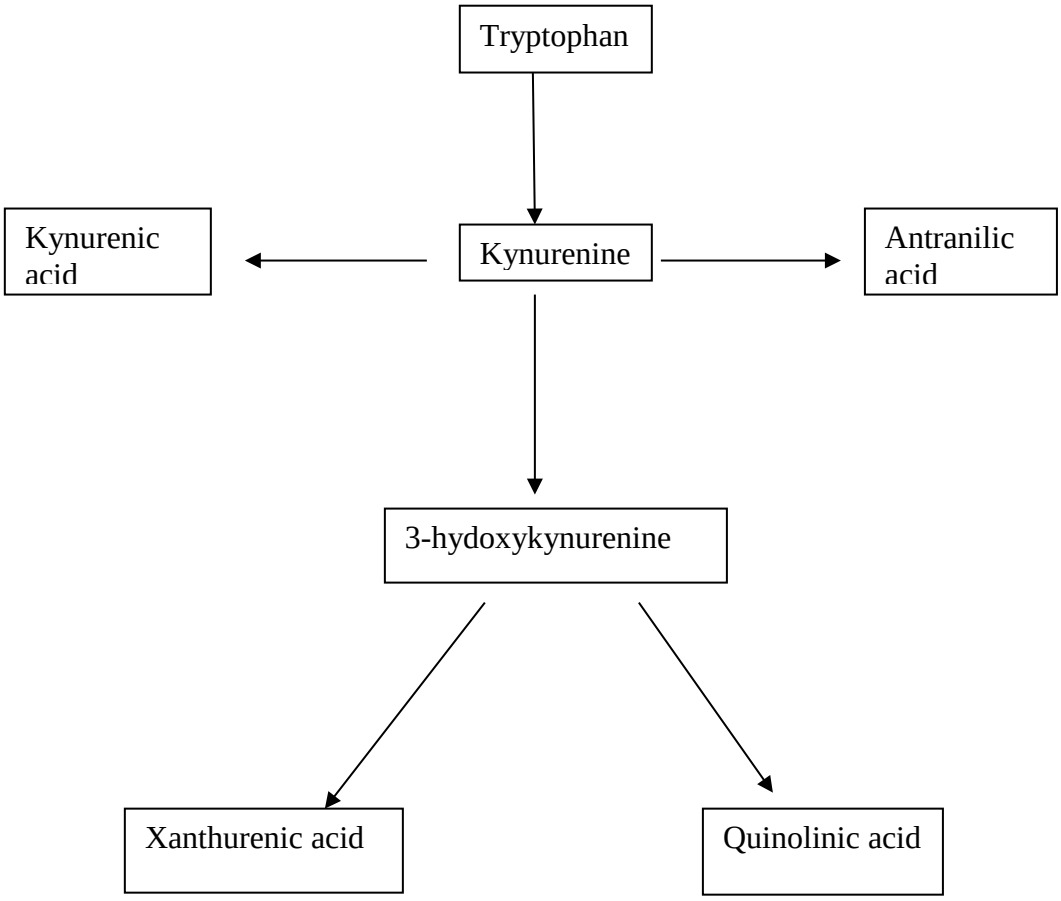


Figure 4.13 Tryptophan metabolism

A previous metabolomic study has suggested that alterations in serum levels of amino acids may be a useful marker of the presence and severity of osteoarthritis in the knee (Zhai et al. 2010), and the urine markers we have found may be indicators of either joint specific degradation processes, or may result from the systemic muscle and tissue changes associated with chronic disease, many of which are mediated through TNFα.

Previous work has investigated predictors of response to TNF antagonists. Analysis of patients in the British Society for Rheumatology Biologics Register found that treatment with methotrexate or NSAIDs predicted response to TNF antagonists (Hyrich et al. 2006). All the patients in our study were on methotrexate and there were an equal number of patients on NSAIDs who had a good response compared to those who did not. Smoking has been associated with a poor response to infliximab (Hyrich et al. 2006) but only one of our patients smoked. Another group has found that the presence of rheumatoid factor or anti-CCP antibodies is associated with a reduced response to TNF antagonists (Potter et al. 2009) but all our RA patients were positive for rheumatoid factor and/or anti-CCP antibodies. Baseline levels of TNF may predict the dose of infliximab needed for optimal response (Takeuchi et al. 2011) and other work has demonstrated that a combination of blood cytokines and autoantibodies can predict responses to etanercept (Hueber et al. 2009). In our cohort there was a significant difference between the CRP levels in the patients that responded to TNF antagonists compared to those that did not. However, the PLSR analysis failed to find an association between CRP and baseline metabolites suggesting that the association between baseline metabolites and response is independent of CRP.

Infliximab and etanercept alter metabolites in the urine differently as there are clear differences in the metabolites at 12 weeks post treatment. Increases in the metabolites hippuric acid, citrate and lactic acid were associated with infliximab treatment and increases in the metabolites choline, phenylacetic acid, urea, creatine and methylamine were associated with etanercept treatment. Infliximab and etanercept are both TNF antagonist but they have different modes of action. Infliximab is a chimeric monoclonal antibody whereas etanercept is a fusion protein. Infliximab binds to soluble and transmembrane TNF α with high affinity and specificity (Wong et al. 2008). Infliximab has murine variable regions which can cause production of anti-mouse antibodies which can lead to diminished efficacy requiring concomitant use of methotrexate. They are known to differ in many ways. Infliximab has a higher rate of activating latent TB (Dixon et al. 2010) and higher risk of anti drug antibodies (Garces et al. 2013;Sandborn and Hanauer 1999). Etanercept is much less effective in inflammatory bowel disease and has been reported to cause inflammatory bowel disease (Flemming et al. 2013). Etanercept also has no evidence for treating uveitis whereas infliximab does not (Elewaut and Matucci-Cerinic 2009). In view of these differences it is not surprising that they both alter metabolites in the urine differently. Further work is required in this area as certain patients obviously respond to one drug over another.

We have also shown that the same metabolites alter in the urine samples of patients with RA and PsA that responded to TNF antagonists. It may therefore be that chronic inflammatory diseases respond by a common mechanism to TNF antagonists.

This is the first demonstration that metabolomic techniques using 1D NMR spectra can predict outcome to TNF therapy in patients with severe RA providing a sensitivity and specificity for response that has potential clinical utility despite a small initial cohort of patients. Our findings are verified by repeat analysis using alternative statistical techniques. There is a pressing need to confirm and extend this finding in a larger cohort of patients, combining metabolomic with CRP and cytokine and autoantibody analyses to develop tests that can predict response without the need for empirical treatment, bringing closer the era of individually tailored therapy.

5.0 Assessment of metabolite profiles of serum from patients with early arthritis

5.1 Introduction

The aetiology of RA is not fully understood but involves both genetic and environmental factors. In addition to synovitis, there are widespread systemic effects mediated by pro-inflammatory cytokines that impact upon metabolism. TNFα, IL1 and IL6 all promote cachexia, which is often associated with RA (Cederholm et al. 1997;Kotler 2000). The extent of the metabolic changes and the types of metabolites seen may therefore be good markers of cytokine mediated inflammatory processes in RA. Low serum levels of a number of specific metabolites, including histidine, have been reported in RA patients (Sitton et al. 1987) and this metabolite discriminated OA from RA (Sitton et al. 1986). Lactate levels in synovial fluids also vary, with higher levels seen in seropositive, compared to seronegative, RA (Gobelet and Gerster 1984). Changes in blood lipids in RA have been widely described (Steiner and Urowitz 2009;Toms et al. 2010). More recently, lipid changes (Myasoedova et al. 2010) and alterations in a range of serum cytokines and chemokines (Kokkonen et al. 2010) have been shown to pre-date the development of arthritis suggesting that changes in metabolites might be observable early in the development of RA.

Altered metabolomic fingerprints have been seen in a number of inflammatory diseases, for example, analysis of faecal extracts differentiated between normal controls and patients with Crohn's disease and ulcerative colitis. Significantly, these two patient groups could be distinguished using metabolic profiling (Marchesi et al. 2007) suggesting that the effects of inflammation on metabolism vary between conditions. This is supported by previous work in our group in which we used metabolite fingerprinting to differentiate

between two otherwise similar inflammatory eye diseases (Young et al. 2009) and between a number of neurological conditions (Sinclair et al. 2010) and also to predict treatment responses in patients with inflammatory arthritis (Kapoor et al. 2013). With this background, we hypothesised that metabolomic profiles may be useful in predicting the development of RA in patients with early arthritis - an area where better predictive tools are currently needed (Raza and Filer 2009). We also wished to determine if this analysis could provide novel insights into disease mechanisms in arthritis as has been the case in other conditions (Sreekumar et al. 2009).

To investigate the potential of metabolite fingerprinting in inflammatory arthritis, we have applied an NMR-based metabolomic approach to the analysis of serum from patients with newly presenting established RA and early arthritis. We initially sought to assess whether the metabolite fingerprint in patients with established RA differed from that of healthy controls and then whether this fingerprint differed in patients with early arthritis in relation to the extent of inflammation and final outcomes.

We were interested to determine if metabolic differences could be observed in early synovitis and also to determine if the metabolic differences persisted on suppression or resolution of the inflammation.

5.2 Patients

Patients were recruited through the inflammatory arthritis clinic at Sandwell and West Birmingham Hospitals NHS Trust, Birmingham, UK. The study was conducted in

compliance with the Helsinki declaration and ethical approval was obtained from the local ethics committee. All subjects gave written informed consent. Serum samples were collected from the patients and stored at -80°C until analysis.

5.2.1 Established RA patients

Serum samples were collected from 16 newly presenting DMARD naive patients fulfilling 1987 ACR classification criteria for RA (Arnett et al. 1988) (due to the higher specificity of these criteria (section 1.1.1)) and with a symptom duration of > 3 months. Symptom onset was defined as the time of onset of inflammatory joint pain and/or early morning stiffness and/or joint related soft tissue swelling.

5.2.2 Early arthritis patients

Serum was collected from early arthritis patients at the time of initial presentation. All patients had one or more swollen joints and symptoms (inflammatory joint pain and/or early morning stiffness and/or joint related soft tissue swelling) of ≤ 3 months duration as previously described (Raza et al. 2005a). Different groups use different definitions of early arthritis in terms of symptom duration (Gossec et al. 2014). Several groups define early arthritis as symptom duration of less than 12 months (Harris et al. 2013;Kuijper et al. 2013). Our patients were recruited within three months as evidence suggests that this time period enables a greater chance of drug free remission for patients with RA (van der Linden et al. 2010). Patients with evidence of previous inflammatory joint disease were excluded. Two groups of patients were studied, one recruited after the other. There were 89 early arthritis patients in group 1 and 127 in group 2. Patients were followed for 18 months and then assigned to their final diagnostic categories. Patients were classified as

having RA according to the 1987 ACR criteria (Arnett et al. 1988) due to the higher specificity of these criteria (section 1.1.1), allowing criteria to be satisfied cumulatively. Patients were diagnosed with reactive arthritis (ReA), psoriatic arthritis (PsA) and a number of miscellaneous conditions according to established criteria. Resolving arthritis was diagnosed if there was no evidence of joint related soft-tissue swelling on final examination and the patient had not received DMARD or steroid treatment within the previous 3 months (Raza et al. 2005a). Persistent joint related swelling or treatment with DMARDs or steroids for inflammatory joint symptoms (within the previous 3 months) defined persistence.

Serum samples were prepared as per section 2.4.3.

5.3 Results

5.3.1 The metabolic profile differs in established RA patients compared to healthy controls.

Details of patients with established RA and age/sex-matched healthy controls are shown in Table 5.1. The median DAS28ESR score in the RA patients was 5.88 (IQR 5.25-6.99). PCA was used to generate an unbiased overview of the major metabolic differences between established RA and control individuals. There was separation of the two groups which was dependent largely on PC2 (Figure 5.1A), the scores of which were significantly higher in the RA group (t-test; $p<0.0001$). To help discover the major discriminatory metabolites, these data were subjected to a supervised analysis using OPLS-DA (figure 5.1B). Discriminatory metabolites are shown in table 5.2.

	Established RA (n=16)	Healthy controls (n=14)	P value
Age (years); median (IQR)	57 (37-79)	54 (40-72)	0.47‡
Female; number (%)	12 (75)	9 (64)	0.69‡‡
Symptom duration (weeks); median (IQR)	31 (18-52)	-	-
NSAIDs; number (%)	7 (44)	0 (0)	-
CRP (mg/ml); median (IQR)	20.5 (7.5-55.5)	-	-
RF positive; number (%)	12 (75)	-	-
Anti CCP antibody positive; number (%)	9 (56)	-	-

Table 5.1: Baseline characteristics of established RA patients and, healthy controls.

‡p value calculated using Mann Whitney test.
‡‡p value calculated using Fisher's exact test.

Metabolite (ppm)	RA vs. controls
LDL-CH3 (0.80)	Low (6.30)
LDL-CH2 (1.21)	Low (7.06)
3-hydroxybutyrate (1.18, 1.19)	High (4.21)
Lactate (1.31, 4.11)	High (54.51)
Alanine (1.46,1.48)	Low (20.00)
Acetylglycine (2.03)	High (48.67)
Methylguanidine (2.81)	Low (10.17)
Taurine (3.26)	High (8.12)
Glucose (3.25,3.88)	High (16.8)
Lipid (5.32)	Low (2.36)

Table 5.2: Analysis of PLS-DA weightings illustrates the metabolites contributing to the differentiation between: established RA and healthy controls "High" implies the metabolite is at higher concentration in the RA disease phenotype. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite. . Variable importance of the projection (VIP) values are shown in brackets for all of the metabolites.

Supervised analysis enhanced the separation of the two groups and the optimised model comprised 4 latent variables (LV) which captured 97.8% of the variance in the metabolic data. The major contributions to the separation were made by LV1 and LV2 (Figure 5.1B). This model was cross-validated using the Venetian blinds approach and identified samples from the RA patients with a sensitivity of 93.3% and a specificity of 100%.

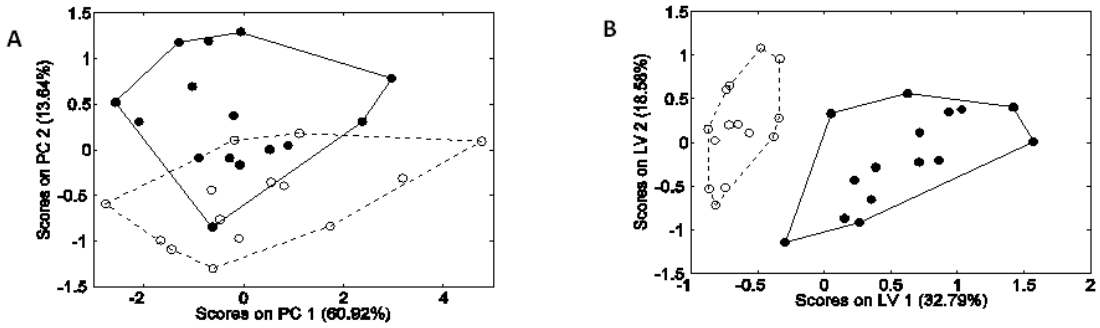


Figure 5.1. Metabolic fingerprinting distinguishes between sera from established rheumatoid arthritis and matched healthy.
1D ^{1}H NMR spectra of serum from DMARD naïve RA patients (●) and control individuals (○) were subjected to PCA (A) and to supervised analysis (PLS-DA) (B). The values on the axis labels indicate the proportion of the variance captured by each principal component.

5.3.2 Resolution of inflammation is reflected in metabolic profile changes.

In 22 patients with early arthritis and active disease at baseline (median CRP 66 (IQR 47-131)), serum samples were available at a follow up time point (a median of 49 weeks (IQR 24-69) from the time of initial assessment and sample collection) at which CRP levels were significantly lower (median <5 (IQR <5 - <5)). This fall in CRP was associated with either spontaneous resolution of disease, or control of disease with therapy. Unbiased

PCA discriminated between samples at baseline and follow-up (Figure 5.2A) and OPLS-DA produced a model comprising of 2 LVs capturing 99.8% of the variance which could discriminate between these samples with 100% specificity and sensitivity (Figure 5.2B) demonstrating the influence of inflammation on the metabolic fingerprint.

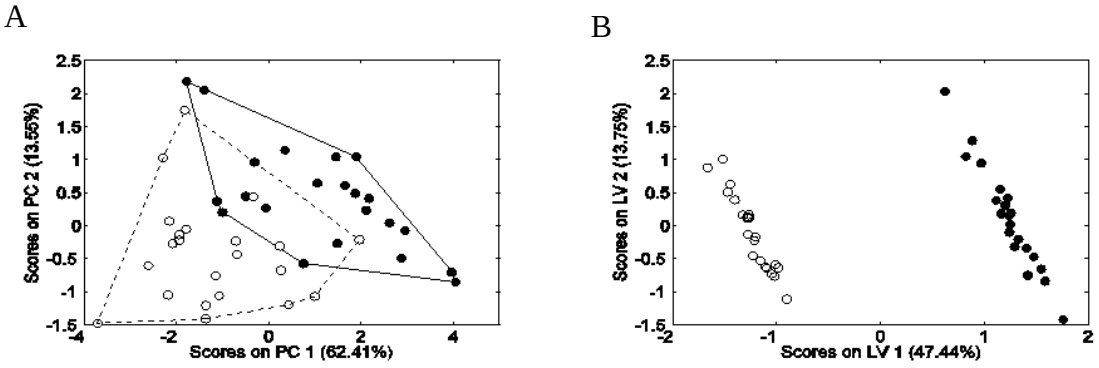


Figure 5.2. Metabolic fingerprinting illustrates that metabolite profiles in patients with early arthritis are altered by control or resolution of inflammation.
1D ^{1}H NMR spectra of serum from a subset of patients with early arthritis before (●) and after (○) the level of CRP had diminished following therapy or spontaneous resolution were subjected to PCA (A) and to supervised analysis (OPLS-DA) (B).

To provide an overall description of which regions discriminated in the PLS-DA analyses, weightings plots derived from the models were generated. Some common features were seen in these weightings plots from PLS-DA models separating RA from controls and from the analysis of the early synovitis patients from whom a second serum sample had been collected at a time when the CRP had significantly fallen (Table 5.3).

Metabolite (ppm)	Active early arthritis vs. less active early arthritis
LDL-CH3 (0.80)	Low (3.03)
LDL-CH2 (1.21)	Low (31.81)
3-hydroxybutyrate (1.18, 1.19)	High (7.90)
Alanine (1.46,1.48)	Low (2.15)
Acetylglycine (2.03)	High (17.41)
Taurine (3.26)	High (9.11)
Glucose (3.25,3.88)	High (12.72)
Lipid (5.32)	Low (2.53)
Urea (5.79)	High (1.32)

Table 5.3: Analysis of PLS-DA weightings illustrates the metabolites contributing to the differentiation between: paired samples in early arthritis patients before and after resolution of inflammation. "High" implies the metabolite is at higher concentration in the active disease phenotype. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite. . Variable importance of the projection (VIP) values are shown in brackets for all of the metabolites.

5.3.3 Metabolic profiles of patients with early arthritis are linked to inflammation.

Details of the 89 early arthritis patients from group 1 and the 127 early arthritis patients from group 2 are shown in Table 5.4.

	Early arthritis patients group 1 (n=89)	**Early arthritis patients group 2 (n=127)**	**P value**
Age (years); median (IQR)	46 (36-61)	50 (35-65)	0.32‡
Female; number (%)	49 (55)	69 (54)	1.0‡‡
Symptom duration (weeks); median (IQR)	5 (2-9)	6 (4-9)	0.02‡
NSAIDs; number (%)	58 (65)	69 (54)	0.12‡‡
CRP (mg/ml); median (IQR)	19 (5.5-54)	19.5 (5.25-44.75)	0.79‡
RF positive; number (%)	12 (36)	30 (34)	0.83‡‡
Anti CCP antibody positive; number (%)	13 (39)	29 (33)	0.39‡‡
Number with persistent arthritis (%)	33 (37)	87 (68)	<0.0001‡‡
Number with persistent arthritis who developed RA (%)	18 (54)	55 (63)	0.41‡‡

Table 5.4: Baseline characteristics of early arthritis patients.

‡p value calculated using Mann Whitney test.
‡‡p value calculated using Fisher's exact test.
a CRP available for 84 patients in group 1.
b CRP available for 126 patients in group 2
c CCP data available for 126 patients in group 2

PCA of NMR spectra of serum from 84 early arthritis patients (group 1) in whom the CRP levels were known, showed a broad spread on the scores plot (Figure 5.3A). When the level of CRP for each sample was superimposed on the PCA plot, it was clear that the

level of inflammation at the time of sampling influenced the distribution. To investigate this further, the relationship between baseline metabolite profiles and CRP was assessed using PLS-R. The analysis employed a forward selection approach to discover those NMR bins that were most predictive. A total of 154 bins created the optimal model with a cross-validated r^2 of 0.671 (Figure 5.3B). Permutation testing with these 154 NMR bins demonstrated that the regression model was statistically valid ($p<0.001$).

To further validate this relationship between the level of inflammation and baseline metabolic profiles, the PLS-R analysis was carried out in a separate group of 127 early arthritis patients. The maximum r^2 for the regression of the real (i.e. ordered) data was 0.416 (Figure 5.3C). Permutation testing with 1136 NMR bins included (as optimised by forward selection) again demonstrated that the regression model was statistically valid ($p<0.001$).

The patients were divided into two groups as the samples were collected over a nine year period due to constraints of recruitment. While the samples were stored under optimal conditions at -80^{o}C, some alterations in some metabolites may have occurred during storage since spectra for two cohorts were acquired 4 years apart. The first group of patients were recruited between 2000 and 2004 and the samples run in 2005. The second group of patients were recruited between 2004 and 2009 and the samples run in 2010. Furthermore, updates in the spectrometer setup occurred over this period and so, for these reasons, the two cohorts showed some differences in the NMR spectra, which made combining them problematic. However, in many ways keeping the two cohorts separate was an advantage, since we were able to validate most of the observations made on the first cohort with those from the second, in spite of the minor differences in the spectra.

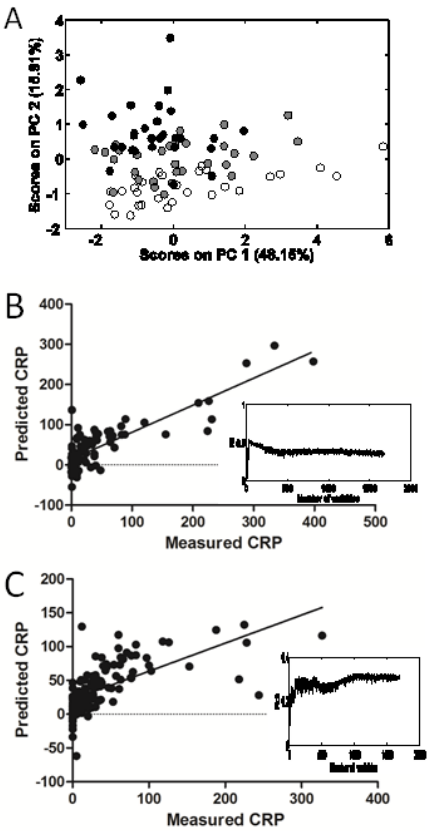


Figure 5.3: The metabolic fingerprints of sera from patients with early arthritis prior to treatment with DMARDs are strongly influenced by the level of inflammation. 1D 1H NMR spectra of serum from patients with very early arthritis (group 1) were subjected to PCA (A). Tertiles of CRP are indicated with high (●), intermediate (●) and low (○) levels. There were strong correlations between measured versus predicted CRP values for group 1 (B) and group 2 (C) ($p<0.001$ for both groups). The predicted values were calculated from the concentrations of a series of metabolites that were discovered using PLS-R. The inset plots in (B) and (C) show the optimisation of the multivariate regression, with the highest correlation between measured and predicted CRP occurring with 154 NMR bins (maximum R^2 of 0.671) for group 1 (B) and with 1136 NMR bins (maximum R^2 of 0.4157) for group 2 (C).

It is known that metabolic status and products of metabolism are influenced by several variables including age, gender, hypertension, diabetes, hyperuricaemia and smoking but when we adjusted our analysis for these confounding effects our results remained significant (Table 5.5). We also excluded the effect of medication used at the time of sample collection (steroids, DMARDs and NSAIDs) and found that the relationship

between CRP and metabolic profile was still significant (Table 5.5 and 5.6). The regression analysis was carried out to look at the relationship between metabolic profiles and CRP without the confounding effect of variables which are known to influence metabolic status by assessing only individuals who were not current smokers (n=67 group 1, n=96 group 2) and individuals without hypertension (n=70 group 1, n=96 group 2), diabetes (n=89 group 1, n=118 group 2) or hyperuricaemia (defined as uric acid level > 340μmol/l) (n=58 group 1, n=67 group 2). The effects of DMARDs and steroids on metabolic status were also excluded by carrying out the regression analyses only for those patients that were not on DMARDs (n=85 group 1, n=125 group 2; the few patients who were on DMARDs were on them for other co-morbidities e.g. inflammatory bowel disease) or steroids (n=86 group 1, n=123 group 2) at time of recruitment into the study. We excluded the effect of NSAIDs by only carrying out the regression analysis for those patients on NSAIDs at time of recruitment as the majority of patients were on NSAIDs (n=60 group 1, n=66 group 2). The significance of the relationship between the CRP and metabolic profiles is shown in table 5.6 and was statistically significant for all analyses apart from when women alone in group 1 were analysed.

	Group 1					Group 2				
	Resolving arthritis	Persistent arthritis	P value	Persistent arthritis who develop RA	P value	Resolving arthritis	Persistent arthritis	P value	Persistent arthritis who develop RA	P value
Steroids; number (%)	0 (0)	2 (7)	0.14#	1 (6)	0.25#	0 (0)	4 (5)	0.31#	3 (5)	0.26#
NSAIDs; number (%)	37 (66)	23 (70)	0.82#	13 (72)	0.78	17 (42)	49 (56)	0.18#	37 (69)	0.11#
DMARDs; number (%)	0 (0)	1 (3)	0.38#	0 (0)		1 (2)	1 (1)	0.52#	1 (2)	1.0#
Age (years); median (IQR)	45 (36-59)	50 (22-64)	0.75‡	56 (23-70)	0.08‡	44 (32-56)	57 (18-67)	0.05‡	60 (47-68)	0.0026‡
Female; number (%)	33 (59)	21 (64)	0.82#	15 (83)	0.089#	23 (58)	47 (54)	0.85#	26 (48)	0.41#
Hypertension; number (%)	15 (27)	3 (9)	0.06#	1 (6) 0.1	0.097#	6 (15)	25 (29)	0.12#	15 (28)	0.21#
Diabetes; number (%)	0 (0)	0 (0)		0 (0)		1 (2)	7 (8)	0.43#	5 (9)	0.23
Hyperuricaemia; number (%)	15 (29)	8 (28)	1.0#	6 (38)	0.546#	10 (31)	21 (32)	1.0#	14 (34)	1.0#
Smokers; number (%)	12 (22)	8 (25)	0.79#	6 (33)	0.36#	9 (22)	22 (25)	0.83#	15 (28)	0.64#

Table 5.5 Number of patients with variables which can affect metabolism in group 1 and group 2.

‡p value calculated using Mann Whitney test.

#p value calculated using Fisher's exact test.

	Group 1 (p value)	Group 2 (p value)
Non smokers	<0.001	<0.001
NSAIDs	0.01	<0.001
No DMARDs	<0.001	<0.001
Females	0.07	0.01
Males	<0.001	0.01
No hypertension	<0.001	<0.001
No DM	<0.001	0.01
No steroids	<0.001	<0.001
No hyperuricaemia	<0.001	<0.001

Table 5.6 Relationship between CRP and metabolic profiles for both groups without the effects of confounding drugs or other variables.

We also used the PLS-R analysis that related the metabolite fingerprint to CRP, to generate a rank order of NMR bins which identified regions of the spectra which most strongly predicted the inflammatory burden in groups 1 and 2 (Table 5.7). Almost identical metabolites were identified for both patient groups suggesting a complex but real relationship between metabolism and inflammatory burden.

Ranked importance	Metabolites identified in patient group 1 (ppm)	Metabolites identified in patient group 2 (ppm)
1	Total choline (3.20, 3.22, 3.23)	LDL-lipids (1.24-1.27)
2	LDL-lipids (1.24-1.27)	Acetylglycine (2.03, 3.71, 3.76)
3	Lactate (1.31, 1.33, 4.11)	Glucose (3.24-3.26, 3.41, 3.48, 3.68-3.69, 3.88)
4	Acetylglycine (2.03, 3.71, 3.76)	Fatty acids (0.8-0.84, 2.22-2.24)
5	Urea (5.77, 5.78, 5.79, 5.80, 5.81, 5.82)	Methylguanidine (2.81)
6	Glucose (3.24-3.26, 3.41, 3.48, 3.68-3.69, 3.88)	Lactate (1.31, 1.33)
7	Methylguanidine (2.81)	Threonine (3.58)
8	Methylhistidine (3.70)	Homocystine (3.86)
9	Cholesterol (0.91)	Glycine (3.55)
10	Taurine (3.42)	Taurine (3.42)
11	Threonine (3.58)	Methylxanthine (3.49)
12	Fatty acids (0.8-0.84, 2.22-2.24)	Total choline (3.20, 3.22, 3.23)
13	Methylxanthine (3.49)	Methylhistidine (3.70)
14	Homocystine (3.86)	Cholesterol (0.91)

Table 5.7: Metabolites most strongly correlated with CRP for early arthritis groups 1 and 2. Metabolites were identified from the PLS-R model and represent the regions of the spectra which had the greatest influence on the correlation with CRP. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite.

5.3.4 Relationships between clinical outcomes and metabolic profiles in early arthritis patients

A PLS-DA model was used to try and separate patients with resolving and persistent outcomes for both patient groups but did so with only moderate sensitivity and specificity of 59.4% and 58.9% for group 1 using 5 LVs (capturing 32% of the variance) (Figure 5.4A) and sensitivity and specificity of 59.5% and 56.4% for group 2 using 6 LVs (capturing 28% of the variance) (Figure 5.4B). There was also no differentiation between resolving disease and persistent RA for group 1 (Figure 5.4C) (sensitivity of 50% and specificity of 69.6% using 2 LVs capturing 74% of the variance) though there was good separation for group 2 (Figure 5.4D) with a sensitivity of 73.1% and specificity of 67.6%

using 3 LVs capturing 98% of the variance. There was no differentiation between patients with RA and non RA outcomes for either cohort (Figure 5.5). Previous data has shown that therapeutic drug use can have effects on metabolic profiles (Kirschenlohr et al. 2006) but there was no significant difference between the number of patients on NSAIDs for either group (Table 5.4) and there was no significant difference in the use of NSAIDs between patients with persistent and resolving disease for group 1 (p=0.13) or group 2 (p=0.1) or between patients with persistent RA and resolving disease for group 1 (p=0.18) or group 2 (p=0.17).

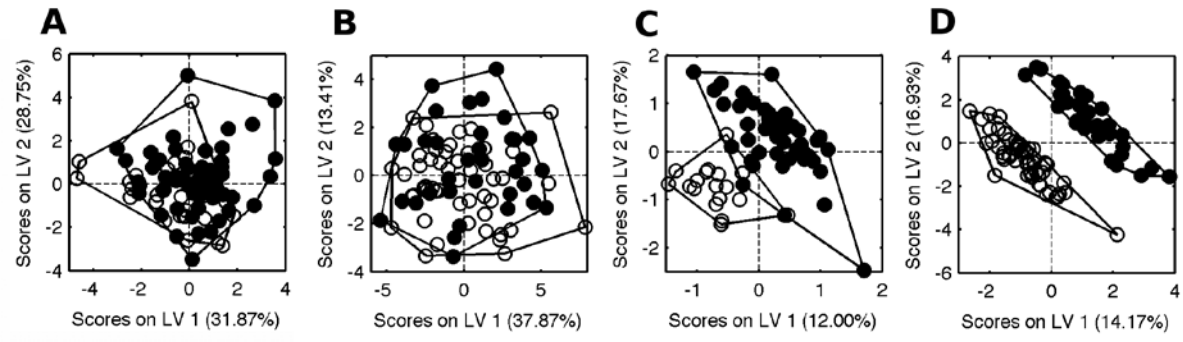


Figure 5.4: The metabolic fingerprints of sera from patients with early arthritis from two different patient groups.
Serum samples from early arthritis patients from group 1 (A) and group 2 (B) at first presentation were assessed using PLS-DA, to separate patients whose disease was resolving (●) or persistent (○). Sensitivity and specificity were 59.4% and 58.9% for group 1 (A) and 59.5% and 56.4% for group 2 (B).
Serum samples from early arthritis patients from group 1 (C) and group 2 (D) at first presentation were assessed using OPLS-DA, to separate patients whose disease was resolving (●) from those that developed persistent RA (○). Sensitivity and specificity were 50% and 69.6% for group 1 (C) and 73.1% and 67.6% for group 2 (D).

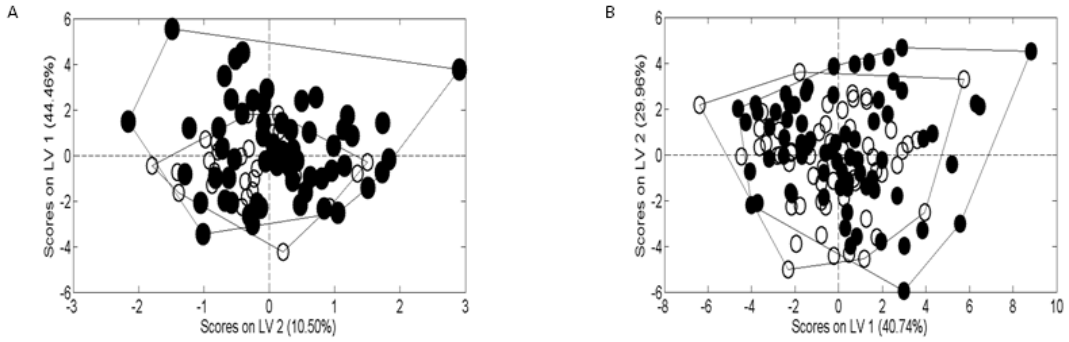


Figure 5.5 The metabolic fingerprints of sera from patients with early arthritis from two different groups separated according to the eventual development of RA or not. Serum samples from early arthritis patients from group 1 (A) and group 2 (B) at first presentation were assessed using PLS-DA, to separate patients who developed RA (○) from those that did not develop RA (●). Sensitivity and specificity were 53.3%and 53.6% for group1 (A) and 62% and 63.6% for group 2 (B).

Metabolites were identified that contributed the greatest to the separation for early arthritis patients split into resolving and persistent disease, and also for resolving and persistent RA for both patient groups (Table 5.8). Although some predictive metabolites were common to both patient groups there were some key differences in metabolites between the groups, suggesting that the metabolomic techniques as applied in this study cannot fully discriminate between resolving and persistent outcomes or resolving disease and persistent RA in early arthritis patients.

Metabolite (ppm)	Persistent vs. Resolving arthritis (group 1)	Persistent vs. Resolving arthritis (group 2)	Persistent RA vs. Resolving arthritis (group 1)	Persistent RA vs. Resolving arthritis (group 2)
LDL-CH3 (0.80)	Low (6.81)	Low (2.87)	-	-
LDL-CH2 (1.21)	Low (7.40)	Low (6.89)	-	Low (1.58)
3-hydroxybutyrate (1.18, 1.19)	-	High (6.87)	-	-
Lactate (1.31, 4.11)	Low (12.85)	High (27.90)	Low (12.74)	High (16.98)
Alanine (1.46,1.48)	-	-	-	Low (3.84)
Acetylglycine (2.03)	High (6.55)	High (6.80)	High (4.57)	Low (1.94)
Methylguanidine (2.81)	High (92.72)	Low (38.15)	High (34.76)	Low (6.51)
Taurine (3.26)	-	High (15.73)	-	High (8.66)
Glucose (3.25,3.88)	-	High (11.55)	-	High (7.49)
Urea (5.79)	High (3.90)	-	High (1.25)	-

Table 5.8: Analysis of PLS-DA weightings illustrates the metabolites contributing to the differentiation between: patients with early arthritis who develop persistent disease or whose arthritis resolves for 2 groups; and patients with early arthritis who develop persistent RA or whose arthritis resolves for 2 groups. "High" implies the metabolite is at higher concentration in the persistent (column 2 and 3) or persistent RA (column 4 and 5) disease phenotypes. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite. . Variable importance of the projection (VIP) values are shown in brackets for all of the metabolites.

5.4 Discussion

Our data demonstrate that the serum metabolite fingerprint of patients with active RA differs from that of healthy controls and that the serum metabolite fingerprint in patients with early arthritis varies depending on the level of inflammation. These results from patients with established RA are broadly consistent with a recent study using a similar experimental approach which focussed on responses to therapy in a cohort of RA patients (Lauridsen et al. 2010). There is also some overlap in discriminating metabolites identified with a recent mass spectrometry-based analysis of established RA (Madsen et al. 2011). However, patients in both of those cohorts had already been exposed to DMARD therapy

and so it is possible that their metabolic fingerprints might be influenced by this. Our cohort of patients was sampled before any DMARD therapy and so was not exposed to this confounding factor.

As with the study by Lauridsen et al (Lauridsen et al. 2010), we found that decreased NMR lipid signals were particularly discriminatory between patients with established RA and controls and Madsen et al (Madsen et al. 2011), using mass spectrometry, also observed changes in cholesterol comparing established RA and controls. Such lipid changes were not seen in a mouse arthritis model (Weljie et al. 2007), probably because the investigators filtered samples to remove protein and the associated lipids. Changes in lipid profiles in the blood of RA patients have been widely described, and have been suggested to be a major contributing factor to the accelerated atherosclerosis associated with RA (Steiner and Urowitz 2009;Toms et al. 2010).

We found that 3-hydroxybutyrate was raised in the established RA patients. This metabolite is known to be present in RA synovial fluid (Naughton et al. 1993a), in pouch fluid from the rat air pouch model (Claxson et al. 1999) and in the blood of mice with experimental arthritis (Weljie et al. 2007). The presence of 3-hydroxybutyrate, a ketone body, suggests an increased level of lipolysis in RA patients compared with controls. This may be another explanation for the decreased levels of lipid that we have observed, and supports earlier spectroscopic studies of synovial fluid (Naughton et al. 1993a) which determined that lipid metabolism may be a predominant source of energy in the hypoxic inflammatory joint.

Our PLS-R analysis revealed a clear correlation between the CRP level and the metabolites present. This was replicated in a separate group of patients. This approach identified that increased concentrations of many of the same metabolites discovered in the PCA and OPLS-DA models, for example LDL lipid, lactate, glucose, taurine, acetylglycine and methylguanidine, were predictive of the inflammatory phenotype. However, a number of amino acids (e.g. choline, threonine, methylhistidine) were also found to contribute strongly to the correlation (Table 5.7). 13 of the 14 metabolites which were most strongly associated with CRP were the same for both groups of patients.

Many of the metabolites that correlated with inflammation, as measured by serum CRP levels, are associated with lipid metabolism and may again contribute to the increased levels of atherosclerosis associated with inflammatory disease. Abnormalities in lipids have been associated with pre-RA (van de Stadt et al. 2012) , early RA (Ahmed et al. 2010;Park et al. 1999) and established RA (Dessein et al. 2002;Kavanaugh 1994;Toms et al. 2011) and lipid levels correlate with CRP even in the absence of clinically apparent inflammatory disease (Hrnciarikova et al. 2009). Furthermore, lipid lowering therapies influence inflammation (Leung et al. 2003;McCarey et al. 2004) and anti-inflammatory therapies influence lipids (Peters et al. 2007) demonstrating a complex relationship between inflammation and lipid metabolites which our data help to dissect.

Blood donors who went on to develop RA at least a decade later have been shown to have significantly more atherogenic blood lipid profiles than those who did not develop RA

(van Halm et al. 2007).While this difference did not correlate with CRP levels it does suggest that lipid profiles early in disease might contribute to prediction of outcome. However, we were unable to discriminate between patients with early arthritis who went on to develop persistent disease and those whose arthritis resolved. Although there was some separation between patients with persistent and resolving outcomes in both groups the discriminating metabolites differed. One contributing factor may be the use of non filtered blood samples in our study as data suggest that filtered blood gives better results due to better resolution of the NMR spectra (Weljie et al. 2008) but we would then lose the effect of lipids which we know play a part in RA (Toms et al. 2010). There was also a delay between our sample collection and sample processing due to samples being collected at City hospital and processing occurring at the University of Birmingham. Samples were not collected on ice and were not spun down promptly and our own quality control work (section 3.1) has demonstrated that the metabolites lactate and pyruvate can alter over time without these precautions being taken. When the metabolites lactate and pyruvate were removed from our NMR spectra there was little change in the prediction of outcome. We decided to include these metabolites in our analysis as most of our samples were processed prior to six hour post collection and also due to the fact that lactate and pyruvate are such key metabolites in cell metabolism. Our work has illustrated that metabolites do not alter too much if samples are collected in an adhoc manner (section 3.1) and the fact that the metabolite data correlate strongly with CRP suggests that the metabolite profile hasn't been perturbed in a too random manner by ex vivo processing issues.

Hence, while we may be able to improve predictive ability if samples are processed more rigorously it is more likely that the lack of discrimination may result from the heterogeneity between the two groups of early arthritis patients for example in terms of the

proportion whose disease persisted (Table 5.4). Group 1 had very few patients whose arthritis persisted and very few of these were ACPA positive. We know that ACPA positive and ACPA negative RA are very different in terms of aetiology (Klareskog et al. 2008) and it may be that they behave very different metabolically. Due to the small number of patients we were unable to do sub analysis of these patients.

Several approaches have been used to predict the outcome of patients with early arthritis as identifying these patients early enables earlier treatment and better outcome (Raza and Filer 2009). The most widely used predictive tool is the 'Leiden rule' which consists of 9 clinical and serological variables: sex, age, localisation of symptoms, morning stiffness, tender joint count, CRP level, IgM RF and the presence of anti-CCP antibodies (van der Helm-van Mil et al. 2007). Genetic susceptibility markers have also been used in the prediction of outcome and the main susceptibility genes, the shared epitope and PTNP22 are only associated with ACPA positive RA. However, SNP markers within the TRAF1/C5 locus, which are also associated with ACPA positive RA, have demonstrated an association with the development of radiological erosions (Plant et al. 2011). The biochemical markers matrix metalloproteinase 3 in the serum and C-telopeptide of type II collagen in the urine have also demonstrated an association with radiographic progression in patients with early RA especially if used as part of a multivariate model (Young-Min et al. 2007). Synovial fluid analysis can also aid outcome prediction and research has demonstrated that certain cytokine patterns are associated with different outcomes in patients with undifferentiated arthritis (Raza et al. 2005b). Imaging has also been used to try and predict outcome in patients with undifferentiated arthritis. Groups have used Magnetic resonance imaging of the wrists and finger joints and illustrated that bone oedema adds to the prediction obtained by serological and clinical factors alone (Tamai et

al. 2009). Ultrasound is widely used in rheumatology and the use of ultrasound counts in our cohort of patients increased the prediction of RA compared to clinical and serological markers alone (Filer et al. 2011). Other groups have not duplicated the additional benefit of ultrasound and this may be due to the inclusion of patients with arthralgia only in their cohorts and the fact that only limited joint examination was done in this study (Pratt et al. 2013). All these measures have shortcomings in predicting outcome in patients with inflammatory arthritis and the prediction outcome may be greatly increased by combining these variables with metabolomic data in the future. Therefore to confirm and extend the observations we have made, these analyses need to be replicated in independent cohorts.

In conclusion, the metabolomic fingerprint reflects inflammatory disease activity in patients with a new onset of arthritis. This suggests that the underlying inflammatory processes drive significant changes in metabolism that can be measured in the peripheral blood. This may give us further insights into the mechanism of disease in inflammatory arthritis as has been the case following the identification of contributing metabolites in other diseases (Sreekumar et al. 2009). Furthermore, metabolomics may prove useful as a measure of the extent of disease, potentially separating low disease activity states from patients in true remission.

6.0 Assessment of metabolite profiles of cultured synovial fibroblasts from patients with early arthritis

6.1 Introduction

Fibroblasts are the major cell in the expanded RA synovium. Their transformation may be dependent on immune cells and so describing the interactions between these cell types is important. Inflammation, and its persistence in RA, results from complex interactions between haematopoietic and stromal cells (Buckley et al. 2001;Parsonage et al. 2005) but research into the pathogenesis of the disease often neglects the role of stromal cells. Activated RA synovial fibroblasts have an important role in determining the site at which inflammation occurs and the persistence of inflammation in the joint microenvironment (Takemura et al. 2001). Fibroblast activation results in the accumulation, survival and retention of leukocytes at sites of inflammation (Iwamoto et al. 2008;Parsonage et al. 2005) by chemokine and cytokine production, thus preventing the resolution of chronic inflammation (Filer et al. 2006). Synovial fibroblasts play a key role in joint destruction in RA and numbers are expanded. They directly invade cartilage and they contribute to bone destruction through activation of osteoclasts (Bartok and Firestein 2010).

In the joint, fibroblasts have limited access to nutrients and oxygen within the poorly vascularised hypoxic synovium and yet expansion of fibroblast numbers still occurs. This suggests fibroblasts may adapt their metabolism to this environment and this process may be involved in driving the persistence of inflammation. It is possible that this adaptation occurs in an incremental manner and so metabolic changes might relate to the stage of the disease. To investigate the potential of metabolite fingerprinting in increasing our understanding of the role of fibroblast in inflammatory arthritis, we have used NMR-based metabolomics in the analysis of synovial fibroblasts from patients with newly presenting

established RA and early arthritis. We sought to identify differences in metabolite fingerprints in fibroblasts from patients with established RA, early arthritis and healthy controls and to determine if inflammatory cytokine production by fibroblasts is related to their metabolic profile.

6.1.1 Cytokines in RA

A broad spectrum of cytokines are involved in the pathogenesis of RA but here I will focus on those produced by synovial fibroblasts since these, together with macrophage cytokines, are dominant in RA. Activated macrophages and fibroblasts interact via secretion of soluble mediators leading to further activation of cells, recruitment of new cells to the joint and increased production of inflammatory mediators (Bartok and Firestein 2010). The cytokines present in the joint may vary dependent on the stage of RA with some evidence that there is a prevalence of IL 4 (Th2 cytokines) in early RA and more established RA being characterised by a Th1 response (Raza et al. 2005b). Several cytokines and chemokines are produced by synovial fibroblasts and different ones are associated with different stages of RA. CXCL9 has been found in cultured synovial fibroblasts from patients with early RA (Tsubaki et al. 2005) and some cytokines have also only been detected in fibroblasts which have been stimulated with, for example, IFN-γ or TNF α (Tsubaki et al. 2005).

6.1.1.1 IL 6

Fibroblasts are the primary source of IL 6 in RA and this cytokine increases differentiation of B cells and their production of antibodies; increases T cell differentiation, proliferation

and survival; activates osteoclasts and chondrocytes and induces acute phase response (Park and Pillinger 2007).

IL 6 binding to its receptor (IL 6R) does not in itself convey a signal. For this to occur a transmembrane accessory molecule (gp 130) needs to associate with the IL 6/ IL6R complex. Gp130 is expressed by most cells but membrane bound IL 6R expression is restricted to hepatocytes, neutrophils, monocytes/macrophages and some lymphocytes. IL 6 can transmit signals using the conventional membrane bound IL6R or in conjunction with a soluble IL 6 receptor (sIL6R). Both forms of signalling can activate gp 130 (Scheinecker et al. 2008). In the rheumatoid joint both IL 6 and sIL6R are abundant and have been shown to mediate osteoclast formation, synovial fibroblast proliferation and cartilage degradation (Kotake et al. 1996).

The importance of IL6 in RA is illustrated by the successful therapeutic use of tocilizumab (TCZ), a humanised antibody to the IL 6R targeting both IL6R and sIL6R. TCZ in combination with methotrexate has an efficacy similar to TNF antagonists in the treatment of RA (Scheinecker et al. 2008;Smolen et al. 2013).

6.1.1.2 VEGF

Vascular endothelial growth factor (VEGF) induces angiogenesis in the RA synovium. VEGF expression is upregulated in both macrophages and synovial fibroblasts (Taylor 2002). Hypoxia is a potent inducer of VEGF (Berse et al. 1999;Jackson et al. 1997).

6.1.1.3 Matrix metalloproteinases (MMP)

Fibroblasts are a major source of proteins of the MMP family which are involved in both the physiological and the pathophysiological breakdown of extracellular matrix as seen in RA. Most MMPs are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. RA synovial fibroblast synthesise and secrete MMP 1, 3, 9, 10 and 13 (Abeles and Pillinger 2006). MMP-1 is a collagenase and degrades collagen type I, II, III, V and XI. MMP-1 is produced in response to cytokine stimulation and is the only MMP which can digest type II collagen, the major collagen in cartilage, if the collagen has not been degraded previously (Abeles and Pillinger 2006). Its production from cultured synovial fibroblasts is increased under hypoxic conditions (Ahn et al. 2008). MMP-3 is involved in the breakdown of extracellular matrix. Its production from cultured synovial fibroblasts is increased under hypoxic conditions and is directly controlled by activation of HIF-1α (Ahn et al. 2008).

MMP9 causes degradation of collagen IV in basement membrane and extracellular matrix and can stimulate angiogenesis (Ram et al. 2006). MMP13 degrades collagen type I, II, III, IV, V, IX, X and XI. It is over expressed in rheumatoid arthritis (Amalinei et al. 2010).

6.1.1.4 Chemokines

Several fibroblast derived chemokines are expressed in the RA synovium (Bartok and Firestein 2010). CCL2 (or monocyte chemotactic protein-1 (MCP-1)) is produced by synovial fibroblasts and recruits monocytes and macrophages to sites of inflammation in

RA (Bartok and Firestein 2010). Its expression by synovial fibroblasts is reduced by hypoxia (Safronova et al. 2003).

CCL3 (or Macrophage inflammatory protein-1α (MIP-1α)), is also produced by cultured fibroblasts. It is involved in the recruitment and activation of T cells, monocytes and natural killer cells (Szekanecz et al. 2010).

CCL5 (or RANTES; regulated on activation, normal T cell expressed and secreted) is produced by cultured synovial fibroblasts (Ahmed et al. 2006). CCL5 is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites (Koch 2005).

Although IL-8 (CXCL8) is produced by macrophages it can also be produced by fibroblasts in culture if they are stimulated with IL-1 or TNF (Szekanecz et al. 2010) , both of which are commonly present in the RA joint. IL-8 is an important mediator in the innate immune response as a neutrophil chemoattractant. Thus it may be responsible for the ingress of these cells into the synovium from where they enter the synovial fluid (where they are often the most prevalent cell) and furthermore it may enhance cell migration by stimulating new blood vessel formation (Bartok and Firestein 2010;Koch et al. 1992).

CXCL10 (or Interferon gamma-induced protein 10 (IP-10)) is secreted by macrophages and fibroblasts in response to IFN-γ. CXCL10 recruits lymphocytes and macrophages (Szekanecz et al. 2010) and inhibits angiogenesis (Bartok and Firestein 2010).

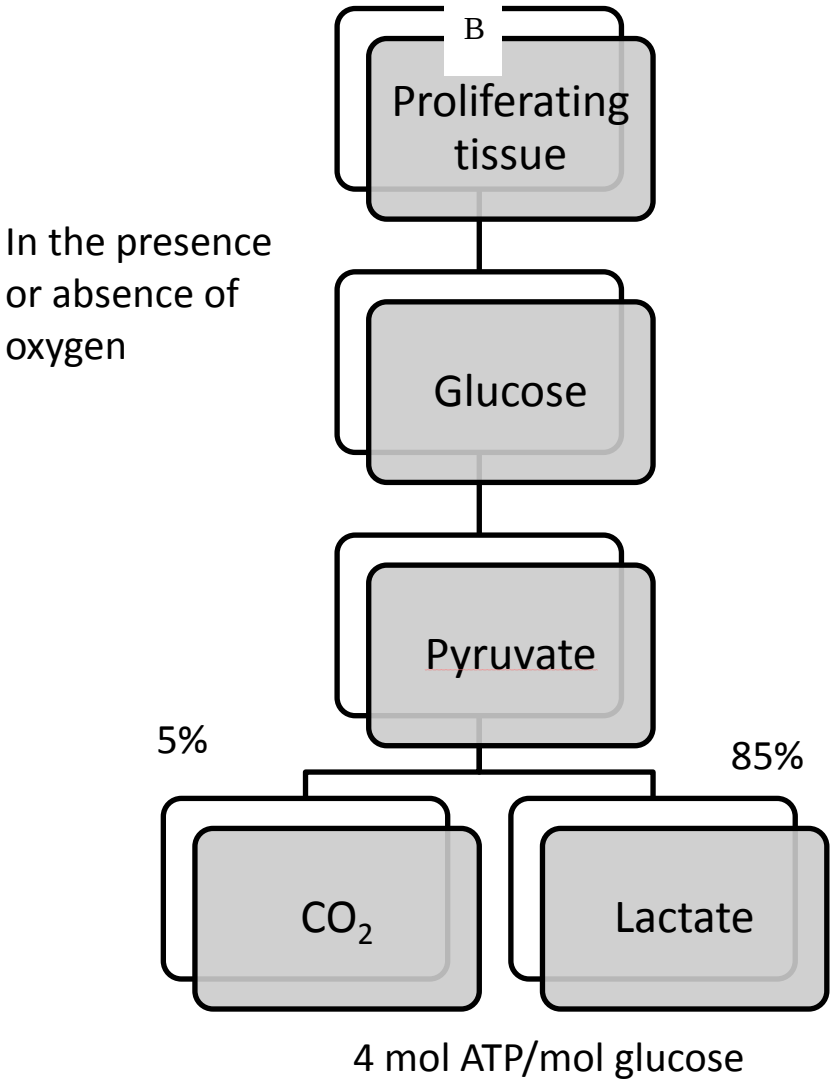
6.1.2 Hypoxia in RA and the Warburg affect

Angiogenesis is present during the earliest stages of RA and leads to increased leucocyte migration (Taylor and Sivakumar 2005), a critical factor underlying the significant increase in the number of macrophages, T, B and plasma cells in the RA synovium. Despite increased vascular supply there is a failure to restore tissue oxygen homeostasis. This is probably due to the metabolically active synovium requiring increased oxygen and the increased intra-articular pressure of joint swelling placing stress on the vascular supply (Ng et al. 2010). Hence rheumatoid synovial microenvironments exist under hypoxic conditions. In the synovial joint there is a Th1 response but the level of IL-2 is low and this may be secondary to hypoxia. Hypoxia upregulates the TNF and IL-1 produced by mononuclear cells but downregulates the IL-2 produced by T lymphocytes. Hypoxia also controls the Th2 to Th1 switch (Bodamyali et al. 1998). Hypoxia may drive inflammation in the joint as hypoxia per se induces cell migration (T cell and macrophage infiltrates) (Ng et al. 2010). Hence, hypoxia mediates persistent synovitis in RA. The reduction in perfusion of the synovial joint, along with the hypoxia and inflammatory cell infiltration lead to synovial fibroblast hyperplasia. This increased metabolic demand causes an increase in glycolysis.

Synovial tissue in RA is hypoxic (Biniecka et al. 2010;Biniecka et al. 2011a;Ng et al. 2010) and has low glucose and elevated lactate levels as hypoxia causes a transition to glycolytic metabolism (Bodamyali et al. 1998). This glycolytic metabolism has been found in cancer cells and is thought to exist in the rheumatoid joint. In normal differentiated cells energy is generated through mitochondrial oxidative phosphorylation. In cancer cells (i.e. proliferating tissues) a process of aerobic glycolysis occurs which is also known as the ‘Warburg effect’ i.e. glycolysis even in the presence of oxygen (Bayley and Devilee

2012;Koppenol et al. 2011). Aerobic glycolysis is inefficient in terms of ATP production but it is thought that it allows production of lipids and amino acids to enable cell proliferation (Vander Heiden et al. 2009) (See Figure 6.1).

A


B
Proliferating tissue
In the presence or absence of oxygen
Glucose
Pyruvate
5%
85%
CO_2
Lactate
4 mol ATP/mol glucose

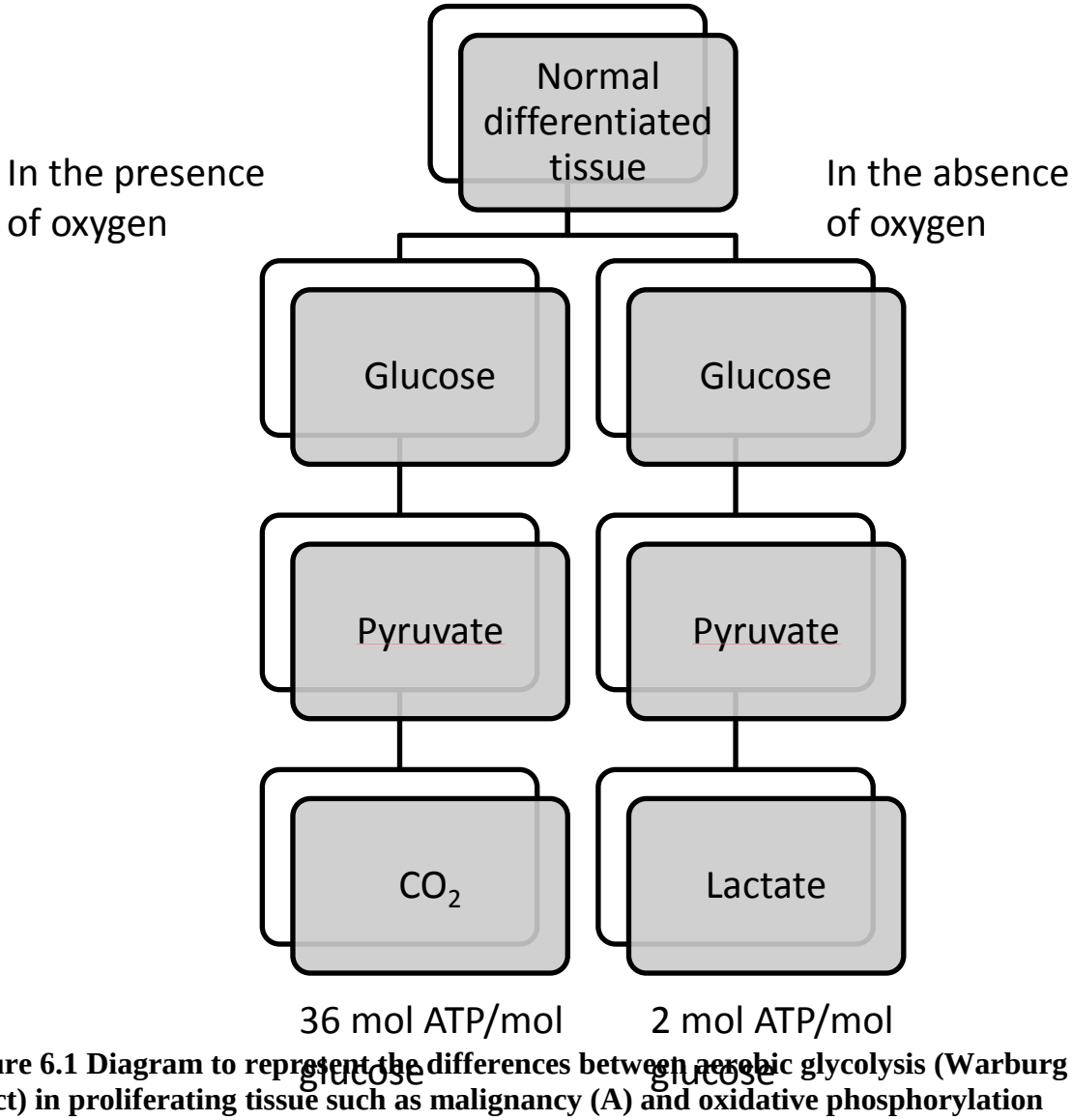


Figure 6.1 Diagram to represent the differences between aerobic glycolysis (Warburg effect) in proliferating tissue such as malignancy (A) and oxidative phosphorylation and anaerobic glycolysis in normal differentiated tissue (B) (Vander Heiden et al. 2009).

6.1.3 Aims within this chapter

Since the level of oxygen in the inflamed joint decreases with increasing synovitis, we hypothesised that synovial fibroblasts taken from joints at different stages of disease would have metabolomic profiles, when cultured in vitro, that may predict the development of RA in patients with early arthritis - an area where better predictive tools are currently needed (Raza and Filer 2009). We also wished to determine if this analysis could provide novel insights into disease mechanisms in arthritis as has been the case in other conditions (Sreekumar et al. 2009). We specifically planned to investigate the relationship between the 'metabotype' of in vitro synovial fibroblasts and the phenotypes and clinical outcomes of patients.

6.2 Methods

6.2.1 Patients

Patients were recruited through the inflammatory arthritis clinic at Sandwell and West Birmingham Hospitals NHS Trust, Birmingham, UK. Patients were eligible for the early arthritis cohort if they had one or more swollen joints and symptoms of ≤ 3 months duration. Patients with evidence of previous inflammatory joint disease were excluded. Those with a joint amenable to ultrasound guided biopsy (Scire et al. 2007) and who gave consent to this procedure were recruited to this study. Biopsies were taken as described in section 2.1.3. Following the biopsy, patients were followed for 18 months and then assigned to their final diagnostic categories. Patients were classified as having RA according to the 1987 ACR criteria (Arnett et al. 1988) due to the higher specificity of these criteria compared with the 2010 ACR/ EULAR criteria (section 1.1.1), allowing criteria to be satisfied cumulatively. As previously described (Raza et al. 2005a) resolving arthritis was diagnosed if there was no evidence of joint related soft-tissue swelling on

final examination and the patient had not received DMARD or steroid treatment within the previous 3 months. Persistent joint related swelling or treatment with DMARDs or steroids for inflammatory joint symptoms (within the previous 3 months) defined persistence.

Established RA and 'normal' patients were studied as control groups. Established RA patients were newly presenting DMARD naive patients fulfilling 1987 ACR classification criteria for RA (Arnett et al. 1988) and with a symptom duration of > 3 months. Symptom onset was defined as the time of onset of inflammatory joint pain and/or early morning stiffness and/or joint related soft tissue swelling. The 'normal' group comprised patients who underwent knee arthroscopy due to unexplained knee pain, with no evidence of inflammatory joint pathology upon clinical examination or arthroscopy. Clinical parameters and blood samples for routine clinical tests such as CRP were collected for all patients with inflammatory arthritis. The study was conducted in compliance with the Helsinki declaration and ethical approval was obtained from the local ethics committee. All subjects gave written informed consent.

Four groups of patients were chosen for this study -patients with established RA, patients presenting with early arthritis who developed persistent rheumatoid arthritis (persistent early RA), patients presenting with early arthritis whose disease resolved (resolving arthritis) and patients with no evidence of inflammation (normal controls). Limited numbers of fibroblasts were available so the patients were matched as closely as possible for autoantibody status (from early and established RA patients), joint biopsied, age and gender. It was felt important to keep the individuals in each group as homogenous as possible, particularly in respect to antibody status as there is considerable debate as to

whether antibody negative RA is a different disease to antibody positive RA (Klareskog et al. 2008). Hence, during matching, autoantibody status was given precedence over the joint from which the biopsy was taken as it has been illustrated that inflammation in one inflamed joint is representative of that in other inflamed joints (Kraan et al. 2002). In this study fibroblasts were derived from 24 different patients (6 from each group). The persistent early RA and the established RA group were all anti-CCP antibody positive.

6.2.2 Fibroblast culture

Fibroblasts were cultured as described in section 2.2.

6.2.2.1 Oxygen status

Fibroblasts were cultured in 20% oxygen as described in section 2.2. Once cells were confluent at P4 fibroblasts were extracted from two T75 flasks as per sections 2.23 and 2.3 and samples prepared as per section 2.4.6. Media were collected as per section 2.2.3 and this was used for NMR analysis as per section 2.4.5 and for analysis via ELISA as per section 2.7 and for Luminex assays as per section 2.8. One flask was trypsinised (section 2.2.2) and split into 3 further flasks. Two of these flasks were then cultured under hypoxic conditions (3% oxygen) for 48 hours prior to extraction and media were collected from these for NMR spectroscopy (section 2.4.5), ELISA (section 2.7) and Luminex analysis (section 2.8). 3% was chosen as a large amount of literature suggests that the rheumatoid joint is hypoxic and that the average oxygen concentration of the rheumatoid joint is 3% (Biniecka et al. 2011b;Kennedy et al. 2011;Ng et al. 2010). My aim was to investigate whether fibroblasts from the four groups could be distinguished using NMR based metabolomic analysis when cultured under conditions of 20% oxygen or 3% oxygen.

6.2.2.2 Treatment with IL 6

Normal fibroblasts were treated with recombinant Human IL 6 (PeproTech) and soluble IL 6 receptor (PeproTech) to determine if a normal 'metabotype' could be converted to an inflammatory 'metabotype' using IL 6. Synovial fibroblasts do not have IL 6 receptor so soluble Il 6 receptor (sIl6R) was also added (Ando et al. 2010;Kawashiri et al. 2009). Six normal fibroblast lines were studied and these were grown to confluence at passage 5 to obtain 8 confluent flasks. Once the fibroblasts were visually confluent the conditioned medium was removed and discarded and the flask was washed with 5mls PBS. The media in four flasks were replaced with 12mls of fresh medium. For the other four flasks from each cell line, the media were replaced with 12mls medium with IL6 at 20ng/ml (Rosengren et al. 2012) and SIL6R at 100ng/ml (Rosengren et al. 2012). There were two time points to the experiment, 24hours and 72 hours. Two of the control flasks and two of the IL6 treated flasks were extracted at 24 hours as per section 2.23 and 2.3 and the other two control flasks and two flasks treated with IL6 were extracted at 72 hours.

6.2.2.3 Treatment with tocilizumab

Synovial fibroblasts from patients with established RA were treated with tocilizumab (TCZ) (Chugai) or ivIG (Gammaplex) as a control. This was to determine if the metabotype found in fibroblasts from rheumatoid patients could be reversed by TCZ i.e. if we could block the effects of the fibroblasts' own IL 6. The method was the same as section 6.2.2.2 but TCZ or ivIG was added in place of recombinant IL 6. Two established RA fibroblast lines were studied for this experiment and these were grown to confluence at passage 6 to obtain 8 confluent flasks. The media in four flasks were replaced with 12mls of media with ivIG incorporated at 500micrograms/ml (120µl) and SIL6R incorporated at 100ng/ml (12µl). The other four flasks from each cell line was replaced with 12mls

medium with TCZ incorporated at 500micrograms/ml (670µl) (Kawashiri et al. 2009) and SIL6R incorporated at 100ng/ml (12µl). There were two time points to the experiment, 24hours and 72 hours. Two of the control flasks (iv IG treated) and two of the TCZ treated flasks were extracted at 24 hours as per section 2.23 and 2.3 and the other two control flasks and two flasks treated with TCZ were extracted at 72 hours.

6.3 Results

6.3.1 Prediction of outcome

Details of the study participants are shown in Table 6.1. Clinical characteristics were matched as closely as possible for all donors and all the parameters were very similar apart from the CRP, which, as expected, differed between different outcome groups.

	Established RA (n=6)	Healthy controls (n=6)	Early resolving arthritis patients (n=6)	Early RA patients (n=6)
Age (years); median (IQR)	55 (46-66)	43 (34-52)	40 (33-54)	50 (48-65)
Female; number (%)	3 (50)	2 (33)	4 (67)	3 (50)
Symptom duration (weeks); median (IQR)	38 (26-152)	-	4 (2-7)	5 (4-10)
NSAIDs; number (%)	4 (67)	0 (0)	4 (67)	3 (50)
CRP (mg/ml); median (IQR)	48 (3.5-66.5)	-	7.5 (0-20.5)	22 (3.75-33.5)
RF positive; number (%)	5 (83)	-	0 (0)	5 (83)
Anti CCP antibody positive; number (%)	6 (100)	-	0 (0)	6 (100)
Clinical assessment score of the swollen biopsied joint; mean	2.17	-	1.5	1.83
Ultrasound grey scale score of swollen biopsied joint; mean	2.67	-	1.67	2.5
Joint biopsied				
Ankle; n (%)	2 (33)	0 (0)	2 (33)	2 (33)
Knee; n (%)	4 (67)	6 (100)	4 (67)	2 (33)
MCP; n (%)	0 (0)	0 (0)	0 (0)	2 (33)

Table 6.1: Demographic and clinical characteristics of study participants.

6.3.1.1 Culture medium from fibroblasts cultured at 20% oxygen

Fibroblasts were cultured and the media were collected from these cells. These media were then investigated using NMR and the results analysed. Fibroblast medium was selected as an appropriate biofluid to study as it contains the metabolites accumulated by cells as is the case for other biofluids such as blood and urine. The metabolic profile of the media from

these fibroblasts cultured at 20% oxygen from patients with inflammatory arthritis was very different from that of healthy controls (Figure 6.2).

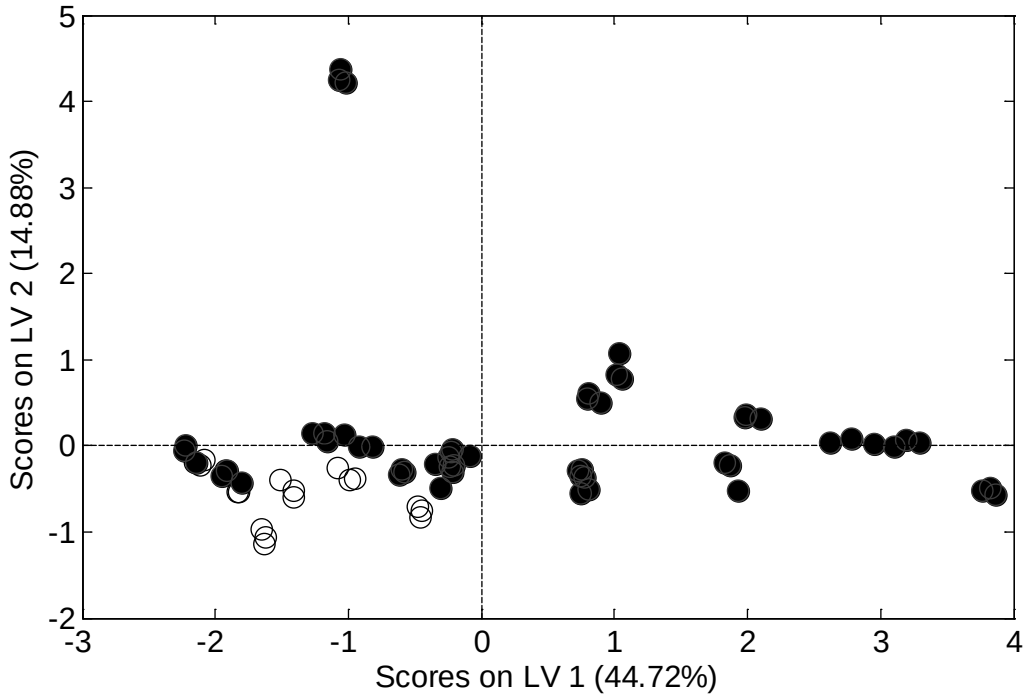


Figure 6.2 Fibroblast medium from fibroblasts cultured at 20% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with inflammatory arthritis (established RA, early RA and early resolving arthritis) (●). PLSDA analysis separated the groups with a sensitivity of 78% specificity of 78%.

There was good separation of the metabolic profiles of the healthy controls and early resolving arthritis (Figure 6.3), healthy controls and early RA (figure 6.4), and healthy controls and established RA (Figure 6.5) using the media from the synovial fibroblasts of these patients.

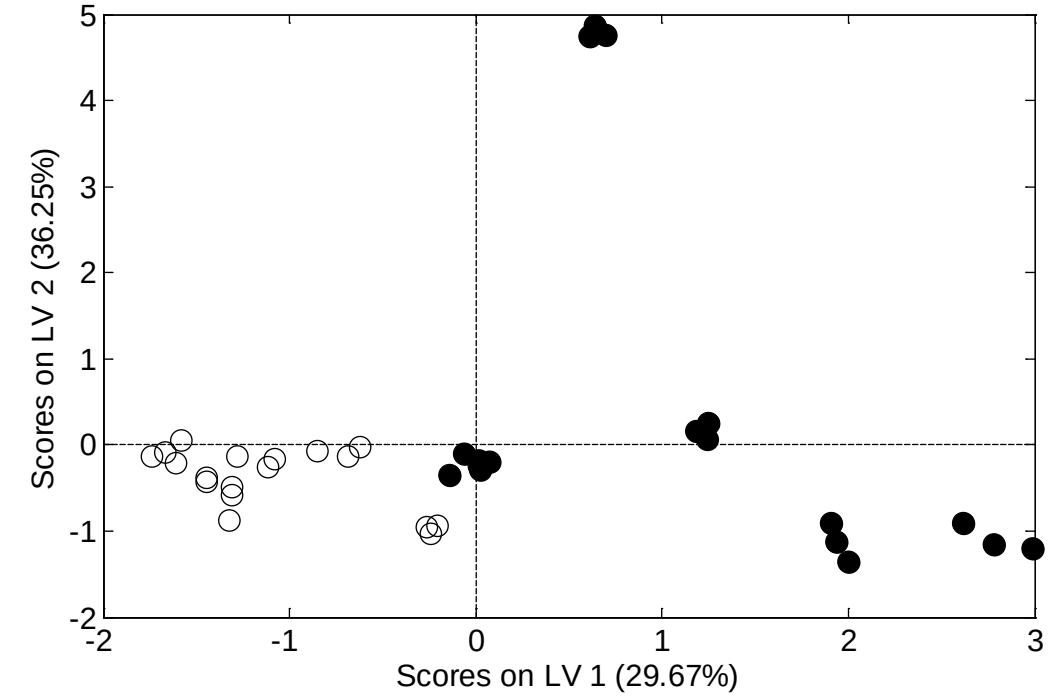


Figure 6.3 Fibroblast medium from fibroblasts cultured at 20% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with early resolving inflammatory arthritis (●). PLSDA analysis separated the groups with a sensitivity of 100% and specificity of 100%

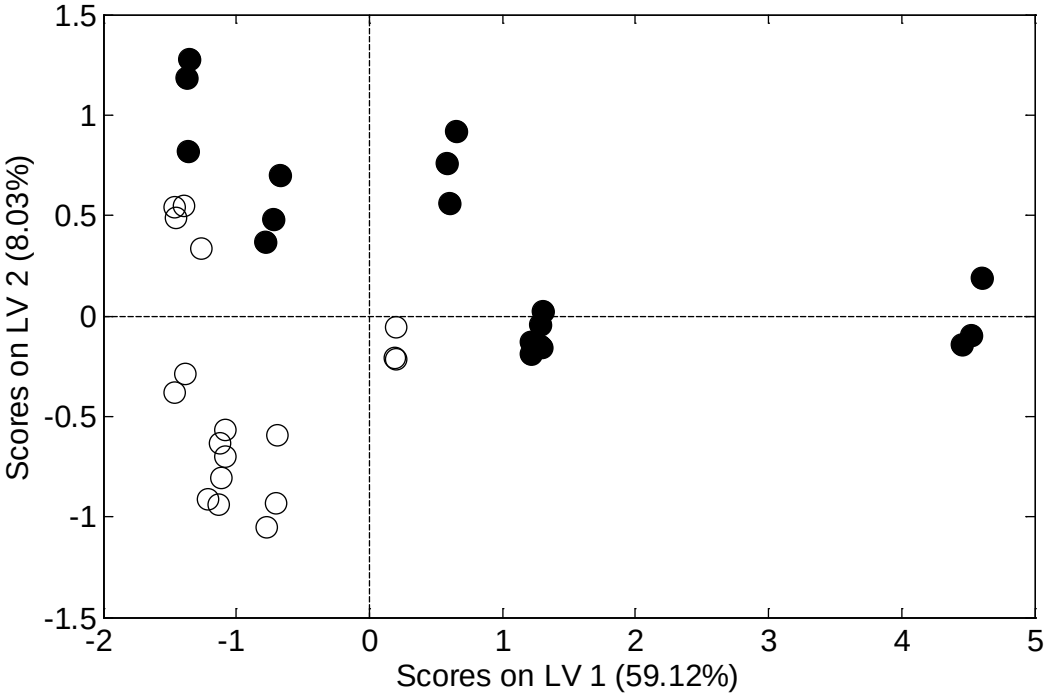


Figure 6.4 Fibroblast medium from fibroblasts cultured at 20% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with early RA (●). PLSDA analysis separated the groups with a sensitivity of 100% and specificity of 100%.

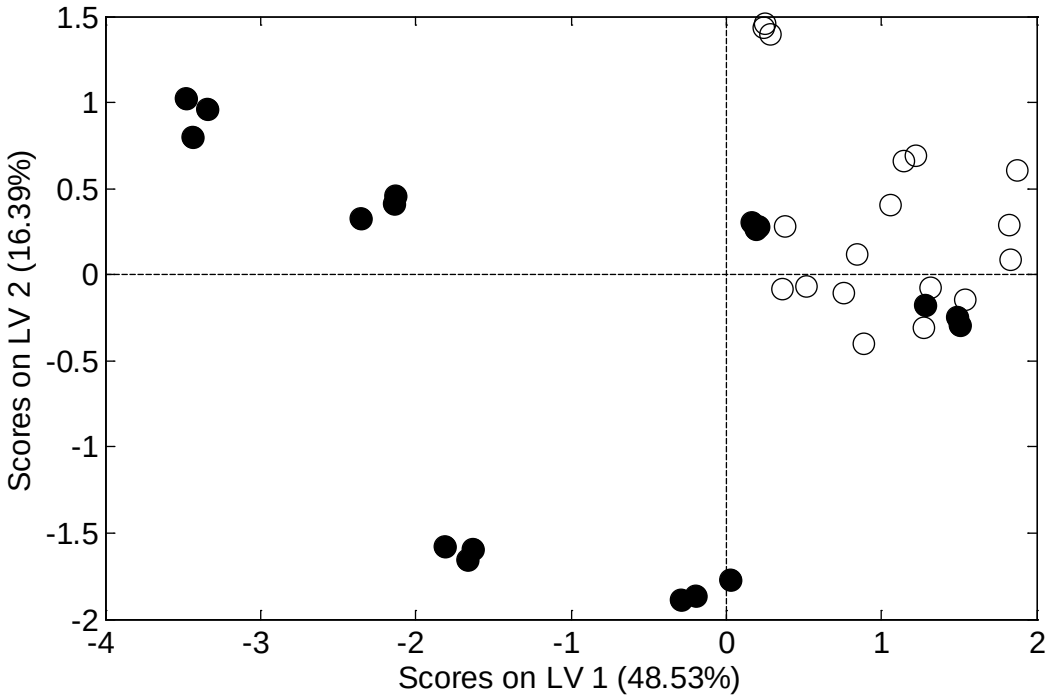


Figure 6.5 Fibroblast medium from fibroblasts cultured at 20% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with established RA (●). PLSDA analysis separated the groups with a sensitivity of 83% and specificity of 89%.

There was also a significant difference in the metabolic profiles of the media of synovial fibroblasts from patients with early RA and those whose early inflammatory arthritis resolved (Figure 6.6). The weightings plot (Figure 6.7) illustrates the regions of the spectra which are responsible for this separation and we used the plot with the VIP (Figure 6.8) to identify the most important discriminatory metabolites. These are shown in table 6.2.

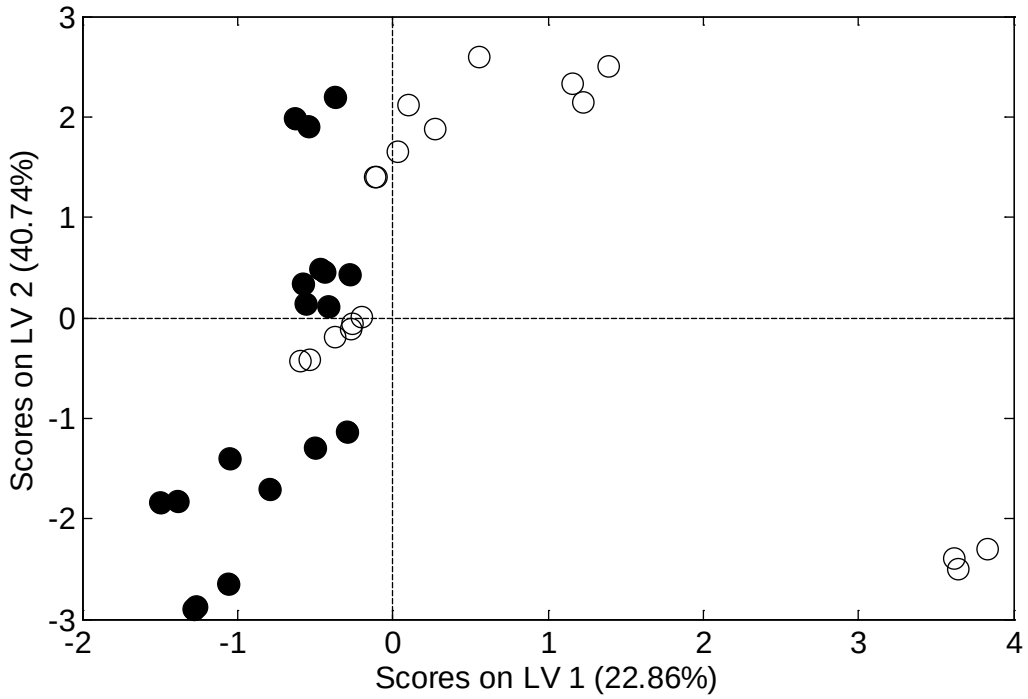


Figure 6.6 Fibroblast medium from fibroblasts cultured at 20% oxygen from patients with resolving arthritis (○) and those with early RA (●). PLSDA analysis separated the groups with a sensitivity of 89% and specificity of 83%.

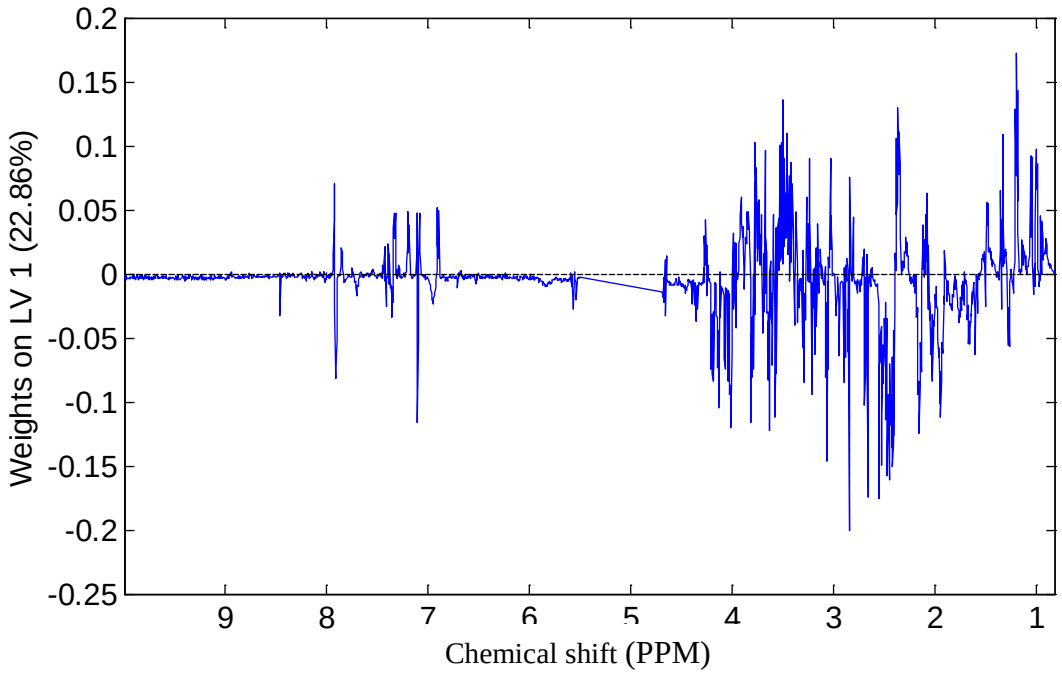


Figure 6.7 Weightings plot of fibroblast media cultured at 20% oxygen from patients with resolving arthritis and those with early RA.

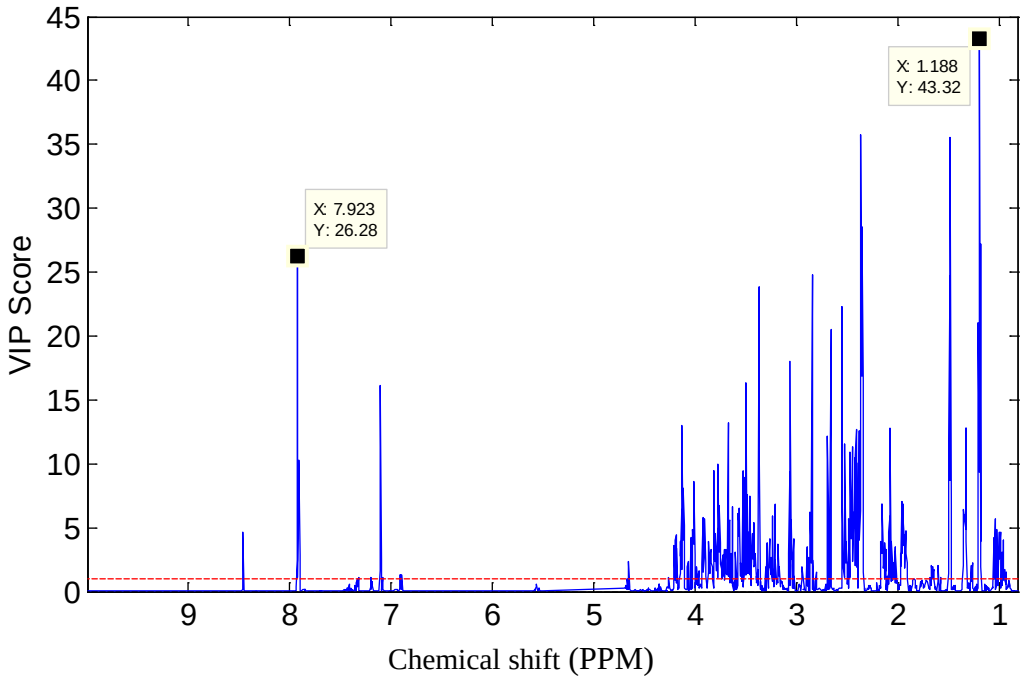


Figure 6.8 Variable importance of projection (VIP) of fibroblast media cultured at 20% oxygen from patients with resolving arthritis and those with early RA.

Ranked importance (VIP)	Metabolites differentiating early resolving arthritis and early RA (ppm)
1 (43)	Ethanol (1.19, 1.18, 1.2) low
2 (36)	Pyroglutamate (2.36, 2.35) low
3 (36)	Alanine (1.48, 1.46) low
4(26)	Histidine (7.92) low
5 (25)	Asparagine (2.84) low
6 (24)	Methanol (3.37) low
7 (22)	Citrate (2.55) high
8 (21)	Aspartate (2.66, 2.69) high
9 (18)	Saccharopine (3.06) high
10 (16)	Glucose (3.49) low
11 (13)	Glycerol (3.672) low
12 (13)	Lactate (4.12, 1.32) high
13 (12)	Glutamine (2.07) low

Table 6.2: Metabolites most strongly associated with the differentiation between the media from synovial fibroblast cultured at 20% oxygen of patients with early inflammatory arthritis that resolves compared to those that develop RA. Metabolites were identified from the PLS-DA model and represent the regions of the spectra which had the greatest influence on the separation. The metabolites for all peaks with a VIP $\geq$ 10 were identified. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite. ”High” implies the metabolite is at higher concentration in the early RA disease phenotype.

6.3.1.2 Culture medium from fibroblasts cultured at 3% oxygen

Using PLSDA analysis I was able to detect a difference between the metabolic profiles of media from fibroblasts cultured at 3% oxygen between the healthy controls and those patients with inflammatory arthritis (Figure 6.9).

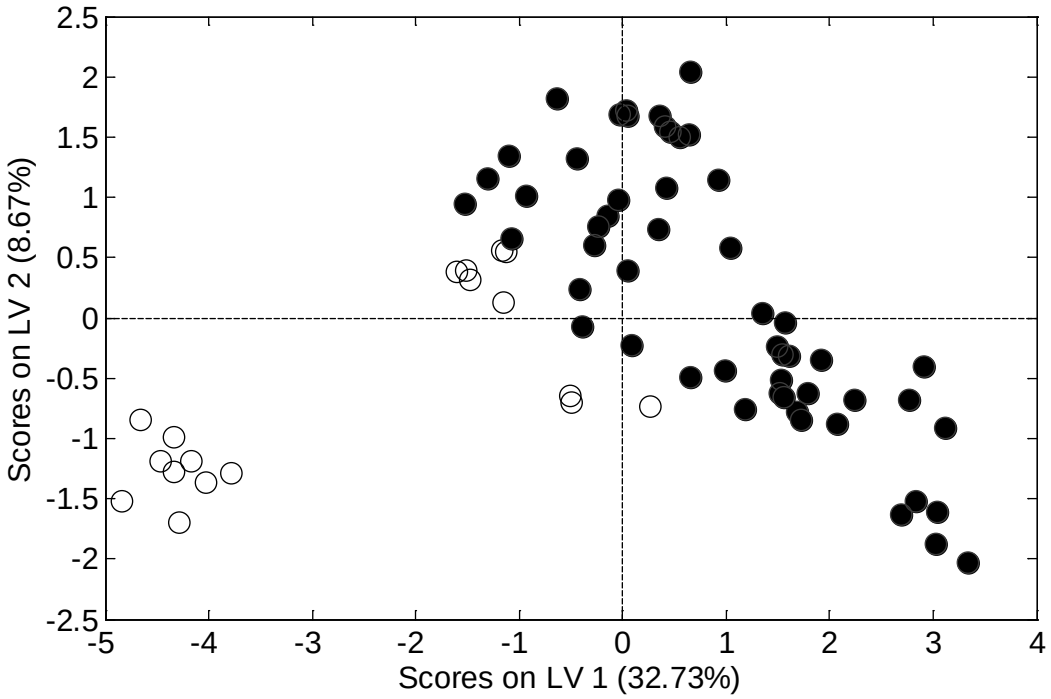


Figure 6.9 Fibroblast medium from fibroblasts cultured at 3% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with inflammatory arthritis (established RA, early RA and early resolving arthritis) (●). PLSDA analysis separated the groups with a sensitivity of 89% and specificity of 94%.

There was good separation of the metabolic profiles of the healthy controls and early resolving arthritis (Figure 6.10), healthy controls and early RA (Figure 6.11), and healthy controls and established RA (Figure 6.12) using the media from the synovial fibroblasts of these patients.

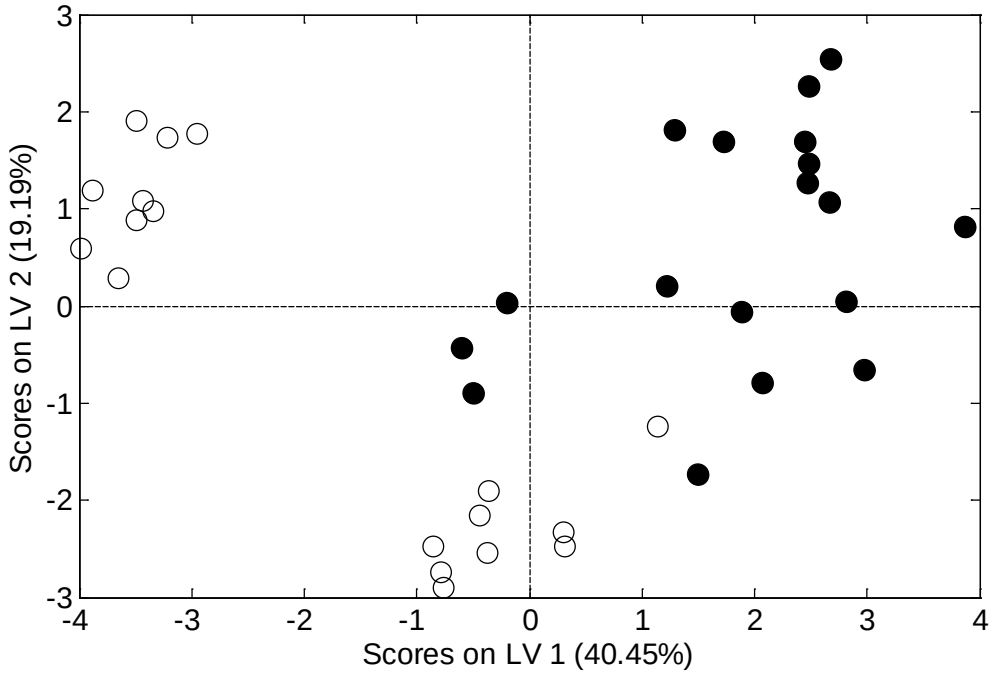


Figure 6.10 Fibroblast medium from fibroblasts cultured at 3% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with early resolving inflammatory arthritis (●). PLSDA analysis separated the groups with a sensitivity of 100% and specificity of 100%.

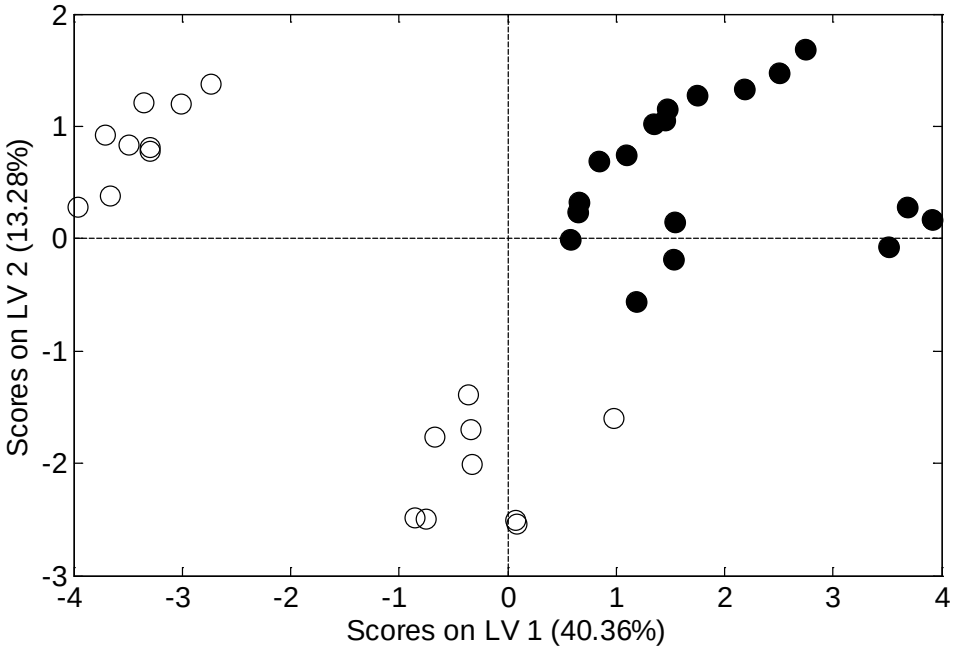


Figure 6.11 Fibroblast medium from fibroblasts cultured at 3% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with early RA (●). PLSDA analysis separated the groups with a sensitivity of 100% and specificity of 100%.

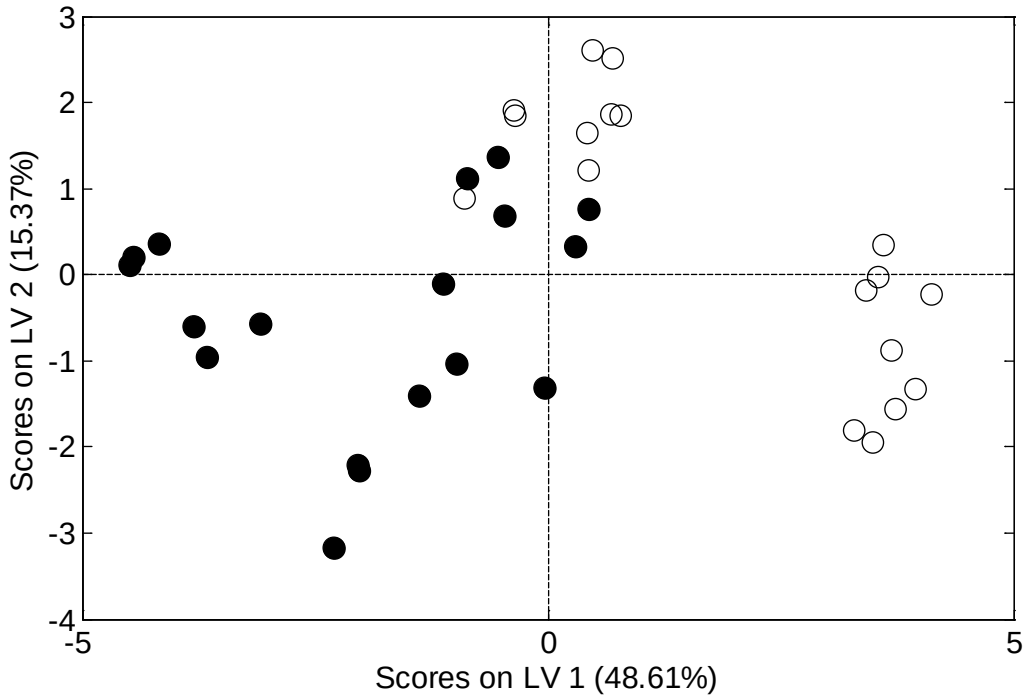


Figure 6.12 Fibroblast medium from fibroblasts cultured at 3% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with established RA (●). PLSDA analysis separated the groups with a sensitivity of 94% and specificity of 94%.

There was also a significant difference in the metabolic profiles of the media of synovial fibroblasts from patients with early RA and those whose early inflammatory arthritis resolved (Figure 6.13). The weightings plot (Figure 6.14) illustrates the area of the spectra which are responsible for this separation and I used the plot with the VIP (Figure 6.15) to identify the most important discriminatory metabolites. These are shown in table 6.3.

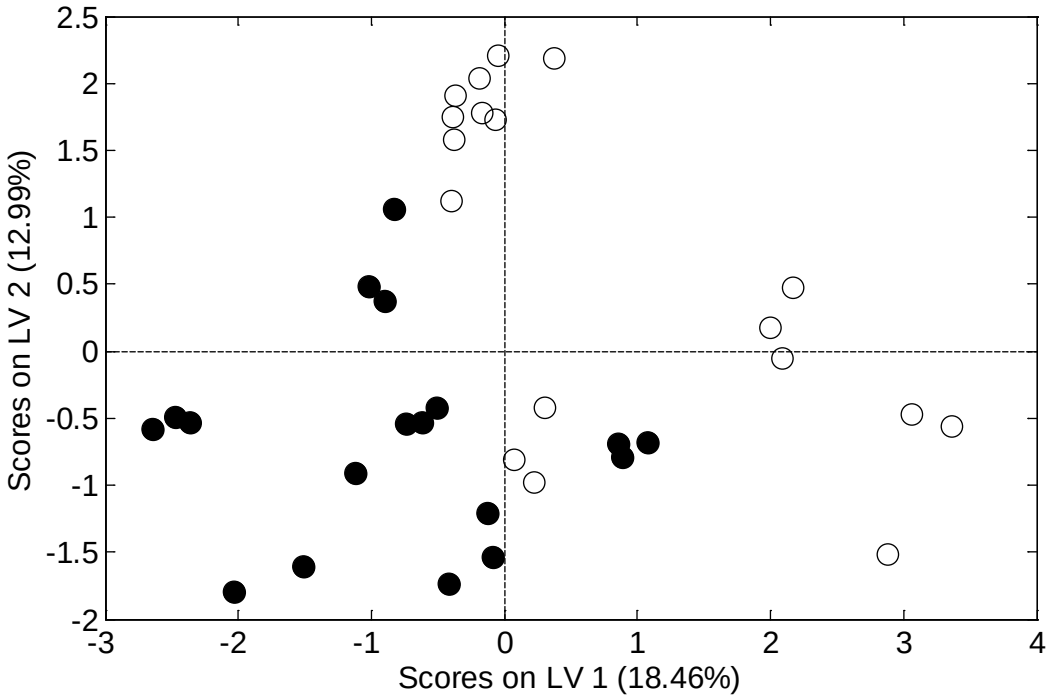


Figure 6.13 Fibroblast medium from fibroblasts cultured at 3% oxygen from patients with resolving arthritis (○) and those with early RA (●). PLSDA analysis separated the groups with a sensitivity of 83% and specificity of 83%.

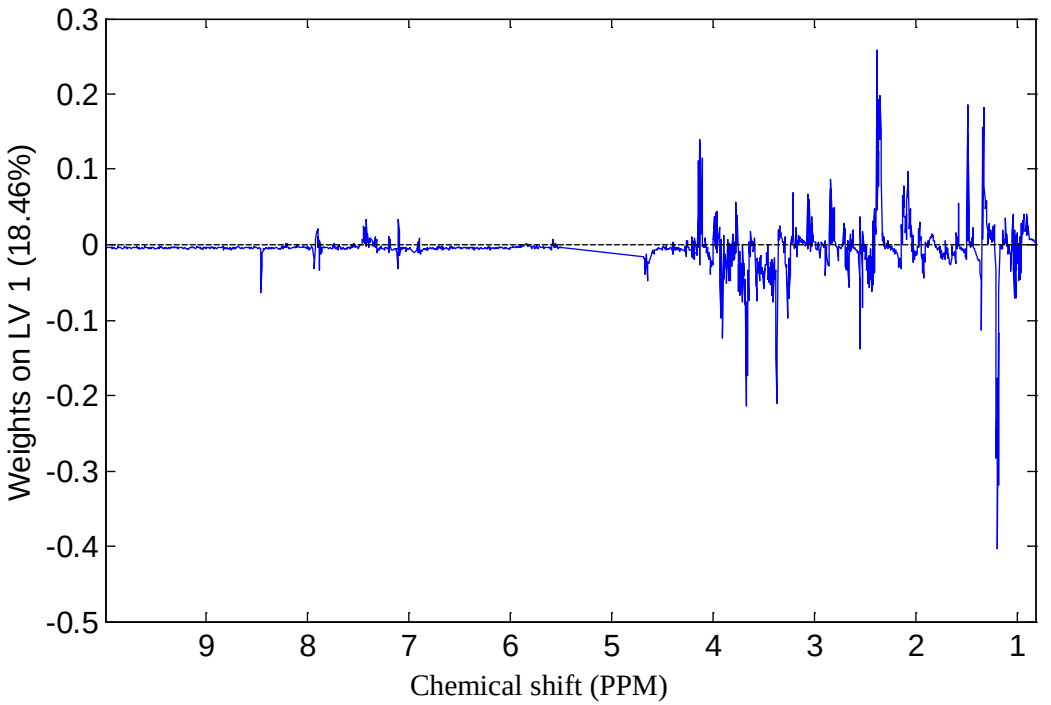


Figure 6.14 Weightings plot of fibroblast media cultured at 3% oxygen from patients with resolving arthritis and those with early RA.

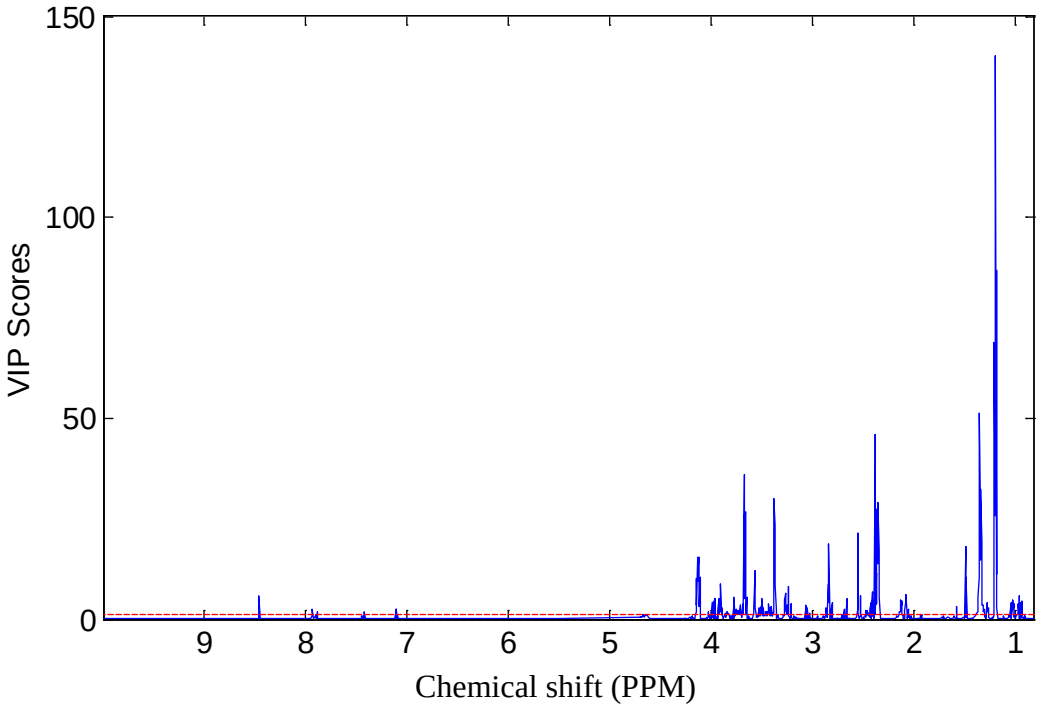


Figure 6.15 Variable importance of projection (VIP) of fibroblast media cultured at 3% oxygen from patients with resolving arthritis and those with early RA.

Ranked importance (VIP)	Metabolites differentiating early resolving arthritis and early RA (ppm)
1 (140)	Ethanol (1.18, 1.19, 1.2) high
2 (51)	Lactate (1.35, 1.33, 4.13) low
3 (46)	Pyroglutamate (2.38, 2.55) low
4 (36)	Glucose (3.38, 3.5, 3.68, 3.66, 3.57) high
5 (30)	Methanol (3.37) high
6 (19)	Asparagine (2.83) low
7 (18)	Alanine (1.49) low

Table 6.3: Metabolites most strongly associated with the differentiation between the media from synovial fibroblast cultured at 3% of patients with early inflammatory arthritis that resolves compared to those that develop RA. Metabolites were identified from the PLS-DA model and represent the regions of the spectra which had the greatest influence on the separation. The metabolites for all peaks with a VIP $\geq$ 10 were identified. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite. ”High” implies the metabolite is at higher concentration in the early RA disease phenotype.

6.3.1.3 Fibroblasts cultured at 20% oxygen

As well as using the media produced by synovial fibroblasts I investigated the metabolic profiles of the cells themselves. Fibroblasts were cultured in vitro and metabolites extracted for NMR experiments. The resulting spectra were then further analysed. PLSDA analysis at 20% oxygen did not show any significant difference between synovial fibroblasts from healthy controls and those with inflammatory arthritis (Figure 6.16).

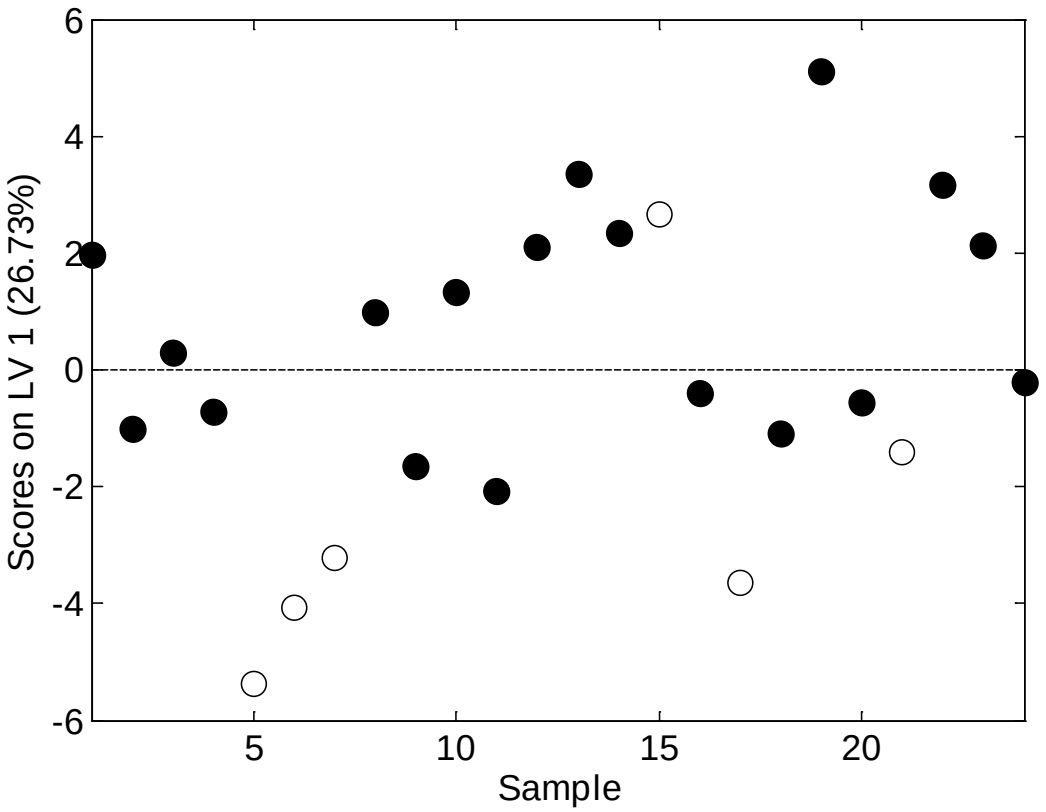


Figure 6.16 Fibroblasts cultured at 20% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with inflammatory arthritis (established RA, early RA and early resolving arthritis) (●). PLSDA analysis separated the groups with a sensitivity of 50% and specificity of 61%.

There was no significant difference in the metabolic profiles of fibroblasts from healthy controls compared with those from patients with early resolving arthritis (Figure 6.17) though there did seem to be some difference between the healthy controls and those that developed early RA (Figure 6.18).

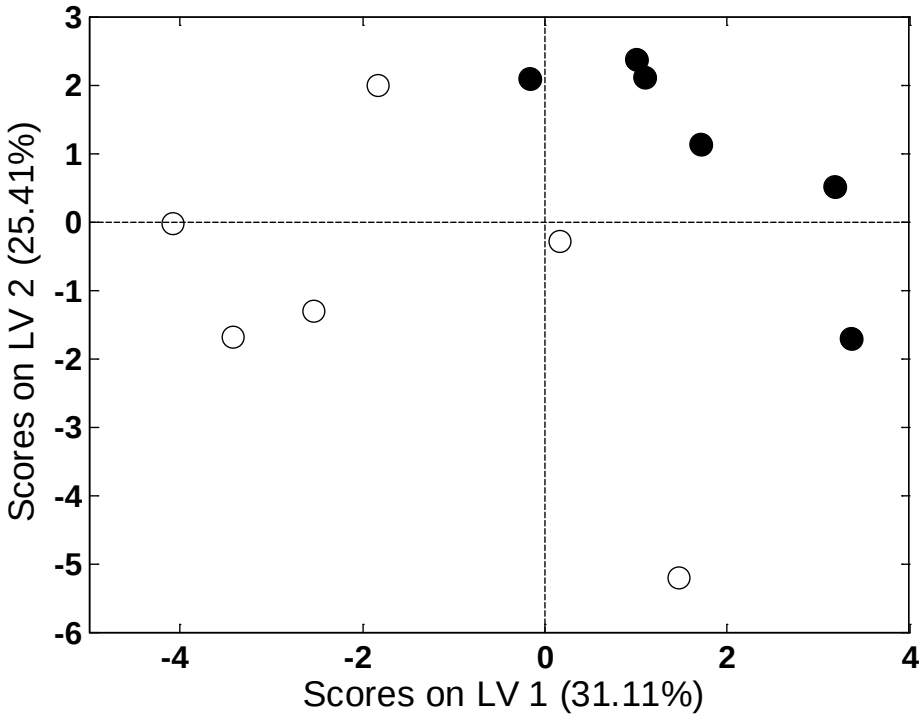


Figure 6.17 Fibroblasts cultured at 20% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with early resolving inflammatory arthritis (●). PLSDA analysis separated the groups with a sensitivity of 100% and specificity of 33%.

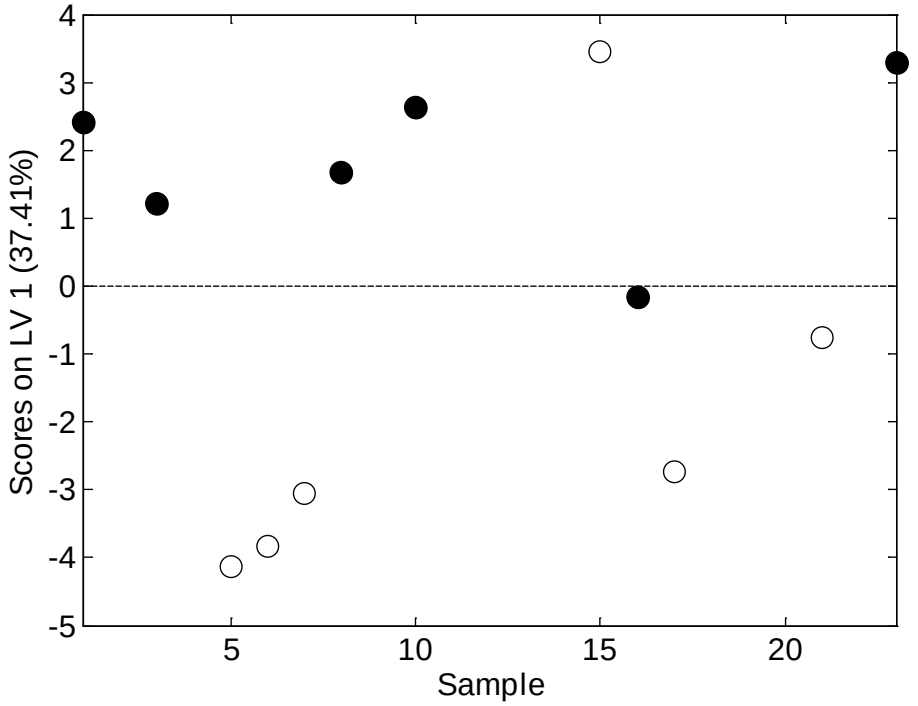


Figure 6.18 Fibroblasts cultured at 20% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with early RA (●). PLSDA analysis separated the groups with a sensitivity of 67% and specificity of 67%.

There was no significant difference in the metabolic profiles of fibroblasts from healthy controls compared with those from patients with established RA (Figure 6.19).

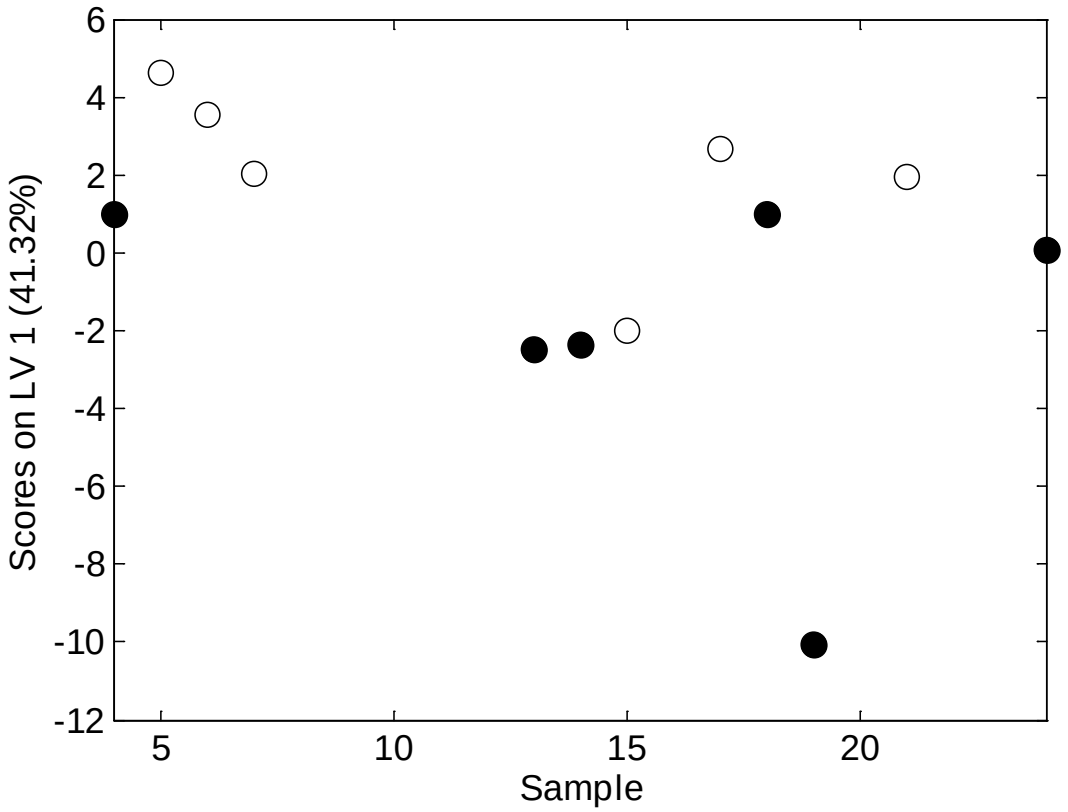


Figure 6.19 Fibroblasts cultured at 20% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with established RA (●). PLSDA analysis separated the groups with a sensitivity of 67% and specificity of 50%.

There was a significant difference in the metabolic profiles of fibroblasts from patients with early RA and those whose early inflammatory arthritis resolved (Figure 6.20). The weightings plot (Figure 6.21) illustrates the regions of the spectra which are responsible for this separation and I used the plot with the VIP (Figure 6.22) to identify the most important discriminatory metabolites. These are shown in table 6.4.

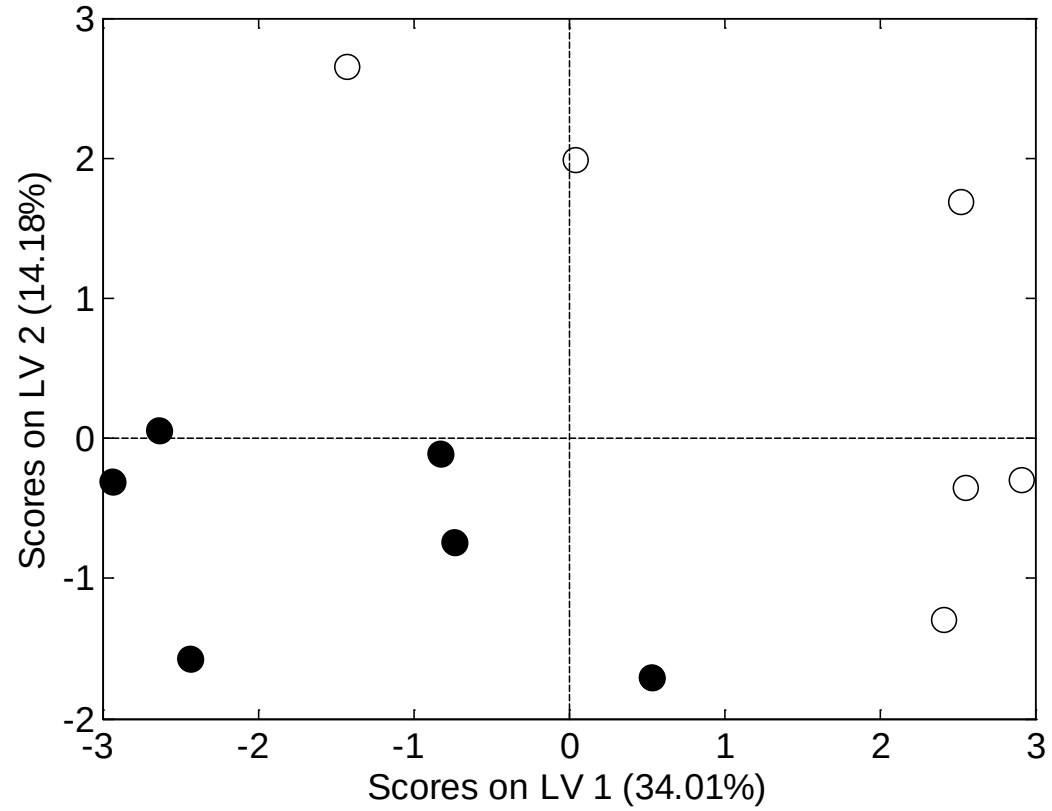


Figure 6.20 Fibroblasts cultured at 20% oxygen from patients with resolving arthritis (○) and those with early RA (●). PLSDA analysis separated the groups with a sensitivity of 67% and specificity of 83%.

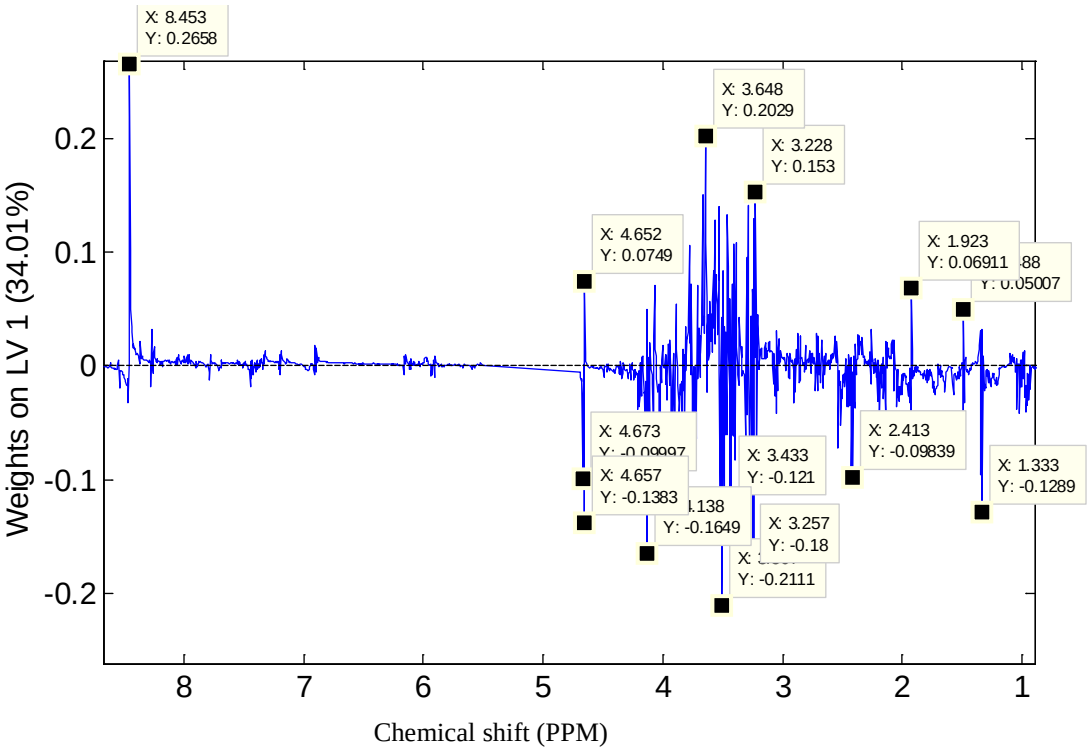


Figure 6.21 Loadings plot of fibroblasts cultured at 20% oxygen from patients with resolving arthritis and those with early RA.

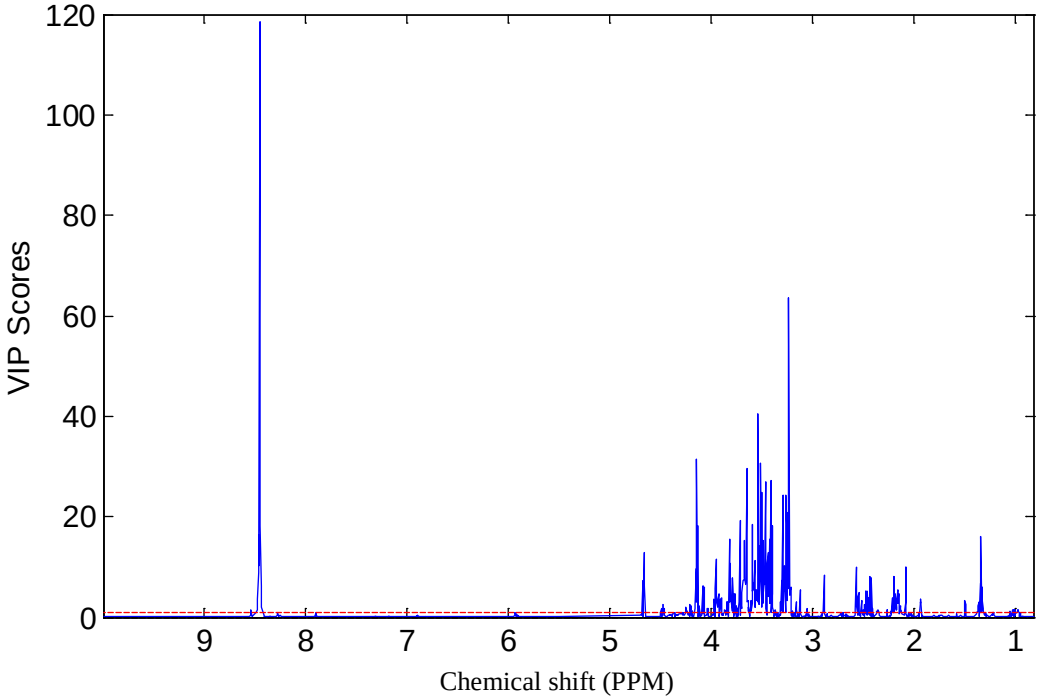


Figure 6.22 Variable importance of projection (VIP) of fibroblasts cultured at 20% oxygen from patients with resolving arthritis and those with early RA

Ranked importance (VIP)	**Metabolites differentiating early resolving arthritis and early RA (ppm)**
1 (119)	Formic acid (8.45) high
2 (64)	Glucose (3.23, 3.26, 3.24, 3.51, 3.41, 3.39, 3.46) high
3 (40)	Glycerol (3.53, 3.65. 3.59, 3.67. 3.81, 3.71) low
4 (32)	Lactate (4.14, 4.13, 1.33) high
5 (24)	Myo-Inositol (3.29) high
6 (16)	2-Phosphoglycerate (3.82) high

Table 6.4: Metabolites most strongly associated with the differentiation between synovial fibroblast of patients with early inflammatory arthritis that resolves compared to those that develop RA. Metabolites were identified from the PLS-DA model and represent the regions of the spectra which had the greatest influence on the separation. The metabolites for all peaks with a VIP $\geq$ 10 were identified. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite.”High” implies the metabolite is at higher concentration in the early RA disease phenotype.

6.3.1.4 Fibroblasts cultured at 3% oxygen

Using PLSDA analysis I was unable to detect a difference between the metabolic profiles of fibroblasts cultured at 3% oxygen between the healthy controls and those patients with inflammatory arthritis (Figure 6.23).

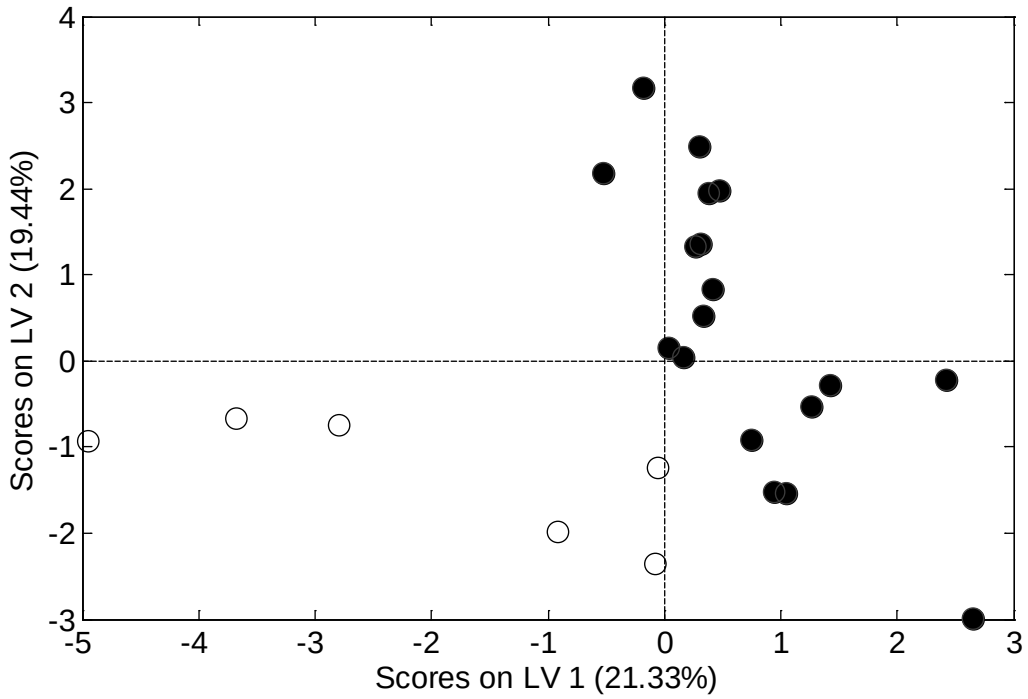


Figure 6.23 Fibroblasts cultured at 3% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with inflammatory arthritis (established RA, early RA and early resolving arthritis) (●). PLSDA analysis separated the groups with a sensitivity of 50% and specificity of 89%.

There was good separation between the metabolic profiles of fibroblasts cultured at 3% oxygen between the healthy controls and those patients with early resolving arthritis (Figure 6.24) and the healthy controls and those patients with established RA (Figure 6.26). However there was poor separation between the metabolic profiles of fibroblasts cultured at 3% oxygen between the healthy controls and those patients with early RA (Figure 6.25).

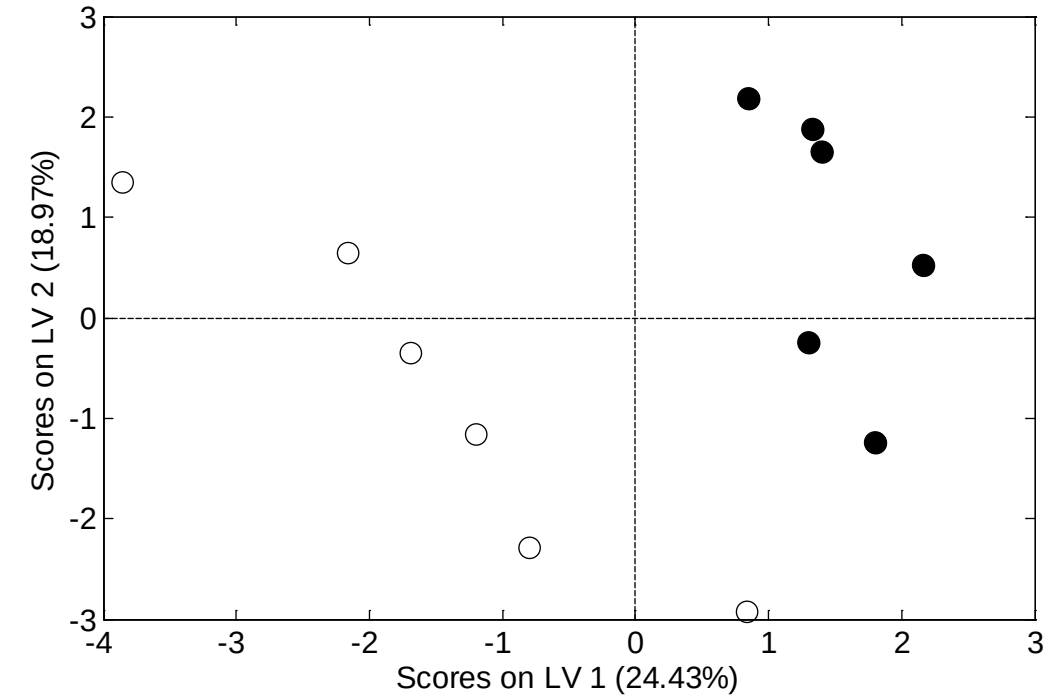


Figure 6.24 Fibroblasts cultured at 3% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with early resolving inflammatory arthritis (●). PLSDA analysis separated the groups with a sensitivity of 100% and specificity of 67%.

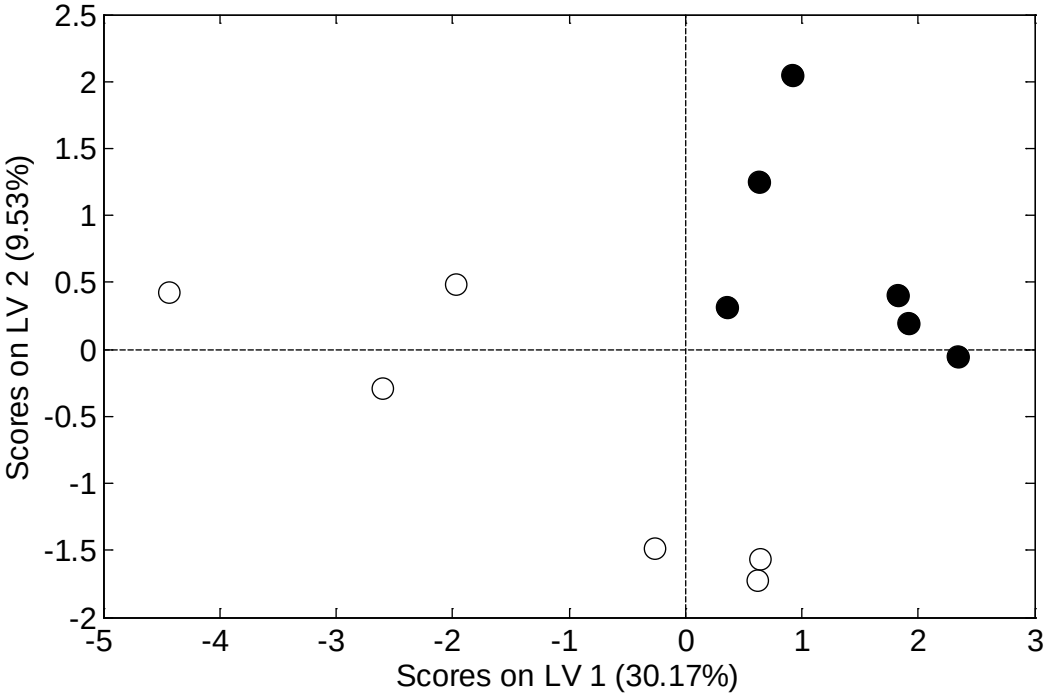


Figure 6.25 Fibroblasts cultured at 3% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with early RA (●). PLSDA analysis separated the groups with a sensitivity of 50% and specificity of 67%.

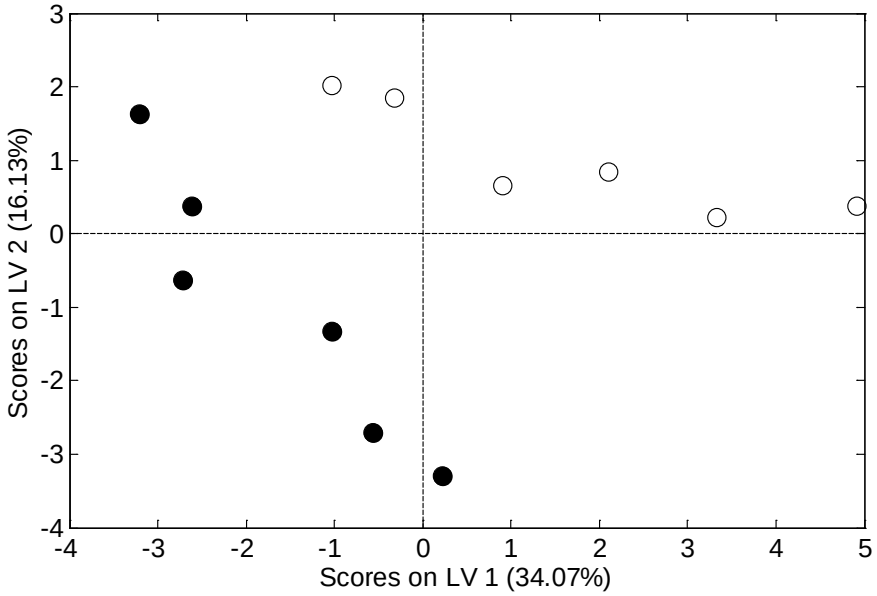


Figure 6.26 Fibroblasts cultured at 3% oxygen from patients with no evidence of inflammatory arthritis (normal) (○) and those with established RA (●). PLSDA analysis separated the groups with a sensitivity of 67% and specificity of 100%.

There was also poor separation between the metabolic profiles of fibroblasts cultured at 3% oxygen between those patients with early resolving arthritis and those who had early RA (Figure 6.27).

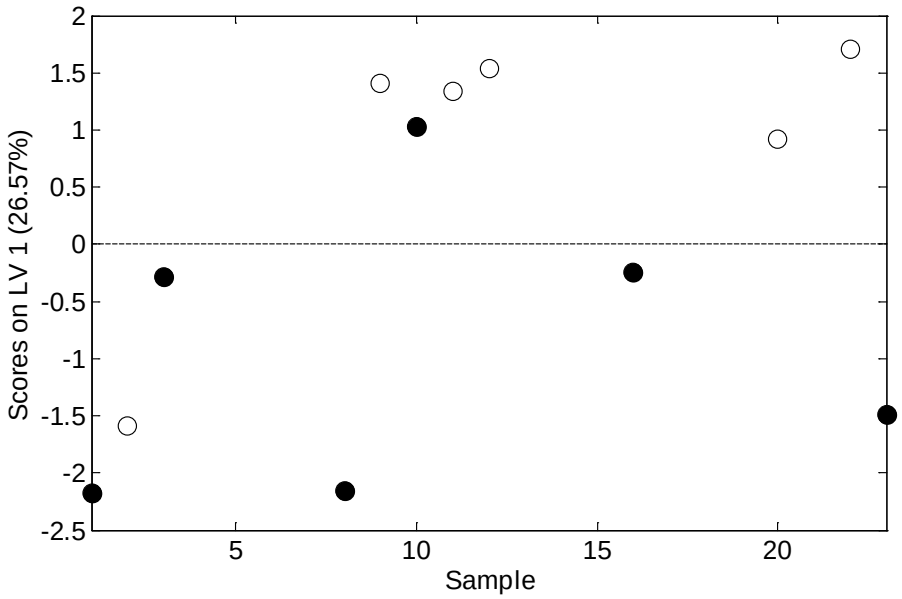


Figure 6.27 Fibroblasts cultured at 3% oxygen from patients with resolving arthritis (○) and those with early RA (●). PLSDA analysis separated the groups with a sensitivity of 67% and specificity of 50%.

6.3.2. Correlation of baseline metabolite profiles with CRP

The relationship between baseline metabolite profiles and CRP (at the time of biopsy) of the patient from whom the synovial biopsy was collected was assessed using PLS-R. The analysis employed a forward selection approach to discover those NMR bins that were most predictive. A total of 204 bins created the optimal model with a cross-validated r^2 of 0.9366 (Figure 6.27). Permutation testing with these 204 NMR bins demonstrated that the regression model was statistically valid ($p<0.0001$).

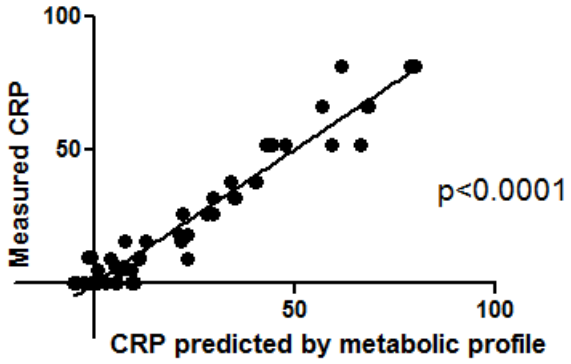


Figure 6.28 Metabolic profiles in cultured synovial fibroblasts from patients' serum CRP level at the time of biopsy

The metabolites identified for this association are illustrated in table 6.5.

Ranked importance (VIP)	Metabolites identified associated with CRP (ppm)
1 (33)	Cholesterol (0.9-1.1)
2 (31)	Fatty acids (0.8-0.84, 2.22-2.24)
3 (29)	Leucine (0.94-0.96)
3 (21)	Citrate (2.54, 2.66, 2.62)
4 (17)	Pyroglutamate (2.52)
5 (16)	Carnosine (2.69)
6 (15)	Alanine (1.48)
7 (13)	Lactate (1.31, 1.33)

Table 6.5: Metabolites most strongly correlated with CRP. Metabolites were identified from the top 50 bins of the PLS-R model and represent the regions of the spectra which had the greatest influence on the correlation with CRP. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite.

6.3.3 Correlation of baseline metabolite profiles with cytokines

6.3.3.1 Correlation with IL-6

The synovial fibroblasts from inflamed joints produced more IL6 in culture than the healthy controls (Figure 6.29). This was significant between the healthy controls and the early RA groups.

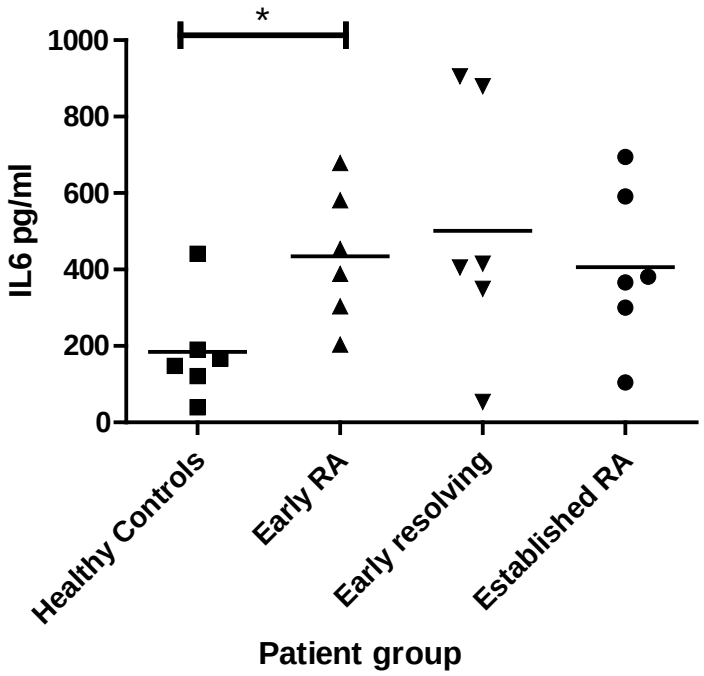


Figure 6.29 Fibroblasts from inflamed joints produce more IL6 in culture

The relationship between baseline metabolite profiles of the media and IL-6 was assessed using PLS-R. The analysis employed a forward selection approach to discover those NMR bins that were most predictive. A total of 111 bins created the optimal model with a cross-validated r^2 of 0.7729 (Figure 6.30). Permutation testing with these 111 NMR bins demonstrated that the regression model was statistically valid ($p<0.0001$). There was also a significant correlation between the patient's CRP and the amount of IL 6 produced in culture ($p=0.03$).

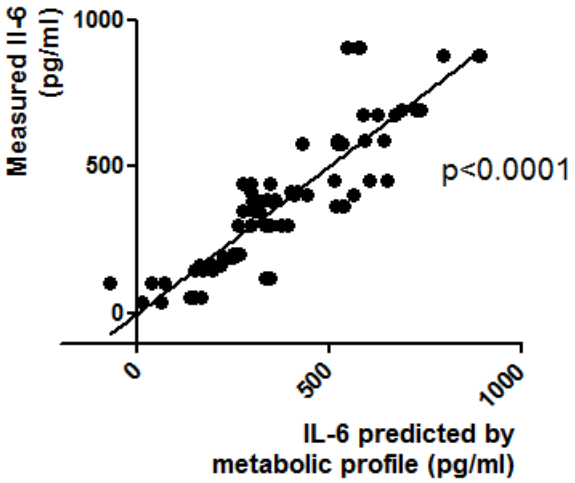


Figure 6.30 Metabolic profiles in cultured synovial fibroblasts from patients correlate with fibroblast IL6 production

The metabolites identified with this association are illustrated in table 6.6.

Ranked importance (VIP)	**Metabolites identified associated with IL 6 (ppm)**
1 (161)	Citrate (2.54, 2.66, 2.62)
2 (123)	Carnosine (2.69)
3 (78)	Pyroglutamate (2.52)
3 (69)	Alanine (1.48)
4 (54)	Lactate (1.31, 1.33)
5 (50)	Glycerol (3.57)
6 (49)	Leucine (0.94-0.96)
7 (44)	Acetylglycine (3.76, 3.77)
8 (37)	Glucose (3.38, 3.5, 3.68)

Table 6.6: Metabolites most strongly correlated with IL-6. Metabolites were identified from the top 50 bins of the PLS-R model and represent the regions of the spectra which had the greatest influence on the correlation with IL-6. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite.

Due to this strong correlation between IL6 and the metabolic profiles I wished to determine whether it was the IL6 that was directly responsible for this alteration in metabotype. Hence, we treated synovial fibroblasts from healthy controls with IL6 and synovial fibroblasts from patients with RA with TCZ. The demographics of the patients used in this experiment are illustrated in table 6.7.

	Established RA (n=2)	**Healthy controls (n=6)**
Age (years); median (IQR)	64 (63-64)	42 (40-52)
Female; number (%)	2 (100)	4 (67)
Symptom duration (weeks); median (IQR)	66 (27-104)	-
NSAIDs; number (%)	1 (50)	0 (0)
CRP (mg/ml); median (IQR)	5 (0-10)	-
RF positive; number (%)	2 (100)	-
Anti CCP antibody positive; number (%)	2 (100)	-
Joint biopsied Knee; n (%) MCP; n (%)	 1 (50) 1 (50)	 6 (100) 0 (0)

Table 6.7: Demographic and clinical characteristics of study participants for the IL 6 related experiments.

Analysis of the media from fibroblast from healthy controls demonstrated a change in the metabolic profile following stimulation with IL6 as illustrated by the PLSDA plot (Figure 6.31). This model comprised of 3LVs which captured 62% of the variance. Metabolites associated with this separation were identified from VIP plots and are listed in table 6.8.

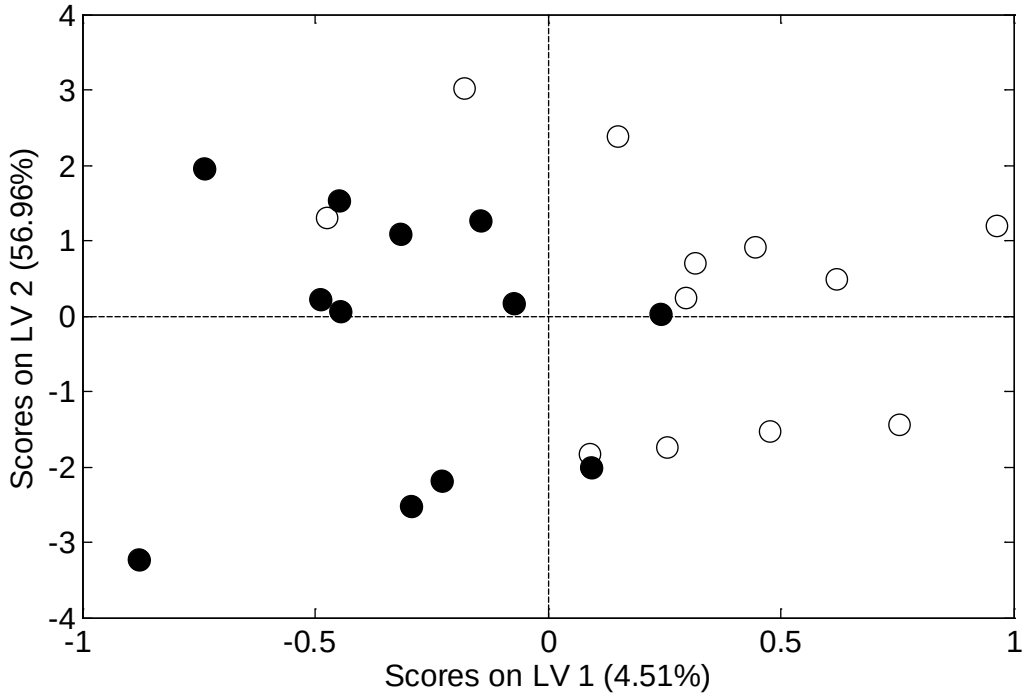


Figure 6.31 PLSDA plot of fibroblast media from fibroblasts cultured at 20% oxygen from healthy controls (○) and those treated with IL6 (●). PLSDA analysis separated the groups with a sensitivity of 67% and specificity of 67%.

Ranked importance (VIP)	Metabolites differentiating healthy controls and those treated with IL6 (ppm)
1 (66)	Glycerol (3.66, 3.65, 3.67, 3.58, 3.57) high
2 (46)	Lactate (1.34, 1.32) high
3(40)	Pyruvate (2.37) high
4 (30)	Ethanol (1.18) low
5 (29)	Glucose (3.45, 3.71, 3.51, 3.91, 3.23, 3.38, 3.70, 3.81) low
6 (24)	Histamine (7.85) high
7 (24)	Methanol (3.36) high
8 (17)	Asparagine (2.84, 2.83) low
9 (12)	Aspartate (2.66) low
10 (12)	Fructose (4.02) low
11 (12)	Saccharopine (3.06) low

Table 6.8: Metabolites most strongly associated with the differentiation between healthy controls and those treated with IL6. Metabolites were identified from the PLS-DA model and represent the regions of the spectra which had the greatest influence on the separation. The metabolites for all peaks with a VIP $\geq$ 10 were identified. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite. ”High” implies the metabolite is at higher concentration in the samples treated with IL6.

Stimulation of synovial fibroblasts from healthy controls with IL6 did not alter the metabolic profile of the fibroblasts themselves (as opposed the media in which they were grown) significantly as the PLSDA plot (model comprised of 3LVs which captured 52% of the variance) did not show any clear separation between the healthy controls stimulated with IL6 and those that were not (Figure 6.32).

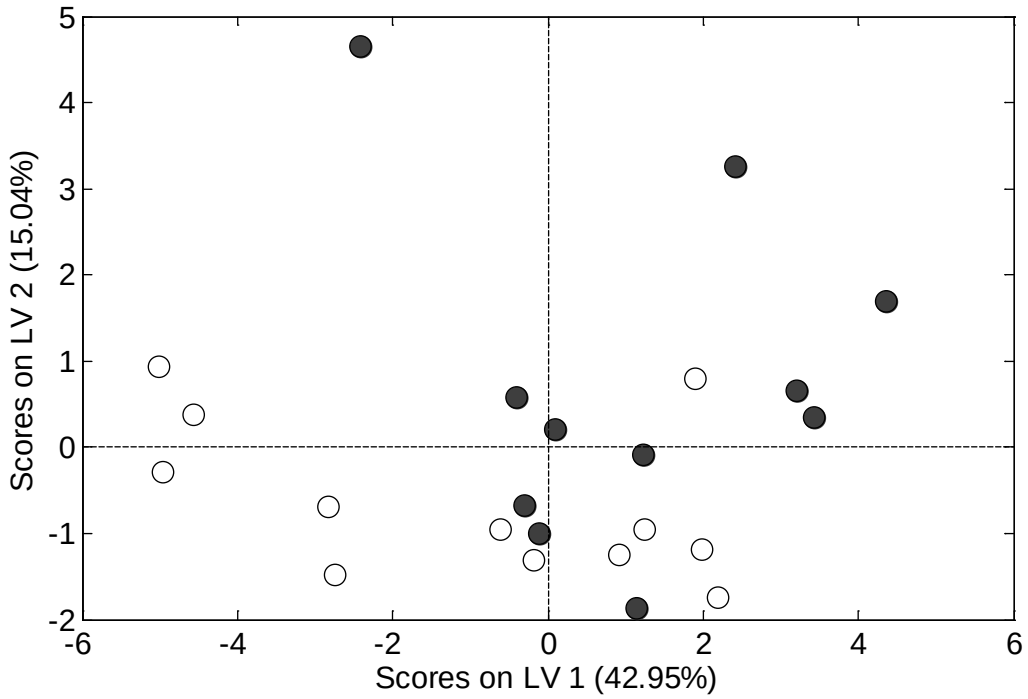


Figure 6.32 PLSDA plot of fibroblasts cultured at 20% oxygen from healthy controls (○) and those treated with IL6 (●). PLSDA analysis separated the groups with a sensitivity of 42% and specificity of 46%.

Metabolic profiles of media from fibroblasts from patients with established RA were altered greatly post treatment with TCZ and this can be illustrated by the PCA plot (Figure 6.33) and the PLSDA plot (Figure 6.34). The PLSDA model comprised of 2LVs which captured 89% of the variance. The metabolites responsible for this separation were identified from the VIP plots and are listed in table 6.9.

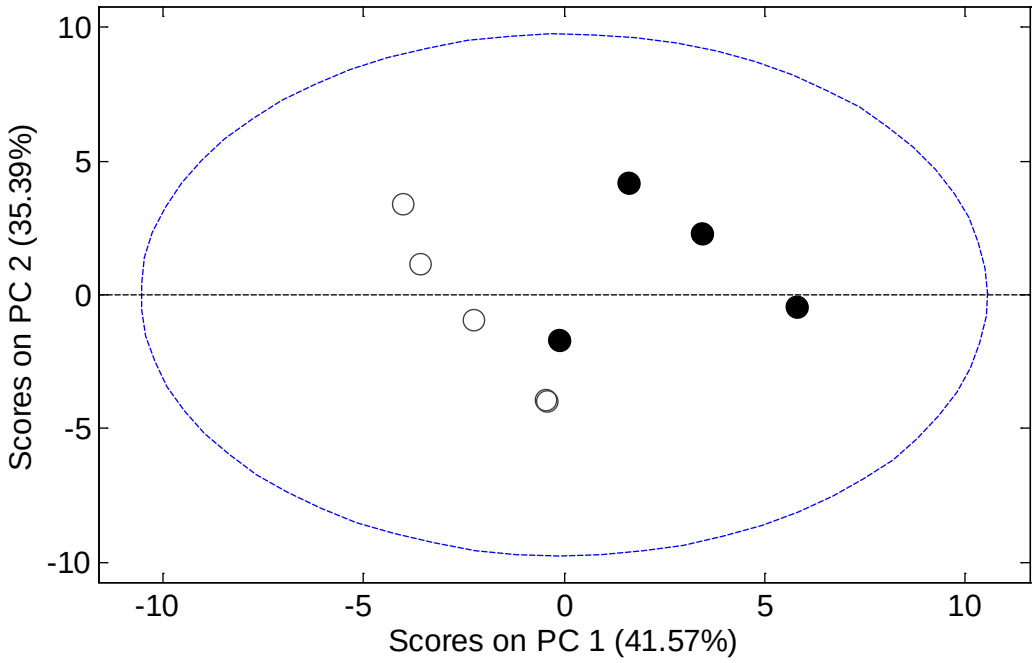


Figure 6.33 PCA plot of fibroblast media from fibroblasts cultured at 20% oxygen from patients with established RA (○) and those treated with TCZ (●)

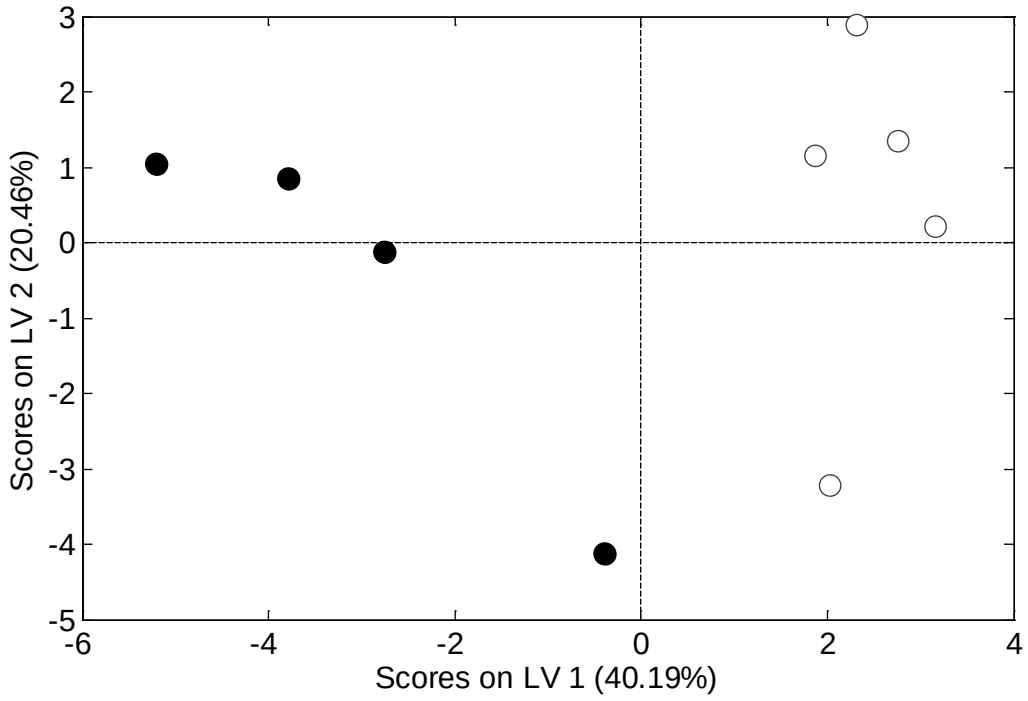


Figure 6.34 PLSDA plot of fibroblast media from fibroblasts cultured at 20% oxygen from patients with established RA (○) and those treated with TCZ (●). PLSDA analysis separated the groups with a sensitivity of 75% and specificity of 80%.

Ranked importance (VIP)	Metabolites differentiating established RA treated with TCZ and those treated with IVIg (ppm)
1 (114)	Sucrose (4.22, 4.23) high
2 (109)	Glucose (3.69, 3.83, 3.85) high
3 (83)	Myo-Inositol (4.06, 4.07, 4.05) high
4 (68)	Glycero-3-phosphocholine (3.65, 3.66) low
5 (45)	Fructose (3.58) high
6 (48)	Ethanol (3.64) low
6 (15)	Arginine (1.92) low

Table 6.9: Metabolites most strongly associated with the differentiation between established RA treated with TCZ and those treated with IVIg. Metabolites were identified from the PLS-DA model and represent the regions of the spectra which had the greatest influence on the separation. The metabolites for all peaks with a VIP $\geq$ 10 were identified. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite. ”High” implies the metabolite is at higher concentration in the samples treated with TCZ.

Treatment of fibroblasts themselves (as opposed the media in which they were grown) from patients with established RA with TCZ also altered the metabolic profile considerably as there is a clear separation on PCA (Figure 6.35) and PLSDA (Figure 6.36). The PLSDA model comprised of 1 LV which captured 92% of the variance. One of the control samples did not ‘lock’ in the NMR spectrometer so we were unable to analyse this sample. The metabolites responsible for this separation are listed in table 6.10.

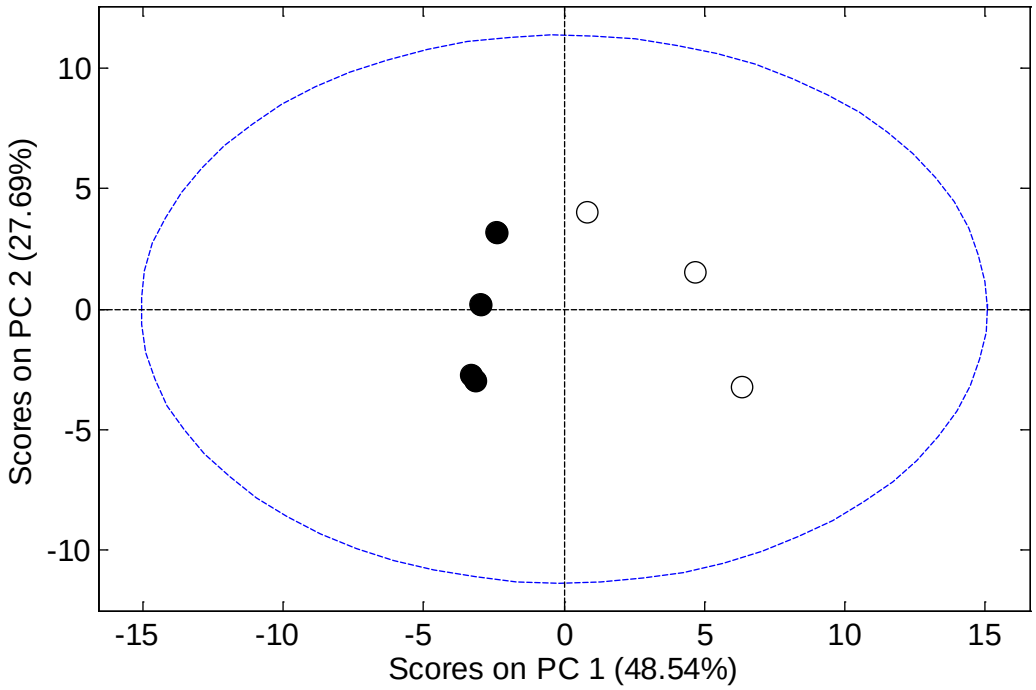


Figure 6.35 PCA plot of fibroblasts cultured at 20% oxygen from patients with established RA treated with ivIG (○) and those treated with TCZ (●)

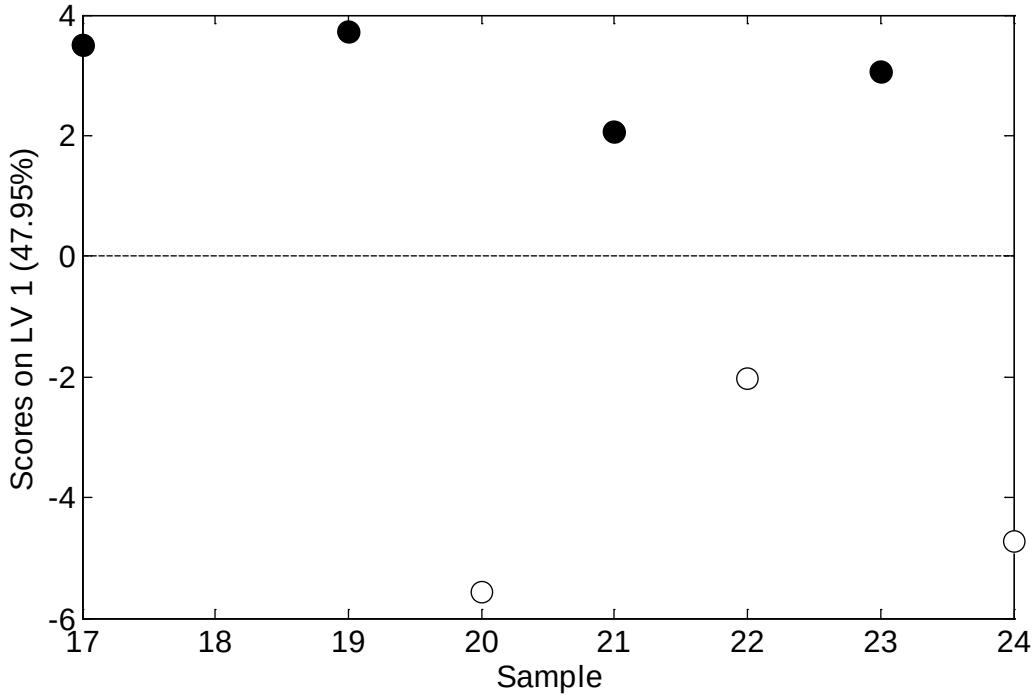


Figure 6.36 PLSDA plot of fibroblasts cultured at 20% oxygen from patients with established RA treated with ivIG (○) and those treated with TCZ (●). PLSDA analysis separated the groups with a sensitivity of 100% and specificity of 100%.

Ranked importance (VIP)	Metabolites differentiating established RA treated with TCZ and those treated with IVIg (ppm)
1 (76)	Glucose (3.69, 3.67, 3.83, 3.47, 3.27, 3.46, 3.52, 3.87) high
2 (57)	Sucrose (4.06, 4.07, 4.22, 4.24) high
3 (30)	Glycero-3-phosphocholine (3.64) low
4 (26)	Lactate (1.34, 1.33) high
5 (22)	Myo-Inositol (3.28) low
6(10)	Arginine (1.93) low

Table 6.10: Metabolites most strongly associated with the differentiation between established RA treated with TCZ and those treated with IVIg. Metabolites were identified from the PLS-DA model and represent the regions of the spectra which had the greatest influence on the separation. The metabolites for all peaks with a VIP $\geq$ 10 were identified. NMR chemical shifts (ppm), which identify the location of the major peaks in the spectra, are shown in brackets for each metabolite. ”High” implies the metabolite is at higher concentration in the samples treated with TCZ.

The IL6 production in culture at 3% oxygen from the different fibroblasts from different outcome groups is illustrated in (Figure 6.37). Again, less IL6 was produced by the healthy controls compared to the cells with inflammatory arthritis.

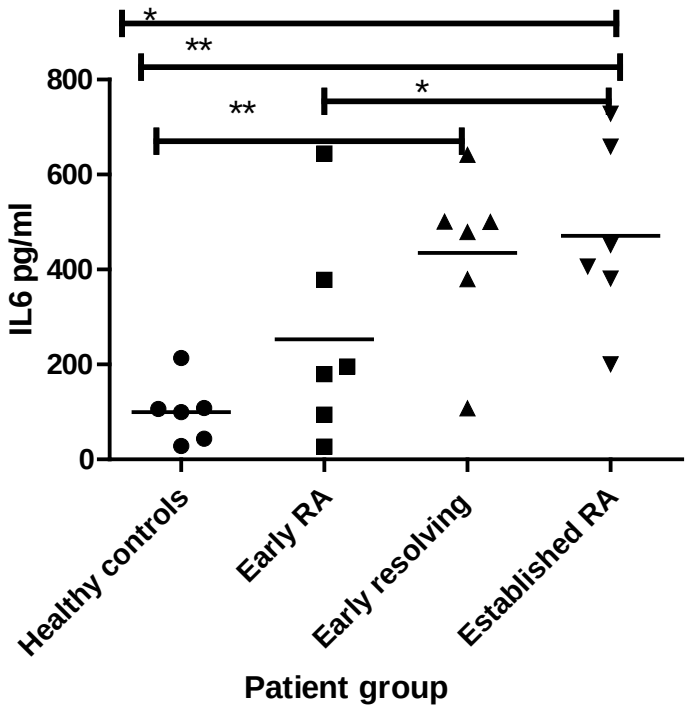


Figure 6.37 Fibroblasts from inflamed joints cultured at 3% oxygen produce more IL6 in culture.

The relationship between baseline metabolite profiles at 3% and Il-6 was assessed using PLS-R but permutation testing with these NMR bins demonstrated that the regression model was not statistically valid (p =0.06).

6.3.3.2 Multiplex analysis

There was no significant difference between any of the outcome group for any of the other cytokines that we tested for at 20% oxygen (Figure 6.38) or 3% oxygen (Figure 6.39). Only five of the twelve cytokines that we tested for were detected in our samples but our samples were all unstimulated and several of these cytokines have only been detected in stimulated samples.

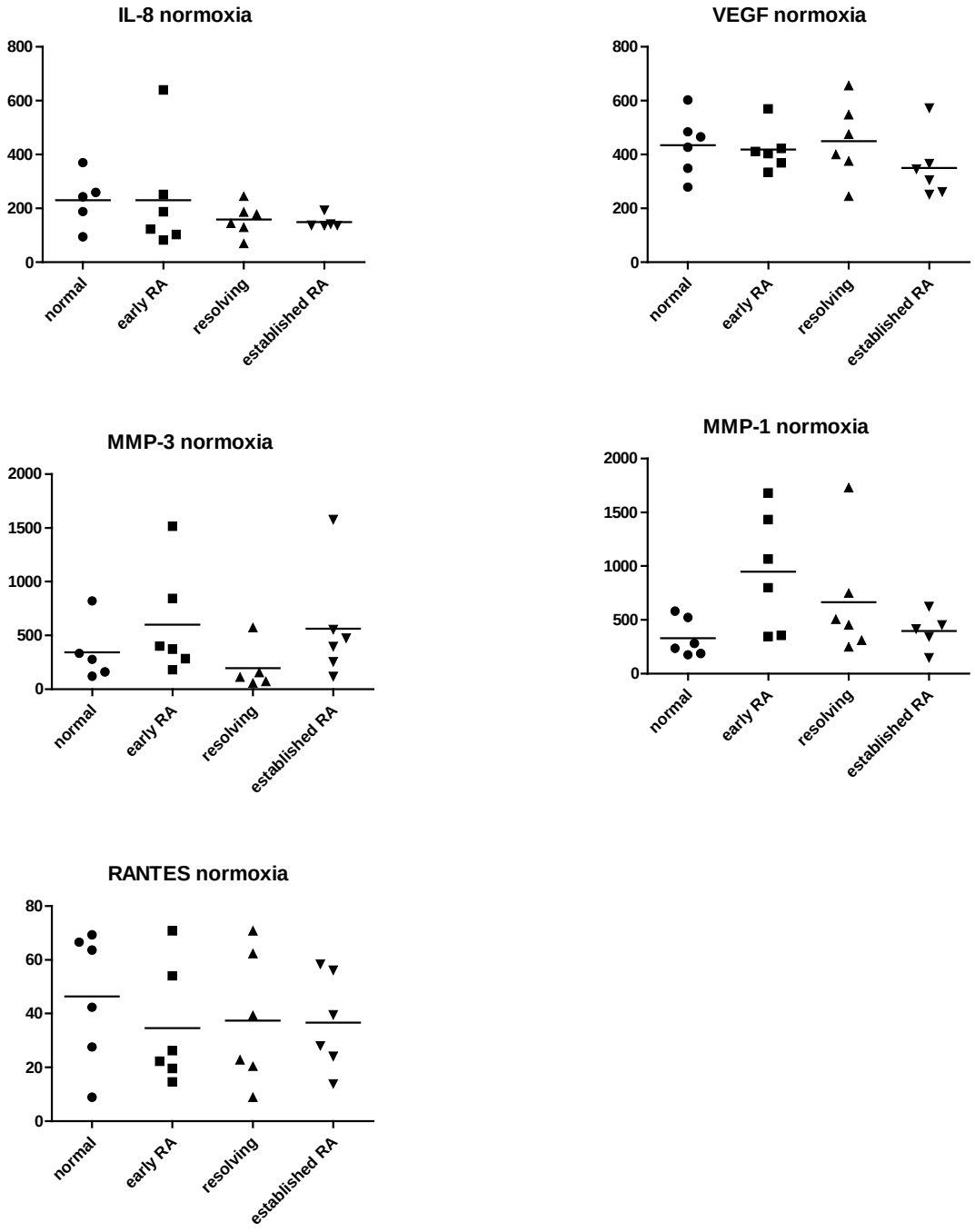


Figure 6.38 Multiplex analyses of cytokines from fibroblasts from inflamed joints cultured at 20% oxygen.

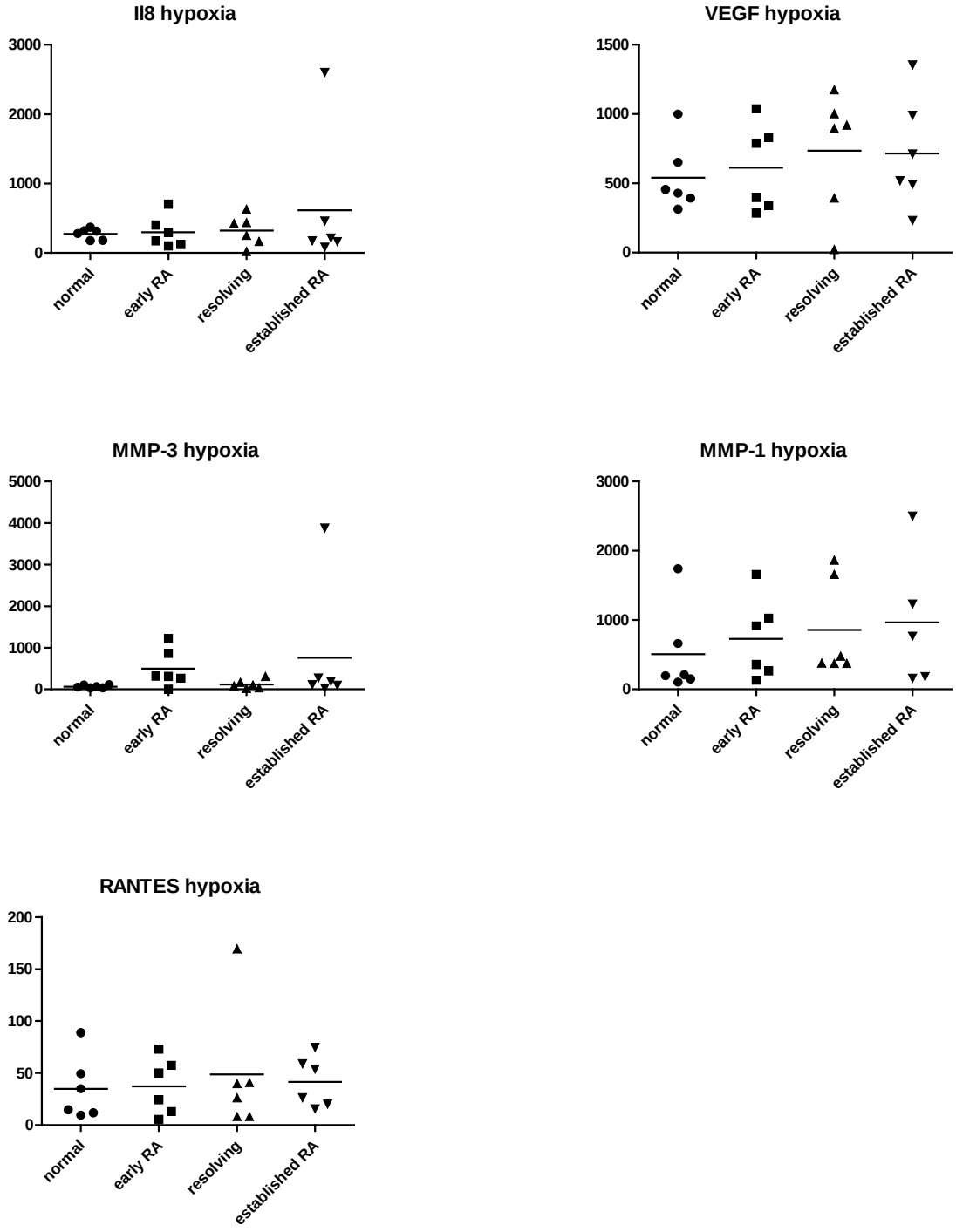


Figure 6.39 Multiplex analyses of cytokines from fibroblasts from inflamed joints cultured at 3% oxygen.

6.4 Discussion

Metabolomics provides a novel "systems" approach to the investigation of disease mechanisms in RA and here we have used it to study metabolism in one of the key cell types found in the synovial membrane. We have discovered that the metabolic profile found in culture media and cell extracts derived from synovial fibroblasts from an individual patient in the earliest stage of inflammatory arthritis can predict the subsequent course of the disease. The cellular metabolic profiles of patients with early inflammatory arthritis whose arthritis resolves could be clearly distinguished from those who developed early RA when we used synovial fibroblasts and their media grown under 20% oxygen (though the media gave better predictive values) and the media of synovial fibroblasts cultured at 3% oxygen. These outcome groups could not be distinguished using profiling of fibroblasts cultured at 3% oxygen. This discrepancy may be a result of the cells of both outcome groups undergoing anaerobic respiration, hence resulting in similar metabolic profiles. 3% oxygen may not have been the most appropriate oxygen sate to use as several studies have used 1% oxygen and low FCS to mimic extreme hypoxic conditions (Ahn et al. 2008). Microarray studies identified differences in gene expression between synovial fibroblasts from healthy controls and those from RA patients but most of these changes were at 0.5% oxygen rather than 3% (Del Rey et al. 2010). However, direct measurement of oxygen shows that the average oxygen concentration in the RA joint is 3% (Biniecka et al. 2011a;Kennedy et al. 2011) and so we are confident that the conditions we have used are the most appropriate. We also cultured our fibroblasts in 10% FCS and the changes may have been more prominent if we had cultured the fibroblasts in 0.1% FCS, as is the case in the microarray studies (Ahn et al. 2008;Rinn et al. 2006).

Different metabolites were identified in the cells and the media of synovial fibroblasts but the media from cells cultured at 20% oxygen and those cultured at 3% gave similar metabolite results. Lactate was a larger contributor from cells cultured at 3% oxygen compared to 20% and this may represent a manifestation of anaerobic respiration and increased glycolysis, usually associated with cancer cells. In cancer cells hypoxia causes an epigenetic and biochemical response to counteract the decrease in energy supply from mitochondrial respiration (Frezza et al. 2011). The metabolites identified and their relationships are illustrated in figure 6.40. The majority of the metabolites identified are involved in glycolysis and are thought to prevent pyruvate from entering the Krebs cycle. This is similar to that seen in the Warburg effect where cells use glycolysis during growth. We are aware that metabolites such as pyroglutamate, which we found contributed to the correlation with CRP and IL6, accumulate if there is a reduced amount of ATP available, which is the case in anaerobic respiration. Our work is supported by other research which has demonstrated that hypoxia causes increased migration and invasion of fibroblasts and that this is mediated by HIF-1α (Li et al. 2013). Research into osteoclast activity in RA has also demonstrated that during hypoxia there is an increase in glucose consumption and lactate production and this switch to anaerobic respiration enables an increase in ATP production during hypoxia which is required due to the increased metabolic demand (Morten et al. 2013).

Several other metabolites were also identified which are related to cell growth. Saccharopine is a lysine metabolite and multiple metabolites such as histidine, alanine, asparagine and glutamine are protein intermediates which may be required for cell growth. The enzyme that is involved in the production of saccharopine (LOR/SDH) has been detected in a number of mammalian tissues, mainly in the liver and kidney, contributing

not only to the general nitrogen balance in the organism but also to the controlled conversion of lysine into ketone bodies (Elpeleg et al. 1990). The metabolite glycerol was also identified which is a fatty acid intermediate and lipolysis is an alternative method which may contribute to the energy required for cell growth (Guppy et al. 2002). Raised levels of ketone bodies have been found in the synovial fluid of patients with RA again supporting the use of lipolysis (Naughton et al. 1993b).

Formic acid was identified as a discriminating metabolite for the fibroblast cells but the cells were extracted using chloroform and methanol and methanol metabolises to formic acid so we feel this was identified simply due to the experimental set up.

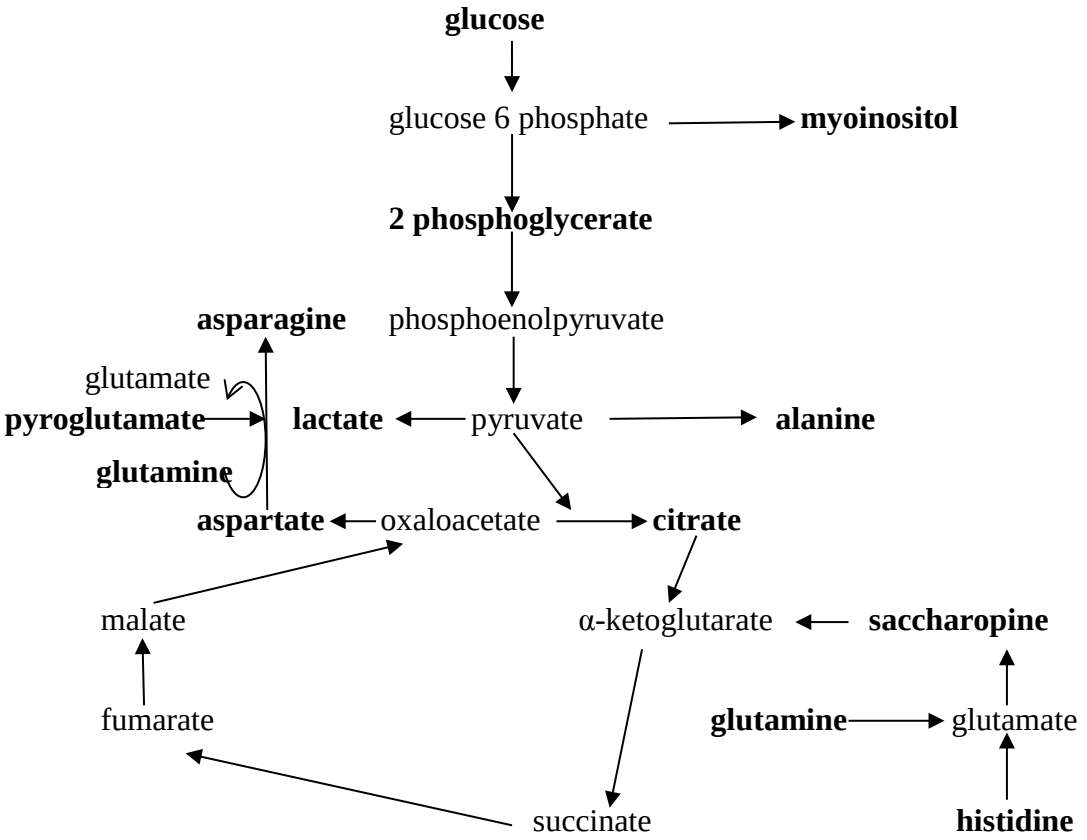


Figure 6.40 Metabolites responsible for the difference in outcome in patients with early inflammatory arthritis. Metabolites in **bold** are the metabolites that were identified.

The medium from cultured synovial fibroblasts was more useful at distinguishing outcome groups than the cells. There was clear differentiation between the metabolic profile of healthy controls and those patients with inflammatory arthritis using the media but this separation was not as prominent using the cells. This may be due to the fact that metabolites accumulate over time in the media whereas the metabolic profile in the cell extracts is just a snap shot of what is occurring in the cell. No previous metabolomic work has been done using synovial fibroblasts and from our control work (section 3.3) we concluded the use of fibroblasts from two T75 flasks was the minimum necessary to derive good spectra but this may not be a sufficient number to identify changes in outcome.

Another reason why the outcome groups may not be clearly distinguished may be due to our definitions of early and established RA. Some of our established RA patients had relatively short disease duration (less than 12 months) and in some cohorts they may be defined as early RA (Harris et al. 2013;Kuijper et al. 2013). We used the shorter disease duration in established RA to prevent potential drug interference in the metabolic profiles. Another possible confounder in our study is the use of healthy controls. Our healthy controls are not really 'normal' but they are the closest to 'normal' that we could use. However, they were experiencing knee related symptoms necessitating arthroscopy and any type of procedure such as an arthroscopy may cause a local inflammatory response.

There was a significant association between CRP levels in the patients' serum and the metabolic profile of their synovial fibroblasts. This implies that fibroblasts from inflammatory arthritis patients retain their metabolic fingerprint during culture ex vivo. There was also a significant association between the metabolomic fingerprint of synovial fibroblasts and their IL6 production, raising the possibility that IL6 production drives or is driven by significant changes in metabolism. Several similar metabolites were associated with the correlation with CRP and the correlation with IL 6 which is not surprising since there was also a significant correlation between the CRP and IL 6. The metabolites that were identified for the correlation with IL 6 were not the same metabolites that were identified for the established RA treated with TCZ or the healthy controls treated with IL 6 even though IL 6 production locally does seem to directly drive changes in metabolism. This is not surprising as the changes in metabolism as illustrated by the metabolic profile

are probably a cumulative effect of the systemic activity of IL6 and other environmental factors.

Our Luminex analysis did not reveal any significant results and this may be because production of several cytokines requires prior stimulation of the cells. The literature illustrates that hypoxia induces expressions of certain cytokines; MMP 1, MMP 3, Il6, IL8, VEGF (Hitchon et al. 2002) and we did find greater amounts of these cytokines in the fibroblasts cultured at 3% oxygen. Certain cytokines which have been found to distinguish OA and RA such as CXCL12 are not detected in early disease (Tsubaki et al. 2005). It would have been interesting to test for CXCL9 and CXCL10 as these have been associated with early RA (Tsubaki et al. 2005) but this was not possible. Culturing synovial fibroblasts for several months in vitro may alter their cytokine production and make prediction of outcome difficult. However, the phenotype of synovial fibroblasts is imprinted for many months when cultured in vivo (Kiener et al. 2010) though primary culture cells markedly differ from cells of passage four (Zimmermann et al. 2001). However, central pathological characteristics such as IL6 expression remain present over many passages using the SCID mouse model (Hirth et al. 2002;Mueller-Ladner et al. 1996). Researchers have found that using microarrays passage 2 to passage 4 is the best window (Neumann et al. 2010) but this may not be the case for metabolomics or cytokine analysis.

In summary, we have found that the metabolic profile of an individual early arthritis patient's cultured synovial fibroblasts (media and to a lesser extent cells) predicts the subsequent course of the patient's disease. We have found a significant association

between CRP levels in the patients' serum and the metabolic profile of their synovial fibroblasts and a significant association between the metabolomic fingerprint of synovial fibroblasts and their IL6 production. Though local activity of IL 6 is only one of the contributing factors to systemic inflammation systemic activity of IL 6 as measured by metabolic profiles may be a useful measure of the inflammatory burden in a patient with inflammatory arthritis.

7.0 General Discussion

Since the start of this project there have been significant advances in the field of metabolomics. Prior to my work there were no publications applying metabolomics to studies of RA and we have now successfully assessed metabolomic profiles of several different biofluids relevant to RA, to detect changes in metabolism. The central aims of my work have been discussed in the individual chapters but I will use this overall discussion to discuss the common themes in further detail. Using urine samples we have shown that two different inflammatory conditions (RA and PsA) can be distinguished using NMR spectroscopy and our work with serum has demonstrated that patients with RA can be distinguished from healthy controls. We have also found clear differences in the metabolic profiles of baseline urine samples of patients with RA who responded well to anti-TNF therapy compared with those who did not. This difference may be important as a novel predictor of responses to TNF antagonists, an area which requires further exploration.

One of the metabolites that contributed to this prediction of response to therapy was histamine, and histidine degradation products have also been found in patients with inflammatory bowel disease. Hence, this may be a generic marker of inflammatory processes rather than being specific to RA. The literature suggests that there are a number of such metabolites which are common to several inflammatory conditions and thus markers of inflammation. Indeed, our work on the serum of patients with early inflammatory arthritis clearly illustrates the strong relationship between inflammation and the metabolic profile and histidine degradation products again contributed to this relationship.

As with data on the serum profiles, we found a strong relationship between inflammation and the metabolites from synovial fibroblasts. Furthermore, there was some overlap between the metabolites in the fibroblast media that correlated with patient CRP and those in serum that correlated with CRP (Table 7.1). However, lipids made a significant contribution to the serum correlation but this was not the case for the fibroblast media. This difference may be because the serum samples were not filtered whereas the media samples were, a process likely to remove most of the lipids since they would be present as micelles or protein-bound.

Metabolites identified in the sera of patient group 1	**Metabolites identified in the sera of patient group 2**	**Metabolites identified in the fibroblast media**
Choline	LDL-lipids	Cholesterol
LDL-lipids	Acetylglycine	Fatty acids
Lactate	Glucose	Leucine
Acetylglycine	Fatty acids	Citrate
Urea	Methylguanidine	Pyroglutamate
Glucose	Lactate	Carnosine
Methylguanidine	Threonine	Alanine
Methylhistidine	Homocystine	Lactate
Cholesterol	Glycine	
Taurine	Taurine	
Threonine	Methylxanthine	
Fatty acids	Choline	
Methylxanthine	Methylhistidine	
Homocystine	Cholesterol	

Table 7.1: Metabolites in the serum and fibroblast media that most strongly correlated with patient CRP.

We found a significant association between the metabolomic fingerprint of synovial fibroblasts and their IL6 production. Local activity of IL 6 alters metabolism (Boettger et al. 2010) and systemic activity of IL 6 and metabolic profiles may be a useful measure of the inflammatory burden in a patient with inflammatory arthritis.

We were able to use metabolic profiles of synovial fibroblasts from the earliest stage of inflammatory arthritis to predict the subsequent course of the disease. The metabolic profiles of fibroblasts from patients with early inflammatory arthritis whose arthritis resolved could be clearly distinguished from those from patients who developed RA. This was true of metabolites from the cells and their media when grown at 20% oxygen and also true of the media from synovial fibroblasts cultured at 3% oxygen. The metabolites associated with this prediction were mainly linked to glycolysis but also related to increased glutamine consumption, lipolysis and protein metabolism. These observations suggest that the "Warburg effect" may be in play in the inflamed joint since it is known that this characterises cells which actively use glycolysis during cell growth rather than mitochondrial respiration. The Warburg effect is usually discussed in the context of cancer but these observations suggest the same metabolic deviation may occur in RA.

In cancer there is an increased requirement for ATP with 10% more ATP being produced by cancer cells compared to normal cells (Koppenol et al. 2011). Traditionally glucose and glutamine are felt to be the major energy sources for cancer metabolism but recent data suggest that glucose and glutamine contribute to less than half of the ATP production (Guppy et al. 2002). One of the methods by which this extra ATP may be produced is through lipolysis (Guppy et al. 2002) and in hypoxia increased metabolites which are lipid

derivatives, particularly acyl-carnitine, have been found (Frezza et al. 2011). We identified the fatty acid intermediate ethanolamine to be an important metabolite contributing to the prediction of responses to TNF antagonists in patients with RA. We also observed the fatty acid intermediate glycerol in our work on synovial fibroblasts. Catabolic intermediates derived from proteins have also been observed in cancer cells under hypoxia (Frezza et al. 2011) suggesting that hypoxic cancer cells require catabolic processes in addition to the increased glycolytic flux to provide the increased ATP required for their proliferation. One of the key metabolites identified in that study on cancer were tryptophan metabolites and we also identified tryptophan metabolites to be key players in the urine in predicting responses to TNF antagonists in patients with RA.

It is likely that the hypoxic nature of the RA joint requires a metabolic switch in fibroblasts to anaerobic respiration in order that they have sufficient energy to drive the bone loss which is seen in RA (Morten et al. 2013). The metabolic changes which we have seen in the RA synovial fibroblasts, with increased glycolysis and increased glutamine consumption, have been seen by others (Chang and Wei 2011;Morten et al. 2013) confirming that significant metabolic shifts occur within the cells of the inflamed joint.

Several groups including ours (Young et al. 2013) have detected an increase in lactate in the blood of RA patients which provides supporting evidence for this shift to anaerobic respiration (Lauridsen et al. 2010;Morten et al. 2013). In cancer, elevated lactate concentrations facilitate sustained glycolysis by the tumour and lead to an acidic tumour environment which is closely correlated with clinical outcome (Bayley and Devilee 2012). Lactate is thought to stimulate angiogenesis via the VEGF/VEGF2 signalling pathway

which is under the regulation of HIF-1α. Recent research has shown that lactate can promote NF-κB activation in endothelial cells, leading to the production of IL-8/CXCL8 as an autocrine signal, mediating the effects of lactate on angiogenesis and tumour perfusion (Vegran et al. 2011). All these events can occur in the presence of glucose thus providing an alternative route through which cell metabolism and angiogenesis may be linked independently of oxygen status and this may occur in RA as well as cancer.

Current data suggest that inflammatory pathways such as those regulated by NF-κB interact with other pathways which are regulated by hypoxia. NF-κB can control the expression of genes for TNF, Il 6, Il 8, VEGF, MMP1, MMP3 and MMP13. Hypoxia can activate NF-κB; hence regulate cytokines in HIF 1 independent or dependent pathways (Konisti et al. 2012). There is now significant evidence for cross talk between HIF-1α and NF-κB pathways and hypoxia can activate both.

Tumour hypoxia has an important role in several aspects of cancer biology but evidence suggests that it is a late-occurring event which may not be the major contributor in the switch to aerobic glycolysis in cancer cells (Vander Heiden et al. 2009). This may be the case in RA too. We have found that synovial fibroblasts from patients with the early stage of inflammatory arthritis undergo glycolysis and produce lactate. Our clinical data of the biopsied joint suggest that these patients have minimal synovitis both clinically and by ultrasound compared to established RA and hence their joints will not be hypoxic as the level of hypoxia is related to the degree of synovitis (Kennedy et al. 2011). The production of lactate by these cells may then cause the joint to become acidic and contribute to the hypoxia and sustain glycolysis. As is the case in cancer, mutations or significant epigenetic

changes might be required to give cells in RA the ability to acquire nutrients and regulate metabolic pathways in the joint.

In cancer, aerobic glycolysis can occur concurrently with mitochondrial respiration. The increase in glycolysis in cancer is caused by damage to the regulation of glycolysis which may result from the activity of oncogenes and tumour suppressor genes. Oncogenic mutations in mitochondrial metabolic enzymes cause increased levels of HIF-1α. MYC is related to the biogenesis of ribosomes and mitochondria (Dang et al. 2009) and can stimulate aerobic glycolysis as MYC targets include glucose and glutamine transporters, glutaminase and glycolytic enzymes. It is also implicated in inducing the gene lactate dehydrogenase-A which generates lactate (Doherty et al. 2014). AKT oncogenes can also induce aerobic glycolysis and increase the activity of HIF-1α. P53 is involved in the regulation of mitochondrial respiration and glycolysis and loss of p53 leads to aerobic glycolysis (Koppenol et al. 2011). Work in the field of ageing has revealed that alterations in the naturally occurring isoform p53 causes changes in amino acids and derivatives of glycolysis (Lin et al. 2013). This alteration may be relevant in RA as well as in ageing and cancer.

RA synovial fibroblasts continue to display an abnormal phenotype when grown in vitro and are able to directly invade cartilage when cultured in vitro. We have demonstrated that fibroblasts cultured in vitro continue to use glycolysis and this leads to increased production of lactate and in vivo would promote hypoxia in the joint. The reason why these features continue in vitro is most likely to result from epigenetic changes in the cells (Ospelt et al. 2011). Many of these have been seen in synovial fibroblasts in RA including

a reduction in methylation in the gene which encodes the chemokine CXCL12 (Bottini and Firestein 2013). CXCL12 stimulates production of MMPs which are partly responsible for the destructive behaviour of RASFs. Further investigation into the epigenetic changes which may be responsible for the glycolysis in fibroblasts could reveal novel and successful treatment targets.

We obtained conflicting results when we used the metabolite profiles of serum from patients with early inflammatory arthritis to predict differences in outcome but we were able to predict differences in outcome using the in vitro synovial fibroblasts of these patients. The metabolites that we identified which were associated with the prediction of outcome using in vitro synovial fibroblasts were different from the metabolites that we identified using serum (Table 7.2). There was some overlap with the metabolites lactate, alanine and glucose but lipids were an important metabolite in the serum. One of the other reasons for this difference in metabolites may be due to all the early RA outcome patients in the in vitro fibroblast study being auto antibody positive whereas the early RA group in the serum study were a combination of antibody positive and negative patients. We were unable to identify lipids in the fibroblast cells as we only analysed the polar layer which did not contain any lipids and we were unable to identify lipids in the media again due to filtering of samples. It is not surprising that the metabolites that we identified are different in the different biofluids as this has been the case in other research (Schicho et al. 2012;Sreekumar et al. 2009) and we are aware that the metabolic profile in serum will also reflect all the systemic changes seen in inflammatory arthritis, in addition to those focussed in the joint.

Persistent RA vs. resolving arthritis (synovial fibroblast media 20%)	**Persistent RA vs. resolving arthritis (synovial fibroblast media 3%)**	**Persistent RA vs. resolving arthritis (synovial fibroblast cells 20%)**	**Persistent RA vs. resolving arthritis Group 1 (serum)**	**Persistent RA vs. resolving arthritis Group 2 (serum)**
Ethanol	Ethanol	Formic acid	Methylguanidine	Lactate
Pyroglutamate	Lactate	Glucose	Lactate	Taurine
Alanine	Pyroglutamate	Glycerol	Acetylglycine	Glucose
Histidine	Glucose	Lactate	Urea	Methylguanidine
Asparagine	Methanol	Myo-inositol		Alanine
Methanol	Asparagine	2-phosphoglycerate		Acetylglycine
Citrate	Alanine			LDL-CH2
Aspartate				
Saccharopine				
Glucose				
Glycerol				
Lactate				
Glutamine				

Table 7.2: Metabolites most strongly associated with the differentiation between persistent RA and arthritis that resolves using synovial fibroblasts and serum.

Metabolomics is still largely a research tool and has yet to move out into the clinical arena. Nevertheless it is an approach that has great potential for widespread clinical application. For example, a large metabolomic study in prostate cancer identified sarcosine as a metabolite that differentiated between benign prostate, clinically localised prostate cancer and metastatic disease (Sreekumar et al. 2009). This group then used this to reveal that sarcosine was a potentially important metabolic intermediary of cancer cell invasion and

aggressivity, thus providing both a novel biomarker in addition to new insights into disease pathology. The work described in this thesis goes a small way to emulating that study since the cellular metabolites that we have identified provide some novel insights into the mechanisms underlying the persistence of inflammatory arthritis and potential therapeutic targets to halt or prevent disease.

7.1 Limitations

Lactate is an important molecule in metabolism and we have demonstrated that it is a key player in the development of persistent arthritis using in vitro synovial fibroblasts. We obtained conflicting results regarding lactate in our serum studies and it is important that clinical serum samples are revisited with better control of collection sampling, as demonstrated by our control work, in order to ascertain whether serum lactate levels could be a biomarker for disease persistence in people presenting with early inflammatory arthritis.

Our in vitro work has focussed on synovial fibroblasts which are an important cell in the pathogenesis of RA but fibroblasts do not act in isolation. Other important cell types in RA include macrophages, T cells and B cells and it may be that we would have obtained a better model of disease if we had used co-cultures of fibroblasts and macrophages for our metabolomic studies. We investigated the action of IL6 on synovial fibroblasts but macrophages not fibroblasts have membrane bound IL6 receptor so a co-culture of macrophages and fibroblasts may have given us further insights into the mechanism of disease in RA. Co-cultures of RASFs and T cells have been used as a model in other studies (Bradfield et al. 2003;Hu et al. 2013) and there has also been published work using

co-cultures of macrophages and fibroblasts in studies looking at bone destruction in RA (Kim et al. 2011;Kwok et al. 2012).

Hypoxia is also important in macrophages and evidence suggests that knockout of HIF-1 α causes cells to develop a M2 phenotype, which is more anti inflammatory. Studies have also demonstrated that knockout of HIF-1 α in a mouse model of CIA caused a reduction of the clinical synovitis symptoms of CIA (Konisti et al. 2012). This suggests that macrophages as well as fibroblasts play an important role in the regulation of metabolism in RA.

7.2 Future work

Conventional treatments target established RA late on in disease and in a non personalised manner. We have demonstrated for the first time that metabolomic techniques using 1D NMR spectra can predict outcome to antiTNF therapy in patients with severe RA providing a sensitivity and specificity for response that has potential clinical utility despite a small initial cohort of patients. There is a pressing need to confirm and extend this finding in a larger cohort of patients, combining metabolomic with cytokine and autoantibody analyses to develop tests that can predict response without the need for empirical treatment, bringing closer the era of individually tailored therapy.

We have used serum and synovial fibroblasts to investigate prediction of outcome in patients with early inflammatory arthritis but our work in established RA has demonstrated what a useful biofluid urine is. Urine samples are extremely user friendly for NMR spectroscopy as they require little preparation, remain stable for several hours and you are

able to obtain very good spectra from these samples. Urine samples are not collected routinely in rheumatology practice which is why our work focussed on serum but over the last two years we have been collecting urine samples in the clinic. It would be extremely useful and interesting to try and use these urine samples to predict outcome in the future.

We have demonstrated an increase in the metabolites related to glycolysis using our in vitro synovial fibroblasts but it would be useful to examine this relationship more closely by the use of metabolic flux. Our results for the synovial media were much more convincing than our results for the cells. The metabolic profile obtained from the cell is just a snap shot whereas the metabolic profile in the media is the result of accumulation over several days at least. For this reason it would be useful to apply metabolic flux to these samples to examine the exact relationships between the metabolites and the temporal relationship for this. This would hopefully give us further insights into the pathogenesis of RA. In vivo Magnetic Resonance Spectroscopy has been used in cancer and may be a useful tool to classify early arthritis in the future using the metabolic differences that I have seen in vitro with the synovial fibroblasts (Vicente et al. 2013).

Targeting the metabolites in glycolysis and reducing the Warburg effect may be ideal options for treating RA, as has been the case in cancer. Changes to signalling of the transcription factor HIF lead to alterations in expression of approximately 1% of all human genes including glucose transporter 1, glyceraldehydes-3-phosphate and VEGF (Konisti et al. 2012). We have found changes in several steps of glycolysis and it would be very interesting and important to identify how much of this was due to HIF. Inhibiting HIF in future experiments would enable us to determine whether these changes were reversed and

would allow us to target HIF in addition to other intermediates of glycolysis in the treatment of RA.

In conclusion, our work has given us significant novel insights into the pathogenesis of RA. Current therapeutic options for RA have improved the outcome for patients with RA but they are still inadequate. They target established RA late on in disease but our research provides novel markers with the potential to justify the early treatment of early inflammatory arthritis and thus prevent the development of RA. This work also suggests that targeting the intermediates of glycolysis, HIF-1α pathway and therefore angiogenesis may be important new therapeutic pathways which will enable us to make further progress in preventing the development of RA.

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8.0 Appendix

8.1 Composition of RPMI

Component	g/l
Inorganic salts	
Calcium Nitrate • $4H_2O$	0.1
Magnesium Sulfate (anhydrous)	0.04884
Potassium Chloride	0.4
Sodium Bicarbonate	2
Sodium Chloride	6
Sodium Phosphate Dibasic (anhydrous)	0.8
Amino Acids	
L-Alanyl-L-Glutamine	—
L-Arginine	0.2
L-Asparagine (anhydrous)	0.05
L-Aspartic Acid	0.02
L-Cystine • 2HCl	0.0652
L-Glutamic Acid	0.02
L-Glutamine	—
Glycine	0.01
L-Histidine	0.015
Hydroxy-L-Proline	0.02
L-Isoleucine	0.05
L-Leucine	0.05
L-Lysine • HCl	0.04
L-Methionine	0.015
L-Phenylalanine	0.015
L-Proline	0.02
L-Serine	0.03
L-Threonine	0.02
L-Tryptophan	0.005
L-Tyrosine • 2Na • $2H_2O$	0.02883
L-Valine	0.02
Vitamins	
D-Biotin	0.0002
Choline Chloride	0.003
Folic Acid	0.001
myo-Inositol	0.035
Niacinamide	0.001
p-Aminobenzoic Acid	0.001
D-Pantothenic Acid (hemicalcium)	0.00025
Pyridoxine • HCl	0.001
Riboflavin	0.0002
Thiamine • HCl	0.001
Vitamin B_{12}	0.000005
Other	
D-Glucose	2
Glutathione (reduced)	0.001
Phenol Red • Na	0.0053

8.2 Early arthritis healthy controls

Information leaflet for healthy individuals

Predicting outcome in patients with early inflammatory arthritis

Invitation to take part

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of the study?

Patients develop inflammatory arthritis because the cells of their immune system start attacking their joints. At the moment we do not know why this happens. What we do know is that in some patients the arthritis settles by itself, whereas in others it can go on to persist and can develop into a condition like rheumatoid arthritis. We are studying why patients develop arthritis and why in some patients the arthritis does not get better. We are doing this by looking at the patterns of patients' arthritis for example which joints are affected. We will assess this by examining the joints and by looking at them with an

ultrasound machine. To understand more about what is going on with the cells of the immune system in patients with arthritis we are studying these cells and what they produce in the blood and urine. In addition we wish to see which genes are working in these cells. Eventually we hope to be able to predict how a patient's arthritis is going to progress from their pattern of arthritis and the characteristics of their inflammatory cells when the patient is first seen in clinic.

Why have I been chosen?

You have been chosen because you have been identified as being a healthy individual. It is important for us to study healthy individuals so we can compare results from such people to results from patients with arthritis. This will allow us to understand how things have changed in patients with arthritis and will help us understand why they have developed problems with their joints (whilst you have not).

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form which you will be given a copy of. If you have been identified by a friend / relative of yours as being a healthy individual who may be interested in taking part in this study and you do not want to take part, the care of your friend / relative will not be affected in any way.

What will happen to me if I take part?

This study has several components. You can choose on the consent form to take part in some parts of the study but not in others.

We will need to ask you a few questions to make sure that you do not have arthritis at the moment and that you do not have any of the following conditions: a current or recent

infection, inflammatory bowel disease, psoriasis, liver disease, inflammatory lung disease or asthma (requiring regular steroids), kidney disease or multiple sclerosis. We would also like to study a blood samples, up to 30 mls (1 syringe). Some blood will be analyzed immediately and some stored for analysis at a later date.

We would also like you to collect the urine that you produce over a 24 hour period. This will allow us to study the levels of natural steroid and other small molecules in your urine. These substances are produced as a normal process in everyone – we wish to see if the level in the urine of healthy people differs from those in people with arthritis.

In addition, we would like to look at your joints with an ultrasound scan. This will take about 1 hour and is an entirely harmless procedure.

You can opt out of either of these last two components of the study, while still taking part in the research study, by allowing us to collect blood samples.

The majority of the samples that we collect will be analysed in Birmingham. Some of the samples may be sent to research groups with whom we work in the UK and Europe including a Biotechnology Company called Cellzome.

Any samples from you will be given a unique code number and no identifiable details about you will be stored. The researchers analyzing your samples will not be able to identify who the sample has come from.

What do I have to do?

If you agree to take part in this study we will ask you a few questions to ensure you do not have any condition listed above and collect the blood sample. We will arrange a convenient time for the ultrasound scan of the joints to be performed. We will give you a

bottle to take home to collect the urine sample which you will need to return to us the following day.

What are the side effects of taking part?

You may develop a bruise around where the needle is introduced into the arm where the blood is taken; these will settle within a few days. There are no other side effects you are likely to experience.

What are the possible disadvantages and risks of taking part?

None besides the possible side effects noted above and the inconvenience associated with collecting a 24 hour urine sample.

What are the possible benefits of taking part?

Whilst there will be no direct benefit to you, the information we get will allow a better understanding of why patients develop arthritis and hopefully lead to improved treatments.

What if something goes wrong?

If you are harmed by taking part in this study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms will be available to you.

Will my taking part in this study be kept confidential?

All information given by you and all results obtained will be treated in the strictest confidence. It would not be possible for the researchers to link the results of the research tests back to you as an individual.

What will happen to the results of the research study?

The results of the research study are likely to be published in a scientific journal. Information from the tests carried out on you will be included but you will not be individually identified.

Who is organising and funding the research?

This study is organised by the Departments of Rheumatology at the Sandwell and West Birmingham Hospitals NHS Trust and the University of Birmingham. This study is being funded, in part, by the Arthritis Research Campaign (ARC) and in part by the European Commission and the Medical Research Council (MRC).

Who has reviewed the study?

The study was reviewed by the Birmingham East, North and Solihull Research Ethics Committee.

Contact for Further Information

A copy of this information sheet and a signed consent form will be given to you to keep.

Title of Project: Predicting outcome in patients with early inflammatory arthritis

Name of Researcher: Dr Karim Raza

Healthy volunteer screening form

Screening questions

If "No" to the following the volunteer is excluded as a healthy volunteer from this study**:**

Do you consider yourself to be in good health? Yes ☐ No ☐

If "Yes" to any of the following the volunteer is excluded as a healthy volunteer from this study**:**

Do you currently have an infection? Yes ☐ No ☐

Have you had an infection in the last 1 month? Yes ☐ No ☐

Do you have a chronic inflammatory disease, including any of the following:

inflammatory arthritis

inflammatory bowel disease

psoriasis

liver disease

inflammatory lung disease or asthma (requiring regular steroids)

kidney disease

multiple sclerosis Yes No

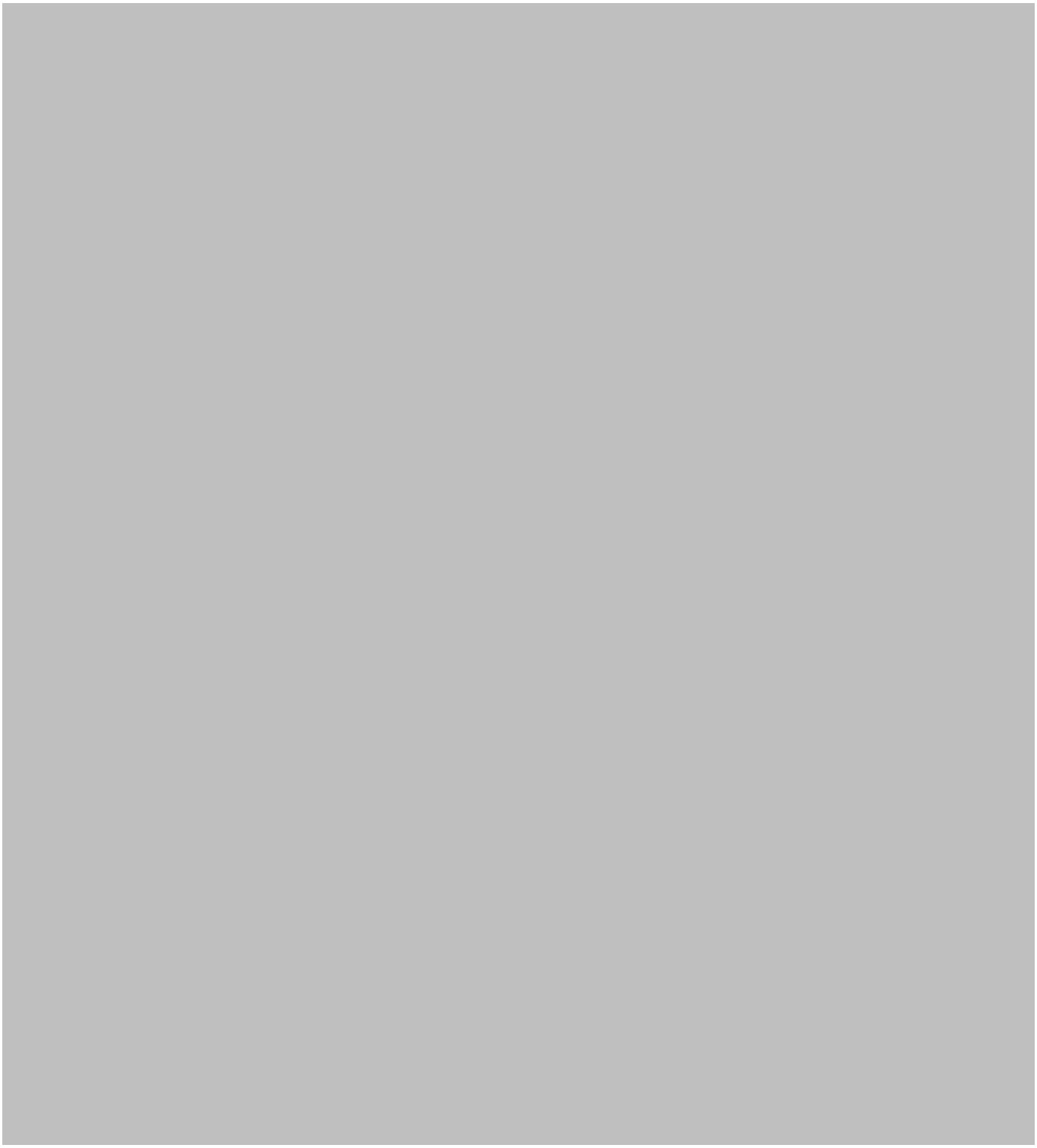
If the volunteer can be included and informed consent has been obtained:

1. Collect blood sample
2. Label sample and this form with Accession number and complete “Transient Research Sample” form
3. Complete the following:

Age (Years):

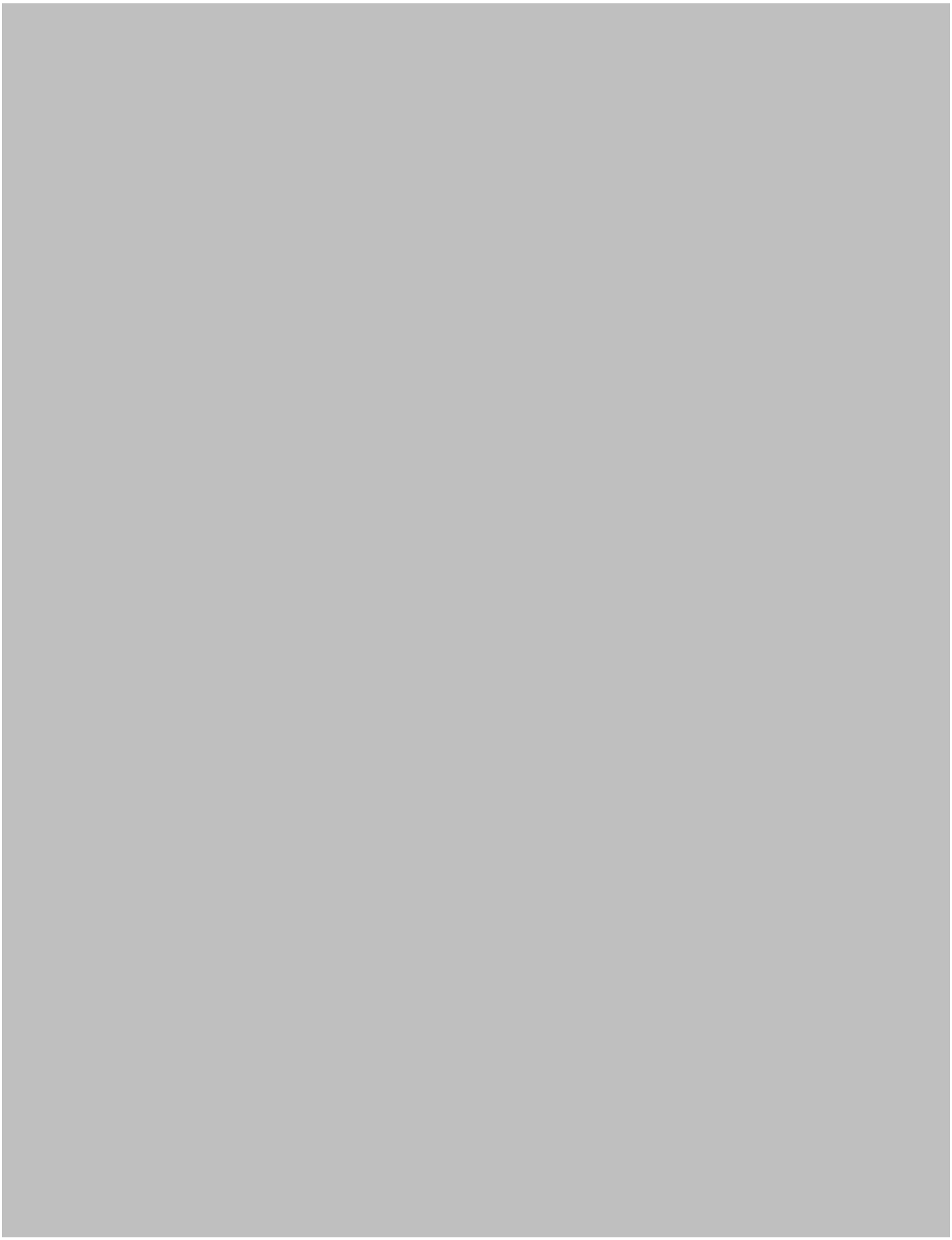
Gender: **Male** **Female**

Consent form for healthy individuals



8.3 Early arthritis study patients

Consent form for patients with ***early*** arthritis



Participant Information Sheet

Study title: Ultrasound guided synovial biopsy in patients with arthritis

Invitation

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. *If you do decide to take part, you are free to withdraw at any time without explanation and your subsequent treatment will not be affected.*

Why have I been chosen?

You have been invited to participate because you have an arthritis, or symptoms suggestive of arthritis, and when your joints were scanned using ultrasound, one of your joints would be suitable to allow a sample (biopsy) of the joint lining (synovium) to be taken.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and

without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care that you receive.

What is the purpose of the study?

We know that in rheumatoid arthritis (RA) considerable thickening of the lining layer (synovium) in the joints occurs. This represents the accumulation of considerable numbers of new cells and tissue. However recently work suggests that the processes going on in the joint in very early RA are different to those occurring in the joints of patients whose arthritis will get better spontaneously. In order to find out what are the "switches" that convert early joint inflammation to chronic arthritis such as RA or get better in others, we need to study small samples (biopsies) of the joint lining. To do this we will use the same ultrasound machine which we used to show the inflammation in your joints to guide a miniature pair of tweezers (2mm thick, the thickness of a pencil lead) to take samples of the lining through a keyhole cut (of the same size, 2mm). These samples will be analysed in studies at the Rheumatology Department in the University of Birmingham, and may be sent to our collaborators within the European Union. Samples from finger joints use a special, even smaller (1.5mm) biopsy needle to obtain tiny samples.

What will happen to me if I take part?

The 'keyhole' procedure known as ultrasound guided synovial biopsy will be performed in a day surgery unit by a doctor trained in the procedure. The whole procedure should take no longer than 90 minutes and uses local anaesthesia. One 2mm incision is made to the upper outer aspect of the joint which allows us to take small samples from inflamed areas guided by the ultrasound probe, which remains on the skin of the joint just as it was during your ultrasound scan. The tiny piece of skin from this incision is also used for research instead of being thrown away. After samples have been taken, the wound will be closed with a dry dressing. If your samples are taken from a knee joint or ankle, then sterile saline (salt water) will be run into the joint until full and then allowed to run out at the end of the procedure. This will be repeated several times to wash away inflammatory liquids from the joint. This is what we call a 'wash-out'. You will be able to walk afterwards but we

recommend you are accompanied home after the procedure. If you cannot be accompanied we will pay for a taxi to bring you and drive you home. The visit should last no longer than 2 hours including rest. The cut will heal leaving a tiny 2-3mm scar (less than 1/8th of an inch).

What are the benefits?

We hope this research will allow us to identify the mechanisms which convert early joint inflammation to established persistent RA in some patients. If your doctor has suggested a knee or ankle joint could be biopsied, then the wash-out of the joint at the end of the biopsy procedure often makes the joint feel better for some months.

What are the risks?

Keyhole-procedures to joints (arthroscopy) are performed under local anaesthetic. Once the local anaesthetic wears off there may be mild, transient pain and discomfort in around a third of patients; temporary swelling of the joint can also occasionally occur. The procedure will leave a tiny 2-3mm scar (less than 1/8th of an inch). The risk of significant complications, such as infection or a thrombosis in the veins of the knee, occurring as a result of the procedure is less than 1 in 500.

What if there is a problem?

[redacted]

Will my taking part in this study be kept confidential?

All information given by you and all results obtained will be treated in the strictest confidence. It will be possible for the researchers to link the results of the research tests back to you as an individual. However you would never be identified individually in any publications.

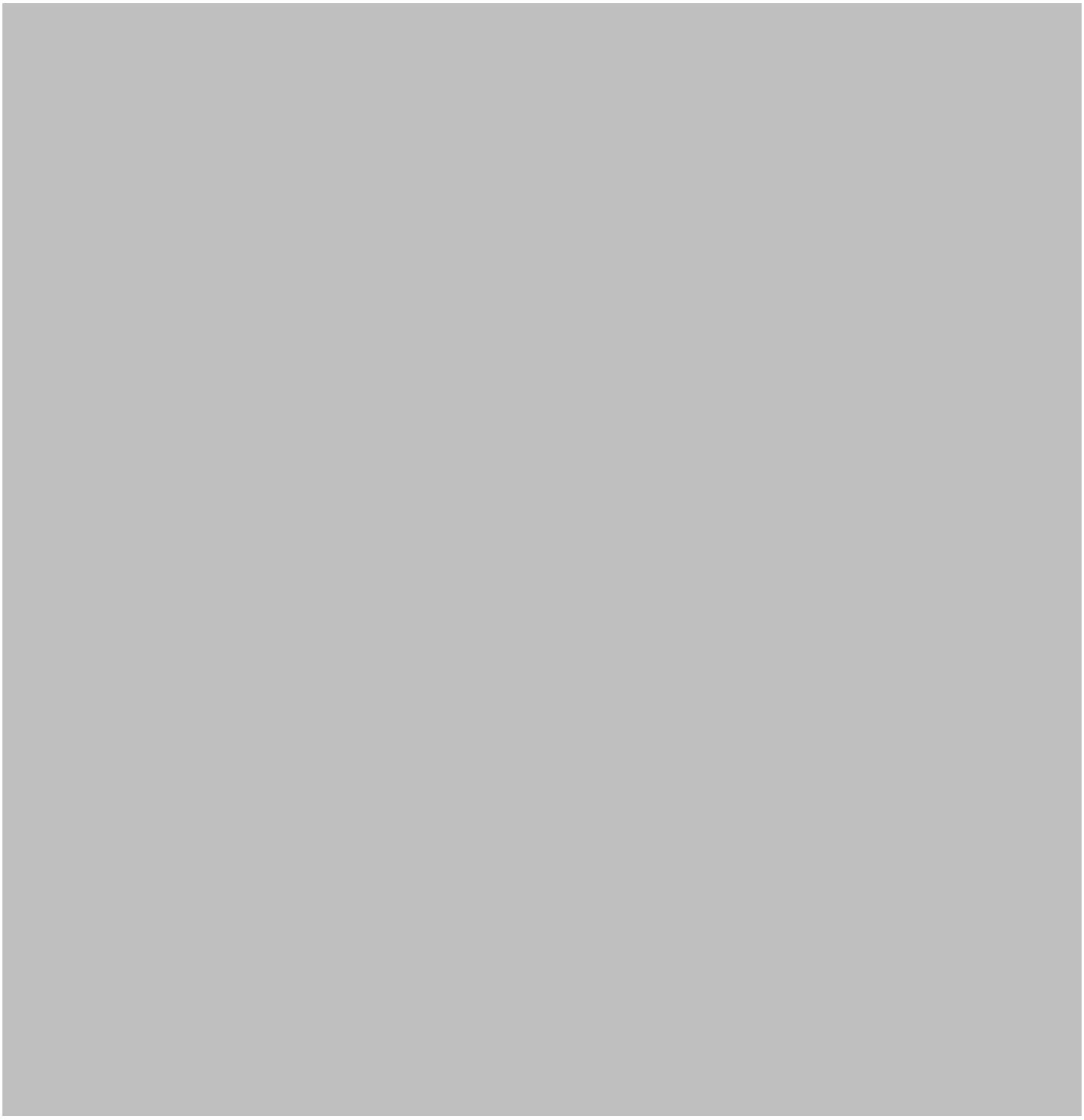
Who is organising and funding the research?

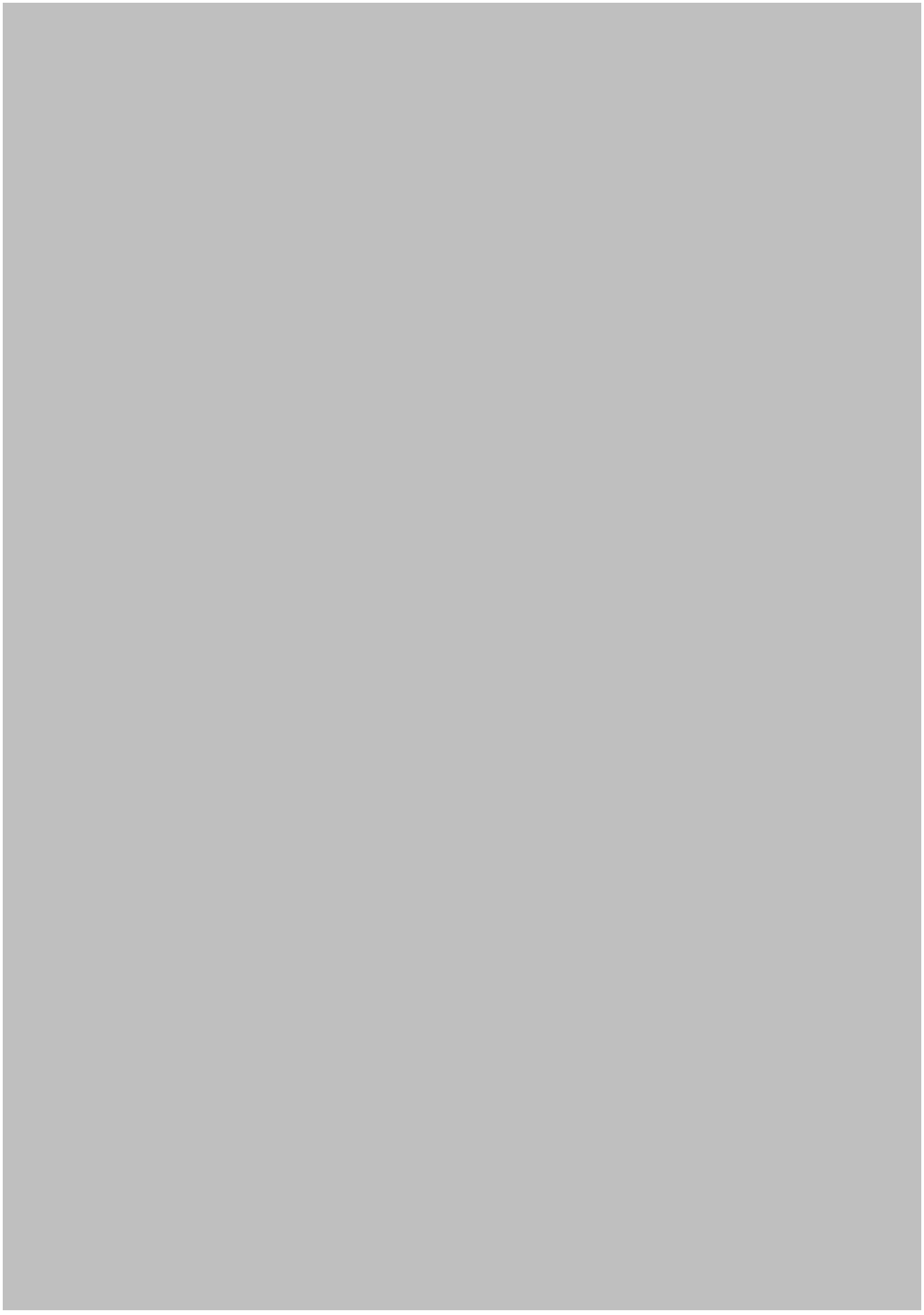
This study is organised by the Departments of Rheumatology at University Hospitals Birmingham NHS Foundation Trust and Sandwell and West Birmingham Hospitals NHS Trust and, and the University of Birmingham. This study is funded by Arthritis Research UK.

Where can I obtain further information?

You will be given a copy of this information sheet and a signed consent form to keep.

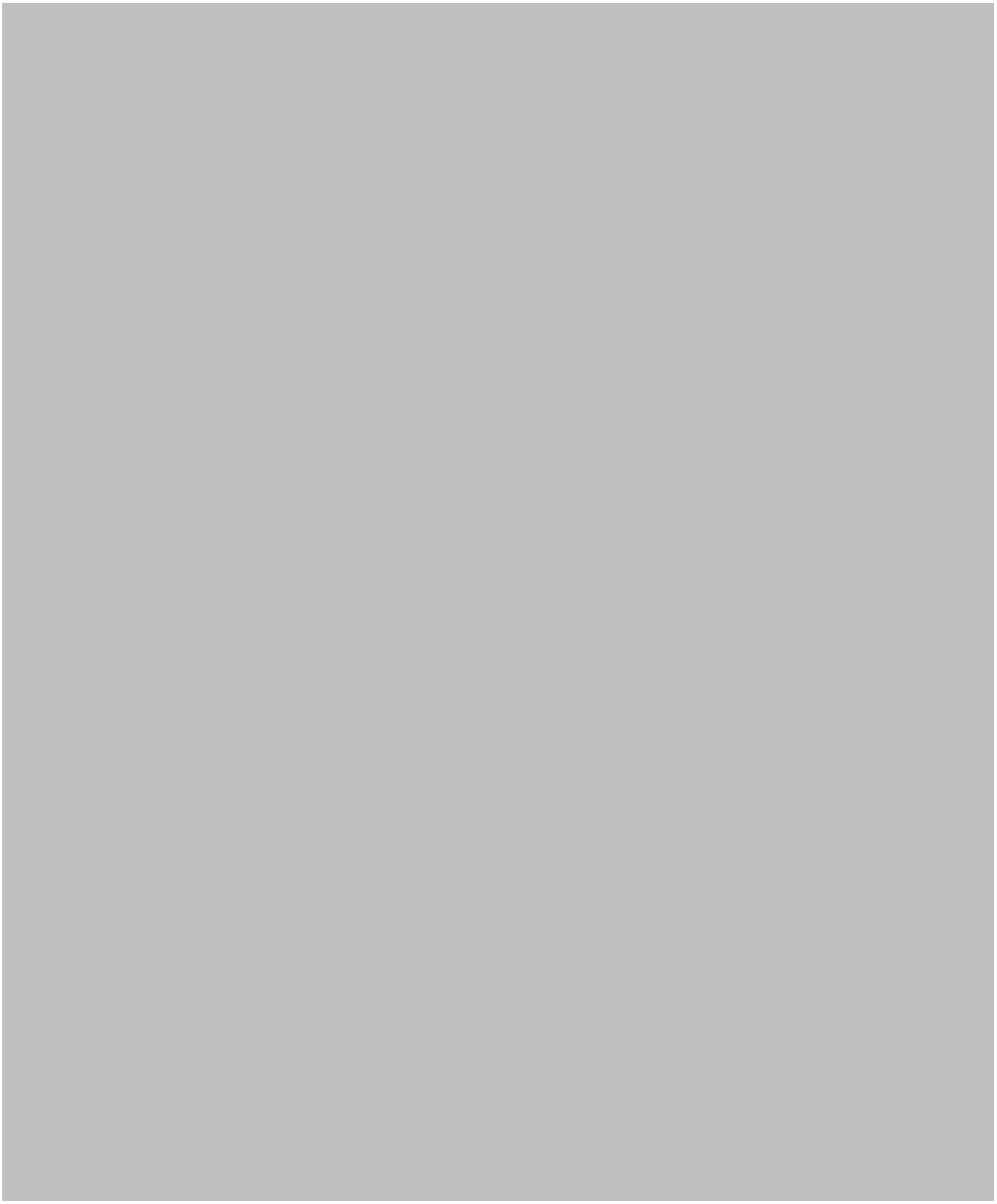
CONSENT FORM

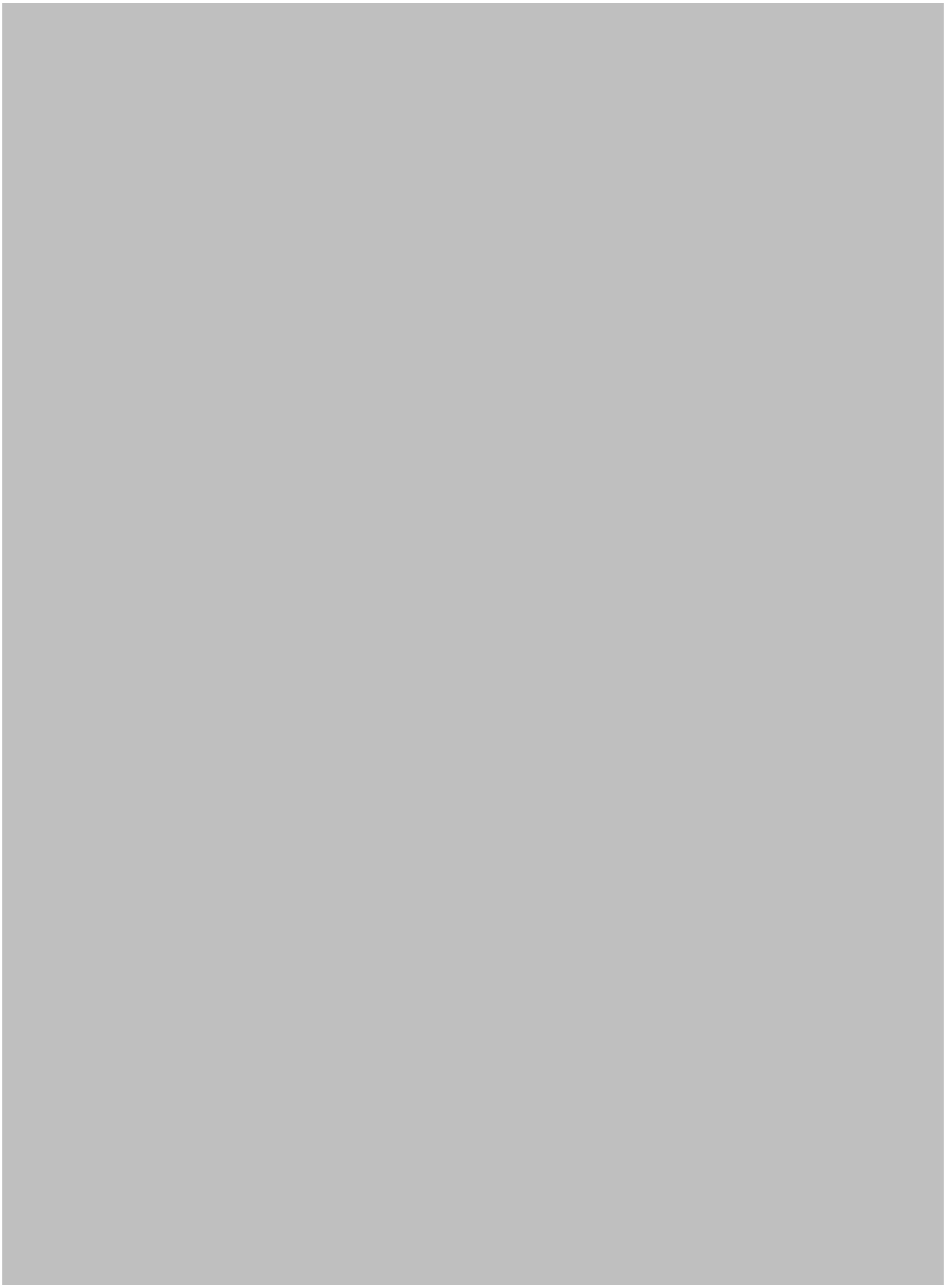




8.4 DATA study consent form

CONSENT FORM





8.5 Publications

ARTHRITIS & RHEUMATISM
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The Impact of Inflammation on Metabolomic Profiles in Patients With Arthritis

Stephen P. Young,[1] Sabrina R. Kapoor,[2] Mark R. Viant,[1] Jonathan J. Byrne,[1] Andrew Filer,[3] Christopher D. Buckley,[2] George D. Kitas,[4] and Karim Raza[2]

Objective. **Inflammatory arthritis is associated with systemic manifestations including alterations in metabolism. We used nuclear magnetic resonance (NMR) spectroscopy–based metabolomics to assess metabolic fingerprints in serum from patients with established rheumatoid arthritis (RA) and those with early arthritis.**

Methods. **Serum samples were collected from newly presenting patients with established RA who were naive for disease-modifying antirheumatic drugs, matched healthy controls, and 2 groups of patients with synovitis of ≤3 months' duration whose outcomes were determined at clinical followup. Serum metabolomic profiles were assessed using 1-dimensional ^{1}H-NMR spectroscopy. Discriminating metabolites were identified, and the relationships between metabolomic profiles and clinical variables including outcomes were examined.**

Results. **The serum metabolic fingerprint in established RA was clearly distinct from that of healthy controls. In early arthritis, we were able to stratify the patients according to the level of current inflammation, with C-reactive protein correlating with metabolic differences in 2 separate groups ($P < 0.001$). Lactate and lipids were important discriminators of inflammatory burden in both early arthritis patient groups. The sensitivities and specificities of models to predict the development of either RA or persistent arthritis in patients with early arthritis were low.**

Conclusion. **The metabolic fingerprint reflects inflammatory disease activity in patients with synovitis, demonstrating that underlying inflammatory processes drive significant changes in metabolism that can be measured in the peripheral blood. The identification of metabolic alterations may provide insights into disease mechanisms operating in patients with inflammatory arthritis.**

The etiology of rheumatoid arthritis (RA) is not fully understood but involves both genetic and environmental factors. In addition to synovitis, there are widespread systemic effects mediated by proinflammatory cytokines that impact on metabolism. Tumor necrosis factor α, interleukin-1 (IL-1), and IL-6 all promote cachexia, which is often associated with RA (1,2). The extent of the metabolic changes and the types of metabolites seen may therefore be good markers of cytokine-mediated inflammatory processes in RA. A novel systems approach to the assessment of metabolic changes in

Dr. Kapoor's work was supported by an Arthritis Research UK Clinical PhD Studentship (grant 18552). The nuclear magnetic resonance data were acquired at the Henry Wellcome Building for Biomolecular Nuclear Magnetic Resonance Spectroscopy at the University of Birmingham; the facility is funded by the Wellcome Trust (grant 066490/Z/01/A).

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Drs. Young and Kapoor contributed equally to this work.

Dr. Kitas has received consulting fees from AstraZeneca (less than $10,000) and speaking fees and/or honoraria for Advisory Board service from Roche, Abbott, Pfizer, Novartis, UCB, and Bristol-Myers Squibb (less than $10,000 each) and has received unrestricted grants from Pfizer.

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disease is metabolomics, which aims to investigate overall metabolic activity, and so takes into account the genetic and environmental background, allowing integration of the effects of these factors (3). Low serum levels of a number of specific metabolites, including histidine, have been reported in RA patients (4), and this metabolite discriminated osteoarthritis from RA (5). Lactate levels in synovial fluids also vary, with higher levels seen in seropositive, than in seronegative, RA (6). Changes in blood lipids in RA have been widely described (7,8). More recently, lipid changes (9) and alterations in a range of serum cytokines and chemokines (10) have been shown to predate the development of arthritis, suggesting that changes in metabolites might be observable early in the development of RA.

Altered metabolic fingerprints have been seen in a number of inflammatory diseases. For example, analysis of fecal extracts differentiated between normal controls, patients with Crohn's disease, and patients with ulcerative colitis. Significantly, these two patient groups could be distinguished using metabolic profiling (11), suggesting that the effects of inflammation on metabolism vary between conditions. This concept is supported by our previous work in which we used metabolic fingerprinting to differentiate between two otherwise similar inflammatory eye diseases (12) and between a number of neurologic conditions (13) and also to predict treatment responses in patients with inflammatory arthritis (14). With this background, we hypothesized that metabolomic profiles may be useful in predicting the development of RA in patients with early arthritis, an area where better predictive tools are currently needed (15). We also aimed to determine whether this analysis could provide novel insights into disease mechanisms in arthritis as has been the case in other conditions (16).

To investigate the potential of metabolic fingerprinting in inflammatory arthritis, we have applied a nuclear magnetic resonance (NMR)–based metabolomic approach to the analysis of serum from newly presenting patients with established RA and patients with early arthritis. We initially sought to assess whether the metabolic fingerprint in patients with established RA differed from that of healthy controls and then whether this fingerprint differed in patients with early arthritis in relation to the extent of inflammation and final outcomes.

PATIENTS AND METHODS

Patients. Patients were recruited through the inflammatory arthritis clinic at Sandwell and West Birmingham Hospitals NHS Trust. The study was conducted in compliance with the Declaration of Helsinki, and ethical approval was obtained from the local ethics committee. All subjects gave written informed consent. Serum samples were collected from the patients and stored at −80°C until analyzed.

Patients with established RA. Serum samples were collected from 16 newly presenting patients who were naive for disease-modifying antirheumatic drugs (DMARDs), fulfilled the American College of Rheumatology (ACR) 1987 classification criteria for RA (17), and had a symptom duration of >3 months. Symptom onset was defined as the time of onset of inflammatory joint pain and/or early morning stiffness and/or joint-related soft tissue swelling.

Patients with early arthritis. Serum was collected from patients with early arthritis at the time of initial presentation. All patients had one or more swollen joints and symptoms (inflammatory joint pain, early morning stiffness, and/or joint-related soft tissue swelling) of ≤3 months' duration as previously described (18). Patients with evidence of previous inflammatory joint disease were excluded. Two groups of patients were studied, one recruited after the other. There were 89 patients with early arthritis in group 1 and 127 in group 2. Patients were followed up for 18 months and then assigned to their final diagnostic categories. Patients were classified as having RA according to the ACR 1987 criteria (17), allowing criteria to be satisfied cumulatively. Patients were diagnosed as having reactive arthritis, psoriatic arthritis, and a number of miscellaneous conditions according to established criteria. As previously described (18), patients were considered to have resolving arthritis if they had no evidence of joint-related soft tissue swelling on final examination and had not received DMARD or steroid treatment within the previous 3 months. Persistent disease was defined as persistent joint-related swelling or treatment with DMARDs or steroids for inflammatory joint symptoms (within the previous 3 months).

Metabolomic analysis. After thawing, serum samples were centrifuged at high speed (13,000*g*) and diluted 1:1 with an aqueous solution containing D_2O (20%), NaCl (150 m*M*), trimethylsilyl 2,2,3,3-tetradeuteropropionic acid (TMSP) (1 m*M*), and sodium phosphate (20 m*M*) (pH 7). One-dimensional ^1H spectra were acquired at 300°K using a standard spin-echo pulse sequence with water suppression using excitation sculpting on a Bruker DRX 500 MHz NMR spectrometer equipped with a cryoprobe. Spectra for patients with early arthritis in group 1 and group 2 were acquired several years apart and so were analyzed separately. Samples were processed and data were calibrated with respect to the TMSP signal. Spectra were read into ProMetab (19), custom written software in MatLab version 7 (The MathWorks), and were truncated to the chemical shift range of 0.8–10.0 parts per million. Spectra were segmented into 0.005-ppm (2.5 Hz) chemical shift "bins" and the spectral area within each bin was integrated. Spectra were corrected for baseline offset and then normalized to a total spectral area of unity, and a generalized log transformation was applied (19,20). Binned data were then compiled into a matrix, with each row representing an individual sample.

Statistical analysis. Data bins were mean centered and subjected to principal components analysis (PCA) using the PLS_Toolbox (version 5.8) (Eigenvector Research) in MatLab (release 2009a; MathWorks). Partial least-squares discrimi-

Table 1. Baseline characteristics of the patients with established RA, healthy controls, and patients with early arthritis*

	Patients with established RA (n = 16)	Healthy controls (n = 14)	Patients with early arthritis in group 1 (n = 89)	Patients with early arthritis in group 2 (n = 127)	P†
Age, median (IQR) years	57 (37–79)	54 (40–72)	46 (36–61)	50 (35–65)	0.32‡
Sex, no. (%) female	12 (75)	9 (64)	49 (55)	69 (54)	1.0§
Symptom duration, median (IQR) weeks	31 (18–52)	–	5 (2–9)	6 (4–9)	0.02‡
No. (%) taking NSAIDs	7 (44)	0 (0)	58 (65)	69 (54)	0.12§
CRP, median (IQR) mg/ml¶	20.5 (7.5–55.5)	–	19 (5.5–54)	19.5 (5.25–44.75)	0.79‡
RF positive, no. (%)	12 (75)	–	12 (13)	30 (24)	0.83§
Anti-CCP antibody positive, no. (%)#	9 (56)	–	13 (15)	29 (23)	0.39§
No. (%) with persistent arthritis	–	–	33 (37)	87 (68)	<0.0001§
No. (%) with persistent arthritis who developed RA	–	–	18 (54)	55 (63)	0.41§

* RA = rheumatoid arthritis; IQR = interquartile range; NSAIDs = nonsteroidal antiinflammatory drugs; RF = rheumatoid factor.
† Patients with early arthritis in group 1 versus patients with early arthritis in group 2.
‡ By Mann-Whitney test.
§ By Fisher's exact test.
¶ Data on C-reactive protein (CRP) were available for 84 patients in group 1 and 126 patients in group 2.
# Data on anti–cyclic citrullinated peptide (anti-CCP) were available for 126 patients in group 2.

nant analysis (PLS-DA) was used to perform supervised clustering of samples (21,22) and on some occasions an orthogonal signal correction was applied (OPLS-DA) to enhance the separation of groups (23). The PLS-DA models were cross-validated using Venetian blinds (21,22), a method which reassigns randomly selected blocks of data to the PLS-DA model to determine the accuracy of the model in correctly assigning class membership. The application of such methods to clinical studies has been demonstrated in many studies and has recently been reviewed by Nicholson et al (24). For all of the PLS-DA analyses, the variances of Y-block explained by individual latent variables (LVs) are available from the corresponding author upon request.

Partial least-squares regression analysis (PLS-R), a regression method that identifies which metabolites can predict a continuous variable, was also used to investigate the relationship between the metabolic fingerprint and inflammatory burden, with C-reactive protein (CRP) being used as the dependent variable. This analysis yields R^2, a measure of the cross-validated goodness-of-fit of the linear regression, while permutation testing (multiple analyses using random data subsets) was used to assess the significance of this prediction.

Lists of metabolites providing the greatest discrimination between groups were then identified for each technique. A diagram demonstrating the methodologies used is shown in Supplementary Figure 1, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38021/abstract. Using multivariate analyses, peaks with large loadings (for PCA), weightings (for PLS-DA), or regression coefficients (for PLS-R) were identified. NMR databases (Human Metabolome Database version 2.5 [25] and Chenomx NMR suite; Chenomx, professional version 4.0 [26]) were used to identify the metabolites. Published data on metabolites identified in human sera were also used to guide identification (27,28).

RESULTS

Different metabolomic profiles in patients with established RA compared to healthy controls. The characteristics of the patients with established RA and age- and sex-matched healthy controls are shown in Table 1. The median Disease Activity Score in 28 joints using the erythrocyte sedimentation rate in the RA patients was 5.88 (interquartile range [IQR] 5.25–6.99). PCA was used to generate an unbiased overview of the major metabolic differences between patients with established RA and control individuals. There was separation of the 2 groups that was largely dependent on principal component 2 (Figure 1A), the scores of which were significantly higher in the RA group ($P < 0.0001$ by t-test). To help discover the major discriminatory metabolites, these data were subjected to supervised analysis using OPLS-DA. Discriminatory metabolites are shown in Table 2 (column 1).

Supervised analysis enhanced the separation of the 2 groups, and the optimized model comprised 4 LVs that captured 97.8% of the variance in the metabolic data. The major contributions to the separation were made by LV1 and LV2 (Figure 1B). This model was cross-validated using the Venetian blinds approach and identified samples from the RA patients with a sensitivity of 93.3% and a specificity of 100%.

Resolution of inflammation is reflected in metabolomic profile changes. In 22 patients with early arthritis and active disease at baseline (median CRP 66

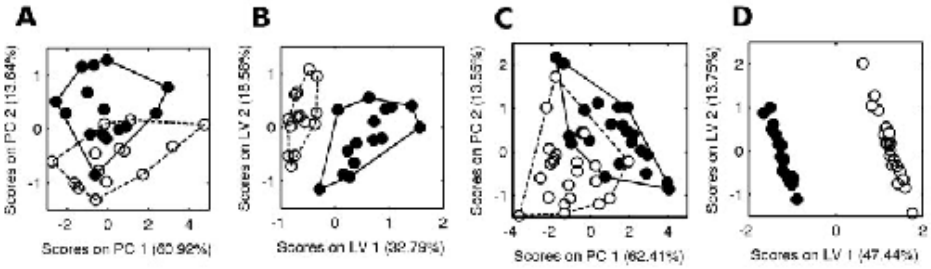

Figure 1. Metabolic fingerprinting distinguishes between sera from patients with established rheumatoid arthritis (RA) and matched healthy controls and illustrates that metabolomic profiles in patients with early arthritis are altered by control of or resolution of inflammation. **A** and **B**, One-dimensional ^{1}H–nuclear magnetic resonance (NMR) spectra of serum obtained from disease-modifying antirheumatic drug–naive patients with RA (solid circles) and healthy controls (open circles) were subjected to principal components analysis (PCA) (**A**) and to supervised analysis (partial least-squares discriminant analysis) (**B**). **C** and **D**, One-dimensional ^{1}H-NMR spectra of serum obtained from a subset of patients with early arthritis before (solid circles) and after (open circles) a decrease in the C-reactive protein level following therapy or spontaneous resolution were subjected to PCA (**C**) and to supervised analysis (partial least-squares discriminant analysis with orthogonal signal correction) (**D**). The values on the axis labels indicate the proportion of the variance captured by each principal component (PC1 and PC2) or latent variable (LV1 and LV2).

[IQR 47–131]), serum samples were available at a followup time point (a median of 49 weeks [IQR 24–69] from the time of initial assessment and sample collection) at which CRP levels were significantly lower (median <5 [IQR <5 to <5]). This decrease in CRP was associated with either spontaneous resolution of disease or control of disease with therapy. Unbiased PCA discriminated between samples at baseline and followup (Figure 1C), and OPLS-DA produced a model comprising 2 LVs that discriminated between these samples with 100% specificity and sensitivity (Figure 1D), demonstrating the influence of inflammation on the metabolic fingerprint.

To provide an overall description of which regions discriminated between groups in the PLS-DA analyses, weightings plots derived from the models were generated. Some common features were seen in these weightings plots from PLS-DA models separating RA patients from controls and in the analysis of the patients with early synovitis from whom a second serum sample

Table 2. Metabolites contributing to the differentiation between groups, determined by analysis of PLS-DA weightings*

Metabolite, ppm	RA patients versus controls	Patients with early arthritis before versus after resolution of inflammation	Patients with persistent arthritis versus patients with resolving arthritis (group 1)	Patients with persistent arthritis versus patients with resolving arthritis (group 2)	Patients with persistent RA versus patients with resolving arthritis (group 1)	Patients with persistent RA versus patients with resolving arthritis (group 2)
LDL-CH3, 0.80	Low (6.30)	Low (3.03)	Low (6.81)	Low (2.87)	–	–
LDL-CH2, 1.21	Low (7.06)	Low (31.81)	Low (7.40)	Low (6.89)	–	Low (1.58)
3-hydroxybutyrate, 1.18, 1.19	High (4.21)	High (7.90)	–	High (6.87)	–	–
Lactate, 1.31, 4.11	High (54.51)	–	Low (12.85)	High (27.90)	Low (12.74)	High (16.98)
Alanine, 1.46, 1.48	Low (20.00)	Low (2.15)	–	–	–	Low (3.84)
Acetylglycine, 2.03	High (48.67)	High (17.41)	High (6.55)	High (6.80)	High (4.57)	Low (1.94)
Methylguanidine, 2.81	Low (10.17)	–	High (92.72)	Low (38.15)	High (34.76)	Low (6.51)
Taurine, 3.26	High (8.12)	High (9.11)	–	High (15.73)	–	High (8.66)
Glucose, 3.25, 3.88	High (16.8)	High (12.72)	–	High (11.55)	–	High (7.49)
Lipid, 5.32	Low (2.36)	Low (2.53)	–	–	–	–
Urea, 5.79	–	High (1.32)	High (3.90)	–	High (1.25)	–

*"High" indicates that the metabolite was at a higher concentration in the rheumatoid arthritis (RA; column 2), early arthritis before resolution (column 3), persistent arthritis (columns 4 and 5), or persistent RA (columns 6 and 7) phenotypes. Nuclear magnetic resonance chemical shifts (in parts per million), which identify the location of the major peaks in the spectra, are shown for each metabolite. Values in parentheses are the variable importance of the projection for each metabolite. PLS-DA = partial least-squares discriminant analysis; LDL-CH3 = low-density lipoprotein CH3.

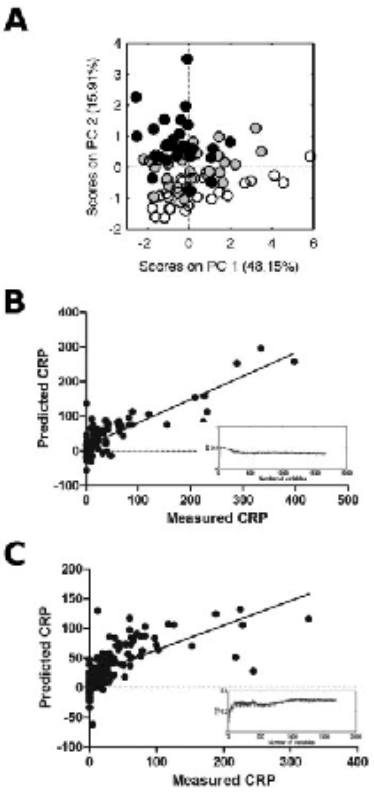

Figure 2. The metabolic fingerprints of sera from patients with early arthritis prior to treatment with disease-modifying antirheumatic drugs are strongly influenced by the level of inflammation. **A**, One-dimensional ^{1}H-NMR spectra of serum obtained from patients with very early arthritis (in group 1) were subjected to PCA. Solid circles indicate C-reactive protein (CRP) levels in the highest tertile, shaded circles indicate CRP levels in the middle tertile, and open circles indicate CRP levels in the lowest tertile. **B** and **C**, Strong correlations between measured CRP and predicted CRP values were found for patients with early arthritis in group 1 (**B**) and those in group 2 (**C**) ($P < 0.001$ for both groups). The predicted values were calculated from the concentrations of a series of metabolites that were discovered using partial least-squares regression analysis. **Insets** show the optimization of the multivariate regression, with the highest correlation between measured and predicted CRP occurring with 154 NMR bins (maximum R^2 of 0.671) for group 1 and with 1,136 NMR bins (maximum R^2 of 0.4157) for group 2. See Figure 1 for other definitions.

had been collected at a time when the CRP had decreased significantly (Table 2 column 2).

Association of metabolomic profiles with inflammation in patients with early arthritis. The characteristics of the 89 patients with early arthritis in group 1 and the 127 patients with early arthritis in group 2 are shown in Table 1.

PCA of NMR spectra of serum from 84 patients with early arthritis (in group 1) in whom the CRP levels were known showed a broad spread on the scores plot (Figure 2A). When the CRP level for each sample was superimposed on the PCA plot, it was clear that the level of inflammation at the time of sampling influenced the distribution. To investigate this further, the relationship between baseline metabolomic profiles and CRP was assessed using PLS-R. The analysis used a forward selection approach to discover those NMR bins that were most predictive. A total of 154 bins created the optimal model with a cross-validated R^2 of 0.671 (Figure 2B). Permutation testing with these 154 NMR bins demonstrated that the regression model was statistically valid ($P < 0.001$).

To further validate this relationship between the level of inflammation and baseline metabolomic profiles, the PLS-R analysis was carried out in a separate group of 127 patients with early arthritis (group 2). The maximum R^2 for the regression of the real (i.e., ordered) data was 0.416 (Figure 2C). Permutation testing with 1,136 NMR bins included (as optimized by forward selection) again demonstrated that the regression model was statistically valid ($P < 0.001$).

It is known that metabolic status and products of metabolism are influenced by several variables, including age, sex, hypertension, diabetes mellitus, hyperuricemia, and smoking, but when we adjusted our analysis for these confounding effects our results remained significant (data are available from the corresponding author upon request). We also excluded the effect of medication used at the time of sample collection (steroids, DMARDs, and nonsteroidal antiinflammatory drugs [NSAIDs]) and found that the relationship between CRP and metabolomic profile was still significant (data are available from the corresponding author upon request).

The regression analysis was carried out to examine the relationship between metabolomic profiles and CRP without the confounding effect of variables that are known to influence metabolic status by assessing only individuals who were not current smokers (n = 67 in group 1 and n = 96 in group 2) and individuals without hypertension (n = 70 in group 1 and n = 96 in group 2), diabetes mellitus (n = 89 in group 1 and n = 118 in

Table 3. Metabolites most strongly correlated with CRP level in patients with early arthritis in groups 1 and 2*

Ranked importance	Metabolites identified in patient group 1 (ppm)	Metabolites identified in patient group 2 (ppm)
1	Choline (3.20, 3.22, 3.23)	LDL lipids (1.24–1.27)
2	LDL lipids (1.24–1.27)	Acetylglycine (2.03, 3.71, 3.76)
3	Lactate (1.31, 1.33, 4.11)	Glucose (3.24–3.26, 3.41, 3.48, 3.68–3.69, 3.88)
4	Acetylglycine (2.03, 3.71, 3.76)	Fatty acids (0.8–0.84, 2.22–2.24)
5	Urea (5.77, 5.78, 5.79, 5.80, 5.81, 5.82)	Methylguanidine (2.81)
6	Glucose (3.24–3.26, 3.41, 3.48, 3.68–3.69, 3.88)	Lactate (1.31, 1.33)
7	Methylguanidine (2.81)	Threonine (3.58)
8	Methylhistidine (3.70)	Homocysteine (3.86)
9	Cholesterol (0.91)	Glycine (3.55)
10	Taurine (3.42)	Taurine (3.42)
11	Threonine (3.58)	Methylxanthine (3.49)
12	Fatty acids (0.8–0.84, 2.22–2.24)	Choline (3.20, 3.22, 3.23)
13	Methylxanthine (3.49)	Methylhistidine (3.70)
14	Homocysteine (3.86)	Cholesterol (0.91)

* Metabolites were identified using the partial least-squares regression analysis model and represent the regions of the spectra which had the greatest influence on the correlation with C-reactive protein (CRP) level. Values in parentheses are the nuclear magnetic resonance chemical shifts (in parts per million), which identify the location of the major peaks in the spectra. LDL = low-density lipoprotein.

group 2), or hyperuricemia (defined as a uric acid level of >340 μmoles/liter; n = 58 in group 1 and n = 67 in group 2). The effects of DMARDs and steroids on metabolic status were also excluded by carrying out the regression analyses only for those patients who were not taking DMARDs (n = 85 in group 1 and n = 125 in group 2; the few patients who were taking DMARDs were taking them for other comorbidities, e.g., inflammatory bowel disease) or steroids (n = 86 in group 1 and n = 123 in group 2) at the time of recruitment into the study. We excluded the effect of NSAIDs by only carrying out the regression analysis for those patients who were taking NSAIDs at time of recruitment since the majority of patients were taking NSAIDs (n = 60 in group 1 and n = 66 in group 2). The relationship between the CRP level and metabolomic profile was statistically significant for all analyses, except for when women alone in group 1 were analyzed (data are available from the corresponding author upon request).

We also used the PLS-R analysis that related the metabolic fingerprint to CRP level to generate a rank order of NMR bins that identified the regions of the spectra that most strongly predicted the inflammatory burden in groups 1 and 2 (Table 3). Almost identical groups of metabolites were identified for both patient groups, suggesting a complex but real relationship between metabolism and inflammatory burden.

Relationships between clinical outcomes and metabolomic profiles in patients with early arthritis. A PLS-DA model was used to attempt to separate patients with an outcome of resolving arthritis from those with an outcome of persistent arthritis for both patient groups 1 and 2. The model separated the patients with regard to outcome but did so with only moderate sensitivity and specificity, of 59.4% and 58.9%, respectively, for group 1 using 5 LVs (Figure 3A) and 59.5% and 56.4%, respectively, for group 2 using 6 LVs (Figure 3B). There was also no differentiation between patients with resolving disease and those with persistent RA in group 1 (Figure 3C) (sensitivity of 50% and specificity of 69.6% using 2 LVs), though there was good separation for group 2 (Figure 3D) with a sensitivity of 73.1% and specificity of 67.6% using 3 LVs.

There was no differentiation between patients with RA and non-RA outcomes for either cohort (see Supplementary Figure 2, available on the *Arthritis & Rheumatism* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.38021/abstract). The variances of Y-block explained by individual LVs are available from the corresponding author upon request. Previous data have shown that treatment can affect metabolomic profiles (29), but there was no significant difference between the groups with regard to the number of patients taking NSAIDs (Table 1), and there was no significant difference in the use of NSAIDs between patients with persistent disease and those with resolving disease for group 1 ($P = 0.13$) or group 2 ($P = 0.1$) or between patients with persistent RA and those with resolving disease for group 1 ($P = 0.18$) or group 2 ($P = 0.17$).

Metabolites were identified that contributed the greatest to the separation between patients with early arthritis with resolving disease and those with persistent

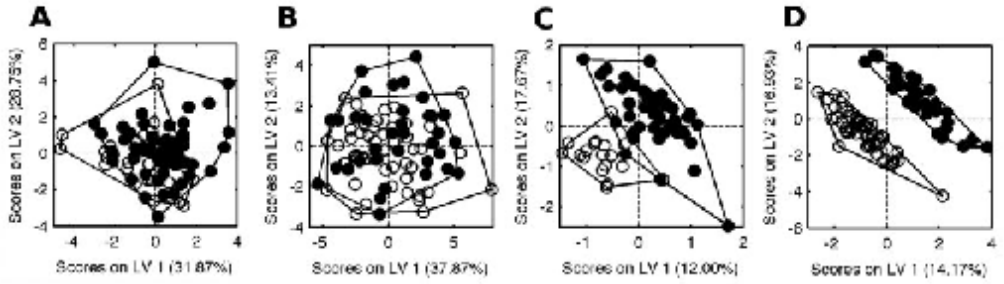

Figure 3. Metabolic fingerprints of sera from patients with early arthritis from 2 different patient groups. **A** and **B**, Serum samples from patients with early arthritis from group 1 (**A**) and group 2 (**B**) at first presentation were assessed using partial least-squares discriminant analysis to distinguish patients whose disease was resolving (solid circles) from those whose disease was persistent (open circles). Sensitivity and specificity were 59.4% and 58.9%, respectively, for group 1 and 59.5% and 56.4%, respectively, for group 2. **C** and **D**, Serum samples from patients with early arthritis from group 1 (**C**) and group 2 (**D**) at first presentation were assessed using partial least-squares discriminant analysis with orthogonal signal correction, to distinguish patients whose disease was resolving (solid circles) from those who developed persistent RA (open circles). Sensitivity and specificity were 50% and 69.6%, respectively, for group 1 and 73.1% and 67.6%, respectively, for group 2. See Figure 1 for definitions.

disease, and also between patients with resolving disease and those with persistent RA for both patient groups (Table 2). Although some predictive metabolites were common to both patient groups, there were some key differences in metabolites between the groups, suggesting that metabolomic techniques as applied in this study cannot fully discriminate between resolving and persistent outcomes or resolving disease and persistent RA in patients with early arthritis.

DISCUSSION

Our data demonstrate that the serum metabolic fingerprint of patients with active established RA differs from that of healthy controls and that the serum metabolic fingerprint of patients with early arthritis varies depending on the level of inflammation. These results from patients with established RA are broadly consistent with a recent study using a similar experimental approach that focused on responses to therapy in a cohort of RA patients (30). There is also some overlap in discriminating metabolites identified in a recent mass spectrometry–based analysis of established RA (31). However, patients in both of those cohorts had already been exposed to DMARD therapy, and so it is possible that the therapy might have influenced their metabolic fingerprints. Our cohort of patients was sampled before any DMARD therapy and so was not exposed to this confounding factor.

Consistent with the findings of Lauridsen et al (30), we found that decreased NMR lipid signals were particularly discriminatory between different groups. Madsen et al (31), using mass spectrometry, observed changes in cholesterol. Such lipid changes were not seen in a mouse model of arthritis (32), probably because the investigators filtered samples to remove protein and the associated lipids. Changes in lipid profiles in the blood of RA patients have been widely described, and have been suggested to be a major contributing factor to the accelerated atherosclerosis associated with RA (7,8).

We found that 3-hydroxybutyrate was elevated in the patients with established RA compared to controls. This metabolite is known to be present in RA synovial fluid (33), in pouch fluid from the rat air-pouch model (34), and in the blood of mice with experimental arthritis (32). The presence of 3-hydroxybutyrate, a ketone body, suggests an increased level of lipolysis in RA patients compared with controls. This may be another explanation for the decreased levels of lipid that we have observed, and is consistent with the findings of earlier spectroscopic studies of synovial fluid (33), which determined that lipid metabolism may be a predominant source of energy in the hypoxic inflammatory joint.

Our PLS-R analysis revealed a clear correlation between the CRP level and the metabolites present. This was replicated in a separate group of patients. This approach indicated that increased concentrations of many of the same metabolites discovered in the PCA and OPLS-DA models, for example, low-density lipoprotein lipids, lactate, glucose, taurine, acetylglycine, and methylguanidine, were predictive of the inflamma-

tory phenotype. However, a number of amino acids (e.g., choline, threonine, and methylhistidine) were also found to contribute strongly to the correlation (Table 3). Thirteen of the 14 metabolites that were most strongly associated with CRP were the same for both groups of patients.

Many of the metabolites that were correlated with inflammation, as measured by serum CRP levels, are associated with lipid metabolism and may contribute to the increased levels of atherosclerosis associated with inflammatory disease. Abnormalities in lipids have been associated with pre-RA (35), early RA (36,37), and established RA (38–40), and lipid levels correlate with CRP even in the absence of clinically apparent inflammatory disease (41). Furthermore, lipid-lowering therapies influence inflammation (42,43) and antiinflammatory therapies influence lipids (44), demonstrating a complex relationship between inflammation and lipid metabolites which our data help to dissect.

Blood donors who went on to develop RA at least a decade later have been shown to have significantly more atherogenic blood lipid profiles than those who did not develop RA (45). While this difference did not correlate with CRP levels (45), it does suggest that lipid profiles early in disease might be predictive of outcome. However, in the present study we were unable to discriminate between patients with early arthritis who went on to develop persistent disease and those whose arthritis resolved. Although there was some separation between patients with persistent disease and those with resolving outcomes in both groups, the discriminating metabolites differed. This may result from the heterogeneity between the 2 groups of patients with early arthritis, for example, in terms of the proportion whose disease persisted (Table 1), and so to confirm and extend our observations, these analyses need to be replicated in independent cohorts.

In conclusion, the metabolic fingerprint reflects inflammatory disease activity in patients with new-onset arthritis. This suggests that the underlying inflammatory processes drive significant changes in metabolism that can be measured in the peripheral blood. This may give us further insights into the mechanism of inflammation in inflammatory arthritis, as has been the case following the identification of contributing metabolites in other diseases (16). Furthermore, metabolomics may prove useful as a measure of the extent of disease, potentially separating patients with low disease activity states from patients in true remission.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Young had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Young, Kapoor, Filer, Buckley, Raza.
Acquisition of data. Young, Kapoor, Filer.
Analysis and interpretation of data. Young, Kapoor, Viant, Byrne, Kitas, Raza.

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Metabolic Profiling Predicts Response to Anti–Tumor Necrosis Factor α Therapy in Patients With Rheumatoid Arthritis

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Objective. **Anti–tumor necrosis factor (anti-TNF) therapies are highly effective in rheumatoid arthritis (RA) and psoriatic arthritis (PsA), but a significant number of patients exhibit only a partial or no therapeutic response. Inflammation alters local and systemic metabolism, and TNF plays a role in this. We undertook this study to determine if the patient's metabolic fingerprint prior to therapy could predict responses to anti-TNF agents.**

Methods. **Urine was collected from 16 RA patients and 20 PsA patients before and during therapy with infliximab or etanercept. Urine metabolic profiles were assessed using nuclear magnetic resonance spectroscopy. Discriminating metabolites were identified, and the relationship between metabolic profiles and clinical outcomes was assessed.**

Results. **Baseline urine metabolic profiles discriminated between RA patients who did or did not have a good response to anti-TNF therapy according to European League Against Rheumatism criteria, with a sensitivity of 88.9% and a specificity of 85.7%, with several metabolites contributing (in particular histamine, glutamine, xanthurenic acid, and ethanolamine). There was a correlation between baseline metabolic profiles and the magnitude of change in the Disease Activity Score in 28 joints from baseline to 12 months in RA patients ($P = 0.04$). In both RA and PsA, urinary metabolic profiles changed between baseline and 12 weeks of anti-TNF therapy. Within the responders, urinary metabolite changes distinguished between etanercept and infliximab treatment.**

Conclusion. **The clear relationship between urine metabolic profiles of RA patients at baseline and their response to anti-TNF therapy may allow development of novel approaches to the optimization of therapy. Differences in metabolic profiles during treatment with infliximab and etanercept in RA and PsA may reflect distinct mechanisms of action.**

The introduction of anti–tumor necrosis factor α (anti-TNFα) treatment has revolutionized the

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Drs. Kapoor and Filer contributed equally to this work. Drs. Raza and Young contributed equally to this work.

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management of rheumatoid arthritis (RA) (1–4). Several agents are available within this class, but response rates are imperfect; only 26–42% of patients achieve a good European League Against Rheumatism (EULAR) response (5) within 6 months (6–8). Given the high cost of these therapies and implications for disease progression in nonresponders waiting 3–6 months for clinical reassessment, the ability to predict treatment responses at baseline is an important goal.

The etiology of RA is not fully understood but involves both genetic and environmental factors. In addition to synovitis there are widespread systemic effects mediated by proinflammatory cytokines that affect metabolism. Muscle wasting is a common feature of RA and its extent is associated with RA disease activity (9), but low body mass index is uncommon as fat mass is preserved or even increased (10). The extent of the metabolic changes and the types of metabolites seen may therefore be good markers of cytokine-mediated inflammatory processes in RA.

Several studies have used metabolomic analysis in patients and animal models of inflammatory disease (11–15). Given the integrated nature of systemic metabolism, the analysis of multiple metabolites may provide a better understanding of the disease-associated changes. Metabolomic analysis, based on nuclear magnetic resonance (NMR) spectroscopy of biofluids, can be used to identify a broad range of metabolites simultaneously. Using this approach, the identification of several metabolites in cancer and cardiovascular disease has provided insights into disease mechanisms and has highlighted their potential as biomarkers of disease activity and response to therapy (16–18).

Systemic changes in many low molecular weight metabolites are reflected by their levels in urine, and, indeed, metabolomic analysis of urine samples has been used in inflammatory conditions such as inflammatory bowel disease (IBD) (19–21), to successfully distinguish different types of IBD, and to identify the presence of ongoing intestinal inflammation. Metabolomic profiles have also been shown to be altered during therapy (16). Consequently, we sought to assess whether metabolomic profiles in the urine may have a role in predicting responses to TNF antagonists in patients with RA and psoriatic arthritis (PsA).

PATIENTS AND METHODS

Patients. Patients were part of a multicenter study (Glasgow Royal Infirmary [PsA patients only], Queen Elizabeth Hospital, Birmingham [PsA patients only], and Charing Cross Hospital, London [RA patients only]) comparing responses to infliximab and etanercept. All patients were age ≥18 years. RA patients were required to fulfill the 1987 revised classification criteria of the American College of Rheumatology (22), to be positive for rheumatoid factor (RF) and/or anti–cyclic citrullinated peptide (anti-CCP) antibodies, and to have a disease duration of ≥6 months and a Disease Activity Score in 28 joints (DAS28) of ≥4.0 (23). The PsA patients were required to have psoriasis at screening, ≥3 swollen and ≥3 tender peripheral joints, negativity for RF and anti-CCP antibodies, and a disease duration of ≥6 months. Treatment with at least 1 disease-modifying antirheumatic drug (DMARD) had failed for all patients, and all patients were treated with methotrexate at a dose of at least 7.5 mg weekly, stable for at least 4 weeks prior to commencing anti-TNFα therapy. No other DMARDs were allowed within the 4 weeks prior to commencing treatment, but prednisolone was allowed provided the dose remained stable and did not exceed 10 mg daily.

Participants (16 RA patients and 20 PsA patients) were randomly assigned to receive 3 mg/kg infliximab at weeks 0, 2, and 6 and then every 8 weeks until week 46, or to receive 25 mg etanercept twice weekly for 52 weeks. Therapy was kept stable for the first 3 months. After 3 months, therapy could be changed as required, including escalation of methotrexate therapy to 25 mg weekly in apparent nonresponders. Clinical data, including erythrocyte sedimentation rate, DAS28, and Health Assessment Questionnaire scores (24), were collected at baseline and monthly up to week 52. A good clinical response in RA was defined as a DAS28 of ≤3.2 and improvement in the DAS28 of >1.2 after therapy (25). A good response in PsA was defined as an improvement in 2 factors (with at least 1 being a joint count) with worsening in none of the following 4 factors: patient's global assessment of disease activity, physician's global assessment of disease activity, tender joint count, and swollen joint count (26). Random urine samples were collected from the patients at baseline and at 12 weeks and were snap-frozen and stored at –80°C. The study was conducted in compliance with the Declaration of Helsinki, and ethical approval was obtained from the West Glasgow Ethics Committee. All subjects gave written informed consent.

Metabolomic analysis. After thawing, urine samples (1 ml) were centrifuged at 13,000*g* for 5 minutes, and samples were prepared using a standard protocol (27). Briefly, urine was buffered with phosphate buffer (100 m*M*), brought to concentrations of 10% D_2O and 0.5 m*M* TMSP, and the pH was adjusted to 7.0 twice over 30 minutes. The sample was then centrifuged and loaded into a standard 5-mm NMR tube for spectroscopy.

One-dimensional (1-D) ^1H spectra were acquired at 300°K using a standard spin-echo pulse sequence with water suppression using excitation sculpting on a Bruker DRX 500 MHz NMR spectrometer equipped with a cryoprobe. Samples were processed and data were calibrated with respect to the TMSP signal. Spectra were read into Prometab (28) (custom written software in MatLab 7; MathWorks) and were truncated to a range of 0.8–10.0 parts per million. Spectra were segmented into 0.005 ppm (2.5 Hz) chemical shift "bins," and the spectral areas within each bin were integrated. Spectra were corrected for baseline offset and then normalized to a total spectral area of unity, and a generalized log transforma-

tion was applied (28). Binned data were then compiled into a matrix, with each row representing an individual sample.

Measurement of metabolites. Glutamine levels were measured in the urine samples using high-performance ion-exchange chromatography. Xanthurenic acid levels were measured using a fluorometric method (29).

Statistical analysis. The data bins from groups of spectra were mean-centered and then assessed using 3 techniques. First, partial least-squares discriminant analysis (PLS-DA) was used to perform supervised clustering of samples using PLS_Toolbox (version 5.8; Eigenvector Research) in MatLab (release 2009a). PLS-DA was cross-validated using Venetian blinds (30), a method that reassigns randomly selected blocks of data to the PLS-DA model to determine the accuracy of the model in correctly assigning class membership. Second, GALGO, a package available in the statistical environment R, was used to further model the relationship between good responders and those who did not respond well using a genetic algorithm search procedure coupled to statistical modeling methods for supervised classification (31). The results of GALGO analyses are presented as principal components analysis (PCA) plots, where the x- and y-axes represent first and second principal components providing the greatest variation between samples and the next largest unrelated variation, respectively. GALGO analysis was cross-validated using K-fold cross-validation, where the original sample is randomly partitioned into subsamples and each observation is used for both training and validation. Finally, we used partial least-squares regression, a regression method that identifies which metabolites can predict a continuous variable. This analysis yields r^2, a measure of the goodness-of-fit of the linear regression, while permutation testing assessed the significance of this prediction.

Lists of metabolites providing the greatest discrimination between groups were then identified for each technique. Using multivariate analyses, peaks with large weightings were identified from the PLS-DA weightings plot. Metabolites were identified using these peaks. GALGO analysis produces a list of "bins" of ranked importance which contribute to the separation between the groups. The partial least-squares regression model represents the 90 "bins" or regions of the spectra which had the greatest influence on the correlation with the change in DAS28. These bins were used to identify the discriminatory metabolites. An NMR database (Human Metabolome Database version 2.5) and Chenomx NMR suite were used to identify the metabolites.

RESULTS

Prediction of response to anti-TNF therapy. After 12 months of anti-TNF therapy, RA patients were divided into 2 groups according to their response, as determined by EULAR criteria (Table 1). Response to anti-TNF therapy was also assessed at 3 months, but only 4 patients had a good response (as determined by EULAR criteria) at this stage. Only 1 PsA patient did not respond to treatment with a TNF antagonist according to the predefined response criteria; it was therefore not possible to look at prediction of response in PsA.

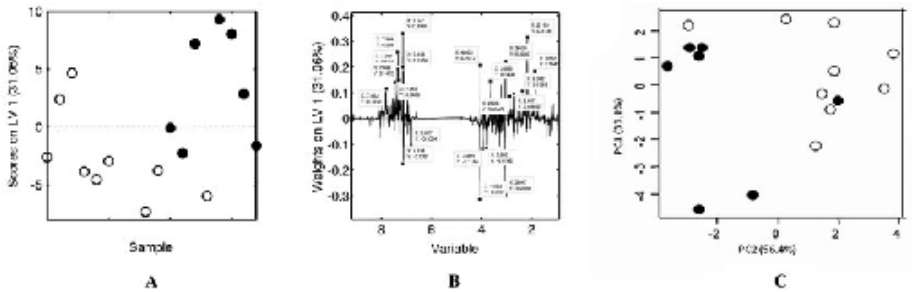
NMR spectra of stored baseline urine samples were acquired and analyzed in order to identify differences between the 2 groups. Supervised PLS-DA analysis (Figure 1A) showed a clear distinction between patient groups segregated according to clinical response. This model distinguished samples with or without a good response with a sensitivity of 66.7% and a specificity of 57.1%. A weightings plot, which indicates regions of the NMR spectra that contribute to this separation (Figure 1B), was used to identify the discriminatory metabolites responsible for the difference in response, and these are shown in Table 2.

The partial least-squares regression model represents the 90 "bins" or regions of the spectra that had the greatest influence on the correlation with the change in DAS28. The GALGO model identifies the bins that have the greatest influence on the separation. For the PLS-DA model, the metabolites were identified from the weightings plot, which indicates regions of the NMR spectra that contribute to the separation. The top 20 bins were identified using GALGO and partial least-squares regression and the metabolites identified from these 20 bins. From the PLS-DA weightings plot the top 20 peaks

Table 1. Baseline characteristics of rheumatoid arthritis patients, by response to anti-TNF therapy at 12 months*

	Good response to TNF antagonists (n = 7)	Not good response to TNF antagonists (n = 9)†
Age, years	50.0 ± 13.40	52.67 ± 12.83
Female, no. (%)	7 (100)	9 (100)
BMI, kg/m^2	26.16 ± 3.75	26.97 ± 5.64
Prednisolone, no. receiving	1	2
NSAIDs, no. receiving	4	4
Baseline methotrexate dose, mg/week	13.57 ± 4.76	15.83 ± 6.12
DAS28	6.041 ± 1.06	6.46 ± 0.91
CRP, mg/ml	21.31 ± 15.97	7.02 ± 7.97
RF positive, no.	6	8
Anti-CCP antibody positive, no.	6	8
Urinary albumin-to-creatinine ratio	1.60 ± 2.71	0.29 ± 0.45

* Except where indicated otherwise, values are the mean ± SD. Except for the C-reactive protein (CRP) level ($P = 0.03$ by unpaired t-test), there were no significant differences between the groups. Anti-TNF = anti–tumor necrosis factor; BMI = body mass index; NSAIDs = nonsteroidal antiinflammatory drugs; DAS28 = Disease Activity Score in 28 joints; RF = rheumatoid factor; anti-CCP = anti–cyclic citrullinated peptide.
† Corresponds to moderate and poor response, as determined by European League Against Rheumatism criteria.



A B C

Figure 1. Metabolic fingerprinting distinguishes between baseline urine samples from rheumatoid arthritis (RA) patients who have good responses to tumor necrosis factor (TNF) antagonists at 12 months and those who do not. **A,** One-dimensional ^{1}H nuclear magnetic resonance (NMR) spectra of baseline urine samples from RA patients with (solid circles) or without (open circles) a good response to TNF antagonists at 12 months were subjected to supervised analysis (partial least-squares discriminant analysis [PLS-DA]). The sample number is plotted against the score in (contribution to) latent variable 1 (LV1), and the percent value on the y-axis shows the proportion of the total variance in the data captured by LV1 in the PLS-DA model. The horizontal dashed line at zero segregates samples with positive and negative scores. **B,** Weightings plot of the PLS-DA model of spectral data from baseline urine samples from the RA patients who did or did not have a good response at 12 months highlights major regions of the spectra that distinguish between the sample groups. The major discriminating peaks are labeled with their chemical shift (in parts per million) on the x-axis and LV weighting on the y-axis. The percent value on the y-axis indicates the proportion of the total variance in the data captured by LV1. **C,** One-dimensional ^{1}H NMR spectra of baseline urine samples from RA patients with (solid circles) or without (open circles) a good response to TNF antagonists at 12 months were subjected to principal components analysis using GALGO analysis. The percent values on the x and y axes indicate the proportion of the variance captured by each principal component (PC1 and PC2).

were identified, and the metabolites were identified from these.

GALGO analysis was then used to reanalyze the data, first in order to verify the results obtained using a

Table 2. Baseline urinary metabolites most strongly correlated with response to anti-TNF therapy using partial least-squares regression, GALGO, and PLS-DA models*

Rank†	Metabolite (chemical shift[s], in ppm), PLS-DA model‡	VIP value§	Metabolite (chemical shift[s], in ppm), GALGO model	Metabolite (chemical shift[s], in ppm), partial least-squares regression model
1	↑ Histamine (7.15)¶	91.54	Trimethylamine (2.87)	Citrate (2.67, 2.53, 2.56)
2	↑ Glutamine (2.16, 2.34)¶	87.72	Thiamine (7.41)	Phosphocreatinine (3.04, 3.94)#
3	↓ p-hydroxyphenylpyruvic acid (4.06, 7.14, 6.82, 7.12)#	72.72	Histamine (7.24, 8.01, 8.00, 7.12)¶	Ethanolamine (3.16, 3.74)¶
4	↓ Phosphocreatine (3.05, 3.94)#	60.58	Thymine (7.37, 7.35, 1.86)#	Creatinine (4.05, 4.06, 3.05)#
5	↑ Thymine (7.35, 1.86)#	61.8	Ethanolamine (3.17, 3.80)¶	Histamine (7.13, 3.28, 7.15)¶
6	↑ Creatinine (3.04, 4.05)#	50.58	Phenylacetic acid (7.29, 7.39, 7.30)#	Glutamine (2.16)¶
7	↑ Xanthurenic acid (7.37, 7.13)¶	36.71	Glutamine (2.12, 2.13)¶	p-hydroxyphenylpyruvic acid (6.82, 6.8225, 4.06, 7.12)#
8	↑ Phenylacetic acid (3.63, 7.44, 7.32)#	21.48	Xanthurenic acid (6.96)¶	Dimethylamine (2.70)
9	↑ Xanthine (7.82)#	12.68	Xanthine (7.90)#	Xanthurenic acid (7.15)¶
10	↓ Ethanolamine (3.80)¶	7.59	Tartaric acid (4.38)	–
11	–	–	3-phosphoglyceric acid (4.14)	–

* The top 20 bins (partial least-squares regression and GALGO models) and peaks (partial least-squares discriminant analysis [PLS-DA] model) were identified; the metabolites these represent and their corresponding chemical shifts in parts per million are shown.
† Extent of contribution to differentiation between responders and nonresponders to anti–tumor necrosis factor (anti-TNF) agents.
‡ ↑ indicates up-regulation of metabolites in urine samples from patients who had a good response to TNF antagonists. ↓ indicates down-regulation of metabolites in urine samples from patients who had a good response to TNF antagonists.
§ Variable importance of the projection (VIP) values were calculated using PLS-DA.
¶ Identified by all 3 methods.
# Identified by 2 different methods.

further supervised analysis technique, and second to use the superior modeling power of the GALGO genetic algorithm, which removes irrelevant variables more effectively. The PCA plot yielded by GALGO analysis showed a clear distinction between RA patients segregated according to clinical response (Figure 1C). The cross-validation of this model was shown to distinguish samples from patients who would not have a good response and samples from patients who would have a good response with a greatly improved sensitivity of 88.9% and specificity of 85.7%. GALGO analysis was further used to identify the discriminatory metabolites responsible for the difference in response as shown in Table 2.

Finally, the relationship between baseline metabolite profiles and the change in DAS28 over 12 months was assessed using partial least-squares regression. This analysis was repeated 100 times with and without randomization of the NMR bin data. There was a significant association between the change in DAS28 and baseline RA metabolites ($P = 0.04$). Permutation testing with 90 NMR bins included (as optimized by forward selection) demonstrated that the regression model was statistically valid ($P < 0.01$).

There was a significant difference between the C-reactive protein (CRP) level of patients who responded to TNF antagonists and the CRP level of those who did not respond ($P = 0.03$). We therefore used partial least-squares regression to further analyze the relationship between CRP and baseline metabolites in order to investigate potential confounding variables; this did not reveal any significant association ($P = 0.52$), suggesting that the difference we found was independent of the inflammatory processes reflected in the CRP levels. Grouping the metabolite data into quartiles according to the CRP values also failed to separate patient groups on PCA or PLS-DA (data not shown). Previous studies have shown that patients with RA have subclinical nephropathy (32,33) and that the urinary albumin-to-creatinine ratio is a sensitive marker of disease activity in RA (32). We measured the albumin-to-creatinine ratio in the urine samples, and there was no significant difference in this ratio between patients who responded to TNF antagonists and those who did not ($P = 0.17$). We also performed regression analysis for metabolic profiles at baseline against the albumin-to-creatinine ratio, and this was not significant ($P = 0.31$), suggesting that the relationship we found between baseline urinary metabolic profiles and the DAS28 was independent of microalbuminuria.

Table 3. Characteristics of the rheumatoid arthritis and psoriatic arthritis patients, by treatment group*

	Patients receiving infliximab (n = 18)	Patients receiving etanercept (n = 16)
Age, years	48.6 ± 10.60	47.46 ± 14.78
Female, no. (%)	13 (72)	13 (81)
BMI, kg/m^2	28.43 ± 4.56	28.36 ± 9.33
Prednisolone, no. receiving	6	3
NSAIDs, no. receiving	10	9
DAS28 at baseline†	6.22 ± 0.95	6.35 ± 1.05

* Except where indicated otherwise, values are the mean ± SD. See Table 1 for definitions.
† Rheumatoid arthritis patients only.

Comparison of metabolites predicting response to therapy in RA. Metabolites that were associated with a change in the DAS28 are shown in Table 2. The metabolites histamine, glutamine, xanthurenic acid, and ethanolamine were identified by all 3 analytic methods. Furthermore, several metabolites were identified by at least 2 of the 3 different methods, including p-hydroxyphenylpyruvic acid, phosphocreatine, thymine, creatinine, phenylacetic acid, and xanthine. These findings cross-validate the analyses used. We were also able to identify glutamine and xanthurenic acid in the urine samples that were used for NMR analysis using ion-exchange chromatography and a fluorometric method, respectively. There was a good correlation between the NMR peak heights and the assayed levels of xanthurenic acid ($r = 0.73$ [Spearman's correlation coefficient], $P = 0.001$) and a strong trend in the results for glutamine ($r = 0.46$ [Spearman's correlation coefficient], $P = 0.07$), which helped to validate our interpretation of the NMR data. However, the assayed levels of glutamine and xanthurenic acid were not significantly higher in the urine samples from the patients who had a good response, which suggests that while these individual metabolites contribute strongly to the discrimination, the whole set of metabolites present in the fingerprints is needed to fully separate the groups.

Effect of TNFα antagonists on metabolite profiles. The details of the patients receiving etanercept and infliximab are shown in Table 3. We investigated the effect of anti-TNF therapy on metabolic profiles longitudinally, comparing baseline and 12-week (during therapy) urine samples using supervised PLS-DA analysis (sensitivity of 71.4% and specificity of 57.1% in RA and sensitivity and specificity of 61.1% in PsA) and GALGO analysis (sensitivity of 100% and specificity of 82.9% in RA and sensitivity of 71.8% and specificity of 69.5% in

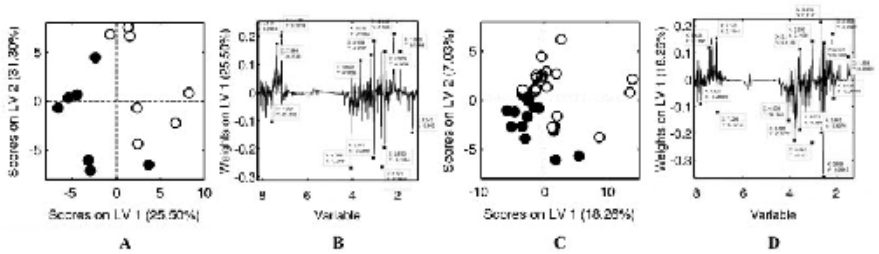


Figure 2. Metabolic fingerprinting enables identification of metabolites that are altered after treatment with TNF antagonists in patients with a good response. **A,** One-dimensional ^{1}H NMR spectra of urine samples obtained at baseline (open circles) and 12 weeks (solid circles) from RA patients with a good response to TNF antagonists at 12 months were subjected to supervised analysis (PLS-DA). The score in LV1 is plotted against the score in LV2, and the percent values on the x and y axes show the proportions of the total variance in the data captured by LV1 and LV2 in the PLS-DA model. The horizontal dashed lines at zero segregate samples with positive and negative scores. **B,** Weightings plot of the PLS-DA model of spectral data from urine samples obtained from RA patients who responded to TNF antagonists highlights major regions of the spectra that distinguish between the baseline and 12-week samples. The major discriminating peaks are labeled with their chemical shift (in parts per million) on the x-axis and LV weighting on the y-axis. The percent value on the y-axis indicates the proportion of the total variance in the data captured by LV1. **C,** One-dimensional ^{1}H NMR spectra of urine samples obtained at baseline (open circles) and 12 weeks (solid circles) from psoriatic arthritis (PsA) patients with a good response to TNF antagonists at 12 months were subjected to supervised analysis (PLS-DA). The score in LV1 is plotted against the score in LV2, and the percent values on the x and y axes show the proportions of the total variance in the data captured by LV1 and LV2 in the PLS-DA model. The horizontal dashed lines at zero segregate samples with positive and negative scores. **D,** Weightings plot of the PLS-DA model of spectral data from urine samples from PsA patients who responded to TNF antagonists highlights major regions of the spectra that distinguish between the baseline and 12-week samples. The major discriminating peaks are labeled with their chemical shift (in parts per million) on the x-axis and LV weighting on the y-axis. The percent value on the y-axis indicates the proportion of the total variance in the data captured by LV1. See Figure 1 for other definitions.

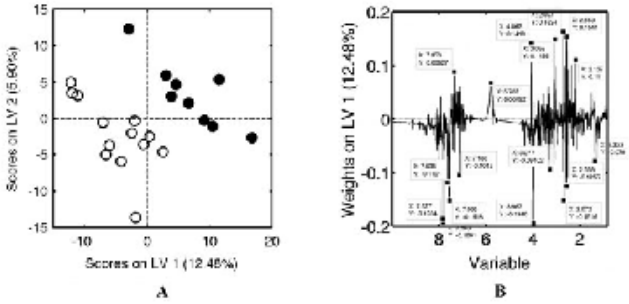


Figure 3. Metabolic fingerprinting of urine samples from RA patients and psoriatic arthritis (PsA) patients. **A,** One-dimensional ^{1}H NMR spectra of urine samples obtained 12 weeks after treatment with infliximab (open circles) or etanercept (solid circles) from RA and PsA patients with a good response to treatment were subjected to supervised analysis (PLS-DA). The score in LV1 is plotted against the score in LV2, and the percent values on the x and y axes show the proportions of the total variance in the data captured by LV1 and LV2 in the PLS-DA model. The horizontal dashed lines at zero segregate samples with positive and negative scores. **B,** Weightings plot of the PLS-DA model of spectral data from urine samples obtained after treatment with infliximab or etanercept from RA and PsA patients with a good response at 12 months highlights major regions of the spectra that distinguish between the sample groups. The major discriminating peaks are labeled with their chemical shift (in parts per million) on the x-axis and LV weighting on the y-axis. The percent value on the y-axis indicates the proportion of the total variance in the data captured by LV1. See Figure 1 for other definitions.

PsA). Using the weightings plot, we determined that in patients with RA who responded to TNF antagonists there were high levels of glutamine, phenylacetic acid, and histamine in the baseline urine samples and higher levels of methylamine and creatinine in the 12-week urine samples. Similar changes in metabolites were also seen in the urine samples from the patients with PsA who responded to TNF antagonists (Figure 2).

Combining RA and PsA patients with a good response, we assessed which urinary metabolites changed after 12 weeks of treatment with infliximab and with etanercept using supervised PLS-DA analysis (sensitivity of 84.6% and specificity of 55.6%) (Figure 3) and GALGO analysis (sensitivity of 86.2% and specificity of 100%). Using the weightings plot, we found that increases in hippuric acid, citrate, and lactic acid were seen with infliximab treatment and increases in choline, phenylacetic acid, urea, creatine, and methylamine were seen with etanercept treatment. Due to the small patient numbers, we could not investigate the effects of etanercept and infliximab in RA and PsA separately.

DISCUSSION

There were clear differences in the metabolic profiles of baseline urine samples of patients with RA who responded well to anti-TNF therapy compared with those who did not. This difference may be important as a novel predictor of responses to TNF antagonists. We have used 3 different data analysis methods to predict response, and each found that similar metabolites contributed. We have used GALGO analysis as well as PLS-DA analysis as it has been shown that genetic algorithms optimize the results by removing irrelevant variables, which dramatically improves the classification ability of models (34). All 3 methods identified histamine, glutamine, xanthurenic acid, and ethanolamine, while both PLS-DA and partial least-squares regression identified creatinine, p-hydroxyphenylpyruvic acid, and phosphocreatine, and both PLS-DA and GALGO identified phenylacetic acid and xanthine. Histamine, glutamine, phenylacetic acid, xanthine, xanthurenic acid, and creatinine were up-regulated in the urine samples from the patients who had a good response to therapy, while ethanolamine, p-hydroxyphenylpyruvic acid, and phosphocreatine were down-regulated.

One metabolite we identified as a strong discriminator in baseline urinary metabolites was histamine. Urinary histamine metabolites have also been suggested as a marker of disease activity in IBD (35), suggesting that it may be a generic marker of inflammatory processes. Histamine is most obviously associated with mast cell–dependent processes such as allergy, and histamine has been identified as a constituent of synovial fluid in arthritis (36). Histologic examination of synovial infiltrates in early RA has shown mast cells to be present (37), suggesting that these cells could be the source of the discriminating histamine. However, an alternative but significant route for histamine generation is via histidine degradation. Histamine arises in many tissues by the decarboxylation of histidine (38). It has long been known that TNFα promotes cachexia associated with chronic inflammatory disease, and this cytokine is known to have direct effects in accelerating muscle breakdown, leading to the release of free amino acids including histidine (39). Consistent with this, levels of histidine have been shown to be considerably higher in patients with RA and patients with systemic lupus erythematosus (40) compared to levels in controls.

Several of the other metabolites that we observed were also associated with catabolic processes and tissue degradation; for example, glutamine, xanthurenic acid, and ethanolamine can result from tryptophan and other amino acid degradation pathways. Tryptophan has been shown to be down-regulated in the plasma of patients with ankylosing spondylitis (AS) compared with the plasma of controls (41). The release of tryptophan from its binding serum protein has been shown to correlate with improvement in disease activity in AS (41), and this may be the same in RA.

A previous metabolomic study has suggested that alterations in serum levels of amino acids may be a useful marker of the presence and severity of osteoarthritis in the knee (42). The urine markers that we found either may be indicators of joint-specific degradation processes or may result from the systemic muscle and tissue changes associated with chronic disease, many of which are mediated through TNFα.

Previous work has investigated predictors of response to TNFα antagonists. Analysis of patients in the British Society for Rheumatology Biologics Register found that treatment with methotrexate or nonsteroidal antiinflammatory drugs (NSAIDs) predicted response to TNF antagonists (43). All the patients in our study were receiving methotrexate, and there were an equal number of patients receiving NSAIDs who had a good response compared to those who did not. Smoking has been associated with a poor response to infliximab (43), but only 1 of our patients smoked. Another group has found that the presence of RF or anti-CCP antibodies is associated with a reduced response to TNF antagonists (44), but all of our RA patients were positive for RF

and/or anti-CCP antibodies. Baseline levels of TNFα may predict the dose of infliximab needed for optimal response (45), and other work has demonstrated that a combination of blood cytokines and autoantibodies can predict responses to etanercept (46). In our cohort there was a significant difference between CRP levels in patients who responded to TNF antagonists and those who did not. However, the partial least-squares regression analysis failed to find an association between CRP and baseline metabolites, suggesting that the association between baseline metabolites and response is independent of CRP.

Infliximab and etanercept alter metabolites in the urine differently, as there are clear differences in the metabolites at 12 weeks posttreatment. Increases in the metabolites hippuric acid, citrate, and lactic acid were associated with infliximab treatment, and increases in the metabolites choline, phenylacetic acid, urea, creatine, and methylamine were associated with etanercept treatment. The presence of choline suggests that etanercept may alter lipid metabolism.

We have also shown that the same metabolites are altered in the urine samples from patients with RA and PsA who responded to TNF antagonists. It may therefore be that chronic inflammatory diseases respond by a common mechanism to TNF antagonists.

This is the first demonstration that metabolomic techniques using 1-D NMR spectra can predict outcome to anti-TNF therapy in patients with severe RA, providing a sensitivity and specificity for response that has potential clinical utility despite a small initial cohort of patients. Our findings are verified by repeat analysis using alternative statistical techniques. There is a pressing need to confirm and extend these findings in a larger cohort of patients, combining metabolomic analyses with CRP and cytokine and autoantibody analyses to develop tests that can predict response without the need for empirical treatment, bringing closer the era of individually tailored therapy.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Young had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Kapoor, Filer, Fisher, Taylor, Buckley, McInnes, Raza, Young.

Acquisition of data. Kapoor, Filer, Fisher, Taylor, Buckley, McInnes, Young.

Analysis and interpretation of data. Kapoor, Filer, Fitzpatrick, Fisher, Raza, Young.

ROLE OF THE STUDY SPONSOR

Merck/MSD had no role in the study design or in the collection, analysis, or interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication. Publication of this article was not contingent upon approval by Merck/MSD.

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11

Metabolomics in the Analysis of Inflammatory Diseases

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1. Introduction

Inflammation is a normal and extraordinarily important component of responses to infection and injury. The cardinal features of swelling, redness, stiffness and increasing temperature are strong indicators of the significant changes in tissue metabolism and the ingress of immune cells into the tissues. The increase in blood flow which underlies many of these changes may result in changes to the supply of nutrients and in particular the level of oxygen in the tissues. Inward migration of immune cells, which is also enabled by the increased blood flow, will put further stress on the metabolic environment of the tissues. The activity of macrophages and neutrophils in clearing infection and repairing tissue damage also have significant metabolic consequences particularly because of the production of cytokines and cytotoxic molecules such as reactive oxygen species and reactive nitrogen species, which are required to kill invading organisms. Production of these molecules will consume considerable quantities of oxygen, ATP and NADPH. These antimicrobial agents put considerable stress on host cells in the surrounding and distal tissues and can lead to significant loss of protective metabolites such as glutathione.

Most infections and traumatic injuries are cleared or repaired relatively rapidly and metabolic homoeostasis is soon restored. However, there is a broad range of inflammatory diseases which involve chronic activation of the immune system and, as a result, chronic persistent inflammation. We have been studying the metabolic consequences of chronic inflammatory diseases with the aim of identifying metabolic fingerprints which may provide clues about why the localised tissue disease persists. For example, why in rheumatoid arthritis does persistent inflammation lead to widespread cartilage and joint destruction? However, the metabolic consequences of chronic inflammation are much more widespread than the localised disease and can lead on to important comorbidities such as accelerated atherosclerosis and cardiovascular disease. Metabolomic analysis may be able to distinguish between localised and systemic metabolic consequences of inflammation and provide novel targets for therapeutic intervention in these important human diseases.

2. Introduction to inflammatory disease

An indication of the strong link between inflammation and metabolic processes is seen in cachexia, the loss of cellular mass associated with disease. The discovery of the involvement of tumour necrosis factor- alpha (TNFα) in this process earned it the name 'cachexin'. While TNFα is now known more generally as a mediator of inflammatory responses, the ability of inflammatory cytokines to have such profound effects on cellular and metabolic processes is informative. Systemic inflammation such as that seen in RA causes changes in metabolism and rheumatoid cachexia is a result of chronic inflammation. This is characterised by the loss of muscle mass and preservation of fat mass (Evans et al., 2008). Classically cachexia is characterised by a low BMI. Muscle wasting is a common feature of RA but low BMI is uncommon as the fat mass is preserved or even increased (Summers et al., 2008). Hence, RA patients may present with either the classic low BMI cachexia (1-13% of RA population) (Munro & Capell, 1997) or more frequently, the rheumatoid cachexia (10-20% of RA with controlled disease and 38% of patients with active RA) (Engvall et al., 2008, Metsios et al., 2009).

The muscle loss that occurs in rheumatoid cachexia is thought to be due to proinflammatory cytokines such as TNFα, IL1 and IL6. TNF promotes proteolysis through the ubiquitin-proteasome pathway. There is also some evidence that cytokines may prevent an increase in muscle protein synthesis in response to feeding (anabolic resistance) (Summers et al., 2010). In rheumatoid cachexia the degree of muscle wasting is associated with the disease activity of RA (Summers et al., 2010).

2.1 The inflammatory process

An acute inflammatory reaction is characterised by the classic cardinal signs of inflammation: heat, redness, swelling and pain. In experimental settings the temporal relationships oedema, accumulation of leukocytes and accumulation of monocytes and macrophages are well established. These events in self-limited inflammatory reactions are coupled with the release of local factors which prevent further release of leukocytes, which allows resolution (Serhan, 2009). The transition from acute inflammation to chronic inflammation is widely viewed as a result of an excess of pro-inflammatory mediators.

2.2 Inflammatory mediators

Cytokines are important regulators of inflammation. Some cytokines such as TNFα and interleukin (IL) 1 promote inflammatory responses by inducing cartilage degradation and promoting a cell-mediated immune response. Other cytokines such as IL-4, IL-10 and IL-13 function mainly as anti-inflammatory molecules (Isomaki & Punnonen, 1997). Key biological targets that have been identified as being involved in a destructive inflammatory reaction are COX-2, pro-inflammatory interleukins, TNFα, migration inhibition factor, interferon gamma and matrix metalloproteinases (Ivanenkov et al., 2008).

Several inflammatory mediators have been identified which are common to several inflammatory diseases. It has been shown that C-reactive protein (CRP) is secreted by several cell types and is capable of directly activating immune cells. This supports a role for CRP as an active inflammatory mediator which has systemic and local effects (Montecucco & Mach, 2009).

White adipose tissue has been shown to secrete several inflammatory mediators called adipokines or adipocytokines. These induce their activities by binding to selective transmembrane receptors. Leptin is the most studied adipocytokine and is thought to have an important role in the inflammatory process (Montecucco & Mach, 2009).

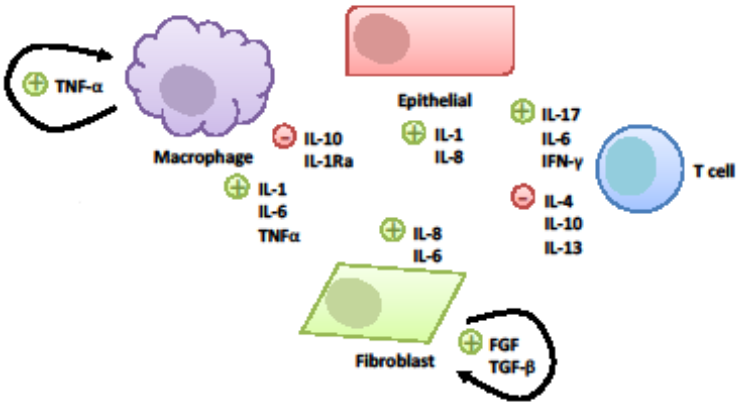

Fig. 1. Key inflammatory cytokines and the inflammatory network. Responses are a balance of pro-inflammatory tumour necrosis factor alpha (TNFα) and interleukin (IL) 1, IL-6, IL-17 and anti-inflammatory IL-1R, IL-4, IL-10 and IL-13. Expression of cytokines is dependent on activation and local signalling driving progression and eventual resolution.

2.3 Metabolic inflammation

Many factors contribute to the complex course of inflammatory reactions. Microbiological, immunological and toxic agents can initiate the inflammatory response by activating a variety of humoral and cellular mediators. In the early phase of inflammation, excessive amounts of interleukins and lipid-mediators are released and play an important role in the pathogenesis of organ dysfunction. Arachidonic acid (AA) is released from membrane phospholipids during inflammatory activation and is metabolised to prostaglandins and leukotrienes. Various strategies have been evaluated to regulate the excessive production of lipid mediators on different levels of biochemical pathways, such as inhibition of phospholipase A2, the trigger enzyme for release of AA, blockade of cyclooxygenase and lipoxygenase pathways and the development of receptor antagonists against platelet activating factor and leukotrienes. Some of these agents exert protective effects in different inflammatory disorders such as septic organ failure, rheumatoid arthritis or asthma, whereas others fail to do so. Encouraging results have been obtained by dietary supplementation with long chain omega-3 fatty acids like eicosapentaenoic acid (EPA). In states of inflammation, EPA is released to compete with AA for enzymatic metabolism inducing the production of less inflammatory and chemotactic derivatives (Heller et al., 1998).

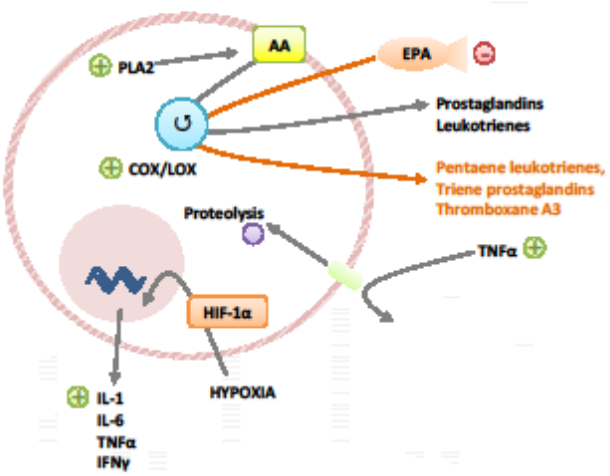

Fig. 2. Some common metabolic responses to inflammation and hypoxia. Arachidonic acid (AA) from cellular membranes is metabolised to inflammatory prostaglandins and leukotrienes. Omega-3 fatty acids (EPA) compete for the same pathway producing less inflammatory derivatives. Hypoxic conditions in the inflammatory site stabilises HIF transcription factor driving production of IL-1, IL-6, TNFα and IFNγ. TNFα in turn drives cellular proteolysis and tissue remodelling.

When investigating inflammation it is important to take into account the many facets of the inflammatory environment that have the potential to play a role in pathology. Hypoxia is known to be prevalent in the inflammatory environments such as those associated with wounds, malignant tumours, bacterial infections and autoimmunity (Eltzschig & Carmeliet, 2011, Murdoch et al., 2005). Increasing hypoxia in the inflammatory site is associated with poorer disease outcome such as increased macroscopic synovitis in rheumatoid arthritis (Ng et al., 2010).

Normal physiological oxygen levels are thought to range between 5-12% oxygen (compared to 21% atmospheric oxygen). However, hypoxic tissue oxygen levels in pathological environments can range from as little as 0.5% oxygen to around 2.5% oxygen. Local hypoxia develops as the result of either blood vessel occlusion by inflamed tissues, or when existing supply is insufficient for increased cellular density caused by infiltrating or proliferating inflammatory cells. Additionally, circulating phagocytes can block blood vessels reducing blood flow into the inflammatory site (Sitkovsky & Lukashev, 2005). Normal tissue structures can lend themselves to hypoxia where they are poorly perfused, such as the synovium or eye. Tissue alteration associated with inflammation can contribute to hypoxia by altering pressure within the blood vessels causing vessel occlusion and increasing distances between blood vessels (Jawed et al., 1997, Mapp et al., 1995).

There is increasing evidence that the inflammatory environment is hypoxic. The tumour environment is known to be hypoxic and extensive angiogenesis reveals the requirement of the tissue for a better oxygen supply. In rheumatoid arthritis, oxygen levels of synovial fluid have been directly measured revealing lower oxygen tensions compared with osteoarthritic patients and patients with traumatic joint injuries (Lund-Olesen, 1970). In systemic sclerosis, direct measurements with sensitive probes revealed lower dermal oxygen levels in fibrotic areas compared to non-fibrotic areas in both patients and healthy controls (Beyer et al., 2009). Metabolomic analysis of eye fluids from uveitis patients has shown increased levels of oxaloacetate and urea, likely derived from anaerobic respiration by locally activated macrophages (Young et al., 2009, Young & Wallace, 2009).

An elegant cellular oxygen detection system is used by cells to respond to changes in environmental oxygen. Reductions in environmental oxygen lead to the stabilisation of the transcription factor hypoxia-inducible factor (HIF), which is otherwise targeted for depletion in oxygen-rich environments. HIF expression is therefore suggestive of hypoxic exposure, and has been detected in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (Gaber et al., 2009, Hollander et al., 2001, Lassmann, 2003). HIF is known to be important in inflammatory development, for example loss of HIF-1a in macrophages is associated with impaired aggregation, motility, invasiveness and killing of bacteria (Cramer et al., 2003).

Hypoxia and HIF stabilisation has a large effect on cellular metabolism. HIF causes a preference for glycolytic metabolism over oxidative phosphorylation by inducing the expression of glycolytic enzymes. This allows ATP generation to continue in the absence of sufficient oxygen albeit at a much reduced efficiency per molecule of glucose. It also induces the upregulation of lactate dehydrogenase A, therefore promoting the conversion of pyruvate (produced during glycolysis) to lactate (Wheaton & Chandel, 2011). Lactate has been detected in many chronic inflammatory conditions such as in inflamed joints (Chang & Wei, 2011, Treuhaft & McCarty, 1971), multiple sclerosis , pulmonary inflammation (Serkova et al., 2008) and is thought to play a role in wound healing (Trabold et al., 2003). Conversely, the acidosis associated with increasing lactate concentrations is thought to play a pathogenic role in cell transformation and autoantigen development in some inflammatory environments (Chang & Wei, 2011). Recently, lactate measurements have been suggested to be useful in the diagnosis of bacterial infections in diabetic foot ulcers compared to non-infected ulcers. Both infected and non-infected ulcers revealed high lactate concentrations, but infected ulcers had significantly higher levels probably due to additional immune and bacterial cell involvement (Loffler et al., 2011). The detection of lactate in metabolomic studies of disease suggests that there may be an inflammatory component, understanding of which may help to direct future treatment.

Immune cells are thought to be highly influenced by hypoxia and HIF stabilisation especially due to the environments they normally act within. In a study performed recently by Gaber et al., peripheral blood CD4+ T cells placed under hypoxia were found to have a large induction of genes involved in metabolism and homeostasis (Gaber et al., 2009). Innate immune cells such as neutrophils and macrophages are thought to be adapted to function best at lower oxygen tensions as they preferentially use glycolysis to provide ATP even at higher oxygen levels (Cramer et al., 2003). Macrophages are known to accumulate in the hypoxic sites of chronic inflammation (Vergadi et al., 2011), and hypoxia is associated with activation of tissue-resident macrophages. Exposure of macrophages to hypoxic conditions

is associated with upregulation of a whole gamut of proinflammatory cytokines such as IL-1 (Scannell, 1996), IL-6 (Albina et al., 1995), IFN-γ (Murata et al., 2002) and TNF-α (White et al., 2004). It is thought that both low oxygen levels and their downstream effects, such as lactate production, may give rise to this macrophage phenotype. That such phenotypic changes are observed in response to the hypoxic conditions of the inflammatory site is strongly suggestive of a role for metabolism in regulation of immune cells. While normal wound resolution is a tightly regulated process, the presence of long-term inflammatory diseases such as rheumatoid arthritis is indicative of the potential for this regulation to go awry. Therefore hypoxia and the resulting change in metabolism may have a profound effect on immune cell behaviour and thus influence disease onset and progression.

Adenosine is another molecule produced in response to hypoxia partly by the hypoxic inhibition of adenosine kinase (Sitkovsky & Lukashev, 2005). It is difficult to detect due to its local action, but expression of CD39 and CD73, two molecules involved in the extracellular generation of adenosine, provide a marker of its presence in the inflammatory environment. Adenosine can have profound effects on immune cells and is generally perceived to be anti-inflammatory. It is a ligand for specific receptors found on many immune and stromal cells. These receptors are upregulated by hypoxia suggesting hypoxia perpetuates both the production and action of this molecule (Hasko et al., 2008, Sitkovsky & Lukashev, 2005). These receptors have varying downstream effects, with the expression of the A2A associated with the anti-inflammatory disease but the A2B receptor expression being implicated in pro-inflammatory conditions such as colitis. Adenosine is known to cause bronchoconstriction when inhaled by asthma and COPD sufferers, but not in healthy controls (Hasko et al., 2008). Higher levels of adenosine A2 receptor are seen in asthma sufferers and these receptors are associated with a pathological role for the molecule in disease (Brown et al., 2008, Hasko et al., 2008).

2.4 Use of metabolomics in inflammatory diseases

Systemic inflammation causes changes in metabolism and many studies have investigated individual metabolites in human disease and animal models of inflammation. From these results it is apparent that the levels of many metabolites are altered by the inflammatory process and this has provided insights into the mechanisms of disease and uncovered several potential biomarkers for disease assessment.

Given these profound systemic and localised changes in metabolism provoked by inflammation and inflammatory cytokines, it is not surprising that metabolomics has been used to investigate several inflammatory diseases. Metabolomics is able to assess the changes in several hundred metabolites simultaneously to build disease metabolites profiles. NMR spectroscopy and mass spectrometry have both been used to derive these multiplexed metabolite profiles.

These metabolic "fingerprints" have proven useful in discriminating between different patient groups or identifying responses to therapy, even if the individual metabolites have not been identified. However, identification of sets of specific metabolites can be derived from these fingerprints and this has led to the identification of novel biomarkers and novel pathways in a number of inflammatory diseases. The use of metabolomic analysis of inflammatory diseases will now be discussed in further detail.

3. The inflammatory diseases

3.1 Aetiology

Chronic inflammatory diseases exist in many forms, and have the ability to affect many systems of the body. These range from localised areas of inflammation such as the gut in Crohn's disease, to more widespread systemic inflammation as in rheumatoid arthritis (RA). Although the mediators and events leading to chronic inflammation are well characterized, the precise conditions under which acute inflammation becomes chronic are poorly understood. Recent developments have highlighted the importance of genetic factors, environmental influences and the interactions between them in the development of chronic inflammatory disease (Rens et al., 2011).

Research into the genetics of inflammatory disease has been accelerated by genome wide association studies (GWAS), which has allowed identification of genetic mutations associated with an increased risk of developing specific conditions. For example, many immunologically relevant genes have been associated with an increased risk of developing RA. These include human leukocyte antigen (HLA) alleles involved in antigen recognition, and the peptidyl-arginine deiminase type IV (PADI4) gene controlling production of cyclic citrullinated proteins (CCP's) commonly seen in RA (Nishimoto et al., 2010). However, in a complex disease like RA, genetics are not the whole story, as illustrated by the fact that twin studies only report a concordance rate of around 60% (MacGregor et al., 2000). Thus the importance of external environmental factors in the development of inflammatory diseases should be considered.

Chronic inflammatory diseases have become more prevalent in recent years, and as major genetic changes are unlikely to have occurred over such a short time period, this is likely to be a result of alterations in environmental exposures and lifestyle factors. To date, several factors have been identified as significant contributors including ageing, infection, poor nutrition and smoking.

Smoking raises an individual's risk of developing inflammatory disease considerably. It has numerous effects on the body including activation of the acute inflammatory response and introduction of large amounts of reactive oxygen species (ROS) (Borgerding & Klus, 2005). It is unclear as to which particular constituent of smoke induces the inflammatory response; however studies have revealed that smoke contains large amounts of lipopolysaccharide (LPS) (Hasday et al., 1999), which could potentially trigger unwanted immune responses seen in chronic inflammatory disease. An increase in ROS is also evident, as indicated by decreased circulating antioxidants found in smokers (Alberg, 2002). This creates a pro-oxidant environment and increases the likelihood of oxidative damage to important cellular components.

It is not surprising given the complex and varied nature of chronic inflammatory diseases that the observed phenotype is a result of gene-gene and gene-environment interactions. For example, it has been shown in mice with a mutation in the Crohn's disease (CD) susceptibility gene Atg16L1 who become infected with murine norovirus develop a Crohn's-like disease (Stappenbeck et al., 2010). There was no evidence of pathology in the wild type mouse, suggesting the presence of two risk factors is required to induce disease. Another example of gene-environment interactions in disease development was found when looking

at the interaction between RA susceptibility genes HLA-DRB1 and PTPN22 and their interaction with smoking (Kallberg et al., 2007). It was observed that the odds ratio (OR) of developing RA with two genetic risk factors was 13.2, which rose to 23.4 if two genetic factors were present and there was a history of smoking. These studies provides sound evidence that gene-gene and gene-environment interactions occur, and risk of inflammatory disease greatly increases with the presence of more than one additional risk factor.

3.2 The gut

Crohn's disease is a chronic debilitating inflammatory disease of the bowel. The exact aetiology is unknown but is thought to be related to the dysregulation of the immune response towards gut microflora (Strober et al., 2007). Urinary metabolite profiling was carried out on a mouse model of Crohn's disease. These samples were analysed using gas chromatography-mass spectrometry and five key metabolic differences were identified between the Crohn's disease model and controls. This suggested that there are alterations of tryptophan metabolism, fucosylation and fatty acid metabolism in Crohn's disease mice and the authors concluded that fucose and xanthurenic acid could be useful markers of gut inflammation (Lin et al., 2009).

Using a mouse model of inflammatory bowel disease (IBD) to investigate urinary metabolites using NMR, it was found that there was an increase in trimethylamine (TMA) and fucose compared to controls. The increase in TMA was parallel to the progression of IBD (Murdoch et al., 2008). A mouse model of Ulcerative Colitis (UC) was used to looked at serum and urinary metabolites (Schicho et al., 2010). These authors found that both serum and urine were equally powerful for detecting colitis but the metabolites responsible for the differences were different for serum and urine.

Metabolomics of faecal extracts have also been used to study inflammatory bowel disease (Bezabeh et al., 2009). It is sometimes difficult to distinguish Crohn's disease (CD) from UC and some cases are labelled as indeterminate. Over time these cases are usually identified by a combination of endoscopic, radiological and histological techniques. Earlier identification could aid treatment and prognostication. Metabolomic analysis of faecal extracts of patients with both inflammatory diseases showed reduced levels of butyrate, acetate, methylamine and TMA compared to control (Marchesi et al., 2007). Comparing the UC and CD samples glycerol, alanine, isoleucine, leucine, lysine and valine were present in higher quantities in CD compared to UC. Acetate was lower in CD compared to UC (Marchesi et al., 2007). Metabolic differences were more marked in CD indicating that inflammation is more extensive in CD compared to UC.

Urinary metabolites have also been used to distinguish CD and UC in humans (Williams et al., 2009). They found that specific urinary metabolites related to gut metabolism differed between CD, UC and controls. Hippurate was lowest in CD and differed significantly between CD, UC and controls. Formate levels were higher in CD than in UC or controls and 4-cresol sulphate was lower in CD than in UC or controls (Williams et al., 2009). Hippurate has been shown to be modulated according to gut microbes and this difference is likely to reflect changes in intestinal microbes.

In summary several studies have looked at IBD. The studies have shown that both in mice and in humans TMA is an important marker of IBD (Marchesi et al., 2007, Murdoch et al.,

2008, Schicho et al., 2010). This has been shown using both urine samples or faecal extracts. Hence, TMA may be a useful biomarker for IBD.

3.3 The eye

As a closed and immuno-privileged site, the eye provides an ideal system for metabolic analysis. Metabolic products of inflammatory infiltrate accumulate in the vitreous fluid of the eye and may be extracted during other corrective surgery.

Metabolomics has been used to look at vitreous humour in order to differentiate ocular inflammatory diseases (Young et al., 2009). Vitreous fluid samples were taken from patients undergoing retinal surgery and analysed using NMR. Patients had various retinal disorders including chronic non-infectious uveitis (CU), lens-induced uveitis (LIU), proliferative diabetic retinopathy, proliferative vitreoretinopathy (PVR), rhegmatogenous retinal detachment, candida endopthalmitis and varicella zoster virus acute retinal necrosis. The different disease groups showed clear separation using principle component analysis (PCA) and partial least squared discriminate analysis (PLSDA). The majority of the patients had LIU and CU. When looking at LIU and CU specifically there was clear separation and individual metabolites from the spectra showed significant differences with urea, oxaloacetate and glucose all being raised in LIU compared to CU. As urea and oxaloacetate are both involved in the urea cycle it suggests that there is more active inflammation in the LIU patients (Young et al., 2009).

NMR has also been used to look at ocular metabolism in pig eyes (Greiner et al., 1985). They used phosphorous NMR and found phosphorous containing metabolites in aqueous and vitreous fluids (Greiner et al., 1985). In addition to quantifying metabolites, phosphorous NMR can be used to monitor the rate of metabolic change in a specific biochemical reaction and the rate of change in the concentration of a particular metabolite (Greiner et al., 1985). Phosphorous NMR provides a non-invasive method to analyse ocular tissues metabolically and detect subtle biochemical changes that precede manifestations of disease. Such detection may allow for early and more effective therapeutic intervention.

3.4 Neurological disease

Multiple sclerosis (MS) is a chronic inflammatory disease affecting the nervous system. Its aetiology is still not completely understood (Ibrahim & Gold, 2005). It is characterised by demyelination, axonal loss and breakdown of the blood-brain barrier (Trapp et al., 1999). It is a heterogeneous, relapsing and remitting disease. Different treatments have been shown to work at different stages of disease (Rieckmann & Smith, 2001) so it is important to identify biomarkers that enable identification of different phases.

Interleukin-1β (IL-1β) and TNF-α, have been found to be associated with a broad spectrum of neurological diseases including MS. Griffin *et al* looked at rat urines to determine whether NMR spectroscopy could detect the presence of IL-1β and TNF-α induced lesions and distinguish between the pathology caused (Griffin et al., 2004). They used an adenoviral vector to induce chronic endogenous expression of either IL-1β or TNF-α. They found significant differences between the groups, with the IL-1β treated group showing increases in leucine, isoleucine, valine, n-butyrate and glucose whilst the TNF-α treated group showed increases of citrate, 2-oxoglutarate and succinate (Griffin et al., 2004).

NMR spectroscopy has also been used to assess cerebrospinal fluid (CSF) in patients with MS. It has been shown that there are increased CSF levels of lactate, creatinine and fructose in MS compared to control patients (Nicoli et al., 1996). Two additional unidentified signals were found to be elevated in MS. The compound responsible for both these signals has now been identified as B-hydroxyisobutyrate (Lutz et al., 2007). This is a typical partial degradation product of branched-chain amino acids. Increased B-hydroxyisobutyrate in urine is thought to be due to respiratory-chain deficiency leading to impaired oxidation of NADH (Chitayat et al., 1992). However the level of B-hydroxyisobutyrate in these experiments was much higher than the level found in CSF from MS patients, and so the precise role of B-hydroxyisobutyrate in MS needs further investigation.

In a study of metabolite fingerprints in the CSF from patients with a range of neurological conditions we have been able to differentiate between some of these conditions by comparing the metabolites found (Sinclair et al., 2010). In particular we were able to identify some novel features of idiopathic intracranial hypertension (IIH) a neurological condition, the pathogenesis of which is poorly understood (Sinclair et al., 2008). Although IIH was not thought to be an inflammatory disease, the elevated levels of lactate we observed in IIH points towards an inflammatory component since lactate has been identified in inflammatory CNS disease previously (Simone et al., 1996). Rabbits with elevated intraocular pressure also show increased levels of lactate which may reflect anaerobic metabolism resulting from decreased blood supply and this may also be an explanation for the lactate in the IIH patients' CSF due to compressed vasculature from the elevated intracranial pressure. Oxaloacetate levels were also increased in IIH and this, together with reduced citrate, suggests alterations in the citric acid cycle. Overall the observations suggest a predominantly anaerobic environment deficient in carbohydrate substrate in patients with IIH, a conclusion supported by the presence of elevated ketone bodies 3-hydroxybutyrate (Sinclair et al., 2010) often observed in hypoxic tissues.

3.5 Lung disease

Pulmonary inflammation contributes to the pathogenesis of a number of lung diseases. There is a growing need for validated experimental models that can help our understanding of disease pathogenesis and therapeutic intervention. Traditionally animal models have been used but they have their own problems in representing human disease. Genetic manipulation can greatly enhance animal models. NMR has had some application in the quantification of experimental lung injury.

Serkova et al used Magnetic Resonance Imaging (MRI) and NMR to try and detect and quantify injury in mice following intratracheal administration of inflammatory cytokines (Serkova et al., 2008). Pulmonary inflammation was induced by intratracheal administration of IL-1β and TNF-α. Lung tissue was used for the NMR metabolomics. They showed that with pulmonary inflammation there was a 50% depletion of ATP and a corresponding elevation of the lactate to glucose ratio suggesting a shift to anaerobic metabolism during inflammation. These returned to control levels at 24 hours (Serkova et al., 2008). These data show that intratracheal administration of IL-1β and TNF-α leads to profound but reversible pulmonary inflammation which is detectable by NMR.

3.6 Osteoarthritis

Osteoarthritis (OA) is a complex disease and has a multifactorial pathogenesis. It has many known risk factors such as age, sex, obesity, activity level, prior joint damage and genetic susceptibility. It is not classically thought of as an inflammatory disease but it may have an inflammatory element. There are currently no disease-modifying drugs for OA and very few are in development.

Synovial fluid (SF) has been used to look at OA via NMR. SF is felt to be a good medium to study as the SF is the first place where the degradation products, enzymes and signal transduction molecules involved in OA are released from the cartilage matrix. The SF should therefore have a higher concentration of metabolites compared to blood, lymph or urine.

Damayanovich et al used SF from a canine model of OA to look at metabolic profiles using NMR (Damyanovich et al., 1999). Metabolites from experimentally induced canine knee OA SF were compared to metabolites from SF of normal canine knees. They found large increases in lactate and sharp decreases of glucose in OA SF compared to normal SF suggesting that the intra-articular environment of an OA joint is more hypoxic and acidic than a healthy joint. They also found increased levels of pyruvate, lipoprotein associated fatty acids, glycerol and ketones in OA SF suggesting that lipolysis may be an important source of energy in OA. There were also elevated levels of N-acetylglycoproteins, acetate and acetamide in OA SF especially with progressive OA (Damyanovich et al., 1999).

In order to understand further the mechanisms behind OA progression, Damayanovich *et al* looked at the effect of joint afferent nerve injury (Damyanovich et al., 1999). They again used a bilateral canine model of OA. Paired SF samples were taken from dogs that had undergone bilateral anterior cruciate ligament transaction, unilateral knee denervation and contralateral sham nerve exposure. NMR was used to look at the SF. Increases in glycerol, hydroxybutyrate, glutamine, creatinine, acetate and N-acetyl-glycoprotein were seen in the SF from denervated compared to control knees. This suggests that the metabolite differences seen in the denervated knees are due to the aggravation of OA caused by joint denervation (Damyanovich et al., 1999). Hydroxybutyrate is also found in SF of RA patients (Naughton et al., 1993) suggesting that it is more of a marker of joint destruction rather than being specific for any joint disease.

Another group used guinea pigs to study OA metabolism (Lamers et al., 2003). They used Hartley outbred strain guinea pigs as they develop spontaneous progressive knee OA with features similar to human disease. The earliest histological features appear at 3 months but progress to extensive cartilage degeneration after 12 months. Urine samples were collected from these OA guinea pigs and from healthy animals at 10 and 12 months of age. They identified a metabolic fingerprint that reflected OA changes in the pigs. Lactic acid, malic acid, hypoxanthine and alanine contributed strongly to the fingerprint suggesting their involvement in OA (Lamers et al., 2003). The metabolic profile largely resembled that found in the guinea pig model. The presence of hypoxanthine suggests that OA may be an inflammatory disease due to the increased oxygen demand and altered purine metabolism.

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Mass spectroscopy has also been used to look for novel biomarkers for knee OA (Zhai et al., 2010). They looked at serum samples of unrelated white women with and without knee OA. Knee OA was defined as radiographic, medically diagnosed or total knee replacement due to primary OA. They found that the ratio of valine to histidine and the ratio of leucine to histidine to be significantly associated with knee OA in humans (Zhai et al., 2010). These ratios have potential clinical use as an OA biomarker. OA branched chain amino acids (BCAA) are raised which may drive the release of acetoacetate and 3-hydroxybutyrate. These can result from the partial oxidation of leucine. BCAA are essential amino acids and therefore cannot be synthesised within the body. An increased level of BCAA may suggest an increased rate of protein breakdown or be secondary to collagen degradation. BCAA increase production of the cytokines IL1, IL2, TNF and interferon (Bassit et al., 2000) which could drive the collagen degradation.

3.7 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a debilitating systemic inflammatory joint disease. An abnormal metabolic profile in the inflamed joint in RA may be due to the impairment of the vascular supply and/or an increase in the metabolic rate of the inflamed joint.

Hyaluronic acid is a major component of the proteoglycan aggregate of articular cartilage which is required for the functional integrity of extracellular matrix. In RA, SF hyaluronate is depolymerised by the action of reactive oxygen radical species (Parkes et al., 1991). Hyaluronidase activity is absent in both normal and inflamed SF. Generation of reactive oxygen species plays a principal part in synovial hypoxic reperfusion injury (Farrell et al., 1992). This occurs as increased intra-articular pressure during exercise exceeds synovial capillary perfusion pressure leading to impaired blood flow (Mapp et al., 1995).

In 1993, The Inflammation Research Group, The London Hospital Medical College looked at the NMR profiles of RA SF and matched serum samples (Naughton et al., 1993). The NMR profiles of SF were markedly different from their matched serum samples. There were high levels of lactate in the SF compared to the serum and low levels of glucose in the SF compared to the serum. These changes are consistent with the hypoxic status of the rheumatoid joint (Naughton et al., 1993). All the SF samples (RA and control) had lower levels of chylomicron and very-low-density-lipoprotein associated triglycerides compared to their matched serum samples. The SF samples also had high levels of ketone bodies compared to their matched serum samples. These results suggest that the intra-articular environment has an increased utilisation of fats for energy even though it is hypoxic (Naughton et al., 1993, Naughton et al., 1993). They were unable to compare the control SF to the rheumatoid SF due to the low levels of SF aspirated.

Serum from mice has been used to identify a metabolite biomarker pattern associated with RA (Weljie et al., 2007). Using NMR they found that uracil, xanthine and glycine could be used to distinguish arthritic from control animals (Weljie et al., 2007). The presence of the metabolites suggests that nucleic acid metabolism may be highly affected in RA and there may be an association with oxidative stress.

More recently, a group in Denmark have looked at the plasma of patients with RA (Lauridsen et al., 2010). They found differences in the metabolites between patients with RA and healthy controls and differences between patients with active RA and controlled RA. The metabolites

that they identified were cholesterol, lactate, acetylated glycoprotein and lipids. The lactate levels represented oxidative damage and thus indirectly reflected active inflammation.

3.8 Atherosclerosis

Atherosclerosis is the thickening of arteries and is the underlying pathological process that affects the coronary, cerebral, aortic and peripheral arteries. Atherosclerosis involves the accumulation of cholesterol particles, cellular by-products, deposition of the extracellular matrix and inflammatory cell infiltration within the vessel wall (Goonewardena et al., 2010). Chronic inflammation has been recognised as one of the key components of atherogenesis (Ross, 1999) but accelerated atherosclerosis is an important confounder of chronic inflammatory diseases such as rheumatoid arthritis (Bacon et al., 2005). Animal models have been widely used to investigate the biochemical basis of atherosclerosis. Using aortas from apolipoprotein-E knockout mice Mayr *et* al concluded that inefficient vascular glucose and energy metabolism coincided with increased oxidative stress in animals with hyperlipidaemia (Mayr et al., 2007). NMR-based metabolomics of mouse urine has been used to look at atherosclerosis (Leo & Darrow, 2009). Using apolipoprotein-E knockout mice they compared untreated mice with those treated with captopril. They found elevated levels of xanthine and ascorbate in untreated mice which may be possible markers of plaque formation (Leo & Darrow, 2009). The interaction between diet and inflammation in promoting atherosclerosis has also been highlighted through metabolomic studies and Kleenmann (Kleemann et al., 2007) suggested that a high cholesterol intake lead to a switch in liver metabolism towards a pro-atherosclerotic state. Another recent example of how metabolomics can provide novel insights into inflammatory disease pathology was the observation that the metabolism of dietary lecithin by gut flora leads to the increased absorption and accumulation of choline derivatives which in turn promote cardiovascular disease (Wang et al., 2011) . Only through the use of the systematic analysis of metabolites using metabolomics was it possible to uncover these complex metabolic relationships underpinning the disease process.

4. Conclusion

As summarised above there is now a growing body of literature describing metabolomic changes in inflammatory diseases, both in humans and animal models. Several distinct metabolic changes have been identified in inflammatory disorders, but there is a core theme of increasing energy requirements coupled with decreasing oxygen supply within the inflammatory environment.

Studies in MS, RA, OA and inflammatory lung disease have all shown an increase in lactate, while studies of inflammatory eye and lung diseases have shown local reductions in glucose. Immunological responses to tissue hypoxia, such as the up-regulation of IL-1, IL-6, IFN-γ and TNF-α seen in macrophages, show the link between local metabolic changes and inflammatory responses. Here transcription factor HIF-1α may play a central co-ordinating role in both normal and pathological inflammation by regulating the underlying cellular metabolism towards anaerobic respiratory pathways and lactate production. Subsequent effects of inflammatory cytokines on tissue remodelling and perfusion further provide a mechanism for feedback driving self-sustaining inflammatory microenvironments, and potentially where resolution is disrupted, a route to chronic inflammatory disease.

Therefore, as both a by-product and mediator of local tissue conditions, metabolites offer a unique opportunity to gain an insight of local and global inflammatory processes. Metabolomics likewise, provides promising opportunities for both diagnosis of inflammatory diseases, and study of the underlying processes that may offer clues as to how the inflammatory process develops.

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