Clinical and Epigenetic factors underlying treatment refractory Rheumatoid Arthritis

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Declaration

The conception and design of this study were based on a protocol originally designed by Professor Iain McInnes and Roche Products Limited, Nutley, USA. This was refined and finalised by myself, Professor Iain McInnes and Dr Duncan Porter.

All clinical assessments were performed by myself (other than several patients in the DMARD good responder group who were evaluated by Dr Eva Ruzicka, Clinical Research Fellow, Glasgow Biomedical Research Centre, University of Glasgow. I performed all statistical calculations following input and advice from Dr Alex McConnachie, Assistant Director of Biostatistics, Robertson Centre for Biostatistics, University of Glasgow. Laboratory biochemistry, haematology and Immunology processing was performed by Greater Glasgow and Clyde regional laboratories (Glasgow Royal Infirmary and Gartnavel General Hospital). MicroRNA sample processing and cytokine processing was performed by Lynn Crawford, Laboratory technician at the Glasgow Biomedical Research Centre, University of Glasgow. Those candidate microRNA examined in Chapter 4 were processed by those individuals acknowledged:- all data analysis and interpretation was undertaken by me.

I declare that this thesis has been composed by myself.

It has not been previously submitted for a higher degree

Ronald Derek Baxter, September 2013

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Abstract/Summary

Introduction

Rheumatoid Arthritis (RA) is a chronic, progressive, multisystem inflammatory disorder for which there is, at present, no cure. It affects up to 1% of the population resulting in chronic pain, disability and, through loss of function, may lead to loss of employment. It is associated with major co-morbidities that account for premature mortality.

There is now extensive published research that suggests early treatment with disease modifying drugs can retard joint damage and improve outcome. In a proportion, drug-free remission is possible. However, there remain both individuals with persistently active disease despite standard drug treatments and those with longstanding disease not exposed to effective early treatment that remain relatively unresponsive to therapy.

There is a growing literature that epigenetic modifications may underpin, or at least accelerate the development of many autoimmune disorders. These include alterations in DNA methylation patterns, histone tail modifications, post-translational mRNA regulation by microRNA and combinations therein. Having established the human genome project and underlying human DNA sequence, the recognition of dynamic epigenetic regulation of the genome has added further complexity. Few data however are currently available in 'real-world' cohorts of patients.

Methods

In order to explore the hypothesis that specific epigenetic changes may underpin differences in response to therapy, I first examined the characteristics of a cohort of 50 RA patients with longstanding and active disease (DAS28 >3.2) despite receipt of standard therapies (disease modifying drugs (DMARD) and biologic therapies). This included a detailed examination of clinical characteristics, immune profile, inflammatory markers and burden of co-morbid complications such as vascular disease and depression. Outcomes such as disability, quality of life assessments and fatigue were evaluated by means of previously validated questionnaires. These groups were assessed at baseline, three months and six months.

I then measured one of the many epigenetic marks, namely microRNA, of this cohort. We analyzed the accessible profile of peripheral RA CD14+ cell microRNAs in treatment resistant RA patients, in healthy controls, DMARD inadequate responders and DMARD good responders in order to determine the presence of a microRNA profile indicative of biologic resistance. An analysis of the serum cytokine profile of the biologic resistant and DMARD resistant groups was also performed. Finally, to extend the analysis beyond conventional clinical and novel molecular biomarkers the influence of additional patient factors such as coping and illness perception were evaluated to determine subjective disease severity in these discrete patient groups.

Results

Active inflammatory disease was present as judged by the DAS28 score and there was some improvements seen over the six-month assessment period reflecting treatment changes in all groups. Substantial disability and impaired quality of life was found, particularly in the therapeutic resistance group and those with inadequate response to DMARD. Clinical variables, quality of life and fatigue were strongly correlated with mood suggesting close interactions. Multiple cardiovascular risk factors were determined and, having applied cardiovascular risk scoring systems, unmet treatment of modifiable risk was detected.

A unique microRNA profile of the biologic resistant group was found. MicroRNA-423 and -1275 were upregulated in the biologic resistant group and reduced in parallel with the DAS28 raising their potential utility as biomarkers. The cytokine profile correlated with composite measures of disease activity and inflammatory markers. An observed reduction also paralleled DAS28 improvements.

Lastly coping strategies favoured were adaptive and problem based. These were unaffected by the high prevalence of mood disturbance. Conversely, illness perception was influenced by mood and both affected subjective disease assessments. The strong influence of mood and fatigue raise the hypothesis that blunted treatment response may be partially driven by these variables.

Conclusions

Ultimately we seek to explain, identify and target those patients with aggressive disease. Much of the variation detected within clinical measures can be explained by co-morbid conditions that have independent treatment options if they are actively sought. Additionally significant cardiovascular morbidity and mortality may be prevented.

The question of true biologic resistance remains open. Undoubtedly residual inflammation exists in longstanding RA but significant ‘disease activity’ may be explained at least in part by subjective clinical variables influenced by both external and internal factors (comorbidites). The identification of a ‘biologic resistant’ microRNA profile may act both as a biomarker of treatment response in longstanding disease, superior to the DAS28 scoring system and, through target identification, better understanding of the regulation of the molecular pathways of inflammation operating in such patients. In this way novel pathways of treatment resistance may be exposed and novel treatment targets revealed. It is also supportive of a ‘resistant’ RA patient. However, mood and thus illness perception also contribute to resistance to therapy and should be sought, characterized, and directly addressed to add to the global improvements in outcome that we seek in the holistic model of care in the rheumatic diseases..

Publications/presentations to learned societies

Baxter D, McInnes IB, Kurowska-Stolarska M. *Novel regulatory mechanisms in inflammatory arthritis: a role for microRNA*. Immunol Cell Biol 2012; 90; 288-92

Addressing Cardiovascular Risk in Treatment Resistant Rheumatoid Arthritis (poster Ref AB0218). Baxter RD, Ricketts HC, McCarey DW, McInnes IB. Ann Rheum Dis 2013;72 (Suppl3):853

Baxter RD, Watt K, McInnes IB. Coping strategies in severe RA; relationship with mood, correlation with clinical variables and implications for practice. Oral presentation to Scottish Society for Rheumatology Spring meeting, April 2012.

Baxter RD, Emerson SJ, Porter D, McInnes IB. Associations and predictors of fatigue in severe Rheumatoid Arthritis. Oral presentation to Scottish Society for Rheumatology Autumn Meeting, 2nd November 2012.

Chapter 1- Introduction and Aims

Chapter One will outline the relevant background to rheumatoid arthritis as a disorder including the epidemiology, those underlying aetiological factors important for development of disease and related pathophysiology. Having described the typical clinical features of RA, the assessment of disease and treatment will then be examined in context. In the same way, an examination of the treatment options and strategies then allows for a discussion of some of the proposed mechanisms whereby individuals may not respond to conventional therapies. This will define the primary resistant study group.

I will then explore the principles of epigenetics as an area of study. I will relate how these changes may link to both autoimmunity and the area of treatment-resistant disease. I will go on to examine specific epigenetic findings demonstrated to date in this field and how these may not only explain the persistent state of inflammation observed in RA but also reveal novel levels of transcriptional regulation. The focus will be on microRNA as the primary epigenetic modification examined in this thesis.

Both these areas will highlight the limitations of current RA management and those challenges posed by developing new therapies to target these complex yet potentially reversible pathways. Within this study, however, is the opportunity to examine a cohort with severe disease and identify novel findings that may improve care.

I will then set out the major hypothesis and aims of this thesis.

1.2 Rheumatoid Arthritis

1.2.1 Epidemiology

RA is the most common inflammatory joint disease. The prevalence of RA is widely quoted as 0.5-1% (approx. 1 in a 100). Lifetime risk of developing seropositive RA is 2.4% (3.6% for RA of all serotypes) for women and 1.1% (1.7%) for men in the USA (Crowson et al. 2011). Their estimate therefore in a US population is 1 in 28 women and 1 in 59 men will develop RA. In context, 1 in 12 women and 1 in 20 men will develop an inflammatory rheumatic condition in their lifetime.

Incidence varies with age: incidence is uncommon below 30yrs and peaks around 50yrs. The presence of other risk factors, such as smoking and family history, confers additional multiplicative and cumulative risk. The incidence of RA may however be decreasing (Doran et al. 2002) related perhaps to changes in environmental influences (protective effect of the oral contraceptive pill?), change in exposure to an unknown infectious agent, reduced rates of smoking or better dental health. Additionally, an aging population and access to better healthcare may affect this prediction. In the same way a *lack* of access to healthcare makes accurate estimation in developing countries more difficult to ascertain and through practical difficulties collecting data.

Worldwide prevalence of RA varies with falling rates in more southern continental Europe. Estimated prevalence is 0.5 cases per 100 population and incidence 16.5 per 100,000 in Southern Europe to 29 in Northern Europe (Tobón et al. 2009). Data is more limited from African and South American areas, however the prevalence may be lower in these areas. The overall global trend is a reduction observed in the Native American Pima Indians (who, with native Alaskan populations, display a particularly high incidence and prevalence) to Japan and Finland (Silman 2002).

1.2.2 Genetics and onset of Rheumatoid Arthritis

Susceptibility, pattern of onset, course and response to therapy might be expected to have an underpinning genetic influence. Indeed, this is suggested in epidemiological studies; RA is more common in first-degree relatives and monozygotic twin concordance is approximately 15% falling to 4% in dizygotic twins (D. M. Lee & Weinblatt 2001). Age at onset varies widely suggesting other influences exist such as environment contributes. HLA-DR4 risk alleles differ between geographical and ethnic populations suggesting further gene-environment interaction. Two main risk genetic loci have been identified.

1.2.2.1 HLA-DRB1

The major histocompatibility (MHC) locus of chromosome 6 may contribute 30-50% of the genetic susceptibility to RA (Deighton et al. 1989). The presence of alleles containing the common amino acid sequence at positions 67-74 (known as the ‘shared epitope’

(SE)) of the HLA-DRB1 molecule are well established risk factors for the development of RA (Gregersen et al. 1987). This forms part of the antigen-binding site, and thus antigen-presenting cells bearing a postulated RA antigenic trigger may interact. Carriage of the SE allele gives an OR of 2.66 increasing to 6.89 if two copies are present (Berglin et al. 2004). Allele types vary between ethnic populations. Furthermore, smoking, in the presence of the shared epitope, and sero-positivity increases this risk (Padyukov et al. 2004). The same group (Lundström et al. 2009) have identified the additional link with ACPA positive status and smoking.
Genetics may also inform prognosis; the presence of the shared epitope remains one of the best predictors of poorer outcome. The presence of two copies of this gene confers poorest prognosis.

1.2.2.2 PTPN22

PTPN22 (protein tyrosine phosphatase, non receptor type 22) is perhaps the best-described non-MHC susceptibility locus. It codes for the protein lymphoid tyrosine phosphatase (Lyp), a negative regulator of T-cell activation, which may act by preventing T-cell receptor signalling in animal models. It has been implicated in a number of other human autoimmune disorders including Graves' disease and systemic lupus erythematosus (SLE) and identified in many ethnic populations. A single nucleotide polymorphism (SNP) and consequent amino acid substitution in the minor allele of PTPN22 (R620W) is associated with RA (Carlton et al. 2005). This change could predispose to autoimmunity by preventing negative thymic selection of auto-reactive T-cell populations. Significant risk of developing RA is associated with PTPN22 1858T carriage and the presence of anti citrullinated protein/peptide antibodies (ACPA) or rheumatoid factor (RF) (odds ratio 132.0). Similarly, the presence of the SE and ACPA gives an odds ratio of 66.8 (Rantapää-Dahlqvist 2009) and carriage of the 1858T allelic variant with the SE allele an odds ratio of 7.85 of developing RA (Johansson et al. 2006).

1.2.2.3 Other genetic factors

Further study with genome wide association studies reveal many other 'non-HLA' RA susceptibility loci. CTLA 4 (cytotoxic T-lymphocyte antigen 4) CT60 polymorphism may confer a small additional risk (Rodríguez et al. 2002). PADI4 (peptidyl arginine deiminase, type 4) identified in Asian (Suzuki et al. 2003) and US cohorts, is of note coding for an enzyme responsible for converting arginine residues to citrullinine (the postulated target for ACPA antibodies). Other risk loci include signal transducer and activator of transcription 4 (STAT4) (Remmers et al. 2007), CD-40 (Raychaudhuri et al. 2008), TNF receptor associated factor-1 (TRAF-1) (Plenge et al. 2007) and interleukin 2 receptor alpha (IL2-RA) (D. Plant et al. 2011) for the Wellcome Trust consortium who also strongly confirmed PTPN22). The additional risk conferred is small compared to those above.

Karlson et al sought those known risk alleles above with environmental risk factors in those developing RA in well-established prospective cohorts (Karlson et al. 2010). Although displaying valid discriminative ability, such an application still makes screening

of the general population impractical. Thus genetic advances have still to be incorporated in daily clinical care and remain of more distant utility in preventative medicine.

1.3 Environment

The presence of genetic findings alone is not sufficient for the development of disease. The presence of an environmental trigger may be the second necessary crucial event before symptoms manifest. Candidates include biomechanical trauma, infectious triggers and vascular dysfunction allowing ingress of inflammatory cells and exposure to novel antigens. Most evidence lies with smoking. Both intensity and duration are important in the period before symptom development and the link strongest in men, RF positive, PTPN22 and possessing the SE (Costenbader et al. 2006; Costenbader et al. 2008). The increased risk may also persist for many years after cessation. In 2006 Klareskog et al put forward their important unifying hypothesis of lung citrullination in smokers possessing the SE (Klareskog et al. 2005). Evidence links both major genetic risk factors above to smoking (A. W. Morgan et al. 2009). The smoking risk does not appear to exist in the same way for ACPA negative RA subsets.

A role for dental disease has been postulated. Porphyromonas gingivalis is the main cause of periodontitis and an observed increased prevalence in RA has been noted. Novel citrullinated antigens generated by such bacteria provide a potential trigger of systemic autoimmunity to endogenous peptides (Wegner et al. 2010). In general, there are number of infectious agents that have been proposed to trigger RA including viral agents such as Parvovirus and Epstein-Barr. These are based on observations that viral material has been isolated in joints but defined mechanisms of triggering have not been elucidated.

A protective hormonal effect may be seen with use of the contraceptive pill (Bhatia et al. 2006) but this finding is not consistent (Pikwer et al. 2009). Prolonged (more than one year) breast-feeding may be protective against the development of RA (Pikwer et al. 2009). RA shows female preponderance however and remission often occurs in pregnancy.

Silica exposure (from dusts such as the building trade) may also be associated not only with RA but other autoimmune disorders (Khuder et al. 2002). Alcohol may be protective within moderation and Vitamin D may a role in disease onset and disease activity (Jin et al. 2013; Gatenby et al. 2013).

1.4 Social Deprivation

The Carstairs Index is a measure of deprivation devised originally by Carstairs and Morris in 1991 (Carstairs & R. Morris 1991). The link between increasing deprivation and mortality is well recognised. Postcodes are assigned a score using combinations of variables from the Census data that are considered detrimental. Specifically, social class (the head of household social class IV or V), overcrowding, employment status (unemployed males seeking work) and proportion of those in a household without car ownership. Results are presented as ‘deprivation categories’ 1 to 7 where 1 is the most

affluent. Results therefore do not take into the account the individual but their population environment.

The alternative Scottish Index of Multiple Deprivation (SIMD) (available from www.scotland.gov.uk/topics/statistics/SIMD) tool allows recognition of areas of most need through the application of the SIMD score. Populations are divided into quintiles where the 5th quintile is the most deprived and 1st the least.

In RA, deprivation has been closely studied in Glasgow owing to the greatest concentration of deprivation in Scotland found within the Greater Glasgow area. Deprivation affects course but not risk of development, although smoking is more common in lower social classes. McEntegart et al identified clear functional outcome differences over five years in a cohort with RA (McEntegart et al. 1997). Harrison et al prospectively followed over 400 RA patients and measured disease activity, disability and quality of life over three years. Using the Townsend deprivation scale they found that those more deprived patients had higher baseline DAS28, poorer function and psychological scores. The same group did however see the most improvements with treatment (M. J. Harrison et al. 2005).

1.5 Pathophysiology and Histology

RA is an autoimmune disorder characterised by a highly coordinated yet dysregulated inflammatory network. The earliest event is currently considered to be a loss of self-tolerance at the central (thymic) and/or peripheral level to unknown citrullinated proteins (antigen). Alternatively, cell types such as dendritic cells, fibroblasts and macrophages that possess antigen-presenting capability may process material of perhaps bacterial, viral or endogenous origin generating an autoimmune response. A long period of immune dysregulation follows characterised by epitope spreading before localisation to the joint and onset of symptoms. In this asymptomatic period, the presence of antibodies to citrullinated proteins can be demonstrated often more than five years prior to symptom onset and prior to any cytokine rises (Rantap Dahlqvist et al. 2003). Subsequent studies have validated these findings in different populations (Rantapää-Dahlqvist 2009). Prior to symptom onset in this pre-articular phase, the ACPA titre is seen to rise but the defining event prior to localisation within the articular compartment is not known (Kokkonen et al. 2010).

Following the T-cell interactions described above, B-cells are recruited and produce immunoglobulins and further cytokine stimulation. T-cells activate and recruit macrophages, which act as effector cells and further act as important producers of cytokines. Finally, fibroblast-chondrocyte-osteoclast interactions mediate bone damage as described below. Local production of joint (neo) antigens may amplify the process.

Accordingly, at the advent of the joint phase the usually relatively acellular synovium, composed of synoviocytes (related to fibroblasts) and macrophages, hypertrophies. At the microscopic level the synovium can be seen to be heavily infiltrated with many cell

types such as CD4 T-lymphocytes predominantly locating in the synovial sub-lining layers. There is prominent plasmablast, plasma cell and B-cell ingress responsible for local autoantibody and cytokine production. In some these may form lymphoid aggregates resembling lymphoid follicles. To facilitate this ingress of cells there is marked increases in vascular permeability and angiogenesis mediated by vascular growth factors such as vascular endothelial growth factor (VEGF). Hypertrophy of the surface epithelial layer and deeper interstitial layer, to which the term 'pannus' is given, invades and destroys underlying bone and cartilage. Together with oedema facilitated by neo-angiogenesis and synovial hypertrophy, the clinical picture of a swollen, synovitic joint becomes evident (D. M. Lee & Weinblatt 2001).
Later the joint phase moves to one of chronicity and a state of self-perpetuance even in the putative absence of auto-antigen. Reasons for this switch are not known but T-cells are typically anergic and insensitive to natural 'brakes' within the inflammatory cascade. Joint destruction is caused by direct fibroblast and mononuclear cell invasion with matrix metallo-proteinase (MMPs) production. This leads to the characteristic loss of function and related co-morbidities that will be demonstrated in Chapter 3.

1.5.1 Immune Cell Types in RA

1.5.1.1 T-cells

The primary role for T-cells in RA is one of antigen recognition and the presence of T-cells in lymphoid aggregates supports this as a central event in disease pathogenesis. T-cells constitute up to 20% of the cells in RA synovium. It is postulated naïve CD4+ T-helper cells (Th cells) and dendritic cells (DCs) interact in the draining lymph node (data communicated via BT-Cure Consortium meeting, Prague 2013 by Professor Danielle Gerlag et al, AMC). MHC Class II/peptide complexes are presented to the T-cell receptor with a necessary co-stimulatory signal. Naïve T-cells (not previously exposed to antigen) may also be activated through the additional activities of the innate immune system through Toll-like receptor (TLR) signalling. This 'initiation' phase proceeds to a 'commitment' phase to produce activated effector T-cells under the influence of cytokines. Finally, and in the presence of on-going antigen exposure, transcription occurs and a specific response mounted. The role of T-cells in joint destruction is less prominent.

Naïve T-cells differentiate upon exposure into two broad T-helper cell types functioning to produce effector cytokines. A dominant Th1 response is seen in RA with production of interferon (IFN) gamma and tumour necrosis factor alpha (TNFa) in the presence of interleukins (IL)-12, IL-23 and IL-6. A diminished Th2 response with reduced IL-4 and IL-13 is seen. The novel discovery of a third subset of Th17 regulatory cells suggests at least a third important effector T-cell type. Th17 cells produce IL-17A, which is pro-inflammatory acting in a similar way to Th1 cells to facilitate B cells, activate fibroblasts and macrophages by cell contact activation. IL-4 is a negative regulator of Th17 cell differentiation.

In longstanding chronic inflammation, T-cells show increased longevity and relative poor responsiveness to further antigen exposure. Treatment with TNFa blockade may restore this reactivity (Cope et al. 1994) as it may restore some regulatory T-cell (T-reg) function (Ehrenstein & Mauri 2007).

1.5.1.2 Macrophages

Macrophages are innate immune cells and the first line of defence against invading microorganisms. Traditionally the pro-inflammatory macrophage phenotype is referred to as M1 and the anti-inflammatory M2 phenotype. They play a central role in the joint through secretion of cytokines and chemokines in a paracrine and autocrine fashion in addition to direct cell lysis (Szekanecz & Koch 2007). In the peripheral blood, as monocytes, they express CD14, CD33, HLA-DR and Fc gamma receptors in addition to TLRs and complement receptors. Amongst many signals, TLR stimulation or TNFa, in the presence of interferon gamma, activate macrophages.
The synovial membrane is heavily infiltrated with macrophages. Upon migration to the joint the macrophage has a number of roles. These range from the production of pro-inflammatory mediators to induce further cell migration, granulocyte/macrophage colony stimulating factor (GM-CSF) which promotes monocyte lineage maturation, further TNFa secretion to prolong cellular survival, matrix degradation products and angiogenesis factors. Secretion of receptor activator of nuclear factor kappa-B ligand (RANKL) stimulates maturation of osteoclast precursors to osteoclasts, which effect resorption of bone. They may also produce reactive oxygen and nitrogen intermediates and prostaglandins and as such contribute to the inflammatory milieu and to the algesic aspect of the disease.

1.5.1.3 B-Lymphocytes

B-cells, plasmablasts and mature antibody-producing plasma cells, are responsible for the production of immunoglobulins (Ig) including IgG rheumatoid factor and ACPA. They are also important antigen presenting cells and thus important for local T-cell activation. They produce further pro- and anti-inflammatory cytokines and have a role in immunological memory. Clustering of plasma cells can occur within the joint in structures reminiscent of ectopic germinal centres in peripheral lymphoid tissue where B-cell maturation and antibody production occurs (Silverman & Carson 2003; Manzo et al. 2010). B-cells may also produce RANKL thus mediating osteoclast activity and angiogenesis and chemokine factors enhancing cellular recruitment.
The importance of B-cells in RA pathogenesis is confirmed by successful therapy with the anti CD20 B-cell depleting agent Rituximab. It is effective at treating the symptoms of RA and preventing radiological erosive damage (Keystone, Emery, et al. 2009a) often for over six months. A reduction in synovial B-cell and plasmablast populations correlates with clinical improvements where peripheral B-cell depletion may not (Thurlings et al. 2007). Furthermore, those RF or ACPA positive patients exhibit a better response to Rituximab. In the absence of other easily accessible ways to predict B-cell

response, this remains one of the most practical ways to aid decision-making (Marston et al. 2010).

1.5.1.4 Dendritic cells

Traditionally, DCs recognise bacterial motifs via TLRs and induce Th1 cell differentiation. They may act as antigen presenting cells within the joint, presenting local antigen to CD4 T-cells and thus participate at a very early stage. They have a role in maintaining self-tolerance (Thomas & Lipsky 1996).

1.5.1.5 Fibroblast-like synoviocytes

Fibroblasts are constitutive cells within the joint with a role in maintenance and repair. They play a key role as both effector cells and coordinators of the other immune cell types above by the nature of the multitude of receptors (TLRs, cytokine and chemokines) and molecules produced such as proteinases, vascular factors and inflammatory molecules (Müller-Ladner et al. 2007).
In RA fibroblasts adopt an aggressive and destructive phenotype contributing to cartilage damage (Müller-Ladner et al. 2005; Pap 2005). The trigger is unknown but TNFa facilitates a change in state to of proliferation, typically more resistant to apoptosis (Müller-Ladner et al. 2005) and ability to migrate and induce disease in hitherto unaffected areas; at least when tested in mouse models (Lefèvre et al. 2009). Once activated, they attract T- and B-cells to the joint and directly secrete matrix-degrading proteins such as MMPs and cathepsins effecting joint damage. They secrete large amounts of RANKL facilitating osteoclast differentiation and activation (Bartok & Firestein 2010).

1.5.1.6 Osteoclasts

Osteoclasts are large multinucleated cells that participate in bone turnover through bone breakdown and remodelling. They are strongly influenced to differentiate from macrophage precursors to mature osteoclasts by IL-1 and TNFa, both directly and mediated by RANK/RANKL binding secreted by fibroblasts and Th1 cells, leading locally to joint erosions and systemic osteoporosis with consequent increased fracture risk (Goldring & Gravallese 2000).

1.5.1.7 Endothelium/blood vessels

Despite significant ‘neovascularisation’, the joint is a relatively hypoxic site. For synovitis to be supported however, an adequate vascular supply is vital and is maintained locally through the production of angiogenic factors and expression of vascular adhesion factors by endothelial cells. This is an important early event needed for the recruitment and retention of leucocytes. The central angiogenic factor is VEGF, which is up-regulated by IL-1 and TNFa (Veale 2006). Therapeutic targeting of VEGF has been undertaken in cancer therapy and anti-angiogenesis in some part explains the action of some biologic therapies (Szekanecz et al. 2009) as treatment with TNFa blocking therapies reduces VEGF (Marrelli et al. 2011). VEGF correlates with C-reactive protein (CRP) and disease activity linking with power Doppler ultrasound (US) and magnetic resonance imaging

(MRI) imaging of the joint as a direct measure of joint vascularity and disease activity (Clavel et al. 2003).

1.6 Cytokines

The coordination of the varied cell types by cytokines above is considered in Chapter 5 in order to present the findings of the study cohort in context.

1.7 Cell signaling cascades

Conceptually, ligand-receptor binding leads to activation of a series of small molecule cascades that will ultimately change mRNA transcription. A variety of cell membrane receptor systems have been proposed to have an influence on the RA pathogenetic state.

The Toll-like receptors (TLRs) are an important member of the family of pattern recognition receptor surface receptors involved in the recognition of self (in this instance joint breakdown products may be relevant) and foreign proteins, particularly highly evolutionarily conserved bacterial wall components and viral envelope proteins (Andreakos et al. 2005). TLRs are present on the surface of most cells involved in antigen recognition and are a key component of the innate immune system. As such they are non-specific yet able to respond to broad patterns of molecular structure, especially those with repeating structures – this allows for a rapid recognition of foreign or local tissue damage associated moieties prior to the elaboration of a peptide specific T-cell adaptive response. The observation that various members of the TLR family are up-regulated in RA has been reproduced on many occasions (McCormack et al. 2009; Brentano et al. 2005). This may lend weight to the bacterial antigen driven hypothesis of RA, although equally one can propose them as an amplificatory pathway working in parallel to autoantigen driven events. The TLRs serve to activate downstream signalling. For example, following TLR4 ligand activation, nuclear factor kappa beta (NFkB) signalling occurs via recruitment of myeloid differentiation primary response gene (88) (MyD88) family of adaptor proteins (with TRAF6 and IRAK1 and TRIF/IRF 3 leading to type 1 interferon (IFN) expression. NFkB is a central pleiotropic regulator of gene transcription key in the inflammatory response. For example, TNFa and IL-6 transcription is regulated through direct binding to the respective gene promoter regions. RANKL signalling (osteoclastic activity) is also NFkB dependent.

Molecular techniques have defined the inflammatory cascade from cytokine binding to generation of (immune) response. Following cytokine binding, downstream triggering subsequently occurs via cascades of small molecules involving the phosphorylation of kinases. One such mechanism is the binding of cytokines to tethered janus kinase signal transducers. This provides a binding site for the signal transducer and activation (STAT) family of transcription factors which can influence DNA binding and thus gene expression. Negative regulation of the STAT signal occurs via the suppressor of cytokine signalling (SOCS) pathway depending on the cytokine binding pattern and feedback inhibition from STAT pathways. This pathway has an important role in the fine-tuning of the inflammatory response and determining T-cell lineage and fate. Other pathways of

interest include the mitogen activated protein kinases (MAP kinases), Bruton's tyrosine kinase, phosphatidylinositide 3-kinase (PI3 Kinase) and potentially Syk (a member of the tyrosine kinases).

Targeting small molecules of such signalling cascades shows therapeutic promise. Concerns over 'off-target' side effects remain. Their most attractive aspect is the oral route of delivery.

1.8 Autoimmunity in RA

Auto-reactivity has long been identified as a key feature of RA however it has become clear there are many postulated protein antigens involved. The finding of varied clinical phenotypes, disease onset and spectrum of severity associated with distinct autoantibody expression is commensurate with this observation.

1.8.1 ACPA antibodies

The anti-citrullinated protein antibodies are directed toward citrullinated peptides including vimentin, fibrinogen, alpha enolase, type II collagen, keratin and filaggrin. They are present in approximately two thirds of patients with RA but are restricted to RA with >95% specificity (Nishimura et al. 2007). They are formed by post-translational deamination of arginine to citrulline, mediated by the family of peptidylarginine deiminases. The humoral response mounted in bone marrow, and ultimately within synovium that is characteristic of RA, is toward these citrullinated epitopes.
There is evidence that citrullination may occur in the lungs as evidenced on broncho-alveolar lavage and demonstration of citrullinated molecules in the joint (Klareskog et al. 2005; Demoruelle et al. 2012). This process is enhanced by smoking and in keeping with the greatly increased risk smoking and ACPA positivity presents. It is proposed that immune complexes may trigger downstream inflammatory cascades.
There is a good deal of evidence for the predictive power of ACPA testing in early arthritis and relative sensitivity and specificity referred to below as useful in predicting disease outcome (Vossenaar & Van Venrooij 2004).

1.8.2 Rheumatoid Factor

Rheumatoid factor has been in use for over 50 years since the original description by Waaler. Rheumatoid factor is a polyclonal autoantibodies directed toward the constant regions (Fc fragment) of IgG. They may be of any immunoglobulin type but enzyme-linked immunosorbent assay (ELISA) conventionally measures RF IgM. Production by plasma cells occurs in the joint where IgM-containing immune complexes can be identified. Around two thirds of patients with RA will have RF and there is evidence that the titre may vary with time and disease stage (like ACPAs, RF may also be demonstrated in the pre-articular phase of RA). Correlation exists between RF and radiological progression, disease severity and presence of extra-articular manifestations.

There are a number of conditions where RF may be demonstrated including other connective tissue disorders, chronic infections, neoplasms and increasing age. RF is pathological through complement activation and presentation of complexes to T-cells and macrophages.

In practice, RF and ACPA provide similar prognostic information in predictive, diagnostic and severity models. ACPA assays are significantly more expensive. In models where pre-test probability is low then neither test helps. If moderate probability then ACPA performs better than RF. If high probability then either test is simply confirming the diagnosis (Chatfield et al. 2009). If both test positive this would inform a poorer prognosis.

1.9 Clinical Features and Natural History

RA is characterised by a period of immune activation preceding overt clinical disease. At some point there is an as yet unknown insult that triggers symptomatic disease and localisation to the articular component. Several studies however, for example the BeST study (Goekoop-Ruiterman et al. 2007), would suggest that progression to chronic arthritis is not inevitable. Either a proportion spontaneously remits or treatment with DMARDs or biologic agents induces a period of sustained remission. Clinical features may be considered in terms of the joint, those extra-articular features related directly to disease and those typically associated with disease.

1.9.1 Diagnosis of RA

RA lacks a single defining characteristic and the diagnosis encompasses a wide range of severity, course and outcome. The first classification criteria were proposed in 1958 by the American Rheumatism Association (ARA) and replaced by the 1987 criteria (Arnett et al. 1988) classify and define individuals with established RA. In such a way, most individuals entering therapeutic trials and epidemiological studies in the decade post-dating this publication have satisfied these criteria. The weakness of these criteria lies in their inability to capture those with early arthritis, in recognising that ACPA positivity is as important as RF status and that erosions may not be present in early disease.

The 2010 ACR/EULAR (Aletaha et al. 2010) criteria capture those features of early undifferentiated arthritis; an important population where early treatment results in most gains. A score of six or higher diagnoses RA in the presence of at least one swollen joint and no alternative explanation. The new criteria recognise the sensitivity and specificity of the autoimmune profile and the insensitivity of plain radiographs in early disease. Finally, the recognition of subtypes of RA has gained prominence in view of their different clinical phenotype and response to therapy (de Vries-Bouwstra et al. 2008).

Criteria		Score
Joint Involvement	1 Large joint	0
	2-10 large joints	1
	1-3 small joints (with/out large joints)	2
	4-10 small joints (with/out large joints)	3
	>10 small joints (and at least one small joint)	4
Serology	Negative RF and negative ACPA	0
	Low + RF or low + ACPA	2
	High + RF or high + ACPA	3
Acute Phase reactants	Normal CRP or ESR	0
	Abnormal CRP or ESR	1
Duration of Symptoms	<6 weeks	0
	>/= 6weeks	1

Table 1-1 2010 ACR/EULAR criteria for diagnosis of rheumatoid arthritis adapted from (Aletaha et al. 2010)

1.9.2 Joint symptoms

RA is characterised by joint swelling, pain and loss of function when the disease enters the articular phase. Tendon and involvement of the bursae may also occur. If persistent, chronic and irreversible changes appear with bone and cartilage destruction, ligamentous and tendon rupture and fibrosis. Loss of articular range of movement then occurs. Morning stiffness and gelling are characteristic and relate directly with disease activity.

The hand and wrist are the most frequently involved joints. Feet, ankles, proximal interphalangeal joints, elbows and shoulders are commonly affected. Symmetrical involvement is characteristic. Sacro-iliac, axial and distal interphalangeal joint involvement however raises the possibility of the related psoriatic sero-negative spondylo-arthropathies.

1.9.3 Extra-articular Disease

Rheumatoid arthritis involves other systems in more severe disease and individuals will often display multiple features. Some manifestations are not clinically evident and post mortem findings of extra-articular disease are more prevalent.

Constitutional features, including fatigue, are some of the most significant extra-articular manifestations to patients. When severe, weight loss and a catabolic state are termed ‘rheumatoid cachexia’. Respiratory involvement includes pleural effusion, pulmonary nodules and interstitial lung disease. Cardiac involvement can include pericardial inflammation and myocarditis. Eye involvement ranges from sicca syndrome (secondary Sjogrens) to episcleritis, conjunctivitis to potentially sight threatening scleritis. Vasculitis may cause skin and nerve involvement. Peripheral nerves may be involved both by compression (eg carpal tunnel) or mononeuritis multiplex. Cervical myelopathy is an important consideration with longstanding neck disease. Rheumatoid nodules typically occur over areas of pressure including extensor surfaces and occur only in those seropositive for rheumatoid factor. Haematological abnormalities are not uncommon including lymphopaenia and thrombocytopaenia (Felty’s syndrome). Lymphoma is more common in RA patients and is associated with high disease activity, especially that which remains over time.

Accordingly, those with extra-articular involvement have premature mortality in addition to disability and quality of life.

1.10 Disease course

RA most often presents insidiously and symmetrically. Other types of onset may include polymyalgic-onset RA (indistinguishable initially from Polymyalgia Rheumatica), palindromic RA (recurrent episodes of usually oligo-articular arthritis) and mono-articular onset. Palindromic presentation is also frequently observed but defies current clear explanation in the context of the preceding discussion.

Factor	Comment
Clinical	
Smoking status	Confers risk of development (Padyukov et al. 2004), is known to increase nodulosis (Schoels et al. 2010) and may worsen X-ray progression
Age at onset	Incidence increases with age.
Gender	Male disease usually worse (Jawaheer et al. 2006; Sokka et al. 2009) until later in life when females fare worse. Female:male=3:1
Baseline erosions	Subsequent risk of persistent disease of 68% in early RA (Thabet et al. 2009). Erosions at baseline =poorer prognosis
Number of joints and higher ESR at baseline	Higher baseline DAS=poorer prognosis
Socio-economic deprivation	Deprivation associated with higher disability
Psychological factors	Low mood and abnormal illness beliefs may confer poorer prognosis. Suicidal ideation up to twice elevated (Tektonidou et al. 2011)
Genetic Risk Loci & SNP's	
HLA DR4	Original identification of HLA-DR4 as significant (Stastny 1978) and associated with seropositive disease.
Twins/Family studies	Up to 60% twin concordance rates (Jawaheer et al. 2006; MacGregor et al. 2000)
STAT4 PTPN22	Regulates threshold for T-cell activation (van der Helm-van Mil et al. 2005; Begovich et al. 2004). Overall effect if present is reduced T-cell receptor signalling so less thymic negative selection.
TRAF-1	Negative regulator of TNF induced nFkB activation (Plenge et al. 2007)
Immunology	
Rheumatoid factor	Presence of RF correlates with poorer prognosis, greater radiological progression (Kaltenhäuser et al. 2001)
ACPA status	ACPA positivity predicts progression to RA from an undifferentiated arthritis (Kastbom et al. 2004). Predicts poorer clinical and radiological course (Kroot et al. 2000; Miriovsky et al. 2010; Agrawal et al. 2006)

Table 1-2 Factors associated with poorer prognosis and disease in RA (abbreviations can be found in text or Chapter 8.1 'Abbreviations used')

1.10.1 Predicting Outcome

In order that treatment can be administered most appropriately and judiciously, attempts have been made to predict disease course and outcome on the basis of clinical factors, biomarkers and genetic profiling. Severity and course reflect those factors in Table 1-2 from genetic, epigenetic, environmental to psychosocial factors.

Clinical and laboratory factors are not accurate when predicting outcome in early arthritis. The Norfolk early arthritis registry examined clinical factors and basic immunological data to seek a responder pattern to Methotrexate (Hider et al. 2008). These factors proved not helpful. Presence of the shared epitope allele tended to increase the likelihood further medication would be required for treat the disease but baseline DAS28 and CRP did not predict outcome.

Large registry data and longer-term follow up of biologic trial patients provide suitable patients to seek predictors of response to TNFa therapy. On the whole these studies are disappointing but individual factors may merit attention (Hyrich et al. 2006). Concurrent methotrexate (MTX) improves biologic response but baseline disease characteristics do not predict response. Smoking impairs response to Infliximab (IFX) and longer disease duration and more severe degree of disability at baseline predict less response. Further discussion of genetic aspects of treatment response and resistance are performed in 1.14.

1.11 Related co-morbidity

When compared to age-matched healthy individuals, RA patients have significantly more comorbidity, sometimes referred to as 'multi-morbidity'. Such conditions may be a primary disease manifestation or independent, but co-exist more frequently in RA patients. These are important in relation to reducing quality of life or premature death. Poor disease control and longer disease duration correlates with increased risk suggesting the cumulative exposure to inflammatory mediators is important. This may manifest as accelerated vascular disease (premature cardiovascular disease and death and incidence of stroke), depression, fatigue and weight loss. Of these I have focussed on cardiovascular risk with mood disturbance and fatigue also addressed in context in Chapter 6.

1.11.1 Cardiovascular

1.11.1.1 Increased mortality in RA patients

In RA, there is an 48% increased risk of incident development of cardiovascular disease (Avina-Zubieta et al. 2012) and x1.5 increased likelihood of death of versus the general population (Aviña-Zubieta et al. 2008). This includes risk of myocardial infarction, stroke and heart failure (Mikuls 2003; Myasoedova & Gabriel 2010). Symmons et al identified around a third of the deaths in long-term follow up of the British Society for Rheumatology biologics registry cohort were related to cardiovascular deaths. Mortality

is increased with standardised mortality ratio of x1.7 (Sokka et al. 2008). Subclinical early atherosclerosis can be identified, by examining carotid intimal thickness or arterial stiffness as a surrogate with ultrasound, and is responsive to tumour necrosis factor alpha inhibitor (TNFi) therapy (Del Porto et al. 2007). An additional surrogate for atherosclerosis is coronary calcification. Chung et al examined calcium scores in an RA cohort and identified the extent of calcium deposition (and thus atherosclerosis) related to longer disease duration and persisted despite allowing for the augmenting effect of smoking and increased age (Chung et al. 2005).

1.11.1.2 Cardiovascular Risk Factors

The observed increased risk in RA is in part due to the increase in conventional vascular risk factors such as hypertension, adverse lipid profiles, obesity and diabetes in addition to reduced ability to undertake physical exercise. Other risk factors include increasing age, ethnicity, gender, smoking status and emotional stress. These can then be further divided into modifiable and non-modifiable risk factors for the purposes of targeted intervention. There is a close link with other diseases such as diabetes, chronic kidney disease, obstructive sleep apnoea and related systemic inflammatory (autoimmune) disorders such as systemic lupus, psoriasis and RA.

Lipid profiling in RA is of interest, as it appears to bear relation to systemic inflammation. The 'total cholesterol:HDL (high density lipoprotein) cholesterol ratio' (TC:HDL Chol ratio) falls in the presence of inflammation (Kitas & Gabriel 2010). Accordingly, effective treatment with the IL-6 blocker Tocilizumab often results in the observation of a more adverse lipid profile; this may simply represent the 'unmasking' of the actual lipid profile. Similar observations, although not as notable, have occurred with the TNFi (Robertson et al. 2013).

Hypertension is also known to have an increased prevalence in RA patients. The driving factors are likely to be multiple including drug-related (NSAIDs, steroids and some DMARDs), inflammation and atherosclerotic disease.

Being overweight has been traditionally defined using the 'body mass index' or BMI. The alternative waist:hip ratio (WHR) attempts to overcome the main weakness of the BMI score in RA. RA patients lose skeletal muscle due to deconditioning and inflammation and replace this with adipose tissue (Kitas & Gabriel 2010). In this way effective measured weight would be unchanged. In the case of preferential adipose deposition in the abdominal area, most cardiovascular risk is observed. "Adipocytokines' may explain this increased risk both through the production of pro-inflammatory cytokines and promotion of insulin resistance.

The combination of adverse cardiovascular risk factors has been referred to collectively as the 'metabolic syndrome' (MetS). There are a number of definitions and typical characteristics are defined by the National Cholesterol Education Program's Adult

Treatment Panel III (NCEP ATP III) is shown in Table 1-3. This accounts for the additional 'metabolic' risk factors but not for smoking, family history, diet, aging and activity.

Abdominal obesity
Elevated blood pressure
Insulin resistance
Pro-inflammatory state
Pro-thrombotic state

Table 1-3 NCEP ATP III characteristics of the metabolic syndrome (adapted from (Grundy 2004)

MetS accounts for a multiplicative effect over individual factors. Presence of the MetS is predictive of the onset of cardiovascular disease. MetS is increased in RA; 42% of those with 'long-standing' RA studied by Chung et al met the NCEP and the World Health Organisation (WHO) criteria (Chung et al. 2008). The prevalence is also increased in cohorts with shorter disease duration (less than two years) although lower (30%). There are a number of suggested target values defining this cluster of features. The National Cholesterol Education Programme report and WHO definitions are commonly referred to and shown in Table 1-4.

NCEP definition (3/5 required)		**WHO definition (2/3 required)**	
Waist	>102cm	Waist	>94cm
	>88cm		>88cm
Dyslipidaemia	TG >150mg/dl	Dyslipidaemia	TG >150mg/dl
	HDL <40mg/dl M		HDL <35mg/dl M
	HDL <50mg/dl F		HDL <40mg/dl M
BP	>/= 130/95	BP	>/= 140/90
	Or on treatment		Or on treatment
Fasting glucose	>110mg/dl	**and**	
		Evidence of Insulin resistance	

Table 1-4 Diagnostic criteria for the metabolic syndrome NCEP(Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2001), WHO (Nishida et al. 2010)

The presence of the MetS is associated with increased atherosclerosis (Chung et al. 2008). However, even allowing for 'traditional' risk factors, excess CV risk remains (del Rincón et al. 2001; Rho et al. 2009) and this may reflect the inflammatory process. When considered together the risk is additive and any CV risk estimation and treatment threshold should reflect this.

1.11.1.3 Endothelial injury explaining increased CV risk

One hypothesis that may explain increased CV risk is that of the presence of similar pathology existing in the atherosclerotic plaque and joint. Endothelial injury by reactive oxygen species (Cai & D. G. Harrison 2000) and influx of inflammatory cells such as macrophages and T-cells (Frostegård 2011) has been observed and may represent the

earliest stages of atherosclerosis. Tumour necrosis factor alpha (TNFa) may be responsible for driving this action and the recruitment of effector leucocytes. Systemic action upon adipocytes may be responsible for abnormal lipid profiles. Additional reasons why patients may experience more events include use of steroids, adverse fat distribution (with a preponderance of central obesity) and use of non-steroidal anti inflammatories (Graham et al. 2005). Finally, patients are less likely to report typical symptoms and have unrecognised events (Maradit-Kremers et al. 2005).

1.11.1.4 Addressing increased CV mortality in RA; risk scoring systems

There are a number of scoring methods by which the cardiovascular risk in RA may be quantified. However the thresholds to treat are more uncertain. NICE guidelines and those below would generally suggest treatment and lifestyle advice should be offered if the 10-year risk score is >20%. This is the suggested threshold for statin therapy.
Of the scoring systems available, each weights CV risk factors differently and is dependent on the source cohort studied in order to determine risk. The original and best-known worldwide is the Framingham score. The Joint British Societies Coronary Risk Prediction (JBSCRP) is based on this cohort and most familiar to physicians as accessible in the British National Formulary. It may underestimate risk by not accounting for strong family history, elevated triglycerides and impaired glucose tolerance.
The ASSIGN score (ASSessing CV risk using SIGN guidelines to ASSIGN preventative treatment) has become more widely adopted in Scotland by nature of taking account of the effect of social deprivation and family history. It also allows for the number of cigarettes smoked.
Within Europe, the SCORE (Systemic Coronary Risk Evaluation) risk calculator is suggested, although also acknowledged as imperfect. It has been derived combining large mixed European cohort studies. Population differences are thus factored in through application of 'lower' and 'higher' risk country of study.

The significance of addressing cardiovascular risk is recognised in the 2010 EULAR guidelines (Peters et al. 2010). The taskforce set out guidance for the management of cardiovascular risk management. The goal is firstly the recognition of excess mortality and education in this area. Secondly, the creation of a framework of guidelines for the management of this excess risk; an annual assessment is suggested irrespective of the presence/absence of risk factors. They note that scoring models such as the SCORE system may underestimate the true risk conferred by traditional risk factors and that inflammatory arthritis be considered an independent risk factor. A x1.5 risk multiplier is suggested. Since the presence of inflammation and risk factors can both be modified, aggressive treatment of both is required.

Blood pressure targets were set by the British Hypertension Society in 2004 and published in conjunction with NICE in 2006. These have been updated in 2011 to include the addition of home or ambulatory BP (NICE CG 127 Hypertension, August 2011).

BHS Guideline (Williams et al. 2004)				NICE guidelines-management of hypertension (Krause et al. 2011)		
	Degree of hypertension	**SBP**	**DBP**	**Degree of hypertension**	**Clinic BP AND**	**Ambulatory Home BP daytime average**
Clinic BP	Normal	<130	<85	Stage 1	≥140/90	≥135/85
	High normal	130-139	85-89	Stage 2	≥160/100	≥150/95
	Mild (Grade 1)	140-159	90-99	Stage 3	≥180	n/a
	Moderate (Grade 2)	160-179	100-109			
	Severe (Grade 3)	>180	>110			
	Isolated systolic hypertension					
	Grade 1	140-159	<90			
	Grade 2	>160	<90			

Table 1-5 Summary of British Hypertension Society (BHS) and NICE hypertension guideline targets (SBP-systolic blood pressure, DB-diastolic blood pressure)

Treatment is indicated for moderate hypertension, mild hypertension with hypertension related/target end organ complications or CV risk of >20% in the next 10 years. In the 2011 guideline, treatment is indicated for stage 2 and 3 hypertension or Stage 1 with end organ complications or CV risk of >20% in the next 10 years.

Finally it is hoped that the biologic therapies may offer the potential to reduce the risk of cardiovascular death through either a drug or class effect or simply through improved disease control (Dixon et al. 2007; Askling & Dixon 2011). Interestingly, effective treatment with TNFa inhibitors may result in increased activity yet weight gain and an alteration toward an adverse lipid profile. A lower threshold for statin use may be justified as by improving lipid profile but also an anti-inflammatory, disease-modifying role (McCarey et al. 2004).

1.11.2 Systemic Co-Morbidities

RA is associated with accelerated bone loss (osteopaenia) and is an independent risk factor for osteoporosis. Bone loss is not only local (disuse of an inflamed joint) but also systemic due to osteoclast activation. Additionally, patients are more at risk of falls, more likely to be female, undertake less physical activity (known to be protective of bone mineral density) and lower skeletal muscle mass/BMI. Long- term 'low dose' steroid employed as a DMARD is an additional risk factor.

1.11.3 Socio-economic impact

The socio-economic implications of RA should be considered. Work related disability as a consequence of RA is seven times that of the general population within five years of diagnosis (Albers et al. 1999) and 32-50% after 10 years of disease (Allaire et al. 2008). It

is assumed these figures will fall with better treatment. Indeed factoring in return to gainful employment is a part of the economic calculations behind TNFi use in the UK. It is difficult to factor in availability of social support and use of informal help.

1.12 Disease Assessment

There is no single measure to assess rheumatoid arthritis. Evaluation remains important to gauge effectiveness of therapy and any interventions. As RA is a chronic, fluctuating and relapsing condition, assessments must continue to be lifelong and encompass not only the disease itself but also the impact upon the individual. Disease activity can be assessed rapidly with clinical and laboratory measurements. Patient reported questionnaires have been in use for over twenty years in order to capture the impact on the individual. Additionally the number of imaging modalities has expanded greatly over the last decade, some of which are employed routinely in clinical care.
Assessment may be considered in terms of subjective and objective outcomes or as patient and physician centred outcomes. Assessment of RA both in clinical care and the research setting may be considered in a number of different domains.

- Assessment of Inflammatory joint disease activity
- Assessment of extra-articular disease manifestations
- Assessment of established articular disease
- Impact upon the individual; social, economic, psychological

1.12.1 Assessment of disease activity

The main reason to assess disease activity is a means to gauging response to treatment change and determines additional therapeutic modifications. Any assessment must serve as a snapshot of disease activity to act as a reference point that can be easily communicated to other health professionals. Assessment must be performed in a rapid, reproducible and quantifiable fashion. I have evaluated the clinical, laboratory, patient reported and imaging outcomes in use in clinical practice to position them in context in this study.

1.12.2 Clinical Assessments

Joint swelling (SJC=swollen joint count) and tenderness (TJC=tender joint count) are physically assessed by palpation. Synovial proliferation and thickening results in swelling and pain may result from both active inflammation and joint damage. SJC may be a stronger predictor of structural damage than the TJC (Emery et al. 2009).
The reporting of joint counts has been standardised in the 28 joint count:- ten metacarpophalangeal joints and proximal interphalangeal joints, wrists, elbows, knees and shoulders (Prevoo et al. 1995; Smolen et al. 1995). Although the feet are not assessed in this way, it is generally considered that those that are assessed are sufficiently representative.

The advantage of a composite score representing disease activity is that it encapsulates disease in one easily recorded and referenced index that can be shared between professionals. The weaknesses of clinical joint exam include the inter-observer reproducibility and the potential impact on DAS28 score (Marhadour et al. 2010). The same authors observed that synovitis, particularly low grade, is underestimated when compared with ultrasound findings in longer-standing arthritis (Jousse-Joulin et al. 2010) It is important that the same observer carries out joint examination where possible. In the longer term, clinical joint findings may not correlate well with radiographic progression and/or functional decline.
Usual clinical care consists of an informal joint examination whereas a movement toward this standardized, reproducible and quantifiable assessment occurring in routine care has been slower to be adopted.

1.12.2 Composite scoring and response criteria

In practice, disease activity measures are combined into composite scores to allow standardised comparison. These scores have the advantage of familiarity to physicians and offer a rapid point of reference. In the absence of alternatives such as biomarkers or a 'molecular DAS' or indeed performing imaging on all clinic attendees then it is likely such assessments will persist. Assessments in a routine clinical care setting continue to differ from those in the trial setting.

1.12.2.1 DAS28 (modified DAS)

The modified DAS28 (DAS='disease activity score') is widely adopted in Europe (Prevoo et al. 1995). Van der Heiijde et al updated this from the original 1990 and 1993 methods for disease assessment. This had composed of the Ritchie Articular index, patient global assessment, erythrocyte sedimentation rate (ESR) and 44-joint count. The 'modified DAS' scores 28 joints and scoring requires a calculator owing to the complex calculation that allows weighting of factors. It requires the input of four variables- the TJC, SJC, patient global assessment of disease activity and either the ESR or C-reactive protein (CRP) (DAS-28 CRP (Fransen et al. 2003). The incorporation of a patient assessment of overall disease is completed using a ten-point Likert scale. The score is a continuous result; a DAS of >5.1 represents high disease activity, moderate activity >3.2-<5.1, low disease activity is >2.6-<3.6 and remission is less than 2.6.
The DAS has both subjective and objective elements. It has been extensively validated and adopted in large-scale trialling acts as a fundamental outcome measure. Furthermore the DAS28 then represents a treatment target and benchmark for satisfying TNFi prescription in the UK (Deighton et al. 2010).

However, the DAS is subject to influence by a wide variety of factors including age, gender, health and illness expectations, mood and co-morbidity. This introduces variability, which must be considered. The TJC and SJC contribute around half of the overall score, the TJC is slightly more weighted. As illustration, a patient global score of 100 may add as much as 1.0 to the DAS score. ESR contributes fifteen per cent of the DAS28-ESR and thus, in other states where the ESR is not suppressed, may result in a

DAS28 not achieving remission (where joints in fact are). Diffuse increases in gamma globulins and non-articular comorbidity often accompany longstanding RA, both of which may increase the ESR.
Patients with concurrent chronic pain syndromes such as fibromyalgia would be expected to demonstrate high patient global assessments and TJC where the SJC and inflammatory markers may not be elevated. In the same way articular damage, as is evident in longstanding RA, may elevate the TJC where inflammatory disease is not present.
An additional weakness of clinical joint exam is inter-observer reproducibility (it is important that the same observer carries out joint examination where possible) and the potential impact on DAS28 score (Marhadour et al. 2010).
The same authors observed that synovitis, particularly low grade, is underestimated when compared with ultrasound findings in longer-standing arthritis (Jousse-Joulin et al. 2010) . Significant disease progression may still occur in these states (Brown et al. 2008; Brown et al. 2006) and for this reason explain the observation thatclinical joint findings in long term follow up may not correlate well with radiographic progression and/or functional decline.

The DAS28 CRP could therefore be considered more specific and sensitive, as it is not affected in the same way as the ESR. CRP is a more direct measure of inflammation. It is rapidly produced in the liver under the influence of other cytokines such as TNFa and IL-6 whereas ESR resolution is more gradual. CRP is a general marker of inflammation but can also be driven by infection or malignancy.
Early standard CRP assays were sensitive to a value of <5mg/l where more commonplace high sensitivity (hsCRP) assays measure as low as 0.3mg/dl. There are several methods of detection of CRP including nephelometry, ELISA and turbidimetry. Values where more than one assay is examined may not be comparable (Roberts et al. 2001). Values >3mg/l may associated with increased cardiovascular risk and discussion around integration in cardiovascular risk algorithms remains or in otherwise ‘healthy’ individuals remains contentious (Hingorani et al. 2012).

The most recent ACR/EULAR definitions of remission suggests incorporation and use of the CRP in clinical trials (Felson et al. 2011). For the reasons above, CRP may underestimate disease activity if the same cut-offs defining disease activity is applied as the DAS28 ESR (Matsui et al. 2007). DAS28 ESR and CRP are not interchangeable.

1.12.2.2 Response Criteria; ACR and EULAR responses

The American College of Rheumatology (ACR) and European League against Rheumatism (EULAR) response criteria are largely used for treatment evaluation, particularly in the trial setting, to reflect change. They can also reflect a desirable target to achieve whereas the DAS is a continuous scale.
In the ACR index, both the 28 TJC and SJC must improve by 20, 50 and 70% respectively to achieve ACR20, ACR50 and ACR70 responses *in addition to* improvements in three of the five remaining domains (patient pain visual analogue scale (VAS), patient global VAS,

physician global score, patient disability assessment (HAQ) and acute phase reactant (ESR or CRP) (Felson et al. 1995). An 'ACR20 response' is the definition of a minimum response observed between placebo and DMARD. The weakness of this composite is that significant improvements in some domains may well be observed but a lack of response in one would still deem the response as a failure. By discriminating in as many domains from placebo this is also a strength.

At least a 20% decrease in number of tender and swollen joints plus a decrease of at least 20% in three of the following core domains
• Patient and physician global assessment of disease
• Patient assessment of pain
• HAQ-DI (health assessment questionnaire disability index)
• ESR
• CRP

Table 1-6 ACR response criteria (Felson et al. 1995)

The EULAR response criteria compares baseline DAS28 evaluation with a second measurement grouped as none, moderate or good according to the DAS change (van Gestel et al. 1996).

DAS28 final score	Improvement in DAS28		
	>1.2	>0.6 to <1.2	<0.6
<3.2	good	moderate	none
3.2 to 5.1	moderate	moderate	none
>5.1	moderate	moderate	none

Table 1-7 EULAR response criteria (adapted from (Hyrich et al. 2006)

1.12.2.3 SDAI and CDAI

The Simplified Disease Activity Index (SDAI) (Smolen et al. 2003) and the Clinical Disease Activity Index (CDAI) (Aletaha & Smolen 2007) are also continuous measures being adopted in continental Europe and form part of more recent recommendations in clinical trialling outcomes.

The SDAI is the sum of the 28 TJC, the 28 SJC, patient and physician global assessment of disease activity (0-10 Likert scale) and CRP (mg/dl). It avoids the need for a calculator but as a CRP result will not be available until after the consultation, an immediate result is not to hand. Similar weighting of the TJC and SJC is made however. The authors suggest remission is an SDAI <3.3, mild disease activity is represented by an SDAI <3.4-11, moderate 21-26 and high >26. A change of -22 represents a major improvement and -10 to -21 a minor improvement.

The CDAI uses the sum of the TJC and SJC, patient's assessment of pain and global disease score. There is no need for an acute phase reactant. Remission is <2.8, low disease activity 2.9-10, moderate disease activity 11-22 and high disease activity >22. Both measures have been validated and show correlation with the DAS28 (Aletaha & Smolen 2007).

The most up-to-date American College of Rheumatology guidelines (developed after the inception of this study) reviewing RA disease measures suggests that the use of the SDAI, CDAI and DAS28 ESR or CRP are all valid, discriminative measures of disease activity (J. Anderson et al. 2012) .

A comparison between the different means of assessment is shown below. Each captures slightly different patient information, time to complete and inherent problems.

	TJC	SJC	PG	PhG	Patient VAS	HAQ	ESR	CRP
DAS28 ESR	✔	✔	✔				✔	
DAS28 CRP	✔	✔	✔					✔
ACR response	✔	✔	✔	✔	✔	✔		✔
CDAI	✔	✔	✔	✔				✔
SDAI	✔	✔	✔	✔				

Table 1-8 Variables assessed by the commonly used disease activity assessment tools in RA (adapted from Fujiwara 2012) PhG-Physician global, PG=Patient Global

1.12.3 Laboratory Measures

1.12.3.1 ESR and CRP

The ESR and CRP are termed 'acute phase reactants' and have been long known to be useful in the assessment of RA patients (Amos et al. 1977). Both are sensitive but lack specificity representing surrogate measures of the inflammatory response. In this way they do correlate with clinical measures of disease activity and may have some value in predicting radiographic progression. For some individuals, the ESR and CRP may be, and remain throughout disease course, resolutely normal. ESR is subject to other confounding variables including anaemia, gender, age and plasma proteins such as fibrinogen and globulins, higher RF titres and immunoglobulins. Secondary Sjogren's syndrome should be considered as a cause of ESR elevation.

CRP correlates with interleukin-6 and TNFa levels and most notably increases with infection. It is not affected by those variables above and in this way the CRP may be of more value (Wolfe 1997). A suppressed CRP correlates with improvements in and

maintenance of function (Devlin et al. 1997) and the CRP level correlates with radiological progression over time (M. J. Plant et al. 2000).

1.12.3.2 Immunology

As addressed above, the presence of rheumatoid factor and ACPA help to inform prognosis when used alongside clinical factors. Autoantibody titres per se do not correlate with disease activity. Routine re-assessment is not usually indicated unless the initial presentation is uncertain.

1.12.3.3 Other variables

A normochromic and normocytic anaemia is often observed in longstanding RA. This may represent a state of reduced marrow production owing to chronic inflammation and increased peripheral turnover. Concurrent iron deficiency owing to gastro-intestinal loss or failure of utilisation may be present. Hypoalbuminaemia is a non-specific finding reflecting increased catabolic protein turnover in the acute phase response. Thrombocythaemia, reflecting increased turnover and marrow production may be demonstrated in acute or chronic inflammation and usually follows the ESR and CRP. Complement consumption can be seen in severe RA with extra-articular disease such as vasculitis. Such peripheral blood findings are supportive but lack sufficient sensitivity to justify informing routine decision-making.

1.12.4 Patient Reported Outcome Measures (PROMs)

The assessment of joint activity through clinical assessments and laboratory measures must be considered alongside a subjective patient assessment of function. Broadly, these are short questionnaires completed by the patient as short accessible questionnaires completed in a few minutes potentially in the clinic waiting area. PROMs are available for the majority of common rheumatological disorders capturing the disease specific dimensions. They correlate well with clinical findings, inflammatory markers and radiographs. They will often reveal information that is of more concern to the patient and family that many often do not feel they can discuss in clinic or time is available for. They are however open to external non-disease related influences. There is extensive published data to support the use of PROMs and the sensitivity of the data they provide (Russak et al. 2003). Several PROMs were utilised in this study to capture these domains and are thus examined.

1.12.4.1 Health Assessment Questionnaire Disability index (HAQ-DI)

The HAQ is an instrument that measures physical functioning. The HAQ-DI (disability index) (Kirwan & Reeback 1986; Pincus et al. 1983; Bruce & J. Fries 2005) is a two page, twenty-activity modified version of the original HAQ which asks the patient to self-assess physical functioning over the last week in terms of ability or otherwise to perform eight daily household tasks (dressing, arising, eating, walking, hygiene, reach, grip, and common activities). These are rated at four levels of difficulty from zero to three representing ‘without any difficulty’, ‘with some difficulty’, ‘with much difficulty’ and

'unable to do' respectively. The need for aids to perform these tasks and need for help from another individual is also included. A scale to assess pain and global assessment of disease activity may also be included (this was assessed as part of the DAS28 evaluation in this study). If a category score is less than two it is increased to a 'two' if help from another person or device in that category is needed. The summary score is an average of the responses such that a higher score represents poorer function. Total administration and scoring time is short. Scoring was carried out in this study as suggested in previous publications listed by the main authors (Bruce & J. F. Fries 2003).

The main weakness of the HAQ lies in an inability to discriminate between the effect of longstanding articular damage and active inflammatory disease. In later disease, whereas the DAS may be relatively constant, the HAQ tends to increase reflecting articular damage rather than inflammatory disease (Welsing et al. 2001). Thus age, pain and patient global assessment of disease correlate with the HAQ. The HAQ is not 'disease-specific' and thus does not evaluate any psychiatric or social dysfunction. These domains and any other co-existent morbidity may of course influence the HAQ. Moreover the scoring system adopted for the HAQ does not equally weight the different components of the score for their true impact: for example inability to walk may equate in the score to a combination of less significant incremental reductions to upper limb function but to most observers the former would constitute a more substantial impediment.

The HAQ-DI is a key outcome in therapeutic drug studies and is routinely assessed in daily UK clinic practice and is a requirement as part of disease follow-up to judge biologic efficacy. The HAQ predicts work disability, healthcare costs, need for future surgery, correlates with disability and with laboratory and clinical variables.

1.12.4.2 EuroQol (EQ5D)

The EuroQOL (www.euroqol.org, (Brooks 1996) is often employed in clinical trialling and can be utilised as part of the assessment of cost utility analysis (QALYs) behind prescribing. It comprises of both a descriptive system of five questions to reflect health state on that day (namely mobility, self-care, usual activities, pain and anxiety/depression) and a visual analogue scale. Each question has three responses:- 1=no problems, 2=some problems and 3=significant problems. These responses may be combined to form a health state ie 11221 would suggest some problems in carrying out usual activities and some pain experienced. The five-digit health state can be converted to a single summary index using value sets from reference populations. This transformation is most useful when measuring change after an intervention or treatment. The 20cm VAS is completed to reflect a general health state from 'best to worst imaginable' whereby 100 represent best and 0 represents worst. Thus the EuroQol is quick to complete and straightforward to use.

The EuroQol has been validated and tested in rheumatic diseases (Hurst et al. 1997; Wolfe & Hawley 1997) and tested in non-UK populations (Luo et al. 2003) and other authors have found good correlation with other HRQoL instruments such as the SF-36 (Mahadeva et al. 2009), HAQ and clinical variables.

1.12.4.3 Short Form-36 version 2 (SF-36v2)

Quality of life in RA may be assessed using the SF-36. The generic SF-36v2 (www.sf-36.org, www.qualitymetric.com) comprises thirty-six questions, thirty-five of which address eight domains (or scales) as shown in Table 1.9.

Domain	Content	Number of items in SF-36v2
Physical functioning (PF)	Performance of physical activities including self-care, walking and vigorous activities	10
Physical role limitations (RP)	Degree to which typical roles ie job, childcare are limited by physical health.	4
Bodily pain (BP)	Intensity, duration and frequency of bodily pain and how limits usual activities	2
General health (GH)	Beliefs and evaluations of one's overall health	5
Vitality (VT)	Feelings of energy and absence of fatigue	4
Social functioning (SF)	Degree to which social relationships are maintained in relation to impairment caused by limitations	2
Role-emotional (RE)	Degree to which typical roles ie job, childcare are limited by emotional health.	3
Mental health (MH)	Emotional, cognitive and intellectual status	5

Table 1.9 Core component domains of the SF-36 v2 questionnaire

These values may also be further combined into a **Physical Component Score** (PF, RP, BP, GH) and **Mental Component Score** (VT, SF, RE, MH), which are often referred to as the 'representative summary outcomes'. Each question may have five response levels and asks patients to reflect on the last four weeks. It is therefore more time consuming but provides a comprehensive snapshot of a number of different health domains and overall quality of life. Version two (1996) is a generic survey that can be used across populations and diseases. Scores are transformed using a linear T-score transformation to a 0-100 score where 0=worst health and 100=best. It is scored using 50 as a reference population 'norm' and standard deviation (10) allowing meaningful comparisons across categories using the reference general US population. In this way a score below 50 represents below average health in that domain. It has been validated in RA (Kosinski et al. 1999).

1.12.4.4 Hospital Anxiety and Depression Scale (HADS)

Depression and anxiety have been recognised as important co-morbid features of medical illnesses for many years. They may both present with physical illness or affect the presentation of a separate medical illness including inflammatory joint disease.

The HADS has been in use for many years (Zigmond & Snaith 1983) is a valid and reliable tool (Bjelland et al. 2002) takes a few minutes to complete and assesses both depression (HADS-D) and anxiety (HADS-A). There are seven items for each mood state and four possible responses scoring 0-3 reflecting mood over the preceding few days. Thus depression and anxiety scores range from zero to twenty-one. Interpretation of these values is suggested by the authors whereby 0-7 represents no further evaluation required, 8-10 representing mild depression, 11-14 representing moderate and 15-21 severe mood disturbance.

1.12.4.5 FACIT-F scale

As part of a series of questionnaires (http://www.facit.org/FACITOrg/Questionnaires) originally developed to assess heath domains in chronic illnesses and in particular Oncology, the Functional Assessment of Chronic Illness Therapy Fatigue Scale (FACIT-F) is a validated (Webster et al. 2003; Cella et al. 2005; Chandran et al. 2007) single page questionnaire to quantify fatigue. There are thirteen items and responses rated 0-4 where 0=not at all, 1= a little bit, 2=somewhat, 3= quite a bit and 4=very much. Recall is over the last week. Scoring is reversed such that a *lower* score represents more fatigue and maximum score 52 and minimum zero as outlined in Appendix 7. The experience and impact of fatigue are evaluated. Alternative measures of fatigue in RA exist but many lack validation (Hewlett et al. 2007). Furthermore, there is no definition of or cut-off that represents 'fatigue' as this is remains subjective.

Fatigue remains one of the most difficult elements of RA to treat. It is a frequent complaint in up to 40% of patient with RA (van Hoogmoed et al. 2010) and the symptom patients would most like treated (Wolfe & Michaud 2004). Pain and mood are often associated whereas neither van Hoogmoed nor Nikolaus found inflammation to be related to fatigue (van Hoogmoed et al. 2010; Nikolaus et al. 2013). Nikolaus et al identified that the experience of fatigue differs according to such fundamental variable as age, gender and daily social roles (Nikolaus et al. 2010). This is important knowledge if treatment is to be targeted and screening done most judiciously.

In order to put the FACIT-F values in context (but not to directly compare), the mean FACIT-F values of recent selected but representative biologic therapy trials are shown below. As fatigue is an important patient outcome it is widely reported in clinical trialling albeit any effect of biologic therapy is disappointingly small (Chauffier et al. 2012). The raises the possibility that factors other than inflammation may be important in driving fatigue.

Mean FACIT-F score baseline	Study (reference)	
30.4	REFLEX	(Cohen et al. 2006)
23.0	GO-AFTER	(Smolen, Kay, et al. 2009b)
27.7	OPTION	(Smolen et al. 2008)
28.4	ARMADA	(Weinblatt et al. 2003)
27.01	DANCER	(Emery et al. 2006)

Table 1-9 Selected representative mean FACIT-F levels from selected published significant biologic trials

1.12.4.6 Rose Angina Questionnaire

In view of the known increased prevalence of IHD and myocardial events in this population, the Rose angina questionnaire was completed at interview at baseline only (Rose et al. 1977). It has since been reproduced and adapted in different forms such as the WHO angina questionnaire. There are a number of cohorts followed up several decades after Rose questionnaire administration that demonstrate higher cardiovascular mortality (Murphy et al. 2006; Graff-Iversen et al. 2008). The questionnaire administered (Appendix 7) is that published by (Lawlor et al. 2003). McEntegart et al identified 30% of a group of seventy-six RA patients were Rose angina chest pain positive versus healthy controls but there are no other publications applying the Rose questionnaire to RA cohorts (McEntegart et al. 2001).

It aims to establish the prevalence of those experiencing chest pain and then goes on to establish if this pain is exertional and consistent with angina. Results could therefore be: no chest pain experienced (no further questions were asked) or if chest pain was present then may be categorised as definite Rose angina (exertional chest pain and typical features prompting Cardiology referral) or possible Rose angina (highlighted to GP) and atypical pain (no further action taken).

The use of PROMs is commonplace in drug trialling (MASSAROTTI 2008) but uptake has been slow in daily practice other than in satisfying the need for economic justification. Patients are usually happy to discuss the domains such questionnaires address and which are arguably of greater importance to the patient. Furthermore they may provide information to the patient beyond simple improvements in clinical and biochemical measures that may inform compliance and establish physician-patent relations (Miedany et al. 2011). In a 2003 evaluation of ACR members, despite 63% considering such evaluation as of benefit, only 48% routinely collected this data (Russak et al. 2003). 61% considered the major barrier time taken to complete/score. A similar survey by Wolfe et al confirmed the higher importance many Rheumatologists place on these evaluations in a trial setting rather than daily clinical care (Wolfe et al. 2003). Greenhalgh et al reviewed the barriers to and evidence against more widespread

implementation (Greenhalgh et al. 2005). These include factors such as physicians not making treatment change on the basis of PROMs data only, that they may not consider discussion of these domains as important, unfamiliarity with the scales and meaningful changes, uncertainty how to interpret the psychological evaluations and the time involved. At present the current positioning of regular PROM data collection is uncertain.

1.12.5 Imaging

Imaging allows quantification of joint damage and thus severity. Effectiveness of therapy can be judged by examining prevention of joint damage with time.

1.12.5.1 Plain X-Rays

The most commonly employed and accessible means to assess joint structural damage is plain radiographs, most often of hands and feet. Joint space narrowing reflecting cartilage loss, and presence and extent of characteristic juxta-articular erosions are assessed by a number of standardised and reproducible scoring systems such as the modified Sharp and Larsen score. Progressive erosions are an indicator of end-organ damage and thus represent inadequate treatment. Plain X-rays may lack sensitivity in early disease, an observation that is particularly in view of current effective therapies starting ideally before erosions are visible. However erosions would be expected to develop in the first few years in poorer prognosis disease. In later disease (and in these older cohorts), the presence of new erosions tends to plateau and sequential radiographs provide less information (Salaffi & Ferraccioli 1989). A further weakness is relatively slow change with time often in the order of six to twelve months. Although they correlate with deformity they do not correlate well with joint counts, predicting work disability or functional outcome (van Riel & Fransen 2007).
There have been a number of methods to score radiographic damage designed to standardize outcomes (Boini & Guillemin 2001) . The original Sharp method in 1971 was modified by van der Heijde in 1989 and is very much the standard for reporting in clinical trials (van der Heijde 1999). The Larsen scoring method is the alternative although both correlate with outcome. Both systems assess joint space narrowing and erosions, quantifying each and scoring accordingly, though differ in the joints assessed. Feet and hands are usually examined.

1.12.5.2 Musculoskeletal ultrasonography

Ultrasound (US) permits rapid, quantitative assessment of synovitis with real-time power Doppler use. It is more sensitive at detecting subclinical synovitis:- for example grade 1-2 synovitis remains clinically undetectable but represents inflammation that may lead to joint damage. Tendinopathy, entheseal assessment, quantification of and guided injection of joint effusions are also possible.

1.12.5.3 MRI (magnetic resonance imaging)

MRI is more sensitive at detecting inflammatory disease than clinical and/or radiographic findings. Reductions in CRP correlate with measures of inflammation from

MRI including bony oedema/osteitis (Emery, van der Heijde, et al. 2011b). It is perhaps the assessment of bony change where MRI supersedes US. Time to train individuals in reporting is considerable and scanning time significant unless focused examination is carried out. It remains prohibitively expensive and not in widespread use.

There is a requirement to diagnose inflammatory disease early to commence treatment. Plain X-rays are an insensitive tool in early disease but may help in detecting progression. Those presenting with erosions at first clinic visit have poorer prognosis disease but often because they present later – established disease being more treatment resistant. Choice of imaging is thus important.

Imaging also presents the possibility of a new paradigm of 'imaging remission'. Radiological progression can progress despite low clinical indices of inflammation. Such patients have demonstrable imaging inflammation yet clinically undetectable disease (Wakefield et al. 2004). Incorporating such sensitive imaging modalities in routine care in such a way as to be time effective and addressing the need for training remains to be resolved in the UK.

1.12.5.4 Disease assessment- discussion

The assessments above may be considered as both short and longer term measurements. Shorter-term assessments in the order of weeks to less than a year would be assessed by the components of the DAS, employment status and inflammatory markers. Longer-term disease assessment in the order of years is best gauged by deformity and need for joint surgery, x-ray scores and progression thereof, functional capacity (which can again be reflected by employment status), number of comorbidities and mortality rate as compared to matched populations.

1.13 Treatment

The treatment of RA involves the coordinated and parallel management of medical and allied health professionals. Involvement of relevant medical specialties such as Respiratory, Neurology, Psychiatry/Psychology and Orthopaedic surgical are necessary to manage co-morbidities.

1.13.1 Multidisciplinary Therapies

Briefly, the involvement of allied health professions such as physiotherapists (joint protection, mobilisation and strengthening, education and treatment options), occupational therapists (provision of aids in the home and workplace to overcome limitations of function), social work involvement (in terms of access to disability entitlement, appropriate housing, social care and employment issues), podiatry and orthotics and psychiatry/psychology are all important in the treatment of this condition (SIGN Guidance Ref No. 123, NICE clinical guideline CG 79 Feb 2009, British Society for Rheumatology guidelines on standards of care (Kennedy et al. 2005).

The role of the Rheumatology Nurse Specialist is key in terms of offering counselling prior to medication start, advice with medication dose escalation, practical injection technique and at times of potential drug toxicity. Furthermore, in many centres the role of the Rheumatology Specialist Pharmacist is increasing.

1.13.2 Disease modifying therapies

With understanding of the pathogenesis o RA as outlined above, treatment options have become increasingly diverse to target molecules within these pathways.
There exists a wealth of data and publications to inform recommendations on the best use of therapies for RA (Smolen et al. 2010). The disease modifying therapies in general use and taken by those included in this study cohort will be outlined.

1.13.2.1 Choice of therapy

Before initiation and having established the diagnosis, choice of therapy is determined by factors such as-

- Efficacy (as determined by randomised controlled trialling and interpreted by the physician. This is further enhanced by expert recommendations in the form of guidelines)
- Safety profile (evidence not only from trialling and registry data but personal experience)
- Individual patient history ie including age and co-morbidities
- RA phenotype (aggressive and poor prognostic factors such as the presence or absence of rheumatoid factor and ACPA)
- Disease stage (early less than 2 years to established disease)
- Patient choice (delivery device of drug, route of delivery, frequency of delivery)
- Drug cost (Kavanaugh 2007)

At present decision-making is not driven by molecular disease classification or predictive biomarkers of response of toxicity.

1.13.2.2 Treatment

1.13.2.2.1 Treatment Principles

The main aim of treatment of inflammatory joint disease is remission, or at least low disease activity as soon as practically possible. This is defined as ‘symptom-free’ through the application of a composite disease activity scores such as the DAS scoring system. Treatment of inflammatory disease is associated with the prevention of radiological damage, the strongest predictor of disability (van der Heijde et al. 2008). The strategy employed to achieve this state is important. Broadly, these fall into several categories often employed together.

- The choice and subsequent order of employment of disease modifying agents

(DMARDs) in step-up, step-down and combination regimens (Möttönen et al. 1999; Landewé et al. 2002).

These have consistently performed better than sequential monotherapy strategies. Combination therapy can yield benefits comparable with TNFi in early arthritis. Therefore if cost is of major consideration, early treatment with a target of low disease activity through frequent follow up is more economically sensible.

- Early (<6 months) intensive treatment strategies to take advantage of the 'window of opportunity' concept (Goekoop-Ruiterman et al. 2007; Grigor et al. 2004).

These are dependent on prompt primary care referral. The benefits in this approach are maintained for many years even when the control of on-going inflammatory disease is allowed for.

- 'Treat-to-target' strategies aiming for remission in early disease (Schoels et al. 2010; Smolen et al. 2010).

By achieving remission it has been suggested that the inevitable progression of RA is halted. Using treat-to-target methods aiming for low disease activity, 65% remission rates were achieved but in current practice this is closer to 33% using available standard of care follow up and treatments (Irvine & Capell 2005; Grigor et al. 2004).

- Subsequent choice after TNFi failure

This is further discussed below.

Decision-making in established disease is less evidence based but follows similar principles to early arthritis. Those with more active disease and features of poorer prognosis disease should be treated with biologic therapies early rather than cycling DMARDs.

1.13.2.2.2 NSAIDs (non-steroidal anti inflammatory drugs)

NSAIDs remain first line *symptom* relieving treatments. Instigation is often early as a bridge to disease modifying therapy and continued until such time as possible to withdraw. Short-term adverse effects include triggering asthma and bronchospasm. Longer-term use has limitations:- daily use may result in peptic ulceration (to include gastric and duodenal irritation), which ranges from asymptomatic effect to dyspeptic symptoms to frank ulceration with bleeding. This is mediated via inhibition of prostaglandin necessary to maintain the gastric mucosal barrier. Owing to inhibition of renal cyclo-oxygenase (COX), hypertension and renal impairment may result.
There is a shift to avoid long-term use of non-steroidals as symptom relieving treatments in view of their long established gastro-intestinal adverse effects but more

recently association with increased cardio and cerebrovascular events. Within drug class there may be 'less' risk (eg Naproxen vs Diclofenac). Their place is very much as symptom controlling treatments and as adjuncts to DMARD therapy. It is of note that disease-modifying properties have been afforded NSAIDs in the spondyloarthropathy spectrum pointing to distinct pathogenesis in the latter.

1.13.2.2.3 Synthetic DMARDs

Despite the heterogeneous nature of RA auto-antigens and cytokine profiles outlined above, treatment for RA is typically less individualised. DMARDs represent a non-selective approach. Methotrexate (MTX) is first choice of the synthetic DMARDs in view of its low toxicity profile and efficacy (Smolen et al. 2010). Sulphasalazine, Hydroxychloroquine, Leflunomide and parenteral Gold remain efficacious particularly in combination strategies. Despite their longer term drawbacks, adjunctive use of oral and intra-articular steroid provide prompt clinical benefit and have a moderate disease-modifying role. In strategies of tight control they are essential. They may be used as a 'bridge' until DMARD effect is captured or in daily low dose (7.5mg or less).
Methotrexate is the so-called 'anchor' drug in RA. It has been used since the early 1980's with a well-characterised risk/benefit profile. Mode of action involves folate antagonism. Current recommendations and reviews emphasise early use and resort to combination with the biologic therapies when disease control is insufficient (Visser et al. 2009).
MTX permits flexible dosing regimens, favourable long-term safety data, the choice of route of delivery orally and subcutaneously and combination with other DMARDs (such as Sulphasalazine and Hydroxychloroquine most commonly), without additional toxicity. MTX however has a relatively slow onset of action at a time when rapid suppression of inflammation is important (Goekoop-Ruiterman et al. 2007). Strategies employing additional steroid in this period are important. The efficacy in suppressing radiological damage is also inferior to biologic agents alone (Genovese et al. 2002). In terms of efficacy, MTX alone is similar when compared with Etanercept (van der Heijde et al. 2006; Emery, Breedveld, et al. 2008a) and Adalimumab (Breedveld et al. 2006) but Tocilizumab may be superior (G. Jones et al. 2010).

1.13.2.2.4 Biologic agents

The advent of biologic therapies has transformed the care of RA for those not responding to DMARD therapies. The identification of relevant molecules involved in disease has allowed the manufacture of drugs specifically targeted to the same. Indeed the biologic drugs are expected to top the sales revenues generated in the USA in 2012 (Anon 2012). The TNFi are the best known and most widely prescribed biologic therapy and represent a cytokine-specific approach to treatment. These therapies are efficacious:- they reduce the signs and symptoms of RA and improve quality of life and return to/maintenance of employment (and hence cost effective). Most notably, they retard bony erosions through inhibition of osteoclast activity:- prevention of erosions is

associated with prevention of disability. There is now a good deal of robust evidence to support their efficacy and safety profile (Nam et al. 2010).

Those biologic agents approved for use in the UK are outlined in Table 1-10. In general these specific molecules are synthesised from human genes and bound to the Fc portion of human IgG to facilitate delivery. Approval for use within the UK is regulated. Currently there is a requirement for active disease (DAS28>5.1 on two occasions) and failure of two traditional synthetic DMARDs to achieve disease control. One requires to be MTX. In addition the response to TNFi must be measured; a DAS28 fall of at least 1.2 or to a DAS of less than 3.2 achieved within six months.
Most recently results from the orally delivered inhibitors of the intracellular signal transducing pathways such as Janus Kinase (JAK) and Spleen Tyrosine Kinase (Syk) have been published. I have not addressed these small molecule inhibitors, nor those drugs in earlier phase trials, as there were no patients recruited in this trial treated with any of these agents.

Mode of Action	Drug	Comments	Route of delivery
Anti TNFa	Infliximab	Chimeric (mouse, antigen bearing region and human, constant region) IgG monoclonal antibody	i.v 8 weekly after loading (dose adjusted)
	Etanercept	TNFa receptor-Fc fusion protein	s.c weekly
	Adalimumab	Fully human monoclonal antibody	s.c fortnightly
'Second generation' anti TNFa inhibitors	Golimumab	Fully human monoclonal IgG	s.c monthly
	Certolizumab	Humanised IgG Fab fragment-polyethylene glycol	s.c fortnightly after loading
B-Cell (CD20+) depletor	Rituximab	Chimeric monoclonal antibody	i.v on 2 occasions, repeat as indicated
Anti IL-6	Tocilizumab	Recombinant humanised monoclonal antibody of IL-6 receptor	i.v monthly (dose adjusted)
CTLA 4 (T-cell co-stimulation)	Abatacept	Recombinant human CTLA4 molecule + portion of IgG	i.v monthly after loading * (dose adjusted)
Anti IL-1	Anakinra	Humanised IL1-Ra (receptor antibody)	s.c. daily

Table 1-10 Mode of action and administration of available biologic therapies licensed to treat severe RA (s.c=subcutaneous, i.v-intravenous) *=subcutaneous formulation recently made available for use

1.13.2.2.5 Biologic limitations and side effects

These therapies are limited in their efficacy owing to primary inefficacy, a secondary loss of response of need to discontinue owing to side effect. Drug retention at 1 year from registry data may be between two thirds to around 80% of individuals.

Owing to molecular differences (even within the TNFi) there are different side effect profiles. In terms of side effects, there is an increased risk of infection with biologics (as there is with DMARDs), particularly in the first few months of usage (Galloway et al. 2011). The risk of reactivation of latent tuberculosis (TB) exists but is reduced by careful screening. Conventional infections are increased, as are more atypical infections, viral infections (such as herpes zoster) and of septic arthritis (native and replacement). The risk of cancer does not seem to be increased per se by drug. There are a number of other more rare phenomena including autoimmune (autoantibody positive) disorders, interstitial lung disease, progressive multifocal encephalopathy and low immunoglobulins (Rituximab).

Condition/Effect	Suggested Action
Major Surgery	Suspend treatment one week before and after (local practice may vary)
Vaccination	Live vaccines are contraindicated
Active infection	Suspend until treated. Presentation may be atypical and organism opportunistic
Active malignancy	Contraindication to starting therapy, suspension if new case detected for at least 5 years
Skin change	Probable increased risk of melanoma and non melanoma skin cancers with TNFi; careful examination pre-treatment and vigilance having started (Askling et al. 2011; Raaschou et al. 2013)
Demyelination (including family history of) or new neurological features	Contraindication to starting therapy/suspend and investigate
Congestive Heart Failure	Contraindication to starting therapy/ discontinue drug
Cytopaenia	Consider other causes, suspend drug and re-evaluate
Generation of auto-antibodies (ANA, DNA)	Evaluate presence of lupus-like features and suspend if present and pathological
Formation of anti-drug antibodies manifesting as loss of clinical effect	Only Infliximab licensed to increase dose (Adalimumab can be off license)

Table 1-11 Important considerations and cautions and with TNFi therapy
(Based on BSR RA guidelines on safety of anti-TNF therapies ((Ding et al. 2010)))

1.13.2.2.6 Biologic therapies in use

The TNFi block the binding of soluble TNFa and block TNF receptor. They were the first targeted biologic therapies after the pioneering work by Feldmann, Maini, Brennan et al and subsequent instructive trials in 1992 with Infliximab.

Etanercept is a fusion of the p75 TNF-α receptor and the Fc fragment of human IgG1. It may also bind less membrane bound TNFa and other members of the TNF superfamily (lymphotoxin-alpha) which may account for some of the differences in terms of side effect profiles. In keeping with Certolizumab it does not bind complement. It has the shortest half-life of the available agents.

Infliximab was the first TNFi licensed for use in 1999. It is considered more immunogenic owing to the mouse components and intravenous route of delivery. MTX reduces this observation thereby preventing secondary loss of effect.

Adalimumab has the longest half-life of the 'first generation' TNFi and can thus be administered subcutaneously fortnightly with a 10-20 day half-life. It is a fully human monoclonal antibody.

Golimumab and *Certolizumab* are the most recently approved TNFi. Their pegylated form allows for more infrequent dosing, while remaining delivered subcutaneously, which is an important patient related consideration.

Rituximab binds the CD20 receptor expressed at certain stages of B-cell development causing cell lysis. Depletion is temporary and return of symptoms coincides with B-cell reconstitution. Rituximab is effective in TNFi failures and may be most effective in those who are rheumatoid factor or ACPA positive.

Abatacept is a recombinant fusion protein that blocks the co-stimulatory signal between CD28 present on naïve T-cells and CD80/86 on antigen presenting cells thus preventing the interaction. This interaction is necessary for subsequent T-cell activation following antigen and T-cell receptor binding. Abatacept is effective at reducing the symptoms and signs of RA (Vital & Emery 2006). Evidence would suggest it is effective no matter the number of prior TNFi treatments. Best response may be when failure of TNFi occurs with a side effect rather than a loss of effect.

Tocilizumab acts to inhibit the pleiotropic cytokine IL-6 (both the cell-bound and free forms) and is effective in those who fail TNF therapy (Emery, Keystone, et al. 2008b). The mode of action additionally improves many of the systemic features of RA including fatigue and anaemia. In the same way notable perturbance of liver function monitoring, neutrophil counts and lipid profiles have been observed.

Anakinra is not addressed as is no longer routinely recommended for use in RA.

1.13.2.2.7 Biologics in Early RA

Infliximab (St Clair et al. 2004), Adalimumab (Breedveld et al. 2006), Etanercept (Emery, Breedveld, et al. 2008a), Abatacept (Genovese et al. 2008) and Golimumab (Emery, Fleischmann, et al. 2011a) were the landmark studies demonstrating superiority to

placebo in MTX failures in *early arthritis* in combination with MTX. Disease duration was longer than conventionally one would wait before commencing therapy in current practice, which may diminish the observed benefits.

The use of TNFi in earlier stages of RA is more controversial. The BeST (Goekoop-Ruiterman et al. 2007) and one year SWEFOT trials (van Vollenhoven et al. 2009) may suggest that early use of biologic therapy in combination with MTX induces remission in higher numbers compared to other strategies. Cost effectiveness is however in doubt (M. H. Y. Ma et al. 2010; Finckh et al. 2009) as the price of TNFi remains high relative to DMARDs and combination DMARDs are very effective for significant numbers.

1.13.2.2.8 Biologics in established disease

The patient with established disease differs from early disease where erosions are established and disability may be less responsive. Improvements in synovitis are still possible but damage is not albeit slowing of progression may thus be an achievable satisfactory target. Evidence for the first generation TNFi in RA comes from the large, placebo controlled trials of infliximab (ATTRACT (Lipsky et al. 2000), Etanercept (Weinblatt et al. 1999) and Adalimumab (Keystone et al. 2004; Weinblatt et al. 2003) in those in whom MTX has failed (biologic +MTX in MTX refractory disease). Rituximab, Abatacept, Tocilizumab, Golimumab and Certolizumab also demonstrate efficacy and cost-effectiveness in longstanding disease refractory to MTX alone (J. C. W. Edwards et al. 2004; Kremer et al. 2006; Keystone, Genovese, et al. 2009b; Smolen, Landewé, et al. 2009a; Smolen et al. 2008).

To date, two head to head biologic studies have been published. Gabay et al studied TCZ vs Adalimumab monotherapy with the former achieving better outcomes and Schiff et al Abatacept vs Adalimumab in MTX inadequate responders where outcomes were comparable (Gabay et al. 2013; Schiff et al. 2013) In meta-analyses these treatments may be roughly equally effective in reducing the signs and symptoms of RA and improving quality of life and psychological measures (Singh JA 2011). Large registry data suggests some difference in side-effect profiles that may inform treatment choices (infection rates, including TB, and incidence of tumours).

In general, study design is such that TNFi are added to those with active disease despite inadequate response to MTX. This trial approach has some weaknesses as in reality the MTX group having failed to respond to MTX would have switched DMARD or had add-in therapy. The inclusion criteria for the TNFi groups are also stringent so it is reassuring that evidence for safety and efficacy comes from large-scale registries.

There are nine biologic agents available and broadly ACR 20, 50 and 70 responses show little difference between all the agents. Around two thirds will obtain and ACR20 response as compared with 30-50% with MTX alone (Salliot et al. 2011; Singh JA 2011).). Choice between treatments is then guided by efficacy, safety profile, disease phenotype and con-morbidities.

These therapies then are judged as effective on the basis they reduce inflammation, improve function (HAQ) and are cost effective when all their benefits are considered.

1.13.2.2.9 Biologics in TNF failures

Following discontinuation of first TNFi directed therapy there are a number of options. The option of switch to different cytokine target or change in DMARD represent the major considerations and a strategy backed by randomised trials. Those drugs studied in randomised trials include Rituximab, Golimumab, Abatacept and Tocilizumab (Cohen et al. 2006; Smolen, Kay, et al. 2009b; Genovese et al. 2012; Emery, Keystone, et al. 2008b).

Response rates in those TNF inadequate responder patients following a switch vary but ACR20 response rates may be between one-two in every five patients. In general response rates are lower and of less magnitude with longer disease duration. There is no data to guide an individualised approach exposing the patient to potentially greater safety issues.

Guidance about switching between TNFi can be inferred from observational data. Primary non-responders have less chance of a response to a second TNFi. Response may be seen if the reason for discontinuation is intolerance but there is a higher chance of intolerance again. Secondary loss of response may also respond to an alternative TNFi (Smolen et al. 2010).

After this the choice is more contentious but a switch to a second appears justified from a wealth of observational registry data (Papagoras et al. 2010). In addition, meta-analysis data would suggest switching from a first to second TNFi irrespective of reason provides clinical benefit after TNFi failure although the magnitude of response is lower (Rémy et al. 2011). If the first TNFi is discontinued for reasons of primary or secondary treatment failure then the chances of a meaningful response are lower than if the reason were side effect (Hyrich et al. 2007). Indeed a switch to Rituximab may offer better response (Salliot et al. 2011). Switching to a third TNFi is least likely to demonstrate a meaningful response in this instance.
Best clinical response occurs with first treatment. In those trials examining options in previous biologic failures, responses are lower than first biologic (a function of those with a more severe arthritis being included and/or longer disease duration)

1.14 Mechanisms of Resistance to treatment

It is not known why some patients with RA respond well to treatment where others fail to do so. This requires to be put in context as not every patient who ‘fails to respond’ to treatment has ‘severe’ disease. Being treatment ‘refractory’ is rather easier to define as defined target variables exist and those that fail to achieve these merit treatment

escalation (Polido-Pereira et al. 2011). There are very few definitions of 'resistant RA' (Kroot et al. 1999) but 'severity' is a more subjective definition and taking into account a number of factors such as those described above such as related disability, co-morbidity, pain or ability to work.

DMARD resistance is likely to be multifactorial and hence why combination drug therapy is most effective. Primary resistance may represent existing genetic polymorphisms and thereby an opportunity to identify and apply 'personalised medicine' if these polymorphisms can be isolated and tested. Secondary resistance is less well understood and the molecular mechanisms not known. Van der Heijden et al have reviewed some of the previously reported mechanisms of drug resistance to specific DMARDs and these are shown in Table 1-12 (van der Heijden et al. 2007). The best-studied mechanisms remain MTX pharmacology (and folate pathways) and the ATP-binding cassette transporters, which are important for several DMARDs.

General mechanism of drug resistance	Examples
Impaired drug delivery to cells	Reduced absorption, binding or increased excretion
Impaired cellular uptake	Transporter effect; reduced uptake or enhanced efflux
Impaired drug activation or increased deactivation	Altered drug phosphorylation or polyglutamylation
Alterations in drug target or downstream of target	eg bypassing to use alternative pathways or protein binding

Table 1-12 Proposed molecular mechanisms of disease resistance
(Adapted from (van der Heijden et al. 2007))

Morgan et al propose the term 'multidrug resistance' to those who have demonstrated inefficacy to three or more DMARD (C. Morgan et al. 2003). In a cohort of 265 patients studied with mean disease duration of 10.7 years, thirteen met these criteria and were more likely to be female and RF positive. The biological phenomena underlying this observation were not determined.

The final challenge having *defined* resistance to therapy is to then measure it in observational cohorts. The observed effect in daily clinical use is often less than that observed in trials (Kievit et al. 2007). This can be explained by the stringent inclusion criteria in trialling that may not be reflective of local populations. Surrogate measures of treatment failure include a switch to an alternative biologic, intensification of dose of concurrent DMARD or increase in dose of biologic agent (Sidiropoulos & Boumpas 2006). Challenges include confounders such as disease duration, defining 'responders', concurrent steroid and DMARD use and dose, study size and differences in ethnicity. Making generalisation to other populations is therefore difficult.

1.14.1 Timing of first therapy

There are multiple publications that support the hypothesis that RA is most treatment responsive early. This window may be a year or perhaps two at the most. At the cellular level this represents immune cells adopting a self-perpetuating phenotype. It is not presently possible to determine which individuals will go on to develop persistent disease but there are factors that suggest disease course may be more severe (Table 1.2).

1.14.2 Response to Biologic therapy

Individual response to biologic therapy is not predictable. 'Resistance' to conventional therapy should be distinguished from those individuals displaying toxicity or adverse events during the course of their treatments. Some individuals may 'acquire' resistance to therapy. Ultimately for the individual the end result is the same but for the purposes of discussion these are separate. Three important responses occur following anti TNF therapy and different molecular mechanisms are at play in each circumstance.

1. Failure to achieve adequate disease response (primary lack of response)
2. An initial good response is lost with relapse (secondary loss of response)
3. Side effect/intolerance

For the first group, it is assumed the molecule targeted is not the primary pathological driver to disease. In the second an important cause may be formation of anti-drug antibodies as discussed below. Alternatively, immunological escape mechanisms are proposed as driven by alternative cell types or pathways. The final situation remains entirely unpredictable but is usually overcome by switching to an alternative drug targeting the same moiety.

Overall best response occurs to first biologic therapy and when employed earlier in disease course. Primary resistance to therapy may occur in up to a third (Hyrich et al. 2007). Overall major response rates of 52-67% with biologic therapies can be expected (Papagoras et al. 2010). Drug persistence also tends to tail with time but overall discontinuation for Infliximab or Etanercept after a year is around 1 in 5 (Sidiropoulos & Boumpas 2006). Most discontinuation occurs within year one and reaches a plateau by year two and thereafter. There may be differences between individual drugs and duration of persistence:- Etanercept demonstrates the longest persistence for individuals and Infliximab lowest of the three first-generation TNFi. Either or both ACPA and RF positive status strongly predicts Rituximab response. In contrast, the same signal is not seen with Abatacept in meta-analyses, TNFi nor Tocilizumab. This has been repeated in a number of studies and registry data.

1.14.3 Methotrexate pharmacology and co-prescription

Methotrexate is the most commonly prescribed first-line DMARD and as such, when examining mechanisms of resistance, is most widely studied.

The complex folate metabolism pathways represent many potential points of pharmacokinetic inter-individual variation from variable first pass liver inactivation to the 7-hydroxymethotrexate form, elevated drug efflux transporter action such as the ATP binding cassette transporters (a mechanism highly studied in Oncology) to altered polyglutamylation (a necessary step prior to downstream action) and variation within folate absorption and metabolism pathways.
Absorption and distribution of methotrexate has been well characterised. Physiologically, there is a plateau of drug that can be absorbed from the GI tract. This may have implications for those with higher BMI and thereby insufficient drug concentration in inflamed tissue. Resistance to therapy therefore may represent inadequate dosing. There is a single clinical trial that suggests some benefit of subcutaneous MTX over oral and a number of observational reports with the same finding (Braun et al. 2008).
Multidrug resistance protein (MDR-1) has been studied extensively in Oncology. It codes for the drug efflux pump that mediates rapid drug expulsion and may have a role in RA (Yudoh et al. 1999). Molecular and genetic study can be applied in this way to create a predictive model combining clinical factors with pharmacogenetic testing. In this way, the authors used gender, baseline DAS28, immunological status, smoking and genotyping (polymorphisms within the folate metabolism pathways) to predict likelihood of response to MTX (Wessels et al. 2007) .

When administered *with* MTX, the biologic therapies represent the most effective treatment available. ACR 20/50/70 responses are broadly similar between therapies however. Both the PREMIER (Adalimumab) and TEMPO (Etanercept) study examined a biologic monotherapy arm. Remission rates were similar between the biologic monotherapy and MTX arms but almost doubled in the biologic/MTX combination arms. This improvement in disease control is reflected in prevention of erosions in addition.
The mechanism is probably a synergistic combination of enhanced prevention of damage at the molecular level, inhibition of those pathways not targeted by the anti cytokine drug and prevention of anti-drug antibody (discussed below)
The *dose* of MTX used in this way is not known. In the early biologic studies in established RA the dose of MTX was lower than may be considered ideal today (TEMPO study (van der Heijde et al. 2006) median dose 10mg, median dose 16mg/week in the IFX study by (Lipsky et al. 2000) and 15mg weekly in the study by (Keystone et al. 2004). The question of a critical MTX dose that may prevent the formation of anti-drug antibody is unanswered. It is not known if a similar effect is seen with non-MTX DMARDs though assumed.

1.14.4 Drug Absorption, dosing and anti-drug antibody

The presence of anti-drug antibodies (acquired resistance to treatment) can be associated with a loss of clinical response through drug neutralisation and enhanced clearance. Measurement of these antibodies is routine in drug trialling yet not in daily practice. Clinically relevant drug neutralising antibodies have been found in all the first

generation TNFi except Etanercept. Both Golimumab and Certolizumab have had anti-drug antibodies studied and identified but no definitive link with reduced efficacy (Vincent et al. 2013).
Not all anti-drug antibodies may be functionally relevant in vivo however. A weakness of current ELISA (enzyme linked immunosorbent assays) based assays is the inability to measure such antibodies in the presence of drug. The presence of antibodies can be measured against disease outcome measures but considered within the overall pharmacokinetic profile (effect on trough and peak drug levels, rate of clearance, influence of individual patient characteristics etc).

1.14.4.1 Etanercept

Of the earliest studies was by Padyukov et al who identified cytokine polymorphisms associated with responders (favoured TNFa production) and for non-responders (alternative IL-1 production) in those treated with Etanercept (Padyukov et al. 2003). Those additional polymorphisms studied as markers of treatment response in Etanercept have been reviewed by Danila M et al, which display some modest positive associations in small groups (Danila et al. 2008).

1.14.4.2 Infliximab

The rates or anti-drug antibody varies by drug:- Infliximab is associated with high levels of anti-drug antibody. The detection of anti-drug antibodies is particularly significant owing to its dosing (rapid serum peaks) and immunogenicity (largest relative portion murine). Wolbink et al identified nearly half of their cohort displayed drug antibodies and was associated with reduced response (Wolbink et al. 2006). Sakai et al prospectively studied a Japanese cohort and found higher discontinuation of Infliximab due to adverse events if anti-drug antibodies were identified (Sakai et al. 2012). Pascual-Salcedo et al found antidrug antibodies in one in three and in all the non-responders (Pascual-Salcedo et al. 2011). This finding was also associated with loss of response and of more infusion reactions. This effect may be overcome temporarily by dose increase and DMARD dose increases (Finckh 2006). In keeping, Infliximab has the lowest treatment retention rate of the three TNFi and is the most likely to require dose increases to sustain efficacy (Hetland et al. 2010).

1.14.4.3 Adalimumab

Anti-Adalimumab antibodies pose similar problems:- after 28 weeks of treatment, 17% had antibodies predictive of less response. By three years just over a quarter had antibodies associated with fewer achieving low disease activity (Bartelds et al. 2007; Bartelds 2011). The clinical utility of this approach has also been studied by (Bartelds et al. 2010). In their cohort, when drug loss of effect occurred, then if no anti-IFX antibodies were found and a switch to Adalimumab made, then a lack of response was often observed. The implication is non-TNFa mediated disease was present in these

individuals. Radstake et al made similar findings whereby clinical response closely followed the serum drug levels as mediated by anti-drug antibodies (Radstake et al. 2009).

In conclusion, it is possible that future decision-making after treatment failure with biologics may be driven by measured immunogenicity in addition to patient related factors.

1.14.4.5 Biologic Dosing

It was observed in the ATTRACT trial (Infliximab) that a proportion of the initial improvements waned with time but could be recaptured with dose increase (Sidiropoulos & Boumpas 2006). A secondary loss of response can be overcome by dose increase particularly in those with anti-drug antibody. Adalimumab and Etanercept are not dose adjusted and there is some suggestion (unlicensed) that increasing dose to 40mg every week of Adalimumab may help capture response in the instance of secondary loss of response. Rituximab early trials examined 500mg and 1000mg doses but some centres do advocate additional dosing if there is a lack of response.

1.14.5 Environmental- Lifestyle

There is good evidence that smoking diminishes response to biologic therapy (Khan et al. 2012; Abhishek et al. 2010). Hyrich et al identified that the Infliximab response is diminished in smokers from the BSR biologics registry cohort (Hyrich et al. 2006). Deprivation and the social environment are relevant and addressed previously.

1.14.6 Genetics

1.14.6.1 Autoantibodies

The presence of the shared epitope confers increased susceptibility but not influence response to TNFi (Potter et al. 2008). A similar finding was observed with PTPN22. However, the presence of RF or ACPA is associated with less response using the DAS28. Genetic variability in drug metabolic pathways may govern response but polygenic inheritance and expression complicate this analysis.

1.14.6.2 Predicting biologic response

Much focus has been on SNPs in biologically plausible candidate genes. One example is the TNF promoters, the best-studied being TNFa 308 G/A SNP polymorphisms. This is a SNP in the TNF promoter gene and thus postulated to increase TNFa messenger RNA (mRNA). Various groups have examined the effect of this polymorphism and TNFi treatment (Emery & Dörner 2011; Wesoly et al. 2006). Prior reviews suggested the -308GG allele is associated with a better response to TNFi than -308AA but more recently this may not hold true as either a class effect or individual TNFi (Pavy et al. 2010) (Krintel et al. 2012) using the DANBIO registry).

There have been a number of other associations identified particularly within the IL-1 pathway, Fc-gammaR IIIA variants (Cañete et al. 2009), the TNF receptor gene (Ongaro et al. 2008), MAP kinase signalling network and transforming growth factor beta 1 (TGF-beta 1) (Kooloos et al. 2007) but sample size and heterogeneity of subjects studied and outcome measures limit the application of these observations. Genome wide association microarray studies (GWAS) have been used to measure the gene output of many thousands of genes prior to and following treatment. Their presence simply by association requires further corroboration however.

Lindberg et al examined synovial tissue pre and post Infliximab treatment and identified those genes and pathways that differ in responders (Lindberg, af Klint, Catrina, et al. 2006a). The aim was to create a genetic profile of a 'good responder'. Higher levels of TNFa pre-treatment were associated with a better response. Synovial biopsy of every patient pre-treatment is not practical and study of circulating mononuclear cells preferable. These cells may not however reflect the articular compartment cytokine expression. Their observation of significant *intra-individual* gene expression is of note. This may underpin the heterogeneous phenotype observed and explanation of the variable response to treatment of a single cytokine.

There are other promising applications of a genetic/biomarker approach. Plant et al examined GWAS data (between zero and six months) for 566 TNFi treated RA patients within the BSR registry and identified seven novel loci influencing treatment response (D. Plant et al. 2011). Hueber et al also identified a predictive biomarker signature in pre-treatment RA patients of cytokines and autoantibodies (Hueber et al. 2009). Badot et al identified a synovial signature predictive of a *lack* of response to Adalimumab (Badot et al. 2009).

1.14.7 Proteomics/Biomarker prediction

Studying the protein output signature is biologically relevant in seeking a profile of resistance to treatment. Fabre et al have studied this in both TNFi (Etanercept) and Rituximab treated patients (Fabre et al. 2008; Fabre et al. 2009). Whereas the former study noted a baseline profile predictive of three-month response, the latter study failed to find a good responder cytokine profile from baseline (the three-month profile differed). Koczan et al measured global mRNA profiles in peripheral mononuclear cells at baseline and after three months of Etanercept in order to determine which genes confer prognostic utility (Koczan et al. 2008). They found gene down-regulation in responders in keeping with TNFa neutralisation. In this way, profiling at baseline would aid decision-making regarding choice of cytokine to target.

Biomarker profiles of treatment response in RA are subject to confounders such as diurnal variation, tissue variation (blood vs synovial fluid vs synovium), intra and inter-individual variation and external influences such as smoking, duration of disease and current therapy. This may explain the slow application and uptake in the clinical setting.

1.14.8 Other factors

1.14.8.1 Individual factors

Having a higher DAS28 at baseline and being more disabled (higher HAQ) make response to biologic therapy less marked (Hyrich et al. 2006; Kristensen et al. 2007). Neither age nor disease duration were important in the BSR biologics registry cohort.

1.14.8.2 Disease characteristics

It is difficult to predict outcome using clinical factors alone (Hider et al. 2008). Anderson et al examined nearly 1500 patients from previously published DMARD trials (J. J. Anderson et al. 2000). They identified four factors from their univariate analysis associated with reduced likelihood of response; longer disease duration, female, being more disabled and prior DMARD use. Mancarella et al examined over 1200 RA patients staring TNFi and from their regression analysis identified lower age, male, RF negative and lower HAQ at baseline as achieving remission at six months more likely (Mancarella et al. 2007).
In the DANBIO registry men with shorter disease duration, despite comparable disease activity pre treatment, were most likely to achieve EULAR responses (Jawaheer et al. 2006).
These are all publications from large-scale registries and the differences are representative of the respective heterogeneous populations, ethnicity and treatment characteristics. Whether or not they represent indicators of poor prognosis or pathological factors is not clear.

1.13.8.3 Compliance

There have been a few attempts to quantify this question. It is well known that compliance with medication is any chronic disease is not optimal. Furthermore patient reporting of compliance is dependent on context (higher if anonymous or by questionnaire than a clinic setting). Biologic trialling does not satisfactorily quantify this problem as discussed below and retrospective studies often self-reported.

Koncz et al performed a recent review of published literature addressing this question (Koncz et al. 2010). There are only a handful of studies (four of the sixteen they reviewed). Compliance is difficult to measure but one method is ‘medication possession rates’. These record ‘collection/prescription redemption’. As the authors identify there is no certainty in this measure that the drug is then actually taken. However, compliance is far from 100% with the net effect difficult to ignore.

Salt et al reviewed non-biologic DMARDs and factors behind drug adherence in addition (Salt & Frazier 2010). They highlight the falling drug ‘persistence’ rates with time. This may reflect compliance, side effect or ‘other’ as the reason is often not recorded. Drug persistence and adherence cannot be considered equal.

Curkendall et al followed a RA cohort for two years and examined this question relating to Etanercept and Adalimumab and the impact of having to pay for these drugs

in the US healthcare model (Curkendall et al. 2008). This had a significant impact leading to early discontinuation and potential later costs relating to untreated disease.

There is evidence that links illiteracy with poorer outcome, perhaps as individuals would have difficulty with printed material (including drug information and prescription) (M.-M. Gordon et al. 2002). Knowledge around the rationale behind treatment and how/when it should be taken is key to achieving compliance. Other authors have reproduced this finding identifying financial concerns (drug prescription rates) in addition to transport costs to appointments that may relate outcome to social class (Garcia Popa-Lisseanu et al. 2005).
In contrast, McEntegart et al also identified poorer outcomes in deprived patient populations and suggest, among other reasons, that more deprived groups are less likely to access health care (McEntegart et al. 1997). At five years there was little difference in drug compliance with Gold therapy when examined by social class.

Finally patient beliefs about medication will influence medication compliance. Prevalent mood and perceived current effectiveness of therapy are important but in a meta analysis of studies performed by Pasma et al examining adherence to medication in RA, the strongest factor related to beliefs about necessity of medication (Pasma et al. 2013). Tied to this is education around disease (and access to means of information such as GP, Rheumatology team and internet multimedia), perceived benefit balanced against lack of harm (van den Bemt et al. 2012) and cultural beliefs which may include ethnic/family values (Kumar et al. 2008).

1.15 Epigenetics and Autoimmunity

1.15.1 Background

The term 'epigenetics' refers to the study of

> *"Modifications of DNA, RNA and associated proteins without a change to the underlying DNA base sequence*"

Importantly, these changes can be either stable or dynamic, meiotically and mitotically retained permitting adaptive changes to persist in a cell lineage in order to maintain integrity in subsequent generations.

Epigenetic processes offer an attractive dynamic mechanism whereby environmental effects such as nutrition, hormonal influence and drugs may interact and influence genomic output (Javierre et al. 2011). For example, despite strong genetic concordance in monozygotic twins the phenotypic picture in disease can markedly diverge. Epigenetic marks demonstrate variance and drift with age that may explain this phenomenon (Lodish et al. 2008). Indeed, it is postulated that epigenetic modifications may explain a number of the disease characteristics not currently explained merely by gene-environment interaction such as age of onset, differences in gender distribution, disease severity and course.

Epigenetic mechanisms allow the cell and organism to adapt to the environment by rapidly influencing gene expression and controlling transcription factor binding. In this way, the precise *degree* to which a gene is expressed is highly regulated. Thus the timing and dynamic integration of epigenetic regulation of gene expression is central to adaptive and developmental processes.

1.15.2 Epigenetic changes driving resistance to Oncology therapies

In cancer therapy it is understood that effective therapy selects for resistant mutant cancer cells that persist despite treatment and account for observed resistance/relapse observed. For example, the phenomenon of transient resistant populations emerging is described related to chromatin alterations and overcome by treatment with histone deacetylase inhibitors (HDACs) (Baylin 2011). In this way, resistant clones are selected for. While TNFi therapy does not induce cell death or apoptosis, it is conceivable that selection for pathways under epigenetic control could be selected (Sharma et al. 2010). In addition transient changes in chromatin methylation was noted following chemotherapy that may mediate resistance. Importantly these changes may be potentially reversible and used in combination with standard chemotherapy.

Conversely, disruption of epigenetic processes leads to inappropriate gene activity or tumour suppressor silencing, a key finding in many cancers. Thus the field of epigenetics has advanced most notably in Oncology (Esteller 2008) and most specifically haematopoietic cancers such as lymphoma and leukaemia owing to the central pathological role of epigenetic modifications (Metzler et al. 2004).

References pertaining to the role of epigenetics and metabolic disorders (including obesity and diabetes (Stöger 2008) in addition to vascular and angiogenic pathogenesis

are important as these processes are not only physiological in health but central to tumour progression and metastasis, inflammation (the provision of adequate blood supply to the inflamed synovium or inflammatory vasculopathies) and accelerated atherosclerosis (Suarez & Sessa 2009). Such processes are in common with rheumatoid arthritis pathogenesis.
Key to the successful study of these changes is their stability and accessibility by being both detectable prior to imaging and accessibility in almost any bodily secretion. Considering the rise in prevalence of many of these disorders, early detection of epigenetic changes offers promise for screening and diagnosis, assessment of response to treatment and later follow up to detect early relapse.

1.16 Epigenetics and other Autoimmune Rheumatic Diseases

The incidence of autoimmune disorders rises with age. One theory may be the accumulation of epigenetic events and/or the influence of cumulative environmental exposure acting on epigenetic processes that explains this observation. In parallel the incidence of cancer rises with age (Goronzy et al. 2010). Immune cells display progressive demethylation with age rendering them less effective but many of these genes are those involved in autoimmunity.
It remains true that the underlying trigger and aetiology for the majority of autoimmune conditions is unknown although many show preponderance in later life and have clear environmental triggers. In RA, genetic factors alone do not explain the heritable characteristics. As epigenetic mechanisms regulate at fundamental levels described above, it is little surprise that a loss of regulation may form part of the pathogenesis of disorders other than rheumatoid arthritis and an area prime to be studied. There are publications that pertain to epigenetic processes in the other autoimmune rheumatic disorders (Ballestar et al. 2006; Y. Tang et al. 2009; Dai et al. 2009; Sonkoly et al. 2007; Alevizos & Illei 2010) but the main focus of this thesis will be RA

1.17 Epigenetic Processes

To date a myriad of epigenetic processes have been identified and the resultant gene expression is the end-product of multiple, dynamic and interacting processes. Broadly there are three epigenetic mechanisms that I have chosen to refer to and are explored below namely DNA methylation, modification of histones and regulatory non-coding RNAs. Breakdown of this regulation is associated with autoimmune diseases. Of the latter, we chose to study microRNAs in depth and more time is thus devoted to exploring this field in detail.

1.17.1 DNA (Cytosine) Methylation

DNA (deoxyribonucleic acid) methylation has a critical role in early embryo development and cell differentiation, and within fundamental processes such as X-chromosome inactivation. Patterns of DNA methylation are not transferred vertically but are maintained in subsequent cell divisions. Early methylation pattern is established in utero where the epigenome is most susceptible to factors such as the influence of maternal

diet or even behaviour. These changes may persist into adult life (Heijmans et al. 2008). Such differences are being actively studied by groups such Gordon et al who are studying twin pairs and recording environmental influences (Novakovic et al. 2011; L. Gordon et al. 2012). Some authors have linked early socio-economic deprivation with later observed DNA methylation patterns in adulthood (McGuinness et al. 2012; Borghol et al. 2012) . Importantly the promoters involved occur in cell signalling pathways including IL-6, MAP kinases, cardiovascular risk markers and cancers. A link between epigenetics and socio-economics can thus be proposed and the increased risk of such diseases in part explained.

One of the most important regions influenced by methylation are CpG-rich islands. These comprise a cytosine base immediately followed by a guanine base in the DNA sequence often immediately upstream of a gene promoter acting to regulate transcription (Saxonov et al. 2006). Within these islands they may not be subject to the same methyltransferase maintenance and regulation catalysed by DNA methyltransferase 1 (DNMT1). In normal human DNA 3-6% of cytosine residues are methylated but the majority of CpG islands are demethylated (Mulero-Navarro & Esteller 2008). **DNA in its methylated state (along with histone conformational state addressed below) *prevents* physical access of DNA binding factors or activation of transcriptional co-repressors thus transcription does not proceed**. The same is true of the methylation state of the CpG islands; methylation of the island located at the promoter region is associated with gene inactivation. Thus, transcriptionally active genes exist in a state of low methylation. Overall global genomic demethylation, along with selective hypermethylation of CpG promoter regions of tumour suppressor genes, is a state associated with many cancers.

Genomic DNA hypomethylation is dynamic and contributes to normal regulation. It occurs with aging but it may be particularly relevant in those genes relating to the immune system. Hypomethylation is also associated with insulin resistance independent of other risk factors. Smoking affects methylation dynamically and reversibly but may account for the persisting adverse risks after stopping (Wan et al. 2012; Zhao et al. 2012). It is not clear if methylation patterns in peripheral white cells may act as ‘markers’ of exposure to toxin and thus of risk for disease (Terry et al. 2011).

Global genomic hypomethylation is a characteristic finding in RA and most likely represents a further contributory factor to the resistant RA patient (Karouzakis et al. 2009). Indeed, the influence of treatment and methylation status in inflammatory arthritis is not a recent concept. Kim et al examined DNA methylation in albeit small numbers of patients with inflammatory arthritis (RA and psoriatic arthritis) treated with methotrexate (Kim et al. 1996). They identified genomic hypomethylation in patients with inflammatory arthritis. This effect was reversed with methotrexate therapy, independent of a folate effect. It is not clear from their data if such patients were those successfully treated with objective improvements disease markers.

Interleukin-6 is one of the central inflammatory cytokines in RA and in recent years proven a valuable therapeutic target. Reduced methylation within a single promoter site for the IL-6 gene is important for regulation of the activity of this gene and methylation was found to be lower in RA patients versus healthy controls (Nile et al. 2008). Finally, methylation of the death receptor 3 promoter was increased in RA compared to OA synovial cells resulting in relative resistance to apoptosis (a prominent feature of many cell lines in RA) (Takami et al. 2006).

Targeting methylation represent a challenge owing to a lack of specificity but is underway in cancer therapy (X. Yang et al. 2010). However more focussed delivery of therapy to gene promoter areas or theoretically even to joints and haematopoietic tissues before symptoms manifest may prove effective.

1.17.2 Histone Modifications

DNA methylation cannot however be considered alone in view of its close relationship to chromatin. DNA exists within the nucleosome in its resting state tightly coiled in the form of chromatin as part of nuclear packaging. In this way DNA is closely associated with core protein subunits known as histones, which together, comprise chromatin. The uncoiling and exposure of gene promoter regions allow access to transcription factors and RNA (ribonucleic acid) polymerase II initiates gene expression. This process of unwinding is mainly mediated through a balance between the activities of acetylases (HATs (histone acetyltransferases) and HDAC (histone deacetylases)). The acetylated state of chromatin is associated with reduced affinity between DNA and the histone component lysine. In such a way, transcription may proceed whereas the deacetylated state is associated with gene silencing. The process demonstrates further complexity and degrees of epigenetic regulation through acetylation of lysine residues of the transcription factors themselves (Grabiec et al. 2008). Further post-transcriptional modifications of histone tails include sumoylation, phosphorylation, methylation and ubiquitination amongst others.

1.17.3 MicroRNA

MicroRNA belong to the family on non-protein coding RNAs transcribed from cellular DNA. Lee et al discovered the first microRNA, a 22 nucleotide, single-stranded transcript of lin-4 in 1993 pertaining to developmental timing in a nematode (R. C. Lee et al. 1993). They noted this short RNA negatively regulated the protein coding gene lin-14 through partial complementarity binding to a sequence in the 3’ UTR of lin-14 and thus influenced developmental timing. From this time there has been an explosion of research and publications within the microRNA field. It has become clear that microRNA are fundamental regulators of post-translational gene regulation. The annotation used microRNA followed by a number, which represents the order of discovery. A letter following denotes only one or two nucleotide sequence changes but the seed region remaining constant (for example microRNA-146a and -146b albeit coded from different chromosomes).

MicroRNA are involved in the regulation of apoptosis, cell differentiation and embryogenesis and have been studied in many animal and plant species demonstrating high evolutionary conservation. MicroRNA may regulate up to a third of human protein-coding genes and have been studied in almost all medical disciplines (Lewis et al. 2005). Typically the pattern of microRNA is cell dependent, with rapid fluctuation but also context dependent such as a state of cellular activation or resting state. It is therefore little surprise that dysregulation of microRNA at any level is implicated in disease pathogenesis. The focus below is upon those microRNA involved in the autoimmune field.

1.17.3.1 Biogenesis

MicroRNA are short (typically 20-26 nucleotides) regulatory, non-coding RNA strands encoded within the host genome. Sequences coding for microRNA are often found in clusters and co-transcribed with the mRNA nearest where they reside (Rodriguez et al. 2004; Weber 2005). Transcription by RNA polymerase II occurs in the nucleus to create a stem-loop shaped primary or pri-microRNA that is cleaved by the enzyme Drosha to create a hairpin shaped pre-microRNA. Active transport through nuclear pores occurs to the cytoplasmic compartment by Exportin-5 where the action of a second RNase termed Dicer acts to further cleave and process the microRNA to its single strand form. This creates the guide strand to which proteins of the Argonaut family bind creating the microRNA-RNA-induced silencing complex (RISC). It is this mature complex that will guide the microRNA guide strand to pair by Watson-Crick base pairing to the target mRNA 3'-UTR (Furer et al. 2010; Bartel 2004). The action of the second microRNA strand generated by Dicer is as yet unknown.

1.17.3.2 Mode of action

The action of microRNA is to act through RNA interference. They act through both translational repression of mRNA (Guo et al. 2010) but also through mRNA cleavage and thus down-regulation post transcription.

Primary binding occurs most often between the seed sequence in the microRNA (positions 2-7 of the 3'UTR end) and target mRNA. Binding in this area is key but binding may also occur in non-seed sequence regions. These include binding of the 5'UTR end (may lead to enhanced translation (Ørom et al. 2008) and 'centred sites' (Shin et al. 2010) where the middle portion of the microRNA binds. Access to the binding site may also be affected by mRNA folding (Ceribelli et al. 2011). The degree of complementary binding is important:- (near) perfect binding allows mRNA to be cleaved and degraded. This is more often the case in plants and more simple organisms. Imperfect binding, which is the more common situation, leads to translational repression (Carthew & Sontheimer 2009). This process facilitates rapid formation and degradation or microRNA within the cell allowing both a rapid response to local signalling and fine-tuning of the cellular response.

Ultimate target down-regulation may occur by translational repression and/or mRNA destabilisation. Translational repression may be carried out by GW182 along with Ago2 sequestered into 'P bodies' within the cell cytoplasm (Jakymiw et al. 2007). This renders the mRNA inactive through mechanisms such as deadenylation (Pasquinelli 2012). Other mechanisms of translational inhibition also prevent mRNA formation.

1.17.6.3 Studying microRNA; MicroRNA-mRNA interactions and target prediction 'in silico'

One of the key questions in microRNA discovery lies in predicting mRNA targets. This stage must precede prediction of the functional importance of microRNA. The significant challenges underlying such exploration are outlined above with reference to complex binding both within the seed sequence and that occurring at additional sites.

Modelling would suggest a single microRNA could influence many different target mRNA to varying extents in a tissue dependent context. For example, Lim et al found that transfection with a single microRNA, microRNA-124, down-regulated at least 174 genes thus altering gene expression toward that of a particular cell type (Lim et al. 2005). In a similar way, the different murine cell types of the haematopoietic system exhibit microRNA profiles according to their stage of differentiation suggesting relative microRNA patterns play an important role in developmental timing, determining and/or maintaining such cell stages (Monticelli et al. 2005).
As a relevant illustrative example, microRNA-155 has become increasingly recognised as central to regulation of the immune and inflammatory process. It is coded from the non-coding transcript of the BIC gene located on chromosome 21. The nucleotide sequence of the mature microRNA is 'UUAAUGCUAAUCGUGAUAGGGG' and that of the seed sequence 'UAAUGCU'. Predicted targets are made based on the knowledge of this sequence and matched mRNAs with conserved complementary binding sequences. Database such as TargetScan (www.targetscan.org) or miRbase (www.mirbase.org) provide such a resource. MicroRNA-155 had around 300 predicted targets at the inception of this study (TargetScan April 2009 release) and 440 by the most recent release 6.2, June 2012).

The identification of post-transcriptional targets can be approached by either an '*in vivo'* experimental microRNA transfection or antagonism within cells or through computational approaches ('*in silico'*). For the researcher, the 'in silico' approach is, to some extent, addressed by the computational predictive databases and open access libraries above (others are outlined by (Mishra & Bertino 2009). These operate on the principal of matching cognate sequences of microRNA. The evolution of databases is dependent on published work and submissions made to reference libraries. The result may be many hundreds of predicted targets and experimental verification will always be necessary to confirm a computationally predicted target. Furthermore, results vary between databases and the use of several databases would be recommended. Therefore considered interpretation of database outputs is necessary.

1.17.3.4 Studying microRNA; 'in vivo'

The weakness of this approach is the loss of cellular context and regulation that lies therein. Experimental approach is designed to try to overcome the weaknesses of the computational method; they work on those algorithms defined by those findings already published and do not address any novel interactions (Ørom & Lund 2007).

In principal, microRNA can be studied in animal models in gain of- or loss of- experiments in order to biologically validate findings. MicroRNA can be introduced by vectors (often adeno-associated viruses) to enforce expression but also as an 'anti-miR' to block action. An 'antagomir' differs by being conjugated to cholesterol to facilitate cellular uptake and, with full-length sequence complementarity, is more specific. Krutzfeldt et al first performed successful experimental blocking of microRNA action in 2005 (Krutzfeldt et al. 2005). Following any experimental manipulation of microRNA, it is necessary to measure target mRNA values. Corresponding reductions in protein may be small but still have a very fundamental net cellular effect. Indeed it may be difficult to tease out the effect from background gene variation. It is therefore necessary the cell be 'under stress' to maximise the microRNA target *availability* (van Rooij & Olson 2012).

1.17.3.5 Studying MicroRNA; within regulatory networks and feedback loops

Many of the experiments in RA referred to below study both microRNA and their tissue in isolation. Having identified a microRNA of interest and post-transcriptional targets, then prediction of mRNA output is possible. However as a single microRNA may target more than one gene and the relative effect of multiple genes must be accounted for, the construction of gene regulatory networks is necessary. Thus the net effect of microRNA-mRNA inhibition may be small within balanced networks and thus more difficult to predict 'in vivo' phenotypic effects.

Inflammatory networks require tight regulation to avoid excessive unchecked and damaging inflammation through a balance of pro-inflammatory and negative regulatory pathways. For example, although the primary action of a microRNA-mRNA interaction may be down-regulation of target protein, the resultant phenotypic cellular or organism response may a positive effect through loss of inhibition. Simple experimental validation can facilitate the modelling of feedback loops. These are illustrated with three important publications shown in 1.17.3.7.

Additionally, the field of bioinformatics can facilitate the construction of complex computational (yet entirely theoretical) networks based on previously published biological interactions. Published theoretical gene-protein networks using the bioinformatics software (www.ingenuity.com), are increasingly common (Volinia et al. 2010; Philippidou et al. 2010). Statistical significance permits confidence that the predicted linkage is a real one. The genes and network can also be fine tuned to display those processes that carry most pathological relevance. Although not directly representative of interactions at the cellular level such networks can reveal novel pathways for additional research in disease pathogenesis.

1.17.3.6 MicroRNA function

Put at its most fundamental level, loss of components of microRNA synthesis leads to death. Dicer deficient organisms die at early stage of embryogenesis (Bernstein et al. 2003). MicroRNA-155 knockout mice are immuno-deficient (Rodriguez et al. 2007). MicroRNA are key to the majority of cellular physiological and developmental processes and, when loss of regulation occurs in this way, are related to disease.

Developmental (Alvarez-Garcia & Miska 2005)	**Physiological**	**Repair**
Skeletal muscle cell differentiation (Seok et al. 2011)	Haematopoietic differentiation (Vasilatou et al. 2010)	After myocardial infarction (Zidar et al. 2011)
Cell cycle progression	Angiogenesis	Bone remodelling (Kapinas 2011)
Cell patterning	Insulin secretion	After stroke (Rink & Khanna 2011)
Cell apoptosis	Cholesterol regulation	
	Osteogenic differentiation (T. Wu et al. 2012)	

Table 1-13 Roles for microRNA in human development, physiology and tissue repair

In the context of this thesis I have made special reference to microRNA involved in **inflammatory signalling networks**. The inflammatory cascade requires tight regulation and microRNA have emerged as important regulators. There is a close link between loss of regulation and autoimmunity and cancers. They achieve this by targeting those fundamental molecules involved in these pathways. These may include the TLRs and downstream molecules, cytokines, transcription factors and gene promoters as examples.

1.17.3.7 MicroRNA regulating TLR signalling

The role of TLRs in inflammation is outlined in Chapter 1.7 and requires close regulation. MicroRNA represent a means to regulate the TLR-mediated inflammatory response and, in this way, represent a means to demonstrate the mechanisms by which microRNA may regulate inflammation. Many microRNA (including those in RA addressed below) such as microRNA-155 are *up* regulated following TLR signalling (reviewed by (O'Neill et al. 2011). McCoy et al demonstrated that TLR signalling also leads to microRNA *down*-regulation (-155 and IL-10 via SHIP-1 (McCoy et al. 2010). The role of microRNA-146 and IRAK1/TRAF6 is further discussed below but is an example of a microRNA targeting key *components* of the MyD88 dependent pathway distal to the TLR leading to eventual nFkB activation. Stanczyck et al observed that microRNA-155 and -146a increase upon stimulation by TLR ligands. They propose that cellular debris, in addition to cytokines, could perform this role within the joint (Stanczyk et al. 2008).

In the same way, microRNA-155 can target and regulate TLR signalling; Ceppi et al show that TAB2, acting distal to IRAK1/TRAF6 is a microRNA-155 target (Ceppi et al. 2009). Finally, Tang et al also identified predicted binding sites for microRNA-155 and MyD88 thus negatively regulating TLR-mediated inflammation in Helicobacter pylori (B. Tang et al. 2010). MicroRNA may also target key *transcription factors* involved in TLR signalling

such as forkhead box P3 (FOXP3) and Taganov et al demonstrated through TLR receptor activation with LPS, nFkB is up regulated (Kohlhaas et al. 2009; Taganov et al. 2006). Finally, the *signalling regulators* such as SOCS1 and SHIP1 have also been shown to be validated targets as referenced below.

1.17.3.8 MicroRNA as biomarkers

There are many hundreds of thousands of protein that may serve as biomarkers but just over a thousand microRNA. To be able to represent valid biomarkers, microRNA must fulfil a number of prerequisites. The requirements and how they are met is described to place their study and results in Chapter 4 in context.

- Stable in storage

Despite the presence of endogenous ribonucleases, microRNA are very stable (Jung et al. 2010) and reproducible between individuals (Chen et al. 2008). MicroRNA have been studied in all body fluid types and secretions and are both stable at -80degrees for a considerable period and to multiple freeze-thaw cycles (Gilad et al. 2008).

- Rapid and simple detection

Measurement in blood is accessible, minimally invasive and acceptable on the whole. Synovial sampling is more invasive and potentially harmful. Polymerase chain reaction (PCR) testing is both sensitive and rapid. However the use of different extraction techniques and both manufacturer's and local extraction policies may interfere with consistent findings

- Reflect the disease or process

One important question is whether the microRNA studied in blood are representative of the condition (in joints). It is therefore ideal the microRNA studied originate from the target tissue. Obtaining synovial tissue is not straightforward in this respect.

MicroRNA act *within* cells and are released into the circulation as exosomes (smaller and endosome derived) or microvesicles (larger) packaged in lipid bilayers. It is presumed that uptake into target cells occurs and that this may be a mechanism of 'cell communication'. Measuring microRNA must account for this and that the secreted profile is reflective of the 'active' intracellular profile.

In Oncology the question of whether circulating microRNAs reflect the cancer or a additional condition present has been extensively addressed and reviewed by a number of authors (Wittmann & Jäck 2010; Heneghan et al. 2010; Cortez & Calin 2009; Ng et al. 2009; K. Wang et al. 2009; R. Wang et al. 2011). This has led to the potential use of microRNA as validated prognostic tools (markers of recurrence or effective treatment). As illustration, Wang et al found a close correlation between the microRNA profile in serum versus that of breast cancer pathological samples (F. Wang et al. 2010a). They also concluded that tumour grading and clinical features correlated with microRNA

profiling. Finally they also reassuringly found the expression profiles reproducible and consistent in both healthy volunteers and those with breast cancer.

In RA, both the serum and synovial profile has been studied and is addressed in detail below. Several studies suggest the peripheral blood signature is similar to that of the articular compartment. However, many of the studies do not allow for individual patient related differences (demographics, disease duration etc) and influence of current treatment. Control groups have included osteoarthritis in which similar microRNA have also been identified. Currently no microRNA reflects disease stage nor of disease response

- Cheap and simple to process

With time the access to and time taken to process samples has fallen greatly although the process remains expensive. Assessment by qPCR or microarrays allows quantification of even tiny levels of microRNA. Challenges of measurement include reproducibly across platforms and local protocols.

1.17.3.9 MicroRNA and Oncology

In this field, the recognition that microRNA are fundamental to cellular processes such as differentiation and apoptosis led to the discovery of distinct microRNA profiles between tumour types versus normal tissue. In addition to DNA methylation and histone modifications, abnormal microRNA expression is characteristic and often distinctive between tumour types. Furthermore microRNA contribute to the transformation from early pre-malignant to the malignant phenotype promoting the tumour line persistence and their observation (Santarpia et al. 2010). Their action in this transformation is primarily mediated by acting as tumour suppressors (a loss of effect of this function) or as oncogenes contributing to the characteristic cancer cellular phenotype with loss of regulation of growth and prolonged survival.

There are a number of detailed reviews of this ever-expanding field with detailed reference to individual microRNA, their targets and associated cancer type (Wiemer 2007; Rodríguez-Paredes & Esteller 2011; Esteller 2008). A relevant example is the association of microRNA-155 with many haematological tumours. The finding of microRNA-155 in inflammation by Pedersen et al (*and discussed in relation to RA below*) is of note as up to a quarter of malignancies may be related to inflammation (Tili & Michaille 2011; Pedersen et al. 2009).

Identification of such microRNA offers great clinical application. MicroRNA are accessible in most bodily fluids; Xie et al studied microRNA-21 in sputum with improved specificity and sensitivity over cytology (Xie et al. 2010). Potential lies in diagnosis (such as expression profiling of undifferentiated tumours in distinguishing origin (Lu et al. 2005), prognosis (Ueda et al. 2010), in detecting metastasis prior to positive imaging findings (Yanaihara et al. 2006) and therapy -responsiveness of tumours.

Thus the demonstration of microRNA in RA subtypes may provide a predictive response profile.

1.17.3.10 MicroRNA polymorphisms

The potential for 'microRNA polymorphisms' at any level in the biogenesis pathway may lead to disease through either loss-of or gain-of-function. In this way, alterations in microRNA could contribute to the variability in disease risk, presentation and outcome.

MicroRNA SNPs may affect function by affecting microRNA primary transcript, affecting intermediate-step processing or the ultimate microRNA-mRNA interaction (Ryan et al. 2010). One relevant example is a variant in the pre-microRNA-146a gene and increased prostate cancer risk (Xu et al. 2009). Chatzikyriakidou et al have examined the variants in microRNA-146a and polymorphisms in it's target IRAK1 and susceptibility to developing RA (Chatzikyriakidou, Voulgari, Georgiou & Drosos 2010a). Their group identified a polymorphism in the 3'UTR of the mRNA encoding IRAK1 (a microRNA-146a target) in RA patients versus controls. They propose this could increase susceptibility to RA. The same group identified the same finding in a smaller cohort with psoriatic arthritis and ankylosing spondylitis (Chatzikyriakidou, Voulgari, Georgiou & Drosos 2010b).

1.17.3.11 What regulates microRNA?

The identification of microRNA not only requires consideration of their functional significance but also consideration of *their* own regulation; namely what are the processes that lead to this observed dysregulation of microRNA? Increasingly it is suggested through functional studies and not simply studying microRNA in isolation that microRNA act within networks of other epigenetic changes.

1.17.3.11.1 MicroRNA is under the control of transcription factors/regulators

Schmeier et al 2009 explored those transcription factors (TFs) involved in controlling microRNA and the differentiation of monocytes to macrophages (Schmeier et al. 2009). By identifying those microRNA expressed during monocyte stimulation they identified the promoter region TF binding sites and both computationally (in silico) and experimentally predicted those TF involved in the regulatory process:- several novel TFs were identified and validated. Fazi et al identified the two competing transcription factors involved in granulopoeisis. Both act at the microRNA-223 promoter gene in opposing fashions to influence microRNA-223-mediated myeloid differentiation (Fazi et al. 2005). Lastly, Sun et al explored the observation that progesterone has a general inhibitory action on the immune system. They identified that progesterone can suppress the microRNA-155 production in LPS treated macrophages perhaps via SOCS-1 (Sun et al. 2012).

1.17.3.11.2 MicroRNA are under the control of other epigenetic processes

There is increasing evidence that microRNA are regulated by DNA methylation patterns. In RA, Nakamachi et al identified reduced microRNA-124a in RA FLS (Nakamachi et al. 2009). Lujambio et al had previously identified the hypermethylated status of the promoter gene for microRNA-124a in colon cancer cell lines and thus the observed reduced levels of -124a (Lujambio et al. 2007). One of the key targets for -124a is the oncogene CDK6 involved in cell differentiation (cyclin D kinase 6) which may thus be epigenetically silenced. Applying the finding of Lujambio et al to the persistently activated FLS would be plausible.

(Niederer et al. 2011) identified the down-regulation of microRNA-34a levels compared to OA samples in RASF unresponsive to further TLR stimuli. In this way, RASF were resistant to apoptosis. They identified the microRNA-34a promoter to be methylated and thus microRNA-34a down-regulated. This was reversible under the action of de-methylating treatments. Similarly, (Stanczyk et al. 2011) also identified elevated microRNA-203 in RASF unaffected by further stimulation. Treating the RASFs with a de-methylating agent reversed this. This suggests promoter methylation contributes to the persistently activated phenotype. Furthermore they postulate the variable levels of microRNA-203 they identified in earlier disease may exist, as the demethylation/ 'persistently-activated' state has not established. The success of early RA treatment and concept of 'window of opportunity' would be very supportive of this concept.

The first study of microRNA and DNA methylation patterns together was recently published (la Rica et al. 2013). They studied RASF versus OA synovial samples identifying altered methylation in key regulatory genes and appropriate changes in expression of microRNA that may explain the RASF activated phenotype. Some genes were regulated in opposing fashions by methylation/microRNA actions. Details regarding treatment and disease characteristics were not known. Reassuringly they identified many of the microRNA listed below in addition to a number of other novel targets. Their study reiterates additional levels of regulation.

In Oncology, several studies/reviews have identified microRNA as tumour suppressors and the methylation status of their promoters being important (Saito & P. A. Jones 2006; Huang et al. 2010). (Toyota et al. 2008) identified low microRNA 34b/c in colonic tumour cell lines (thus acting as tumour suppressors). The neighbouring CpG island was found to be hypomethylated. Finally, similar interacting epigenetic processes can be found in the related rheumatic disorder SLE. (Pan et al. 2010) identified microRNA-21 and 148a as being overexpressed in SLE CD4+ cells driving DNA hypomethylation, a characteristic finding in SLE cells compared to controls.

1.17.3.11.3 Pseudogenes

Pseudogenes may play a role in regulation of microRNA. These are non-coding competing RNA sequences with microRNA binding sites that bind and prevent target inhibition (Salmena et al. 2011).

1.17.3.12 MicroRNA as therapeutic targets

The attraction of microRNA as treatments is that they represent important regulators in the inflammatory pathway. However, expression is cell-type, time- and tissue-dependent meaning unintended side effects may be unpredictable with systemic delivery. The first human trials have been carried out in hepatitis C treatment with anti-miR-122 with successful Phase IIa trials and a number of others in Oncology (Zhang et al. 2010). MicroRNA regulate important repair and remodelling processes in ischaemic heart disease/infarction and the early phase trials underway have been reviewed by (van Rooij & Olson 2012). This emphasises the promise of novel microRNA as targets and unravelling the molecular mechanisms if disease.

1.18 Epigenetics and Rheumatoid Arthritis

To date there have been a number of important studies published which, when considered together, suggest epigenetic modifications underpin RA pathogenesis. This field has evolved rapidly from the conception of this study and the use of high throughput methods and predictive bioinformatics has advanced the field. Epigenetic modifications are consistently demonstrated not only in the development of RA but may also explain the finding of persistent inflammation observed in RA (Ammari et al. 2013). I will briefly consider DNA methylation and histone modifications before focussing on microRNA.

1.18.1 DNA methylation

Within studies of DNA methylation in RA, the synovial fibroblast remains the best-studied cell type.

Kim at al first studied methylation status in 1996. In small numbers with inflammatory arthritis, the finding of DNA hypomethylation was made. Importantly, this state was reversible with treatment with MTX irrespective of dose. They did not relate this to an assessment of disease activity nor to whether such change is reflected in RA therapies that do not target folate metabolism (Kim et al. 1996).

Karouzakis et al 2009 studied the RASF from RA and OA patients and attribute the characteristics of the RASF to methylation status (Karouzakis et al. 2009). Inducing global demethylation induced a RASF-like state in normal SFs and production of typical pro-inflammatory molecules. They confirmed global genomic demethylation in RA patients. In addition the levels of Dnmt-1 were reduced in RA contributing to the persisting demethylated state. This may be a part of the contribution to the chronic state observed.

Nakano et al 2013 have built upon this observation by demonstrating that the differentially methylated loci are key regulatory loci involved in RA (Nakano et al. 2013). Similarly, Gowers et al examined the methylation status of the TNF promoter by examining PBMCs in healthy individuals. With age, the promoter was increasingly demethylated, a finding that may relate to the increasing incidence of RA with age (Gowers et al. 2011). A related more recent paper by Liu et al examined over 300 individuals at diagnosis of RA. They found that the methylation state of two regions in the MHC was present and this may confer some of the increased risk observed (Yun Liu et al. 2013)

Nile et al identified a single demethylated CpG promoter regulating IL-6 production in RA PBMC's. Whether this represents a primary susceptibility finding or mere disease characteristic requires further examination. Study in early RA or even individuals prior to symptom onset would be of interest(Nile et al. 2008).

Takami et al examined death receptor 3 (DR3) gene regulation and promoter methylation status in RA patients and OA patient's synovial samples in addition to PBMCs. Methylation of the promoter reduced DR3 gene expression and contributes to the observed resistance to apoptosis (Takami et al. 2006).

Schwab et al studied B-cells and identified that the CpG island related to the CD21 molecule (present on mature immunoglobulin producing B-cells) is demethylated in both peripheral mononuclear cells (PBMC) and in the synovial compartment (Schwab & Illges 2001). CD21 is necessary for recognition of C3d as part of immune complexes and dysregulated expression in the synovial compartment could be anticipated in the joint.

1.18.2 Histone Acetylation

Relatively fewer publications have addressed histone modifications perhaps due to the plethora of different types of tail modifications (and more than one) that may be present. There a number of animal model experiments confirming the important potential of HDAC inhibitors.

(Huber et al. 2007) identified differences (reduced) in relative histone acetylase and deacetylase activity in RA synovial tissue when compared to OA. RASFs have a hyperacetylated genome, owing to the low activity levels of total histone deacetylase (HDAC) enzymes, possibly resulting from reduced levels of HDAC1 and HDAC2. Low-level HDAC activity might therefore contribute to the activation of pro-inflammatory transcription factors.

(Kawabata et al. 2010) investigated nuclear HDAC/HAT expression and cytoplasmic TNFa in RA synovial tissue versus OA. HDAC activity was found to be higher in RA, consistent with increased gene expression. This is at odds with the results of Huber however. The authors suggest this may in part be due to some of the patients in the study by Huber to be treated with TNFi.

(Grabiec et al. 2012) also examined RA synoviocytes and examined the effect of treatment with an HDAC inhibitor in suppressing IL-6 production. This effectively blocked IL-6 by influencing IL-6 mRNA stability. This expanded on the same group's prior

observation in 2010 where HDAC inhibitors blocked macrophage activation (Grabiec et al. 2010).

As with other epigenetic modifications, acetylation is potentially reversible. Notably, Givinostat is an HDAC inhibitor and effective in the treatment of juvenile idiopathic arthritis (Vojinovic et al. 2011). There were no safety problems of note at short follow up.

1.18.3 MicroRNA

Having established that microRNA offer an attractive role in the pathogenesis of RA, it is necessary to review the evidence to date that shows microRNA are the most studied and reported epigenetic marks in RA. Publications have increasingly focussed on their biological role and integration in inflammatory networks. I have reviewed the initial identification of microRNA and then linked them to crucial molecules within the inflammatory pathway.

1.18.3.1 MicroRNA in RA- Specific examples

The best-studied microRNA in RA are microRNA-146a, -155 and -223. These have a role in inflammation and tissue destruction and, in many ways, contrasting actions. Yet, despite identifying their fundamental identification in disease pathogenesis a role in onset, course or treatment response is not known.

1.18.3.2 MicroRNA-155 and inflammation (RA)

MicroRNA-155 is a 'multifunctional microRNA' having been identified in many immune cell types and diseases (Faraoni et al. 2009). The nuclear precursor is transcribed from the BIC gene (chromosome band21q21.3) and microRNA-155 is regulated both transcriptionally and post transcriptionally. It has a fundamental role in the immune system and can be found expressed by activated immune cells. Both pro- and negative regulatory roles have been identified which are cell type/context dependent (Stanczyk et al. 2008; Spoerl et al. 2013).

MicroRNA-155 is central within myeloid physiology from germinal centre B-cell maturation and memory cell formation to myeloid and T-cell differentiation (Tili et al. 2009; Thai et al. 2007; Vigorito et al. 2007). The observation of a close link with many haematological malignancies such as lymphoma and leukaemia is of note. MicroRNA-155 levels are elevated in these conditions. Tili et al propose not only persistent production of microRNA-155 (perhaps by oncogenes) but also a failure of normal negative feedback mechanisms (usually activated in parallel with T and B-cell maturation and Toll-like receptors stimulation). Net effect is an increased downstream transcriptional targeting effect of microRNA-155, perhaps acting on tumour suppressor genes.

In general, inflammatory ligands increase microRNA-155. Tili et al 2007 confirmed mIcroRNA-155 as a positive regulator of inflammation in mice. It is up regulated (under

nFkB control) in response to LPS and may enhance the TNFa transcript stability and thereby serum TNFa. It is in this way a key part in the regulation of the innate immune system in response to bacterial infection. Teng et al and Thai et al also demonstrate microRNA-155 is induced by inflammatory stimuli (Teng et al. 2008; Thai et al. 2007).

Initial observational studies in RA confirm a constitutive increase in both PBMCs and synovial joints in RA (Pauley et al. 2008; Stanczyk et al. 2008). RASFs, upon stimulation by pro inflammatory mediators, produce microRNA-155 and -146a. Such mediators make up the inflammatory milieu observed in the articular compartment.

Within our laboratory microRNA-155 has been studied in detail (Kurowska-Stolarska et al. 2011). Importantly, double staining for microRNA-155 and macrophages demonstrates clear localization in the synovium. MicroRNA-155 is up-regulated in synovial CD14+ cells compared with PBMC. This was validated by transfection of PBMC with a microRNA-155 mimic, which leads to production of pro-inflammatory mediators. This would suggest CD14+ cells acquire an inflammatory phenotype when entering the articular compartment. Importantly, microRNA-155 deficient mice were resistant to collagen-induced arthritis.

FLS also produce elevated microRNA-155 and further increases with exposure to the pro-inflammatory milieu. This observation would be consistent with an autocrine positive feedback loop un-reliant on the need for on-going exposure to antigen.

These observations place microRNA-155 in the centre of the regulation of the inflammatory process and in key cell lines involved in RA pathogenesis.

MicroRNA studied	Cell type studied	Regulation	Murine/ human	Predicted targets/ effect (if sought)	Notes	Publication
155	Synovial cells and PBMC	Increased	Human	SHIP-1	SHIP-1 expression reduced by microRNA-155 thus increased inflammatory mediators. Administration of a -155 'antagomir' reduced LPS-induced TNFa production.	(Kurowska -Stolarska et al. 2011)
	Draining lymph node cells and osteoclasts	Absence of	Murine	n/a	MicroRNA-155 deficient mice do not develop clinical findings of collagen induced arthritis (nor at cellular level=unable to generate adaptive T- or B-cell response)	(Blüml et al. 2011)
	PBMC	Increased	Human		Increased -155 (and other) x1.8 fold relative to healthy controls (see also microRNA-146a table). Some correlation with disease activity but none with age nor treatment.	Pauley 2008
	RASF, synovial cells and peripheral mononuclear cells	Increased	Human	MMP1 and 3 (indirectly? as not predicted targets)	Synovectomy or arthroplasty specimens, monocytes from peripheral blood and synovial fluid. Few details about RA patient's disease characteristics. Synovial fibroblasts then stimulated with TNFa and measured microRNA-155 and -146a higher in RA than OA (though TNFa still induced 155 production in OA cells). -155 higher in RA SF monocytes (CD14) than PBMCs by x4-fold. No difference between healthy and RA levels in peripheral blood They suggest -155 has a repressive effect on MMPs in RASFs (through enforced expression experiments)	Stanczyck 2008
	Marrow macrophages	Increased	Murine	n/a	MicroRNA-155 induced by a variety of inflammatory ligands	(O'Connell et al. 2007)

Table 1-14 Selected microRNA-155 publications in RA

1.18.3.3 MicroRNA-146a

Much interest surrounds 146a as a primary ***negative*** regulator (Ammari et al. 2013). As such it represents an attractive therapeutic target. It has many varied roles in physiology and disease from regulation of granulopoesis to oncology, sepsis and autoimmunity (J.-F. Wang et al. 2010b). In general it is ***up-regulated*** in many of the cell types and compartments studied in RA, including blood and joint, by pro-inflammatory signals. It should act to down-regulate downstream inflammatory pathways, one example being inhibition of apoptosis (Curtale et al. 2010), but fails to act in this way. Biologically validated targets are key players within the inflammatory cascade including TNFa, IL-6, type 1 interferon, TLRs and COX-2. Other important downstream targets are key regulatory (Curtale et al. 2010; Y. Tang et al. 2009; B. Tang et al. 2010; Jazdzewski et al. 2008).

The first study to link microRNA-146 and RA (in the joint) was Nakasa et al (Nakasa et al. 2008). Their group's findings are shown Table 1-15 and built upon the findings of Taganov et al who had identified TRAF6 and IRAK1 as downstream targets of microRNA-146a and -146b. TRAF6 and IRAK1&2 are members of the Toll/Interleukin-1 signalling pathway and IRF5 and STAT1, members of the type 1 IFN pathway. In this way the role of microRNA is notable in the innate immune system.

The paper by Pauley et al in 2008 is the second important early paper that was the first to examine peripheral blood findings (Pauley et al. 2008). They examined patients (n=16) and correlations with clinical factors; they demonstrated significant differences in microRNA irrespective of treatment or not, DMARD or biologic use and no correlation with age. Their data did suggest some correlation with inflammatory markers. Finally they noted the relative levels of TRAF6 and IRAK1 mRNA and protein were not decreased versus control. This suggests an unregulated effect and explaining persistently elevated TNFa via elevated microRNA-146a.

Li et al 2010 demonstrates that microRNA-146a expression is correlated with TNFa in blood and synovium but not disease activity. (Abou-Zeid et al. 2011) have also demonstrated elevated microRNA-146a in peripheral monocytes. Furthermore, they did demonstrate correlation with TNFa and *were* able to demonstrate some correlation with ESR and DAS-28 in 70 RA patients. Of note there did not seem to be a difference in microRNA-146A levels in those treated with TNFi and conventional DMARDs.

Systemically administered microRNA-146a inhibits osteoclastogenesis and bone destruction in a collagen induced arthritis model (TNFa being one of the greatest drivers for). This raises the possibility of microRNA-targeted treatments. Notably in this study, the synovial pannus was relatively unaffected (Nakasa et al. 2011).

MicroRNA-146 is also not limited to RA. It is increased in OA (Yamasaki et al. 2009; Okuhara et al. 2011) along with -155, -181a and -223 adding further complexity to

interpretation of longstanding arthritis where both inflammatory and degenerative arthritis often co-exist.

Therefore elevated microRNA-146a is consistently demonstrated in RA (and other autoimmune diseases) and in the pathogenesis by targeting key molecules. The close correlation with TNFa but not other cytokines is of interest; TNFi act through TNFa and are effective at inhibiting bony erosions. MicroRNA-146a has been identified both peripherally in mononuclear cells and in synovium. There may also be some correlation with disease activity and therapeutic potential.

MicroRNA studied	Cell type studied	Regulation	Murine/ human	Predicted targets/ effect	Notes	Publication
146a	RASF	Increased	Human	n/a	Methods; see microRNA-155 entry. 146a x4 fold higher versus OA. Induced by LPS and IL-1B but not TNFa	(Stanczyk et al. 2008)
	PBMC	Increased	Human	TRAF6, IRAK1	Examined PBMC in RA. Some correlation with disease activity between -146a and -16 (active disease is above 'normal' ESR or CRP). Levels higher in monocytes vs lymphocytes. PBMC signature close to SF profile. Also elevated; -155, -132 and -16 versus healthy controls. TRAF6/IRAK1 levels not proportionally increased as might expect with -146a increase. ?reason why lack of inhibition/TNFi response	(Pauley et al. 2008)
	RASF	Increased	Human	n/a	5 RA patients vs 5 OA vs 1 control. Low CRP levels but long disease duration (9-28 years) -146a up-regulated in RA synovium along with elevated TNFa. -146a also present on normal and OA tissue but much lower levels. Expressed mostly in CD68+ macrophages and induced by TNFa and IL1-Beta. In those with lower disease activity -146a/b and TNFa were lower	(Nakasa et al. 2008)
	PBMC and SF cells	Increased	Human	n/a	Subjects varying activity (ESR 8-74mm/hr, disease duration 2-17years, mixed treatments). -146a /b, let-7a, -26, -150 and -155 identified by initial microarray. -146a highest in most inflamed cellular synovium and correlated with IL-17 expression, disease activity and shorter disease duration. -150 highest in those with most joint destruction	(Niimoto et al. 2010)
	SF, PBMC, CD4+ T-cells	Increased	Human	FAF1	Microarray of 2 treatment-naïve seropositive RA patients (DAS 6.85-7.51) to identify eight microRNAs more than two-fold up-regulated versus healthy controls. Confirmed by qPCR in 33 RA patients (mean ESR 68mm/hr and disease duration 58 months, DAS28 6.35). -146a up-regulated and correlated with disease activity and TNFa levels (both in blood and synovial fluid) but NOT inflammatory markers, cytokines nor clinical markers of disease activity. -146a target FAF1 that modulates T cell apoptosis.	(J. Li et al. 2010)
	Human THP-1 cells	Increased	Human		Aim; analyse microRNA expression of monocyte cell line following LPS (thus via TLR-4, innate mechanism). Examine -146a profile in detail and inducers/regulators.-146 up-regulation is through nFkB mechanism. IRAK-1 and TRAF-6 identified targets representing a feedback mechanism	(Taganov et al. 2006)
	PBMC	Increased	Human	N/a	40 RA patients versus healthy controls. Increased -146 and -16, correlation with disease activity (ESR, CRP and DAS-28)	(Feng et al. 2011)

Table 1-15 selected microRNA-146 publications in RA

1.18.3.3 MicroRNA-223

MicroRNA-223 is a key regulator of differentiation within the haematopoietic system. Johnnidis et al identified a binding site within the transcription factor Mef-2c suggesting a role in the fine-tuning (down regulation) of granulocyte activation building on earlier findings by Fazi et al (Johnnidis et al. 2008; Fazi et al. 2005). MicroRNA-223 also has a role alongside cytokines and transcription factors in the regulation of osteoclast differentiation from marrow macrophages (Sugatani & Hruska 2009). As an important effector cell in joint erosion, this is a key finding in RA. In RA, microRNA-223 is increased in both blood and synovial compartments yet action is cell type-dependent. The important publications are shown in Table 1-16 below.
Additionally, Murata et al noted a difference in -223 vs osteoarthritis in both plasma and synovial fluid but no correlation with clinical factors other than an inverse correlation with tender joint count (Murata et al. 2010). Fulci et al identified up-regulation of microRNA-223 in naïve CD4+ peripheral T-lymphocytes and were the first group to study this cell line. MicroRNA-223 was not expressed in Th-17 cells (Fulci et al. 2010). Shibuya et al overexpressed microRNA-223 and noted both suppressed markers and histology of osteoclastogenesis (Shibuya et al. 2012). Li 2012 went on to administer microRNA-223 in a therapeutic fashion and demonstrated two interesting findings in a mouse CIA model. Firstly, that microRNA-223 is elevated *prior* to the demonstration of overt arthritis in the ankle joints. Secondly, the administration of a lentivirus-mediated suppression of microRNA-223 reduced both bony erosion and histological synovitis (Y.-T. Li et al. 2012).
MicroRNA-223 has also been observed in osteoarthritis (Okuhara et al. 2011). This group demonstrated some correlation between microRNA-223 and Kellgren score (severity of joint narrowing and OA findings) and cartilage degradation products. Both microRNA-146a and -223 levels were higher at an earlier disease stage.

These findings suggest that microRNA-223 may have a role in cartilage destruction and be a potential therapeutic target in RA.

1.18.3.4 MicroRNA-34 cluster

MicroRNA-34 may have a role in megakaryocyte differentiation and cell cycle regulation (Ichimura et al. 2010). It acts as a 'tumour suppressor' under the influence of p53 leading to cell apoptosis. In RA, (Niederer et al. 2011) identified the down-regulation of microRNA-34* accounting for the observed resistance to apoptosis in synovial fibroblasts. They went on to identify the methylation status of the microRNA-34a* promoter to be relevant.

1.18.3.5 MicroRNA in RA; other examples

Selected other microRNA studied in RA are summarised in Table 1-17.

MicroRNA studied	Cell type studied	Regulation	Murine/ human	Predicted targets/ effect	Notes	Publication
223	Synovial tissue (vs OA synovium)	Increased	Human	Nuclear factor I-A	Patients at arthroplasty or synovectomy Disease duration 3-17 years and mixed treatments, all destructive and erosive disease.-223 present in joint sub-lining, macrophages, mononuclear cells and CD4+ T-cells. NFI-A mRNA not down-regulated but is at the protein level suggesting potential target. Controls osteoclast differentiation; high levels in RA leads to suppressed osteoclastogenesis	(Shibuya et al. 2012)
	CD4+ naïve T-lymphocytes, serum	Increased	Human	n/a	Hypothesis; T-lymphocytes involved in early stages of Ag presentation. Either on no treatment or steroid <10mg only but had *established* disease. No correlation with disease activity examined (were all 'active' and double antibody positive.) -223 up regulated in peripheral blood CD4+ naïve T cells. Healthy naïve cells did not express -223 after TCR stimulation suggesting a disease phenomenon.	(Fulci et al. 2010)

Table 1-16 selected microRNA-223 publications in RA

Reference and MicroRNA	Significant findings and Comments
(Nakamachi et al. 2009; Kawano & Nakamachi 2011) **MicroRNA-124a**	Synovial cells of 16 RA patients (at time of joint surgery, disease duration 2-32years) vs OA. Examined microRNA profile and targets MicroRNA 124a significantly reduced; predicted targets=CDK-2 (role in cell cycle control) & MCP-1 (attracts memory T-lymphocytes and NK cells and role in angiogenesis via VEGF); increasing respectively If transfect cells with precursor microRNA-124a then cells arrest at G1 phase Could not identify cytokine that reduced -124a expression. Hypothesis that other epigenetic processes may be regulating (for example the microRNA promoter gene?)
Murata 2010 **MicroRNA 16** **MicroRNA 132** **MicroRNA 146a** **MicroRNA 223**	30 patients each group. PBMC vs synovial fluid (SF) vs synovial 'tissue' and looked at microRNA 16, 132, 146a and 223. Synovial fluid concentrations in plasma lower than SF. SF and plasma concentrations differed RA vs OA. Negative correlations with disease activity (TJC only) No correlations with MMP, CRP or ESR *Overall didn't perform well as biomarkers*
(Stanczyk et al. 2011) **MicroRNA-203**	MicroRNA-203 increased in synovial fibroblasts vs OA samples. Some variation according to disease stage (more *variable* in early disease). No correlation with treatment. Disease activity not examined Showed *methylation status of the upstream promoter* region important and this in turn increased MMP-1 and IL-6 via nFkB. Suggests may be important in the persistently activated fibroblast state. -203 unresponsive to TNFa or LPs stimulation; therapeutic demethylation leads to an increase in responsiveness again to pro-inflammatory ligands
(Trenkmann et al. 2013) **MicroRNA-18a** (Pandis et al. 2012) **MicroRNA-323-3p**	Studied microRNA-18a in RA synovial fibroblasts from those undergoing joint surgery TNFa induced production of the microRNAs from the 17-92 cluster. In turn transfection with microRNA precursors leads to production of MMPs. The action of microRNA-18a was to remove the inhibition of nFkB by TNFAIP-3 Propose a *positive* feedback loop Attempted to 'overcome' patient and disease heterogeneity using a mouse model of synovial fibroblasts Increased 323-3p identified. This may target and enhance Wnt/cadherin signalling which induces cartilage breakdown
(Semaan et al. 2011) **MicroRNA-346**	Objective; to identify the microRNA involved in observed finding of increased TNFa mRNA but not TNFa in LPS activated FLS Studied FLS derived from 4 RA patients at time of joint surgery. Already resistant to further TNFa secretion after LPS yet there is increased TNFa mRNA (proposed to be unstable and subject to cytoplasmic breakdown, a process usually stabilised by Bruton's tyrosine kinase) In LPS activated FLS, -346 co-expression with TNFa mRNA inhibits TNFa production, acts via Bruton's tyrosine kinase inhibitor. -346 'trying' to act as anti-inflammatory brake in FLS.

Table 1-17 Additional selected relevant microRNA publications in RA

1.18.3.6 MicroRNA targeting key inflammatory molecules

1.18.3.6.1 Validated microRNA-155 targets

MicroRNA-155 targets are many and varied from regulatory genes to key signalling and binding proteins (Faraoni et al. 2009). Although the predicted number of 'in silico' targets is far greater, some validated examples are shown.

c-MAF (repressed by microRNA-155, dendritic cell maturation and haematopoietic maturation) (Rodriguez et al. 2007)

SHIP-1 (reduced by -155, acts as an inhibitor of inflammation by inhibiting myeloid proliferation (O'Connell et al. 2009; Pedersen et al. 2009). Increase observed in myeloid tumours ?reduced tumour suppression.

Bach1 (a transcription factor regulator) (Skalsky et al. 2007)

Pu.1 important for early B-cell development (Vigorito et al. 2007)

AID (activation induced cytidine deaminase) is a regulator of antibody diversification and targeted by -155 (Teng et al. 2008; Dorsett et al. 2008). It is down-regulated by -155; in this way it acts as a brake on un-regulated new antibody formation .

SOCS-1 (suppressor of cytokine signaling). Is part of the *negative* regulation of the LPS-induced inflammatory response. SOCS-1 has been identified as a target by a number of authors and may also act as a 'tumour suppressor gene' in this way. Androulidaki 2009 demonstrated LPS stimulated microRNA-155 production in macrophages is suppressed via Akt-1. MicroRNA-155 may act to suppress SOCS1 via Akt-1 (one of the known negative feedback pathways to reduce TNFa). (Androulidaki et al. 2009). Jiang 2010 identified high microRNA-155 and suppressed SOCS-1 in breast cancer. In turn the unregulated production of inflammatory cytokines contributes to cell growth (Jiang et al. 2010). Wu et al concluded that microRNA-155 modulates TNFa driven osteogenic differentiation by targeting SOCS-1 expression. TNFa may increase microRNA-155, which, via SOCS-1, leads to osteoblast differentiation (J. Wu et al. 2011).

SHIP1/C/EBPbeta in mice are both targets of microRNA-155 and act as regulators of IL-6 signaling (Costinean et al. 2009).

SMAD2 microRNA-155 repressed SMAD 2 protein and in this way reduced TGF-B action (Louafi et al. 2010)

TAB2 (TAK1 binding protein 2, a signaling molecule downstream of TRAF6). Ceppi showed that LPS induced microRNA-155 inhibits TAB2 expression (a part of TLR4 signaling) and thus acted as an *inhibitory* regulator in monocyte derived dendritic cells (Ceppi et al. 2009)

FADD (the anti-inflammatory and pro-apoptotic molecule Fas-associated death domain protein) Involved in TLR signaling.

p53 Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development (Gironella et al. 2007)

MyD88 (myeloid differentiation protein 88) is a key adaptor protein component of most TLRs. Predicted binding site examined and confirmed by (B. Tang et al. 2010).

1.18.3.7 MicroRNA targeting key inflammatory signalling pathways and within regulatory networks

JAK/STAT pathway

(Kutty et al. 2010) et al studied retinal epithelium and inflammatory signalling. IFNg regulates gene expression via STAT1 activation (a transcription factor) through JAK/STAT signalling. The BIC/microRNA-155 promoter has two probable STAT1 binding elements such that the administration of a JAK kinase inhibitor blocks microRNA-155.

nFkB pathway

MicroRNA-146 and NFkB signalling is discussed above in relation to the work performed by Taganov et al 2006. (X. Ma et al. 2011) review microRNA in nFkB signalling.

JNK pathway

O'Connell 2007 et al identified inhibition of inflammatory mediator triggered production of microRNA-155 by inhibiting JNK.

Three example regulatory networks are described below:

- *McCoy et al propose that IL-10 down-regulates microRNA-155*. The binding of LPS produces microRNA 155 leading to an increase in SHIP1 (McCoy et al. 2010) Furthermore the LPS-induced production of IL-10 inhibits microRNA 155 and thus further fine-tunes the response.
- *MicroRNA 146a negatively regulates TLR signalling*. Taganov et al in 2006 demonstrated following TLR receptor activation with LPS, nFkB is up regulated. MicroRNA-146a is also transcribed in parallel creating a negative feedback pathway acting through IRAF1 and TRAF6 (Taganov et al. 2006).
- *MicroRNA-187 regulates IL-10 driven anti-inflammatory response.* Whereas the TLR are the important mediators of inflammation, IL-10 is an important negative regulator. Rossato et al sought to identify the microRNA involved in the IL-10 mediated reduction of inflammation. They identified microRNA-187 as being elevated and a corresponding reduction in TNFa and IL-6 (Rossato et al. 2012).. IL-10 leads to a fall in micro-RNA-155 (in keeping with McCoy et al) and a rapid and transient increase in microRNA-146b in addition. There is a also predicted binding site for microRNA-187 in TNFa mRNA.

1.18.3.8 MicroRNA are demonstrated in key cell types and compartments

Within these publications and in order to summarise, the observation of an individual microRNA such as -155 can be seen in common between cell types from monocytes and macrophages to synovial fibroblasts. Additionally, these microRNA are present in key

immune cell types and principal cellular components in RA (previously referenced);

- Blood 155; Niimoto, Stanyczk, Pauley
  223; Fulci
  146; Pauley, Niimoto, Murata
- Synovial environment 146; Murata, Li
  124; Nakamachi
  155; Stanyczk
- Osteoclast 155; Stanyczk, Bluml
  146; Nakasa
- Fibroblast 346; Alsaleh
  155; Stanyczk
  146; Nakasa, Stanyczk
  203; Stanyczk

- Plasma; Murata, Li, Kurowska-Stolarska, Fulci, Pauley, Niimoto, Filikova
- RASF; Stanyczk , Nakamachi, Alselah
- SF; Murata, Nakamachi, Nakasa, Niimoto

1.19 Summary

1.19.1 Epigenetics, microRNA and RA

In summary, the small human and animal studies published to date confirm epigenetic modifications are central findings in the pathogenesis of RA. Initial observations suggest microRNA are dysregulated and form a key regulatory role in the inflammatory feedback loops. Modifications in key effector cells may also provide an explanation for the persistent state of activation within the immune system (Ospelt et al. 2011). Notable observations relevant to the findings and methodology of this study therefore include;

- That the link between both microRNA-155 and -146a and TNFa is strong and potentially pathological. It is not clear if this observation reflect a primary disturbance of microRNA regulation or is merely a marker of other dysregulated immune/epigenetic processes.
- MicroRNA are involved in inflammation and tissue destruction (-155, -146a, -223) They regulate and participate at multiple steps in the inflammatory cascade and network.
- That those microRNA observed are not only central to the molecular pathological processes in RA but also present within those cell types demonstrated within the joint.
- MicroRNA may act in a disease-specific fashion; for example microRNA-146 is reduced and involved in IFNg regulation in SLE but increased in RA and regulates TNFa
- That similar microRNA profiles have been replicated between research groups
- That the peripheral blood compartment signature is similar to that of the articular compartment. Correlation between peripheral blood analysis and disease activity is inconsistent- a finding that may/not be of use in overcoming the inherent weaknesses in the DAS composite scoring system.
- A difference in epigenetic profile between RA, OA and healthy immune cells exists. Both degenerative and inflammatory disease often co-exits in RA but the potential remains that microRNA represent a biomarker.
- MicroRNA may represent therapeutic targets in the future

At present, no studies have addressed the study of microRNA in disease onset, disease stage nor related profiling to drug response.

1.19.2 Challenges in RA management and potential application of examining epigenetic modifications

There exist shortfalls in the current treatment of RA that can be approached by examining the disease course of the individual. At the earliest stages, it is not possible to either screen for or identify 'pre RA'. Intervention prior to symptoms would be the ideal scenario but faces ethical and practical challenges. Genetic risk loci alone do not adequately predict risk of developing RA owing to the unpredictable influence of environmental risk.

Once symptoms have become present and persistent then treatment choices remain imperfect. Although treating to low disease activity is key attempts to define disease subsets based on autoantibody or treatment-response profiling. A personalised bio-molecular approach would determine to which drug (s) the individual would best respond at any part in disease course.

There exists a cohort of patients with severe disease resistant to conventional therapy that pose a significant challenge. It is unclear what the primary mechanisms are driving this finding. At present therapy remains trial and error with cycling through therapies and exposure to risk from treatment side effect.

Research in the field of epigenetics, most notably microRNA, suggests a potential role for these processes in RA.

- Epigenetics offers an appealing mechanism to tie together the principal established risk factors of genetic predisposition and environmental factors.
- In addition, through their appreciable relative contributions, epigenetic modifications could offer an explanation for the range of phenotypes, disease severity and responses to therapy seen in RA.
- Epigenetic changes could explain biologic resistance such as the example of the persistently activated fibroblast. Persistent methylation may explain this phenomenon and why RA persists.
- Through their potential for therapeutic reversal, epigenetic modifications may act as a potential future treatment target.

1.20 Hypothesis under Investigation in this thesis

That networks of microRNA mediate the observed resistance to standard available therapies in rheumatoid arthritis and that these changes are accessible in peripheral leucocytes.

1.20.1 Primary Aims

The primary objective is to firstly define the phenotype of and carry out a detailed examination of patients with severe RA with active inflammatory joint disease demonstrated by the DAS28 clinical scoring system (DAS28>3.2). Having characterised such a cohort I will seek a microRNA signature in this cohort.

1.20.2 Secondary aims and means to achieve

- Quantifying the co-morbid burden of this cohort and burden of unmet need
- To explore the stability and variability over time of the clinical and standard laboratory characteristics of the resistant RA patient group (longitudinal analysis)
- MicroRNA profiling according to disease stage by comparing the primary cohort with control groups at differing disease stage (cross sectional analysis) .
- Stability with time of observed microRNA
- Relationship between microRNA and disease activity (biomarker potential)

2.1 Chapter 2; Patients and Methodology

2.1.1 Chapter Introduction and Aims

Within this chapter I will outline the methods used in this study. I will first describe the clinical methods including study design, ethical considerations and application, recruitment and assessments performed at each visit.

A careful characterisation of the 'resistant' patient phenotype will be performed. Information collected will include disease history to examine onset, previous and current therapy including reasons for drug termination. Employment status and demographics including postcode were collected. Previous joint surgery and presence of extra-articular features were examined.
Measurement of co-morbidities, non-RA related and RA-related were collected. Importantly, more in-depth assessment of cardiovascular and mood assessment were examined. Fatigue and psychological parameters were assessed, level of functioning and quality of life were also assessed using the using the questionnaires detailed in Appendix 1. Amendment 2 to the original ethical application sought to examine illness perception in RA and coping with the effects of severe RA in view of the domains examined above.

I will then describe the laboratory methods employed and principles to those performed to study microRNA profiling and proteomics. I will also outline the haematology, biochemistry and Immunology assays used.

2.2 Clinical Methods

2.2.1 Patient Recruitment

2.2.1.1 Ethical Considerations and Approval

Ethical approval for the study, and subsequent amendments, was obtained from the Local Research Ethics Committee (West of Scotland) REC Ref Number 10/S0703/4 (R&D Ref: GN09RH669) in Mach 2010 and was obtained prior to the study commencing in August 2010. Recruitment ran until September 2011. All patients provided written informed consent prior to their study participation. Several amendments were required during the study to reflect slow recruitment and evolving methods/aims.

- Amendment 1- May 2010 concerned re-wording of the Patient information sheet to reflect samples potentially being processed outwith the UK.
- Amendment 2- February 2011 was the addition of Stobhill Hospital as a third recruitment site and the two additional questionnaires that formed the basis of Chapter 6.
- Amendment 3- June 2011 was the addition of a fourth control group (the DMARD good responder group).

2.2.1.2 Recruitment- General considerations and challenges

Prior to patient recruitment starting, potentially suitable patients were identified at the Glasgow Royal Infirmary and Gartnavel General Hospital. A third centre, Stobhill General was later added to help recruitment. Patients were initially opportunistically identified from records of those regularly attending respective Day Units for infusions. These patients were sent Patient Information Sheets (see PIS appendix) prior to contact. Disease activity was assessed on the day and inclusion/exclusion criteria revisited prior to consent being provided. Where possible and time allowing, questionnaires were completed during the study visit. If not suitable then addressed envelopes were supplied to return questionnaires or returned to ward staff at a later date in the case of those regularly attending for infusions.

The second and third clinical visits were carried out at three months and six months where practically possible. Many patients endured multiple other hospital visits and study visits were often earlier or later to accommodate this but all +/- three weeks. Furthermore, drug safety monitoring is undertaken in primary care and so as to avoid duplication, study visits were arranged on occasion to coincide with such time, as venepuncture would have been otherwise due.
In order to minimise errors in joint assessments, DB had attended a EULAR joint assessment workshop and thus standardised techniques followed. DB carried out all clinical assessments other than patients 416 to 421 inclusive (recruited from the

Glasgow Royal Infirmary and clinical assessments were carried out by Dr Eva Ruzicka, Clinical Research Fellow (also Glasgow Biomedical Research Centre, University of Glasgow)

2.2.1.3 Recruitment- study groups

Four study groups were characterised and examined. Recruitment and study visits for all four groups were carried out between August 2010 and September 2011. Blood sampling was undertaken at the same visit as clinical assessments. Primary care monitoring does not stipulate that inflammatory marker monitoring is undertaken but if it had been undertaken in the preceding week, and assuming there was no major clinical change from that time, then this was not repeated so as to reduce the volume of blood drawn. Blood sampling for epigenetic analysis and clinical assessments were always carried out on the same day.
Characteristics of each study and control group, recruitment, inclusion and exclusion criteria are addressed. The following also expands on that information in Appendix 4 Study Protocol.

2.2.1.3.1 Biologic therapy resistant group

Inclusion Criteria	Exclusion Criteria
-Rheumatoid Arthritis (meeting 1987 ACR criteria)	-Known active cancer
-DAS28 ESR (or CRP) >3.2	-Active infection
-Previous treatment with 2 or more previous biologic therapies	-Recent surgery

Table 2-18 Inclusion and Exclusion criteria to biologic resistant study group

Recruitment- patients meeting inclusion criteria were identified from the Rheumatology databases (Gartnavel General Hospital and Stobhill Hospital) employed in Greater Glasgow and Clyde centres. Further information was then obtained in the drug administration areas where more detailed examination of biologics prescription files could be carried out. Additional patients were opportunistically identified from colleagues and Biologic follow up outpatient clinics (Glasgow Royal Infirmary and Gartnavel General Hospital).

Group Characteristics- Such individuals may have demonstrated primary or secondary loss of efficacy to standard synthetic disease modifying medication and to at least two

biologic therapies (including for reasons of primary failure (lack of effect), secondary failure (loss of effect), side effect and 'other' reasons). This includes all biologic agents approved for use in the Scotland including Etanercept, Adalimumab, Infliximab, Rituximab, Abatacept, and Tocilizumab. Several individuals had been treated with Anakinra (no longer recommended for use in Scotland) who were eligible for inclusion. Several patients received a biologic drug within the remit of a trial. In this instance, the drug employed and response to therapy was available.
At the time of assessment, **active inflammatory disease** was present. This is defined as a DAS-28 ESR of greater than or equal to 3.2. This is in keeping with most recent recommendations that a DAS-28 of this level be applied when starting anti-TNFa therapy than the 2005 guidance of 5.1 (Deighton et al. 2010; Ledingham et al. 2005).
Insofar as can be best ascertained from clinical records, response to and/or side effects to standard disease modifying therapy was collected. Response to previous biologic therapy was collected. A primary lack of response is defined as failing to achieve a DAS-28 reduction of 1.2 or residual disease activity failing to suppress to less than or equal to 3.2 within six months treatment. Secondary loss of effect was defined in those demonstrating an initial response to their biologic therapy within the recommended six months but in whom a subsequent increase in DAS-28 occurs to at least baseline values. Toxicity encompasses all side effects from infusion reactions to symptoms attributable to the drug necessitating withdrawal.

2.2.1.3.2 DMARD Resistant group

Recruitment- Eligible patients were those in whom DMARD therapy has either not been tolerated or has failed to adequately suppress inflammatory joint disease (also known as inadequate responder, DMARD-IR). In such patients, current treatment recommendations are that biologic therapy would be the next therapeutic intervention[96].

Group characteristics- The ORBIT (**O**ptimal management of patients with **R**heumatoid **A**rthritis who require **B**iolog**I**c **T**herapy, REC Ref 09/S0703/109) study is an on-going twelve-month study across Scotland and North England randomising treatment to either anti-TNFa therapy or B-cell depletion with Rituximab. The primary outcome compares efficacy and cost-effectiveness. Inclusion and exclusion criteria were met (see below) and within the consent for this study was permission to examine peripheral blood samples at baseline, three and six months. No other assessments of patients were undertaken nor change to treatment that remained under the study physician. Access to the study database to draw results was permitted.

Inclusion Criteria	Selected Exclusion criteria (see Appendix 4)
Those eligible for biologic therapy according to BSR criteria Rheumatoid factor **OR** CCP antibody positive	Active infection, septic arthritis within last year NYHA grade 3 or 4 cardiac failure Demyelinating disease Malignancy

Table 2-19 Inclusion and Exclusion criteria to the ORBIT study

2.2.1.3.3 DMARD Good responder group

Recruitment- Recruitment took place at the above named centres. Medical staff at local clinics and DB identified patients and if the criteria were met below then further consenting took place with clinical assessment and blood sampling on the same day.

Inclusion and Exclusion Criteria-

Inclusion Criteria
-Rheumatoid Arthritis (meeting ACR 1987 criteria)
-DAS-28 ESR (or CRP) less than 3.2
-Disease duration greater than ten years
-Prior therapy (defined as 'therapeutic' and greater than three month trial) with two or fewer DMARDs

Table 2-20 Inclusion criteria to DMARD good responder study group

Group characteristics- this comparator group aims to mediate the effect of disease duration upon observed findings. A good response to treatment with two or fewer standard DMARDs should be demonstrated. Thus at the time of examination low disease activity (as demonstrated by a DAS28 of less than or equal to 3.2) should be present.
Disease duration of greater than ten years was chosen pragmatically for two reasons. Definitions of 'longstanding' RA have not been formally defined. 'Early' rheumatoid is generally accepted as disease of less than one year and after the second year

'established' and thus persistent. Symmons et al chose between five and twenty years as inclusion to a study of 'established RA' in 2006 whereas by 2012 this period was defined as 'greater than six months' by the American College of Rheumatology consensus guidelines (Symmons et al. 2006; Singh et al. 2012). In this way, ten years would represent 'longstanding' disease with a stable phenotype. Secondly, the cohort disease duration would approximate the main study group.

Additional clinical data collected included age, gender, and medical co-morbidities including treatment where relevant, smoking status, height and weight to allow calculation of BMI.

2.2.1.3.4 Healthy controls

Recruitment-healthy controls were age matched where possible. Recruitment using age-matched relatives was used where possible and supplemented by the use of volunteers based at the three local recruiting hospitals.

Inclusion and Exclusion criteria- to this group is stipulated by a lack of current inflammatory, degenerative or otherwise symptoms at the time of interview. Those with a known diagnosis of arthritis were excluded. Those with a current or recent joint injury were excluded. Those with a family history of arthritis were considered. Those with an established diagnosis within first/second degree relatives were excluded as the role of hitherto asymptomatic epigenetic modifications has yet to be elucidated. CCP antibodies can be demonstrated prior to symptomatic joint disease (Rantapaa-Dahlqvist S 2003) and epigenetic modifications could be expected.
Additional clinical data collected included age, gender, and medical co-morbidities including treatment where relevant, smoking status, height and weight to allow calculation of BMI.

2.3 Data Collection

Data was collected and entered in a structured pro-forma later entered in spreadsheet format for analysis. All clinical DB undertook all assessments other than those patients assessed by Dr Rusicka as referred to in the DMARD good responder group. The following assessments were made:

2.3.1 Clinical Data

The main biologic therapy resistant group had the following data collected at the time-points shown.

Assessments	Further details	Study Visits (months)
Demographics	Age, gender, ethnicity, height, weight, smoking status, employment status, postcode	0,3,6
Medical comorbidities	Active and inactive medical condition (treatments) from case notes, GP referral documentation and Rheumatology databases (Gartnavel and Stobhill patients)	0,3,6
RA phenotype	Disease duration, extra- articular disease, previous surgery, immunology	0
Current and prior drug therapy	Previous DMARDs and biologic therapies, reasons for failure, steroid use	0,3,6
Disease activity	Clinical and biochemical assessments (DAS-28 ESR and/or CRP, ESR, CRP), SDAI and CDAI	0,3,6
Quality of life assessments	See Table 2.4 and 2.5 below	0,6
Cardiovascular assessment	Lipid profiles, blood pressure, ECG, CV medications and CV personal and family history, smoking status, BMI, WHR	0
Psychological comorbidity	See Table 2.4 and 2.5	0,6

Table 2-21 Assessments performed on biologic resistant study group

Of note from the table above;

- Smoking status was defined as never, non-smoker (>1year) and current smoker
- Height and weight were measured using regularly calibrated clinic scales and wall-mounted measuring tapes within local departments.
- Waist:hip measurements were carried out as outlined (Han et al 2006)
- Blood pressure was carried out using local calibrated equipment.
- ECG was performed at local Cardiology departments or if carried out within the last six months then the interpretation of this used.
- BMI (body mass index) was calculated using the standard calculation
- Case records were examined when available; relevant medical comorbidity was drawn in this fashion supplemented by patient recall at study visit. Patients at Gartnavel General have medical comorbidities recorded in a Rheumatology database. Records were accessed and confirmed with patients.

The following questionnaires were administered in this study in order to study quality of life measurements and co-morbidities and are thus examined.

Questionnaire	Time to complete	Time to score	Main health dimensions assessed	Study Administrations
HADS	<5mins	3mins	Psychological	0&6 months
HAQ-DI	<5mins	3mins	Physical function	0&6 months
SF-36v2	<10mins	5mins	Physical function Psychological Social functioning Pain Energy/Fatigue	0&6 months
FACIT fatigue scale	<5mins	5mins	Energy/Fatigue Social functioning	0&6 months
EQ-5D	<3mins	2mins	Physical functioning Pain Psychological	0&6 months
Rose angina questionnaire	0-3mins	2mins	Specific medical health domain	Once at baseline

Table 2-22 Questionnaires administered to biologic therapy study group

The PROM questionnaires administered during this study are scored as shown in Table 2-23. DB performed all scoring.

Questionnaire	Outcome
HADS	Score 0-21 (>8 suggestive of morbidity, >11 then morbidity very likely)
HAQ	Responses converted to a score 0-3 rising in increments of .125 where 3 is most disabled
SF-36v2	8 domains (4 physical, 4 mental) scored. Uses norm based scoring system (assume population mean=50, <45 then health status less than 'average'). Thus 10 results; 8 individual domains with summary physical and mental component scores
FACIT fatigue scale	13 items; weighted and used to calculate total score. Negatively worded items are reversed. Higher score=less fatigued. Score 0-52
EQ-5D	5 scoring dimensions, rated 1='no problems', 2='some problems', 3='extreme problems' and a visual analogue score 0-100.
Rose angina questionnaire	Result is 'no angina', 'possible angina' or 'atypical chest pain' (angina unlikely)

Table 2-23 Patient related outcome measures used in biologic resistant group

Patients from the DMARD resistant group (ORBIT patients) had clinical data collected from the trial website (www.glasgowctu.org/ORBIT). Where necessary, data was supplemented from clinical case notes or Rheumatology database.

Assessments	Further details	Study Visits (months)
Demographics	Age, gender, ethnicity, height, weight, smoking status	Baseline only
Medical comorbidities	Active and inactive medical condition from case notes, GP referral documentation and Rheumatology database (Gartnavel and Stobhill patients)	Baseline only
RA phenotype	Disease duration, immunology	Baseline only
Therapy	Current and previous DMARDs, current biologic therapy arm Steroid use between visits	Baseline, three and six months
Disease activity	Clinical and biochemical assessments (DAS-28 ESR and/or CRP, ESR, CRP)	Baseline, three and six months

Table 2-24 ORBIT study assessments imported and examined for DMARD resistant group

Blood sampling was undertaken at ORBIT study visits as per protocol. Additional samples were provided for this study when practically possible (transport time and laboratory space availability).

2.3.1.1 Cardiovascular risk calculators

The Framingham CV risk score (risk of developing CV disease over the next 10 years) was evaluated using the University of Edinburgh free to access online calculator (http://cvrisk.mvm.ed.ac.uk/calculator/calc.asp) This utilizes the Joint British Societies equation and links to calculate ASSIGN scores, risk of myocardial infarction, stroke and death from either in the next ten-year period.
Overall risk of future events was calculated using the SCORE risk calculator (peters 2010) using the high-risk chart and the suggested x1.5 multiplier. Perk et al (eur heart 2012 guideline) suggest if the score result (risk) >5% then those patients 'may' benefit from drug treatment if any other target value is not met. If risk >10% then treatment is 'frequently required'. Whilst not prescriptive, this serves as a guide.

2.3.2 Blood Sampling

See Appendix 3 for details of sampling and transport. This collection procedure was carried out on all patients recruited to this study.
Carstairs deprivation scores were calculated using the charts available (http://www.sphsu.mrc.ac.uk/publications/carstairs-scores.html) based on data from the 2001 census.

2.4 Discussion of Study Design

The frequencies of clinical visits are defined in Table 2.7 below. We chose to examine the biologic and DMARD resistant groups across three visits to allow a cross sectional analysis. It would be anticipated that within the ORBIT group there would be good responders and non-responders that may allow an analysis of the effect of therapy and any observed changes. The biologic group were considered likely to undergo alterations to therapy during the course of the study as guided by their treating physician. This

would facilitate some correlation between disease activity, treatment change and epigenetic findings.

Study Group	Baseline	Three months	Six Months
Biologic Resistant N=50	✓	✓	✓
DMARD Resistant N=50	✓	✓	✓
DMARD Sensitive N=25	✓		
Healthy Control N=25	✓		

Table 2-25 Proposed study numbers and study visit timetable

2.5 Sample size

The choice of 50 in the primary study group has been based on pragmatic judgement in the absence of any systematic cohorts or publications at the time of study design upon which to base power calculations.

2.6 Laboratory Method

2.6.1 Haematology and Biochemistry panels

CRP testing was undertaken using a highly sensitive CRP (hsCRP) assay undertaken at Greater Glasgow Biochemistry Laboratories. One healthy control patient (227) was recruited from NHS Lanarkshire where local Biochemistry facilities were used. CRP results are a standard CRP assay and unable to quantify below 6mg/dl. This value was thus excluded. Haemoglobin assay was undertaken in the same accredited laboratories.

2.6.2 Immunology-General

All Immunology testing was undertaken at the local Immunology Laboratory at Gartnavel General Hospital serving Greater Glasgow. This laboratory undergoes regular accreditation by the Clinical Pathology Accreditation regulatory body.

2.6.3 Immunology-Rheumatoid Factor

The Siemens Healthcare Diagnostics UK N-Latex-RF kit was used to quantify RF. The manufacturer's guidance, reagents and method were followed. The nephelometer used to quantify the antigen-antibody complex was the Dada Behring model BNII.
The Latex testing principle is as follows; when serum containing RF is mixed with polystyrene beads coated in human immunoglobulin and sheep anti-human IgG then aggregation (agglutination) occurs. This scatters a beam of light passed through the sample and the intensity is proportional to the concentration of RF. This is then compared against a reference of known concentration.
The reference range suggested by the manufacturer and on the basis of local agreed cut-offs at the Gartnavel Immunology Laboratory were determined as follows; <15IU/ml=negative, 15-34IU/ml=equivocal, 35-350IU/ml=positive and >350IU/ml as strong positive.

2.6.4 Immunology-ANA

ANA slides and conjugate were manufactured by Menarini Diagnostics. The analysis was carried out on a Zenit processor model SP+. The technique used was an immunofluorescence assay and HEP-2 slides. The principle is as follows; patient serum is incubated with mitotic human epithelial cells (Hep-2 cells) and if ANA's are present then binding will occur forming antigen-antibody complex. To this fluorescent antibody is bound which is visible by microscopy. The degree of and pattern of binding form the basis of the result. In terms of reported binding avidity then 1/40 and 1/80 is considered very weak, 1/160 and 1/320 weak positive, 1/640 and 1/1280 moderate positive and 1/2560 strong positive. These cut-offs have been determined combining the manufacturer's guidance and those of the laboratory performing these tests.

2.6.5 Immunology-CCP2 assay

The product used was ELiA^{TM} manufactured and distributed by Phadia (Thermo Fisher Scientific) using the principle of ELISA. Analysis was undertaken using the Phadia ImmunoCAP 250 automator.

This is a second generation CCP antibody test using a mix of citrullinated proteins (exact mix proportions undisclosed). The manufacturer details a sensitivity of 87.8% and specificity of 96.7% based on in house testing. The measured range is 0.4U/ml to 340U/ml. The manufacturer suggests a cut-off guide of <7U/ml as negative, 7-10U/ml as indeterminate and >10U/ml as positive.
In general, anti-CCP2 assays have a sensitivity of 55% to 80% and a specificity of 90% to 98% for established RA.

2.6.6 MicroRNA

Method is described in detail in Chapter 4.

2.7 Statistical Analysis

2.7.1 Chapter 3

Descriptive (univariable) analysis was performed including calculation of mean (and standard deviation), median (and inter-quartile range (IQR)) where applicable. Distribution of all data was first calculated and non-parametric testing was appropriate for the majority of variables. Spearman's correlation coefficient was calculated between variables to test the strength of their association and either the Mann-Whitney test or Kruskal-Wallis test were chosen when comparing distribution of variables between or within groups unless stated otherwise. Wilcoxon signed rank test was used as the non-parametric test to compare two related results at subsequent timepoints (e.g. baseline with 3 or 6 month disease activity). Power calculations were not performed when this study was designed for the reasons stated (lack of published body of evidence). Statistical significance was assumed where p-value was less than 0.05 (ie less than 5% chance the result is non random). Bonferroni correction for multiple variables was applied in 3.3.9.3.5.
All data was analysed by DB and outputs produced using SPSS version 19 for Windows software.

2.7.2 Chapter 4

DB, using SPSS statistical package for Windows version 19, performed all statistical analysis. Analysis of descriptive clinical variable was presented using median values as the clinical values were skewed. Log transformation of microRNA relative values was undertaken. Mann Whitney U test was used to compare differences between the clinical variables and the groupings. Spearman's correlation coefficient was used to test associations between microRNA copy number or relative expression and clinical/biochemical variables.
Analysis of differentially expressed microRNA was performed by a third party vendor, as referenced in Chapter 4.3.7.4, and statistical method referred therein.

2.7.3 Chapter 5

DB, using SPSS statistical package for Windows version 19, performed all statistical analysis. Data quality was highly variable and distribution curves followed non-normal

distribution. Log transformation was performed on all data to correct for skew to enable parametric testing. A number of analytes remained non-normally distributed after visual inspection of histograms and application of Shapiro-Wilks to confirm (EGF, IL-6, -10, -13, -17, -5, -4, -21, -23, -7, TNFa, IFNg, GM-CSF and MIP1-alpha). Independent t-testing was used with the Bonferroni correction method to allow for multiple comparisons for parametric data or Kruskal Wallis testing with pairwise comparisons for non parametric results.

For cross sectional analysis the null hypothesis was considered that there was no difference between the four study groups.

For longitudinal analysis at baseline, three and six months in the biologic resistant group, the student's paired t test was used. Significance was assumed where the p-value was <0.05.

2.7.4 Chapter 6

DB scored half the returned questionnaires and the other half by Kelsey Watt, 4th year medical student, University of Glasgow as part of a clinical attachment. DB using SPSS statistical package for Windows, version 19, subsequently carried out all analyses. HADS items were scored as outlined in Chapter 1 with a cut-off of greater than or equal to 8 applied as representing 'possible depression or anxiety' used so as to represent a categorical variable. Disability was determined likewise with a value of greater than 2.000 representing 'severe disability'.

Again, cohort descriptive data was analysed using non-parametric tests as the data did not follow normal distribution. The Mann-Whitney U test was used to assess differences between groups. In order to relate variables and seek any relationship/causality, bivariate correlations were made between clinical variables, fatigue and disability with HADS anxiety/depression scores and Spearman's correlation coefficient used. For the Brief COPE questionnaire where each item has two questions, internal consistency coefficients (ICC) were calculated using Cronbach's alpha between dimension items.

Multivariable analysis was performed to assess the independent contributions of selected clinical, patient related outcome variables and illness perception in evaluating mood and fatigue as dependent continuous variables. Only those significant bivariate correlations (where $p<0.05$) were entered into regression models. Testing for co-linearity was performed.

3.1 Chapter 3; Results- Clinical Cohort

3.1.1 Introduction

Within this chapter I will set out the main clinical findings of this study. The primary focus is the main biologic resistant study group upon whom the most detailed data collection was performed. The aim of examining disease characteristics such as patient demographics, disease history and markers of severity in addition to current disease activity will place the molecular findings in context.

Quantification of the burden of medical co-morbidities and patient related outcome measures is captured by the questionnaires administered herein and aims to quantify those dimensions of RA not captured by clinical examination and rarely sought in the time-limited clinic setting.

More limited data was collected from the three control groups; demographics, disease activity and immunology were available for comparison. A discussion of the clinical and biochemical markers of inflammation will be performed.

Finally, discussion of the importance of considering the external influences on clinical assessments, addressing co-morbidity and how patient related outcomes might influence subjective response to treatments is undertaken in light of the findings.

3.2 Overall Study Recruitment

The final numbers recruited to all groups in this study are shown.

	Baseline	Three months	Six months
Biologic resistant	50	49	48
DMARD resistant	26	21	24
DMARD good responder	21	X	X
Healthy controls	27	X	X

Table 3-26 Total study recruitment numbers
(X=not applicable as no visit)

In the DMARD sensitive group, 2 patients were later excluded after DAS calculation and two patients from the Healthy controls group (reasons referred to below)

3.3 Biologic Therapy Resistant Group

50 patients were recruited from three sites across Glasgow as described in Chapter 2.

22 patients were recruited from the Glasgow Royal Infirmary
21 from Gartnavel General Hospital
7 from Stobhill Hospital

Baseline visit 50 clinical assessments were completed and 48 sets of questionnaires returned.

Second Study visit 49 completed a second (three month) visit where clinical assessment was carried out and 48 provided blood samples. One patient was unable to have blood successfully drawn.

Third Study Visit 47 clinical assessments were carried out (six months) and 4 patients did not return questionnaires. The same patient from the 2nd study visit was unable to have venepuncture carried out again and thus 46 blood samples were available for analysis. 43 questionnaires were thus available for scoring.

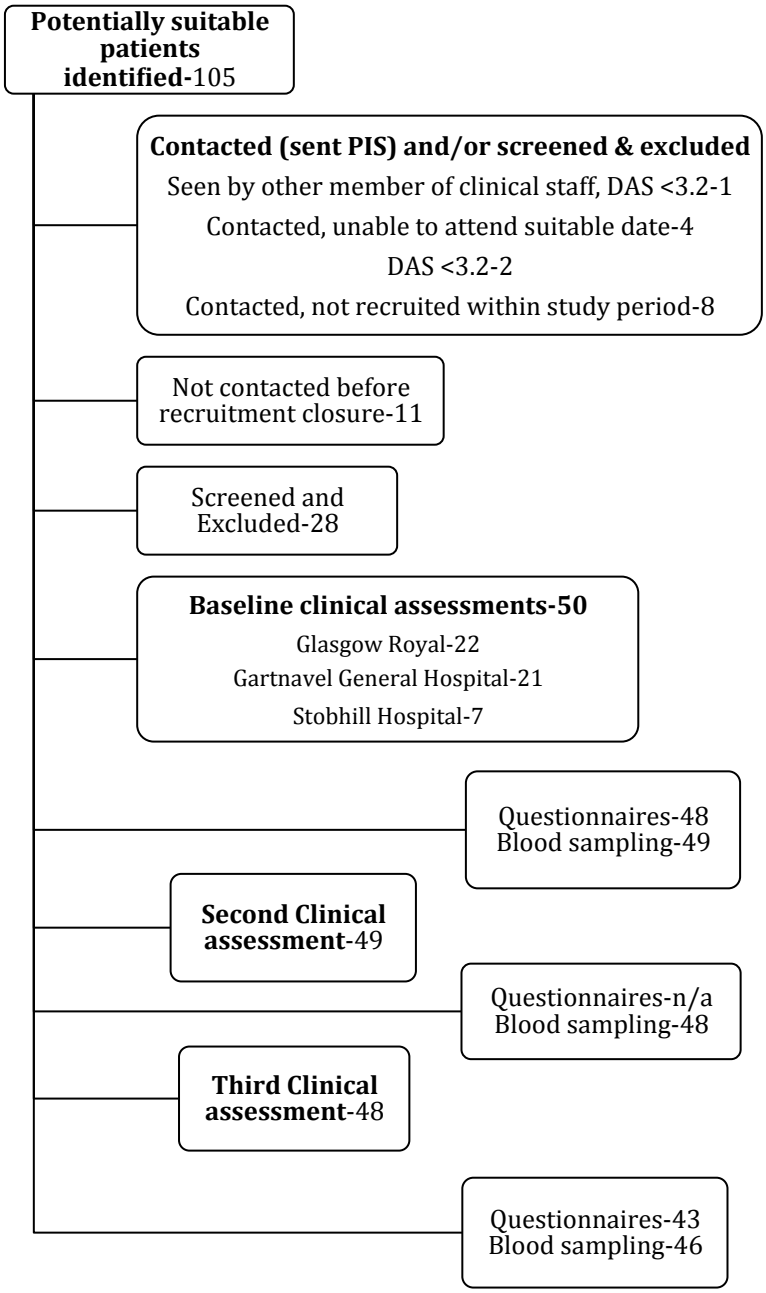

Figure 3-1 Biologic resistant study group recruitment consort diagram

3.3.1 Demographics

3.3.1.1 Overall Summary Findings

The summary findings of the resistant group were as follows:

Gender	42/50 (84%) of patients were female
Age (median)	59 yrs (range 36-78yrs)
Ethnicity	50 patients; White Caucasian
Smoking status	7 patients were current smokers (14%) 10 ex-smokers, for greater than one year 33 had never smoked *Smoking status remained unchanged through the three study visits.*
Education	All patients had completed school level education 3/50 had completed higher education (degree or equivalent)
Employment	7/50 were employed at baseline visit

Table 3-27 Summary descriptive findings of biologic resistant group

3.3.1.2 Social Deprivation

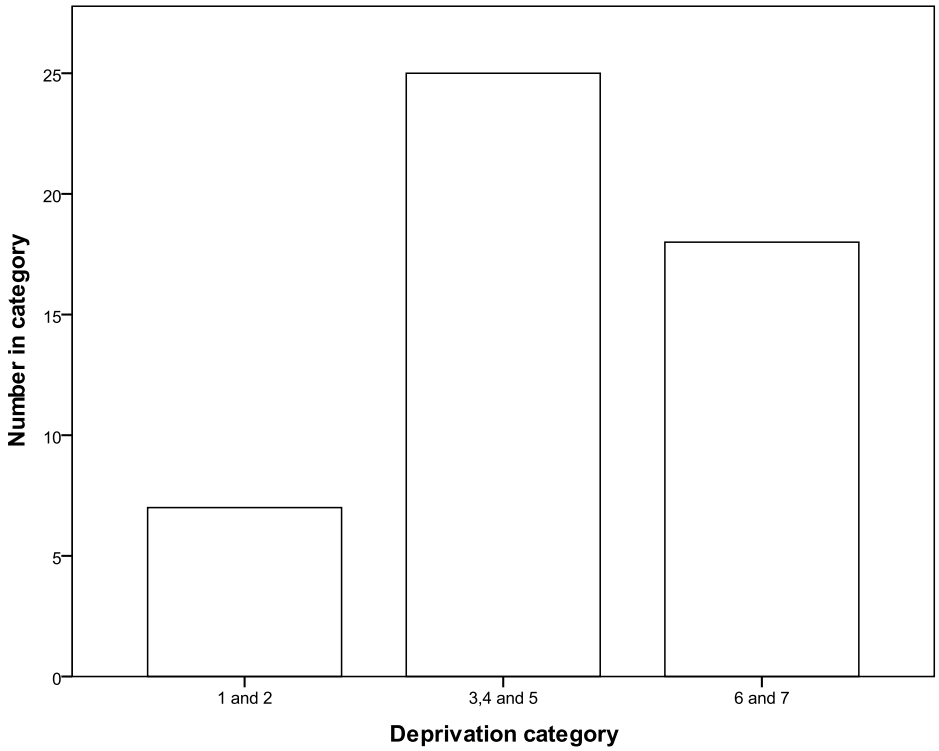

Figure 3-2 Carstairs deprivation categories, biologic resistant RA group at baseline assessment

Deprivation is shown in Figure 3.2. 86% were in category three or above and 36% in the most deprived category. These findings are in keeping with observations of other RA studies drawing patients from this area. The effect of deprivation on disease outcome is not exclusive to rheumatoid arthritis.

3.3.2 Disease history and Phenotype

Disease duration (median)	213 months (range 72-537) or 17.75years
Disease duration (mean)	223 months
Non-joint replacement surgery	0.5 (range 0-8)
Joint replacements	1.5 (range 0-7) 25/50 had not had any joint surgery 60% had at least one joint replaced 11 had neither surgery nor joint replacement
Extra-articular disease manifestations	1 (range 0-3) Most common manifestation was secondary sicca symptoms
'Phenotype' of resistant study group	
Weight (median) n=50	70.5kg (range 47-155)
BMI (mean) n=49	26 (16.25-50.0)
WHR (n=32)	1.04 (0.87-1.21)

Table 3-28 Disease history, extra-articular manifestations and phenotype of biologic resistant study group

Significant disease duration was recorded of over seventeen years. Additionally, 58% reported a family history of rheumatic joint diseases (not limited to RA but also including osteoarthritis).

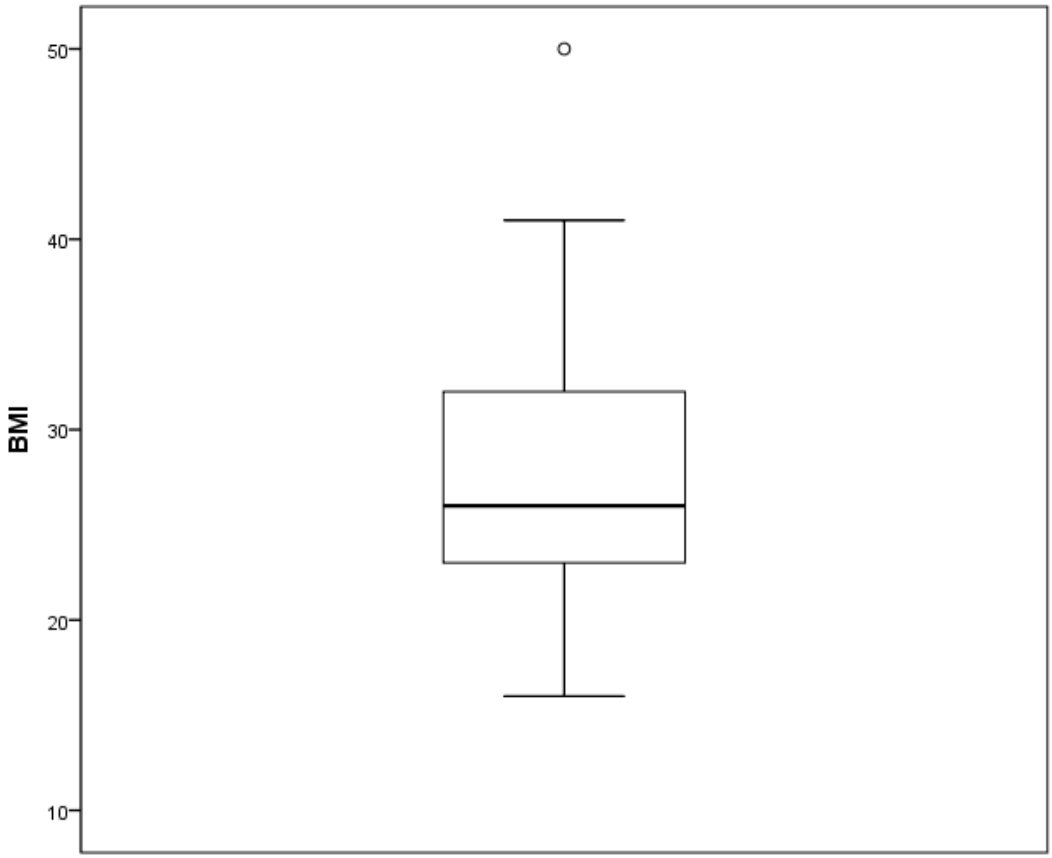

Figure 3-3 Mean body mass index biologic resistant study group
(95% CI)

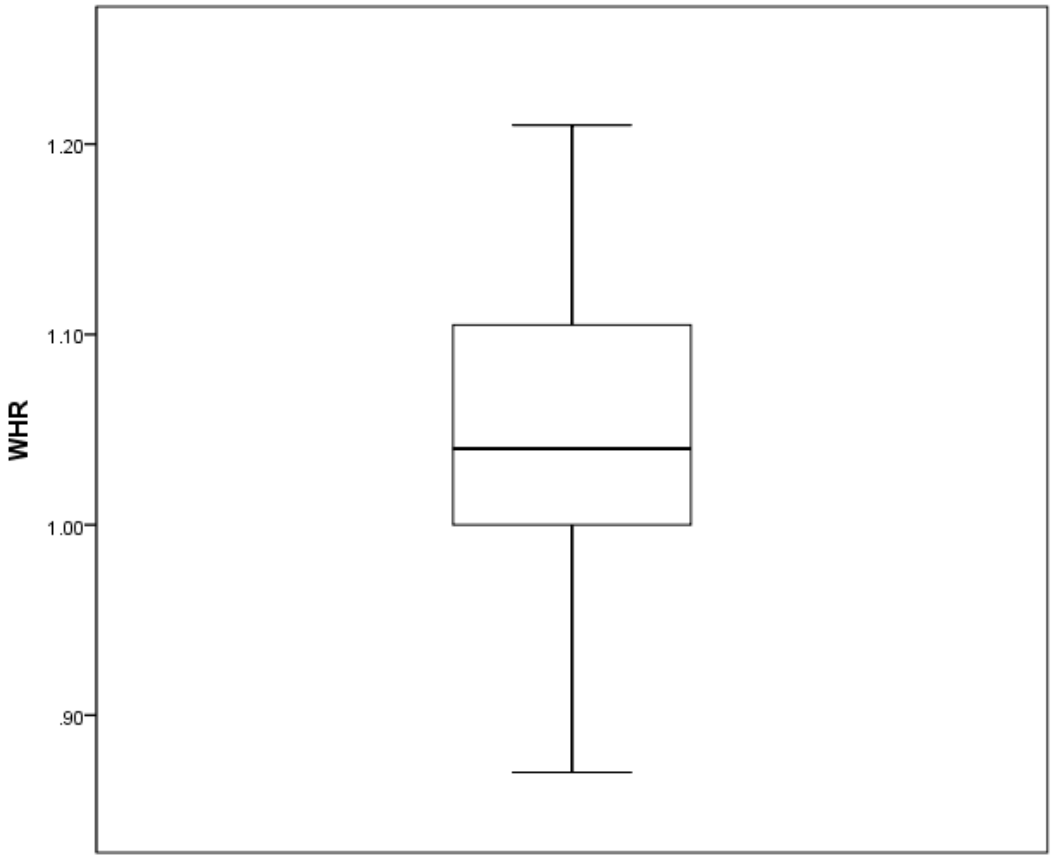

Figure 3-4 Median waist:hip ratio of biologic resistant study group
(95% CI)

3.3.3 Prior therapy

At baseline visit, median number of DMARDs was 6 (range 2-9) and mean 5. At baseline 30/50 were presently treated with MTX but all patients had received MTX at some stage in their disease.

3.3.3.1 Reason for stopping first biologic

As response to first biologic therapy is important, the reasons for discontinuation of first biologic therapy are shown in Table 3-29.

Reason for discontinuation of first biologic therapy	Number	Number later switched a 3rd Biologic therapy
Loss of effect (secondary failure)	15	93% (14/15)
Lack of (primary failure)	13	69% (9/13)
Side effect	18	61% (11/18)
Unknown	2	
'End of trial' drug	2	

Table 3-29 Reasons for discontinuation of first biologic therapy

Results show a mixed distribution of causes in this group. Significant numbers go on to need a third biologic therapy reflects how difficult treatment remains for this cohort. The majority of those that display a loss of effect with their first drug need more treatment changes than the group that experience a side effect with their first treatment in keeping with published literature.

3.3.4 Current therapy

3.3.4.1 Current DMARD

The nature of the condition and potential for drug toxicity requires frequent drug switches and dose adjustments. At the second visit there were five DMARD dose changes, four DMARDs were stopped and one new DMARD therapy started. At the final visit, no new DMARDs had been started but six patients required DMARD dose changes and two were currently suspending therapy.
The final median and mean DMARD was thus 6 (range 3-9) at last study visit.

3.3.4.2 Steroid treatment

In keeping with the observation of a need for treatment changes is the use of, and need for, steroids. This was used as a 'rescue' therapy when given intramuscular (IM) or intra-articular or as a 'bridge' to onset of action of DMARD therapy through the same routes. Daily oral steroid therapy was also used as a DMARD and IV methylprednisolone administered as a pre-treatment with Rituximab administration to avoid adverse drug reaction. By the second study visit, twelve (24%) patients had steroid in this fashion and fifteen (30%) by the third visit.

3.3.4.3 Biologic therapy- baseline, between visits and final

At baseline there was a wide range of current biologic therapies. Twelve patients were not treated with a biologic agent. The most common therapy was Rituximab (twenty-four patients) with varying individual treatment intervals and a mixture of biologic treatment and modes of action.
By the second study visit, eight patients had required a change in biologic therapy and a further two patients by final study visit in keeping with their active disease. Both mean and median final number of biologics was three (range 2-6).

	Current Biologic	1st biologic	2nd biologic	3rd biologic	4th biologic	5th biologic
Adalimumab		22	17	4	0	0
Etanercept	4	19	15	4	1	0
Infliximab		5	4	7	1	0
Rituximab	24	0	14	14	1	1
Abatacept	2	1 (Trial)	0	0	1	2
Tocilizumab	8	0	0	4	3	1
Campath		1 (Trial)	0	0	0	0
Anakinra		1 (Trial)	0	1	0	0
None	12					

Table 3-30 Current biologic therapy at baseline study visit and prior biologic therapies of total group
(trial)=administered in context of a drug trial

For the majority of the cohort, first biologic therapy was targeting TNFa followed by either a switch to an alternative TNFi or switch to Rituximab. By third choice of treatment the most common switch was to B-cell inhibition followed by anti IL-6. This is in keeping with a need to target an alternative mode of action of treatment.

Figure 3-5 shows how biologic treatment changed for each individual and is summarised in Table 3-31.

Study Number	baseline biologic	three months	six months
101	tcz	none (TCZ susp)	tcz
102	tcz	tcz	tcz
103	ritux	ritux	ritux
104	tcz	tcz	tcz
105	ritux	ritux	ritux
106	ritux	ritux	ritux
107	none	none	none
108	ritux	ritux	ritux
109	none	tcz	tcz
110	tcz	tcz	tcz
111	ritux	ritux	ritux
112	none	none	none
113	ritux	ritux	ritux
114	none	tcz	tcz
115	abatacept	abatacept	abatacept
116	tcz	tcz	none (TCZ susp)
117	abatacept	abatacept	abatacept
118	ritux	tcz	tcz
119	ada	ifx	ifx
120	ritux	ritux	ritux
121	tcz	none	none
122	ritux	ritux	ritux
123	none	none	none
124	ritux	tcz	tcz
125	ritux	abatacept	abatacept
126	ritux	ritux	ritux
127	etan	none (etan susp)	etan
128	ritux	ritux	ritux
129	none	none	none
130	none	abatacept	abatacept
131	ritux	ritux	ritux
132	etan	etan	etan
133	tcz	tcz	tcz
134	none	none	none
135	etan	etan	etan
136	ritux	ritux	ritux
137	ritux	tcz	tcz
138	ritux	ritux	ritux
139	ritux	ritux	ritux
140	ritux	ritux	ritux
141	ritux	ritux	ritux
142	ritux	ritux	ritux
143	tcz	tcz	tcz
144	ritux	ritux	ritux
145	etan	etan	etan
146	ritux	ritux	ritux
147	none	none	none
148	none	abatacept	abatacept
149	ritux	ritux	ritux
150	ritux	ritux	ritux

Figure 3-5 Biologic therapy use between study visits

Totals	Baseline	Three months	Six months
None	10	7	7
Adalimumab	1	0	0
Etanercept	4	3+1	3+1
IFX	0	1	1
Rituximab	25	21	21
TCZ	8	11+1	11+1
Abatacept	2	5	5

Table 3-31 Distribution of biologic treatments at study visits
(those shown +1 refer to those treatments prescribed but suspended at the time of venepuncture)

3.3.4.4 Treatment changes during study

In keeping with the presence of disease activity, changes were made to therapy between visits. By study visit two thirteen patients had treatment escalation. By visit three an additional fifteen patients required treatment escalation, three of which had also had changes between baseline and visit three. Overall 50% (25/50) patients had therapy escalation during the study period. Table 3-32 and 3-38 demonstrates that meaningful improvements in DAS28 were achieved whereas the HAQ remains less responsive.

	Baseline				6months			
Patient study No.	**DAS28ESR**	**SDAI**	**CDAI**	**HAQ**	**DAS28ESR**	**SDAI**	**CDAI**	**HAQ**
101	6.53	70.60	120.60	1.875	4.87	29.90	101.30	1.750
106	6.50	91.20	95.80	2.625	6.38	40.60	96.50	2.500
107	6.92	52.60	125.00	3.000	5.77	30.90	108.30	2.500
109	4.12	131.30	51.40	3.000	2.69	10.10	59.20	3.000
111	4.98	28.30	46.70	2.250	3.77	12.70	38.00	2.250
114	5.28	47.70	69.40	2.000	4.49	27.40	75.70	2.125
119	3.62	18.80	20.30	1.875	2.04	3.30	3.20	1.000
122	7.05	62.90	134.00		2.22	11.60	11.60	0.750
123	3.75	19.10	15.40	2.500	4.15		28.70	2.375
124	6.07	111.10	58.10	2.875	4.27	95.00	8.60	
125	6.31	35.50	117.40	2.500	3.37		23.10	2.625
127	5.48	33.60	85.30	2.375	3.88	13.80	50.50	
128	6.01	45.50	72.10	1.875	3.58	11.70	34.60	2.125
129	6.85	99.60	112.10	2.375	5.88	43.20	81.70	2.125
131	3.34	21.80	21.80	0.875	2.35	9.30	15.30	1.125
132	4.28	65.70	25.00	2.000	2.10	4.10	7.40	2.250
134	4.85	37.00	82.90	1.625	4.68	33.20	79.40	2.000
137	5.61	46.30	104.80	2.375				
138	4.69	40.60	97.80		4.02	25.30	76.40	2.250
141	5.59	55.30	53.70	1.750	5.72	36.70	72.30	1.750
144	3.67	15.60	32.20	1.375	2.27	13.20	17.70	1.625
145	5.27	25.50	82.00	1.875	6.15	34.10	125.60	1.875
146	4.95	82.20	56.50	2.250	3.43		32.90	2.375
148	6.09	64.30	83.20	2.000	7.14	105.90	117.30	2.000
149	5.26	60.20	53.90	1.375	3.00	19.90	22.30	
Mean value	**5.27**	**53.82**	**70.70**	**2.13**	**4.06**	**29.10**	**51.58**	**2.03**

Table 3-32 Responsiveness of disease activity and disability (HAQ) in those biologic resistant patients that had treatment escalation between baseline and six-month study visit

In contrast, three and four patients respectively had treatment reductions between visits. No reductions were made for reasons of good disease control, but rather suspensions occurred in view of drug toxicity, inter-current illness or need to avoid drug side effect with respect to steroid dose reduction.

3.3.5 Immunology

3.3.5.1 ACPA status

ACPA status was determined using the methods described in Chapter 2 and available in 48 patients. Using the manufacturer's suggested guidance of CCP titre cut-offs, 38/50 patients were 'positive' and 8 patients CCP negative.

CCP status	Number	%
Negative	8	19
Indeterminate	2	5
Positive	38	88
Not known (not tested)	2	n/a

Table 3-33 CCP antibody status of biologic resistant study group

The 2010 EULAR/ACR guidelines to classify RA (Aletaha et al. 2010) would suggest alternative cut-off values for positive status as <20 as negative, 21-59 as weak positive and >60 as strong positive. The group acknowledge the difficulties of non-standardised methods to assay CCP and assume similar predictive weighting of RF and CCP in their scoring algorithm. They also highlight the heterogeneity of RA, with probable disease subtypes, and the use of this distinction for analysis purposes. Their criteria could be applied to longer standing cases of RA although it has not set out to address this. We have not set out to apply these criteria to this group.
Of the 38 CCP positive patients, 6 were current smokers and 8 ex-smokers.

3.3.5.2 Rheumatoid Factor

For analysis purposes the manufacturer's guidance was followed to determine the antibody status and for RF titre. RF status was available for all 50 patients.

RF status	Number	%
Negative	15	30
Equivocal	3	6
Positive	24	48
Strong positive	8	16
Total positive	*32*	*64*

Table 3-34 Rheumatoid factor status of biologic resistant study group

The proportion of seropositive patients was again in keeping with expected values noted in published literature. In this group, seven were current smokers and 7 seven ex-smokers.

3.3.5.3 Combined antibody status

Combined Autoantibody status	Number (n=50)	%
Double negative	10	20
Single positive	17	34
Double Positive	23	46

Table 3-35 Combined autoantibody status of biologic resistant study group

Of the fifteen patients who were RF negative, nine of these were CCP positive (weak positive or positive). The presence of either autoantibody confers a poorer prognosis and, as expected, this group often exhibit either or both. As expected, 40/50 (80%) was positive at significant titres for either or both antibodies. However, these numbers suggest it is not a prerequisite. The double-negative cohort display similar baseline composite measures other than ESR and, by extension, DAS28 (Table 3-36). This suggests the influence of autoantibody status at this stage of disease is less significant.

Clinical Parameter (Median value)	Double Antibody negative n=10	Either Antibody positive n=40	Significance (Mann-Whitney U test)
DAS28	3.97	5.70	**P=0.017***
ESR (mm/hr)	6	32	**P=0.002****
CRP (mg/dl)	5.8	14	P=0.078
TJC	8	8	P=0.566
SJC	10	10	P=0.294
Disease duration (months)	184.5	240	P=0.126
Age (yrs)	62	56.5	P=0.698
Number of joint replacements	2	1	P=0.623
Number of biologic therapies	3	3	P=0.178
HAQ	1.94	2.25	P=0.136

Table 3-36 Influence of autoantibody status and selected clinical variables

3.3.5.4 ANA

36% (18/50) of patients had a positive ANA titre of levels of 1/160 or higher. This observation is in keeping with expected values observed in RA populations.

3.3.6 Disease Activity

Disease activity was assessed using the composite scoring methods referred to in Chapter 1.

		DAS-28 Baseline	DAS-28 Three month	DAS-28 Six month
N	Valid	50	46	47
	Missing	0	4	3
Mean		5.31	4.54	4.17
Median		5.36	4.36	4.09
Minimum		3.12	2.60	1.40
Maximum		7.15	7.86	7.30

Table 3-37 DAS28 ESR score of biologic resistant study group at all study visits

Using both composite disease activity measures and examining the component variables, a reduction in disease activity was seen. Although ESR and CRP numerically fell these did not achieve statistically significant differences.

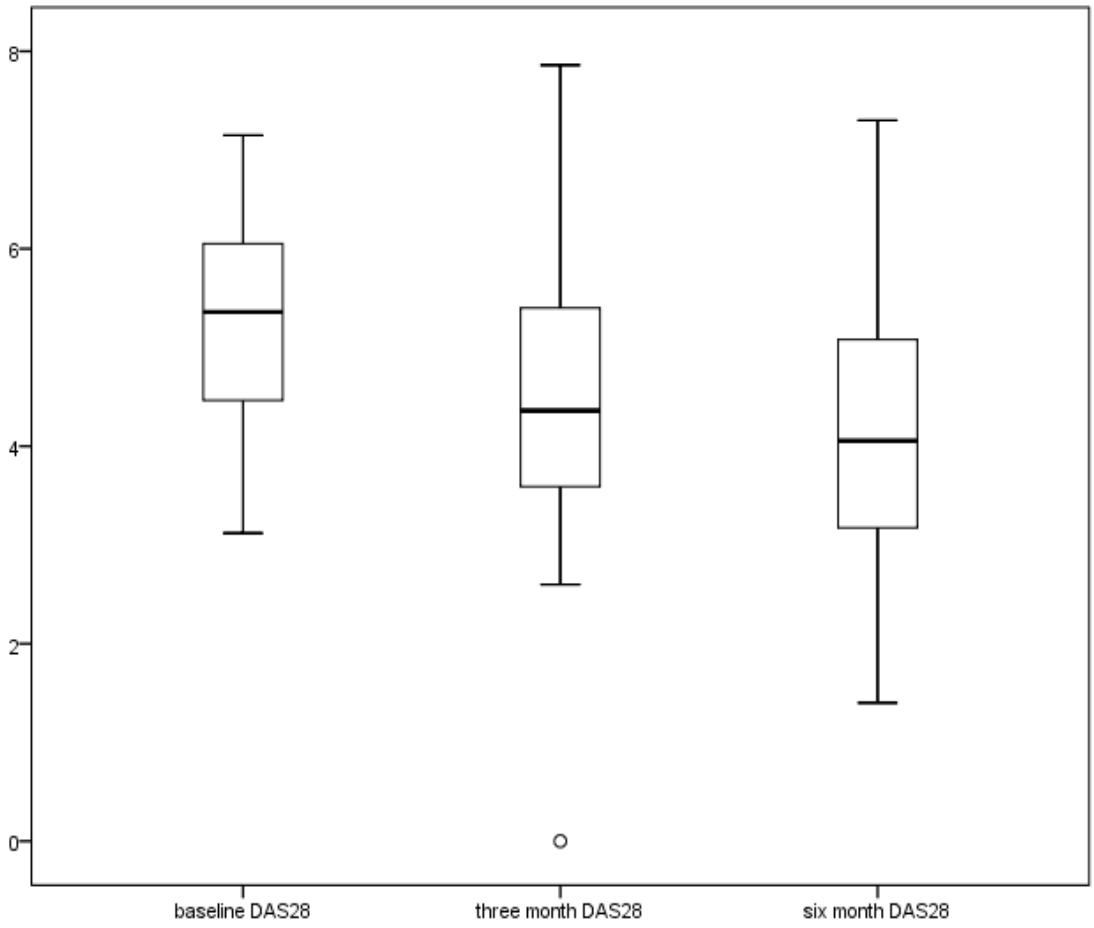

Figure 3-6 'box and whisker' plot of DAS28 ESR biologic resistant study group between study visits

Where DAS-28 ESR was not available, a DAS-CRP was calculated. A reduction in disease activity was apparent between visits in keeping with therapy changes described. In order to confirm this change, this information was combined with laboratory measures and other composite disease activity measures to confirm.

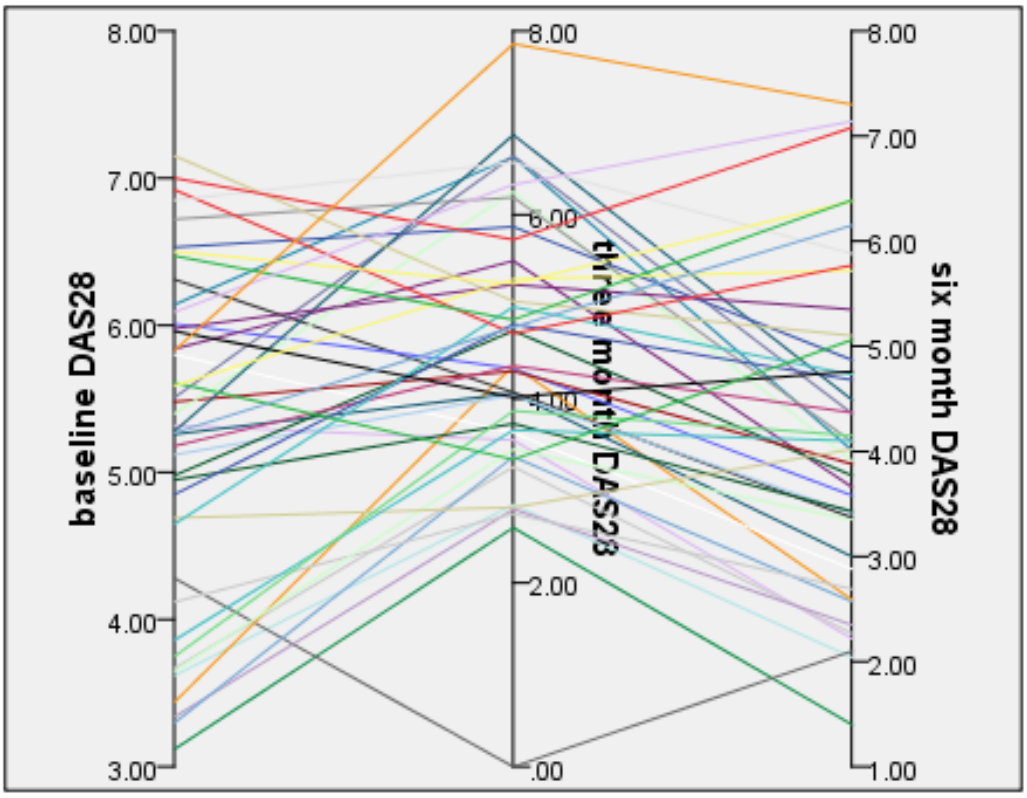

Figure 3-7 Representation of DAS28 ESR of biologic resistant study group change by patient between visits

Study Visit		DAS28 ESR	SDAI	CDAI	ESR (mm/hr)	CRP (mg/l)
Baseline visit n=50	Median	5.36	45.35	70.75	24	11
	Max	7.15	131.30	134.0	95	116
	Min	3.12	15.10	15.40	2	0.10
Three month n=46	Median	4.36	24.50	51.30	21	9.1 (n=46)
	Max	7.86	180.90	126.40	86	132
	Min	2.60	8.40	8.70	4	0.2
Six months n=47	Median	4.09	25.40	50.50	14	4.25 (n=39)
	Max	7.30	114.0	125.60	122	90
	Min	1.40	3.30	3.20	2	0.3
WSRT	Zero-three	P=<0.0001**	P=<0.0001**	P=0.005**	P=0.083	P=0.136
	Zero-six	P=<0.0001**	P=<0.0001**	P=0.004**	P=0.019*	P=0.053
	Three-six	P=0.077	P=0.035*	P=0.475	P=0.315	P=0.048*

Table 3-38 Combined assessments of disease activity at three study visits
(WSRT =Wilcoxon signed rank test)

3.3.6.1 Component analysis of DAS28

In order to examine trends in the components of the DAS28 score and to illustrate the burden of and severity of disease numerically, a breakdown of the variables collected was examined. Statistical improvements in clinical variables are shown other than pain (p=0.330 from baseline to six month assessments).

	Baseline Median (range)	Three month Median (range)	Six month Median (range)	Statistical difference (Wilcoxon matched pair signed rank)	
EMS (mins) N=49/48/46	60 (0-300)	60 (0-270)	40 (0-360)	Zero-three Zero-six Three-six	P=0.420 P=0.077 P=0.467
SJC N=50/50/47	10 (3-20)	5 (0-15)	4 (0-18)	Zero-three Zero-six Three-six	P<0.0001** P<0.0001** P=0.005**
TJC N=50/50/47	8 (0-24)	6.5 (0-20)	3 (0-19)	Zero-three Zero-six Three-six	P=0.011* P=<0.0001** P=0.055
Pain N=50/50/47	44.5 (3-91)	32 (0-85)	32 (2-98)	Zero-three Zero-six Three-six	P=0.136 P=0.330 P=0.650
Patient Global N=50/50/47	53 (4-100)	35.5 (0-94)	39 (2-96)	Zero-three Zero-six Three-six	P=0.028* P=0.195 P=0.453
Physician Global N=50/50/47	50.5 (12-85)	25 (0-77)	16.5 (0-76)	Zero-three Zero-six Three-six	P=<0.0001** P=<0.0001** P=0.181

Table 3-39 DAS28 score component analysis between study visits
(Whole group, n=50)

3.3.7 Patient Related Outcome Measures

3.3.7.1 EQ5D

Complete data for questionnaire and VAS was available for 46 patients at baseline and 43 at six months. The descriptive data is presented.

3.3.7.1.1 Health State

The subjective 'presence or not' of problems (answered either category 2 or 3) is shown in Table 3-40. Patients identified the domains of mobility, self care, usual activities and pain as being affected by their arthritis in 98%, 85%, 96% and 98% of instances respectively. Those describing problems in the anxiety/depression category were lower at 54%. Similar findings were observed at the third visit.

Study Visit		Mobility	Self Care	Usual Activities	Pain	Anxiety/Depression
Baseline n=46	Problems	98%	85%	96%	98%	54%
	No problems	2%	16%	4%	2%	46%
Six-month n=43	Problems	95%	88%	95%	93%	42%
	No problems	5%	12%	5%	7%	58%

Table 3-40 EQ-5D questionnaire results of biologic resistant group (% experiencing problems)

3.3.7.1.2 Pain VAS (EQ5D)

The VAS median was 45.5 (IQR 24) at baseline and 60 (IQR 30) at the third study visit representing a reduction in pain.

		VAS Baseline	VAS Six month
N	Valid	46	43
	Missing	4	7
Mean		47.13	55.95 (p=0.030)
Median		45.50	60.00
Minimum		20	5
Maximum		95	97

Table 3-41 EQ-5D pain VAS score at baseline and six months in biologic resistant group

(higher value represents less pain, paired t-test used)

A paired t-test was applied (p=0.030). The whole group median pain scores (collected with the DAS28 score) at visit one of 32-44mm and three of 41-46mm are consistent with the EQ5D evaluation.

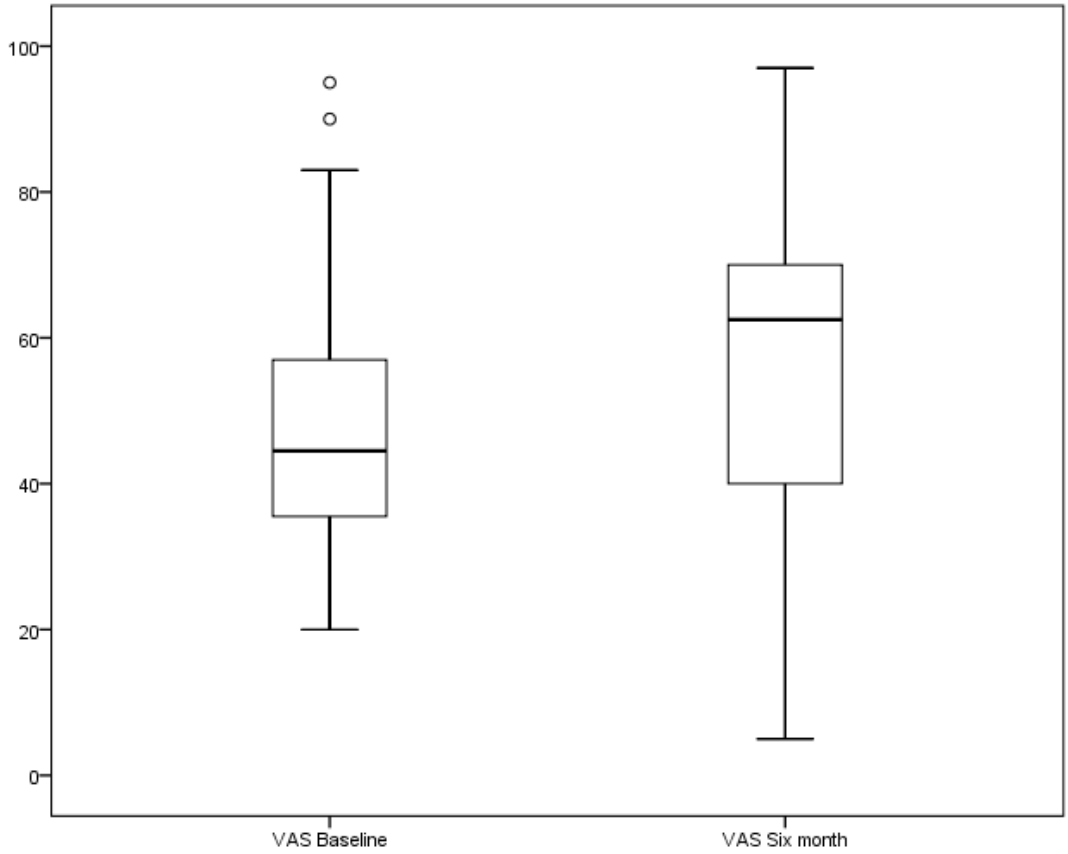

Figure 3-8 'Box and whisker' plots of EQ-5D VAS pain score baseline and six months

3.3.7.2 Physical function (HAQ-DI)

The HAQ-DI values were high in this group in keeping with significant disability. Median HAQ-DI at baseline was 2.125 (range 0.875-3.000) and at unchanged at six months.

		HAQ baseline	HAQ 6 months
N	Valid	45	42
	Missing	5	8
Mean		2.12	2.015
Median		2.125	2.125
Minimum		0.88	0.50
Maximum		3.00	3.00

Table 3-42 Disability (HAQ) at baseline and six months

3.3.7.2.1 HAQ-DI responsiveness

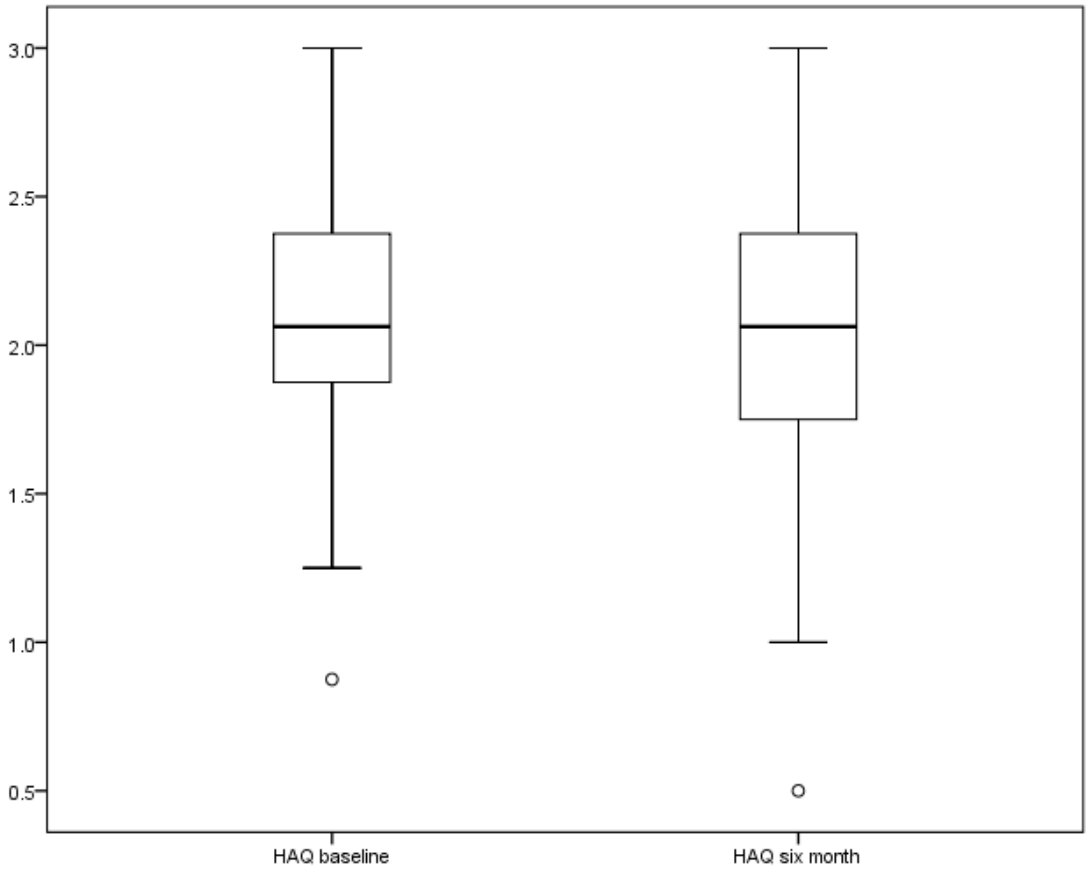

Figure 3-9 'Box and whisker' plots of HAQ at baseline and six months

Over the short follow up period, relatively little change in disability was observed.

3.3.7.2.2 HAQ-DI influences

In order to determine those factors influencing disability, the HAQ scores were analysed with a variety of clinical variables as shown in 3-43.

Variable	HAQ baseline	HAQ six months
Early morning stiffness	**0.012***	**0.001****
SJC	0.190	0.406
TJC	0.683	0.318
Patient Global	**0.001****	**0.006****
Pain	**0.037***	**0.011***
ESR	0.140	0.410
CRP	0.064	0.988
Physical Component Score (SF-36)	**0.006****	**0.006****
DAS28 ESR	**0.028***	0.060

Table 3-43 Bivariate analysis of disability (HAQ) and clinical and biochemical variables
(Spearman's correlation coefficient, where $p<0.05^{\star}$ $p<0.01^{\star\star}$)

As shown, the most important influences are early morning stiffness, patient global assessment of disease and pain with some correlation with disease activity at baseline only. The correlation with patient global estimate of disease activity and physical component score reflects consistent responses, rather than direct influences.
There was a lack of association with tender joint count and with laboratory markers of inflammation and the DAS28 score.

3.3.7.3 Quality of life (SF36 v2)

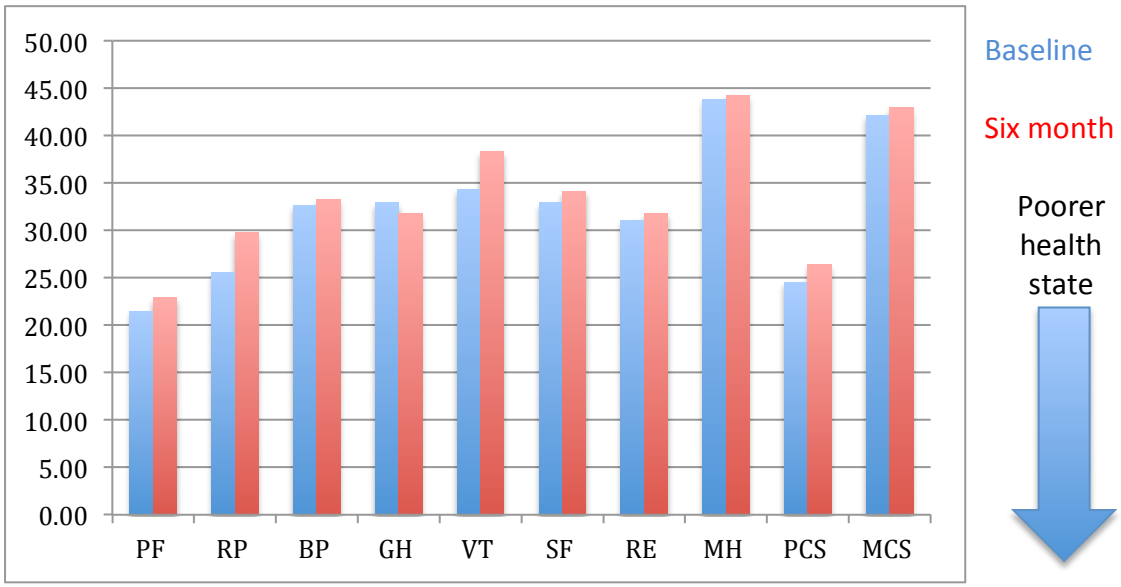

Figure 3-10 Baseline and six-month SF36 questionnaire domain and summary scores

Responses were available for forty-six patients at baseline and forty-three at six-month visit. Patients scored substantially worse on physical measures. Mean PCS score was 26.4 (SD 7.97) and mean MCS 42.94 (SD 14.99). The physical functioning mean score of 21.44 and role physical of 25.59 are most notably low. General health and vitality scores are substantially below the population norm of 50. Responses are broadly consistent between visits.

3.3.7.3.1 SF-36 influences

In view of the strong influence of mood and other assessments (further discussed in 3.3.6.3), correlation between the components of the SF-36 and responses to the HADS questionnaire were examined. Significant correlations were seen in some individual physical domains although correlation with the summary physical score was not demonstrated. Social functioning and emotional role showed strong statistical correlation with anxiety. Mental summary scores correlated with prevailing mood again suggesting consistency of response. Indeed, 55% merit screening for depression and 48% in the six-month report as compared with an 18% population norm (analysis tool provided by SF36v2 software designer, Quality Metrics®)

SF-36 component	HADS-D (R, p value)	HADS-A (R, p value)
Physical Functioning	-0.314 (0.036)	-0.280 (0.063)
Physical Role limitations	-0.589 (<0.0001)	-0.369 (0.013)
Bodily Pain	-0.384 (0.008)	-0.440 (0.002)
Global Health	-0.383 (0.009)	-0.312 (0.035)
Vitality	-0.273 (0.067)	-0.134 (0.375)
Social Functioning	-0.451 (0.002)	-0.447 (0.002)
Role Emotional	-0.623 (<0.0001)	-0.574 (<0.0001)
Physical CS	-0.051 (0.737)	0.090 (0.555)
Mental CS	-0.688 (<0.0001)	-0.718 (<0.0001)

Table 3-44 Bivariate correlations between SF-36 components and HADS-D/HADS-A scores
(Spearman's correlation coefficients (p value)) CS=summary component score

PCS values are considerably lower than population averages in this group. There is a strong correlation between the HAQ-DI and PCS of at both time points (shown above). This again implies consistency between the questionnaire responses and construct.

3.3.7.4 Fatigue (FACIT-F)

		FACIT-F baseline	FACIT-F 6 month
N	Valid	47	44
	Missing	3	6
Mean		**21.5**	**24.3**
Median		21.0	24.0
Std. Deviation		11.755	12.142
Minimum		0	3
Maximum		50	52
Percentiles	25	13.50	13.00
	50	21.00	24.00
	75	28.00	33.00

Table 3-45 FACIT fatigue results at baseline and six-month study visits

The mean FACIT-F score was 21.5 at baseline and 24.3 at visit three. This finding of significant fatigue is in keeping with the poor SF-36 vitality domain (VT) scoring shown above.

3.3.7.4.1 Fatigue and clinical variables

In order to examine the factors that may relate be important in fatigue, bivariate correlation with a number of individual clinical variables was performed across the whole group.

Variable (At baseline or 6 months)	FACIT-F baseline R value (p value)	FACIT-F six months R value (p value)
TJC	**-0.321 (0.028)**	**-0.392 (0.009)**
SJC	**-0.405 (0.005)**	**-0.321 (0.036)**
EMS	**-0.351 (0.017)**	**-0.461 (0.002)**
Pain	**-0.533 (<0.0001)**	**-0.496 (0.001)**
Patient Global	**-0.543 (<0.0001)**	**-0.619 (<0.0001)**
ESR	0.003 (0.985)	-0.277 (0.072)
CRP	0.034 (0.827	-0.142 (0.409)
DAS28	**-0.560 (0.006)**	**-0.554 (<0.0001)**
SDAI	-0.198 (0.183)	**-0.493 (0.002)**
CDAI	**-0.560 (<0.0001)**	**-0.518 (<0.0001)**
HAQ	**-0.311 (0.037)**	**-0.347 (0.024)**

Table 3-46 Correlation between fatigue and clinical, biochemical variables and composite disease activity scores

(Spearman's correlation coefficient (p value))

Clinical variables and fatigue are strongly correlated whereas inflammatory markers are not. Table 3-47 shows the strong correlation between mood and fatigue.

FACIT-F baseline	HADS-A baseline	HADS-D baseline
	-0.435 (0.002)	**-0.513 (<0.0001)**
FACIT-F six month	HADS-A Six month	HADS-D Six month
	-0.561 (<0.0001)	**-0.353 (<0.0001)**

Table 3-47 Bivariate correlation between fatigue and mood
(Spearman's correlation coefficient (p value, significance assumed at p<0.05 level))

There were strong correlations with a number of variables including disability, pain, TJC, patient global and composite disease activity. Fatigue does not appear to be related to readily available biochemical markers of inflammation in this cohort.

These correlations are illustrated in Figures 3-12 to 3-14.

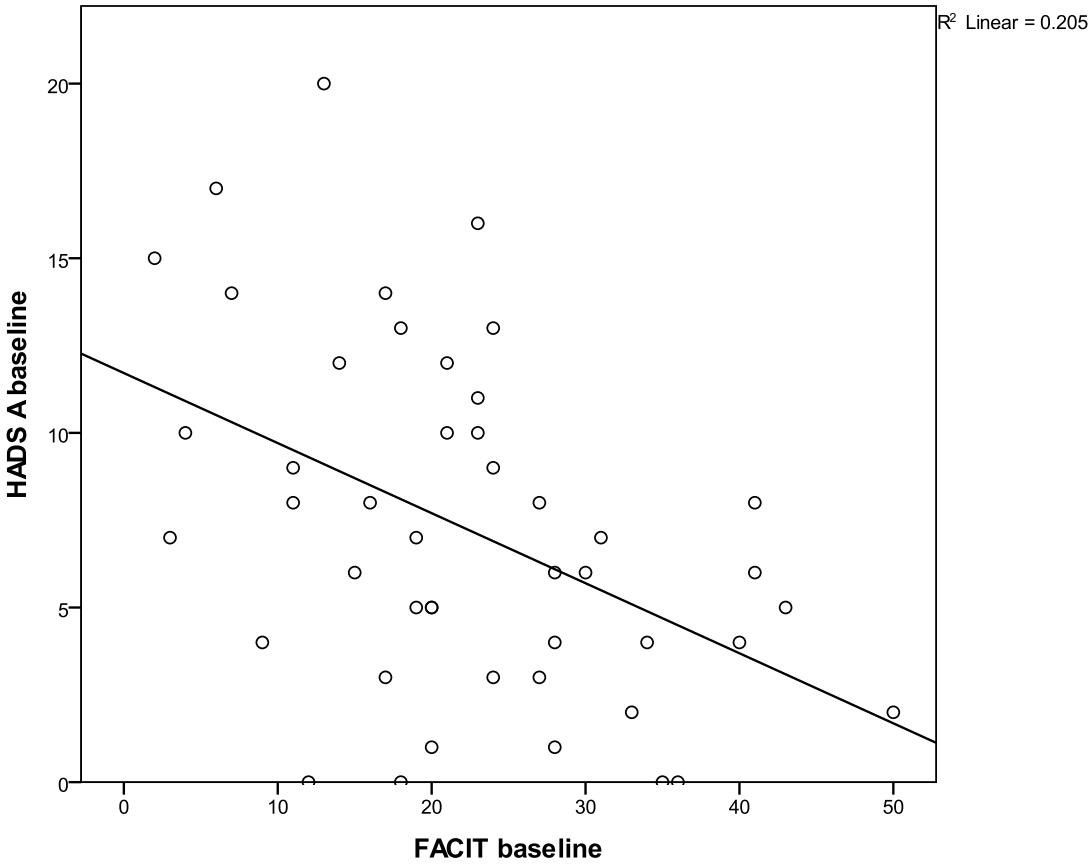

Figure 3-9 Scatterplot of anxiety and fatigue in biologic resistant study group at baseline study visit

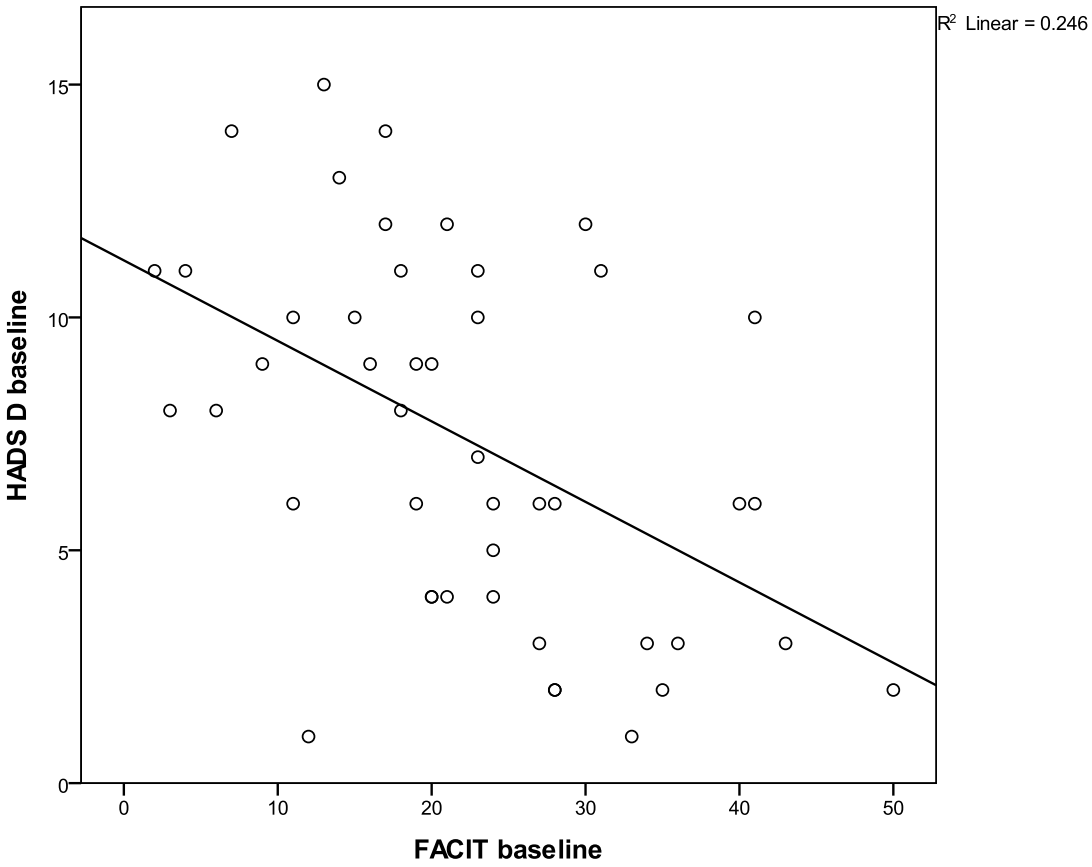

Figure 3-10 Scatterplot of depression and fatigue in biologic resistant study group at baseline study visit

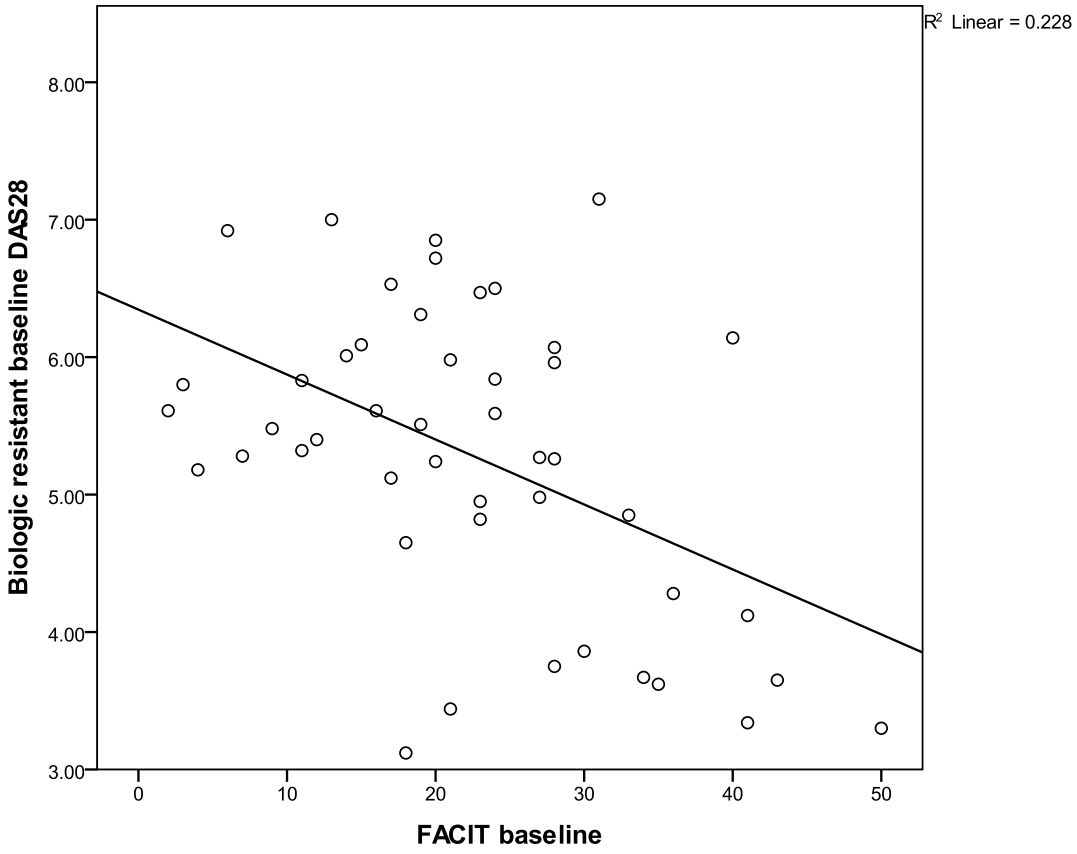

Figure 3-11 Scatterplot of disease activity and fatigue in biologic resistant study group at baseline study visit

3.3.7.4.2 Severity of fatigue

In order to further quantify this observation, and in view of the inherent limitations of the DAS28, fatigue was further stratified by severity and then correlation with clinical factors, disability and mood sought.

Fatigue level (Baseline FACIT-F score)	Not fatigued (≥30) n=11	Mild-moderate (15-29) n=26	Severe Fatigue (≤15) n=10	Significance (Kruskal-Wallis test)
Clinical variable				
SJC	9	11	12	**0.021***
TJC	8	8	8	0.220
Pain (mm)	19	44.5	59	**0.004****
Patient Global	18	53	60.5	**0.002****
DAS28 ESR	3.86	5.60	5.55	**0.016***
Function and QoL (median)				
HAQ	1.88	2.25	2.38	0.050*
SF-36 vitality	45.85	30.24	25.55	**<0.0001****
SF-36 physical functioning	27.57	21.26	17.05	**0.047***
Mood (median value)				
HADS-D score	3	7	9.5	0.086
HADS-A score	4	7	9.5	0.17
Inflammatory Markers (median)				
ESR (mm/hr)	17	30	23.5	0.563
CRP (mg/dl)	7.8	16.5	7.5	0.256
Other variables				
Haemoglobin (g/dl)	125	124	118	0.268
Age (years) (Mean +SD)	61.6 (7.2)	60.8 (10.5)	52.5 (9.3)	0.077
Disease Duration (Mean +SD)	247 (104)	238 (116)	184 (59)	0.284

Table 3-48 Selected clinical factors associated with severity of fatigue at baseline in biologic resistant RA

Correlation was observed between severity of fatigue and the DAS-28 ESR (and it's components) and with disability. There was no correlation with Haemoglobin values, inflammatory markers or age. Although not achieving statistical significance, age in the more fatigued group was numerically younger and disease duration shorter. Recent thyroid function was available and normal for nineteen patients within the last fourteen months. One patient had known treated hypothyroidism.

3.3.7.4.3 Variables associated with fatigue

In order to explore those factors that may influence fatigue and confirm those observations above, exploratory bivariate correlations at baseline visit based on Table above and additional factors was constructed.

Variable correlated with fatigue (FACIT-F baseline) n=47	Variable	Correlation coefficient	p-value
Clinical	**TJC**	**-0.321***	0.028
	SJC	**-0.405****	0.005
	Pain	**-0.533****	<0.0001
	EMS	**-0.351***	0.017
	Patient Global	**-0.543****	<0.0001
	Age	0.257	0.081
	Disease duration	0.234	0.113
Disease Activity	**DAS-28 ESR**	**-0.398****	0.006
Biochemical	ESR	0.003	0.985
	CRP	0.034	0.827
	Haemoglobin	0.105	0.481
PROMs	**HAQ**	**-0.311***	0.037
	HADS-A	**-0.435****	0.002
	HADS-D	**-0.513****	<0.0001

Table 3-49 Individual bivariate correlations between clinical, disease, patient outcome measures and fatigue (FACIT-F) at baseline
(Spearman's correlation coefficient where *p<0.05, **p<0.01)

3.3.7.5 General questionnaire conduct comments

The high number of questionnaires returned at baseline reflects an overall willingness to participate in studies furthering RA research. The reduction in number returned by visit three may reflect 'questionnaire fatigue' but could reflect a perceived lack of personal benefit in doing so (unlike in therapeutic drug trials). Therefore a degree of returner bias cannot be excluded in the group at visit three completing questionnaires.

3.3.7.6 Discussion

The PROMs described above not only quantify the degree of daily difficulties this cohort with severe disease experience but also the complex interaction between physical, biochemical and psychological factors involved that constitute severe disease.

3.3.7.6.1 Disease Activity

As expected, even achieving moderate disease activity levels is difficult in this group with longstanding disease. The treating physician made changes to therapy in an

unstructured fashion in half of the study group. Meaningful improvements in inflammatory disease were observed.

Analysing the component values of the DAS provides insight into those elements that remain responsive to treatment changes. Early morning stiffness improved (reflective of inflammation) and improvements observed in both tender joint counts, swollen joint counts and inflammatory markers are in keeping with reduced inflammation. Furthermore the reduction in disease activity is apparent and confirmed by the examining physician (also suggesting consistency of examination between visits).

3.3.7.6.2 Quality of Life and Disability

Physical function may be considered by examining the HAQ, PCS of the SF36 and mobility category of EQ5D. Quality of life may be assessed by the SF36. Assessing and quantifying the burden of health related quality of life outcomes (HRQoL) measures are important not only in assessing treatment efficacy but also for healthcare budget planners.

In view of the lack of a comparison control group to establish differences, I have examined selected published results of other authors to put results in context. The majority of larger drug trial studies report *differences* in SF-36 domains between two time points after drug administration. As such raw values are not available.

SF-36 domain	**This study (baseline)**	Rigby 2011	Lillegraven 2007	Lempp 2011	Salaffi 2009	Strand 2011	Kvien 1998
Disease duration	**18.6yrs**	**0.92yrs**	**<3yrs**	**<2years**	**6.1yrs**	**6.2yrs**	**12.9yrs**
PF	21.44	27.72	52.5	33.95	41.8	32.9	47.3
RP	25.59	30.04	32.4	16.11	29.8	14.2	27.0
BP	32.63	30.98	43.9	33.98	30.1	30.8	41.0
GH	32.96	37.10	47.5	45.41	44.0	34.3	42.0
MH	43.76	38.64	70.2	60.53	50.3	38.1	68.1
RE	31.00	32.63	56.9	42.33	38.2	49.3	52.0
SF	32.9	29.34	69.4	51.32	46.9	31.4	63.7
VT	34.35	37.02	42.0	32.93	41.9	52.9	39.4
PCS	24.55	30.9		31.42	32.5	31.1	
MCS	42.17	37.0		42.48	39.5	39.6	

Table 3-50 selected publications where SF-36 data published (mean values)

Table Refs (Rigby et al. 2011; Lillegraven & Kvien 2007; Lempp et al. 2011; Salaffi et al. 2009; Strand et al. 2011; Kvien et al. 1998)

Direct comparisons are not possible yet the heterogeneity of patient reporting is evident. Even in early disease, Rigby et al reported substantial impairment across many health domains after treatment with Rituximab and, in some areas, more marked than this cohort (BP, MH and SF) (Rigby et al. 2011). The inference is that disease duration is

not the sole determinant of impaired QoL. Overall, the MCS score is better in this biologic resistant cohort yet PCS substantially lower compared with cohorts of shorter duration. A process of acceptance and adaptation could be proposed in this group despite significant disability.

When considered together, two important observations can be made. Firstly, longstanding RA-related disability remains relatively refractory to modest improvements in inflammatory disease. This suggests that a considerable component of disability is unresponsive and may be related to existing joint damage and muscle deconditioning. This phenomenon may be a 'ceiling effect' of disability achieved in these patients (Russak et al. 2003; Voshaar et al. 2011). Conversely, further evaluation of a therapy change with these methods may lack sensitivity to demonstrate change. As illustration, six of the eight patients starting a new biologic therapy between first and second study visit to study completion are shown.

Study patient	HAQ baseline	HAQ 6 months
109	3.000	3.000
118	1.625	1.750
119	1.875	1.000
125	2.500	2.625
130	2.125	1.875
148	2.000	2.000

Table 3-51 Disability (HAQ) pre- and post-introduction of new biologic therapy

Results shown in Table 3-51 are mixed but there are individuals who can still achieve meaningful HAQ reductions. Selection of the clinical and treatment factors that determine those with greatest gains to be made would determine most judicious use of treatments.

Secondly, mood is seen to influence quality of life domains but not physical component domains (disability) and this is discussed later. There is a lack of association between the TJC and disability. However pain and disability correlate and this may imply a disconnect between overall bodily pain (causing disability) and joint pain. This may be explained by other causes of pain causing disability such as neuropathic pain or fibromyalgia. At this advanced stage of disease however, inflammatory disease when judged by ESR and CRP, does not correlate with disability.

3.3.7.6.3 Fatigue

In this cohort, highly significant differences in clinical variables were observed when examined by degree of fatigue. Fatigue is not only a symptom of RA but will influence reporting of RA symptoms and influence quality of life. It is one of the more challenging

symptoms to treat and the assumption commonly held is that fatigue relates to disease activity. The observations herein highlight a number of potential implications for daily clinical practice when considering this important patient symptom.

Firstly, levels of fatigue are comparable if not higher than similar RA cohorts (see Chapter 1). Fatigue in RA is also multifactorial:- sleep disorders, prescription medication, pain and medical comorbidity (thyroid dysfunction, renal and cardiac failure) may also influence severity. Fatigue must also be considered in context of social stressors such as employment, young family and availability of support. Future assessment of these variables would compliment those factors assessed above.

Secondly, many clinical variables correlate with fatigue, the strongest of which are depression and disability. Pain also correlated with fatigue and is potentially treatable if the nature of the symptom is carefully considered. In this cohort there is a lack of correlation with laboratory markers of inflammation yet association with composite disease activity scores. This may be explained by the relative influence of clinical variables on the DAS28 score outweighing that of inflammatory markers. The SDAI includes CRP thus introducing a variable that may account for the lack of association at baseline with fatigue.

Fatigue thus reflects in higher DAS28 scores and lower physical and emotional functioning. In addition, fatigue is correlated mood (depression) and disability but direction of causality is not established. A complaint of high fatigue should prompt careful evaluation of inflammatory disease activity, attention to managing pain and evaluation of mood.

3.3.8 Influence of Deprivation and outcomes

Baseline value (Median)	Carstairs Category			
	1 and 2 (n=7)	3, 4 or 5 (n=25)	6 and 7 (n=18)	Kruskal-Wallis test
DAS28	4.98 (2.27)	5.32 (1.58)	5.81 (1.46)	0.102
EMS	60 (80)	60 (101)	90 (90)	0.170
SJC	9 (2)	10 (6)	11 (4)	0.393
TJC	8 (3)	8 (8)	10 (12)	0.316
Global	29 (26)	49 (39)	64 (30)	0.053
Pain	**28 (33)**	**40 (26)**	**60 (31)**	**0.008**
ESR	25 (25)	32 (26)	21 (28)	0.485
CRP	7.8 (15.3)	10.0 (23.2)	19 (52.5)	0.229
FACIT	**28 (14)**	**21 (15)**	**18 (13)**	**0.024**
HAQ	1.625 (0.875)	2.250 (0.563)	2.000 (0.656)	0.090
HADS-A	**4 (4)**	**5 (9)**	**8 (6)**	**0.039**
HADS-D	**3 (4)**	**6 (8)**	**9 (3)**	**0.040**
PCS	27.6 (9.3)	23.4 (8.4)	25.1 (5.9)	0.105
MCS	**56.4 (22.9)**	**42.7 (16.0)**	**36.4 (10.4)**	**0.043**
Weight (kg)	68 (25)	71 (17.3)	80 (27.4)	0.495
BMI	24 (13)	27 (6)	28.5 (11)	0.535
WHR	1.08 (0.22)	1.03 (0.08)	1.04 (0.14)	0.740
Joints replaced (n=)	3 (4)	2 (3)	0.5 (2)	0.182
Joint surgeries (n=)	1 (2)	1 (3)	0 (3)	0.383
Number of previous biologic therapies	2 (2)	3 (1)	3 (1)	0.586
Number of previous DMARDs	**6 (4)**	**6 (4)**	**4 (3)**	**0.015**

Table 3-52 Influence of deprivation and clinical factors
(Median values and IQR between groups. Significance assumed where p<0.05)

Table 3-52 confirms that deprivation may influence a number of assessments and outcomes in keeping with previous observations. Reported pain scores increase with worsening deprivation. Similar findings are suggested with higher anxiety and depression scores and reported fatigue. In contrast, tender/swollen joint counts, and thus function, and biochemical measures of disease activity are not statistically different (although numerically the median DAS28 score was 4.98 in the most affluent group and 5.81 in the most deprived).

Consideration of the patient's background social situation is therefore important when assessing prevalent mood and fatigue.

3.3.9 Co-Morbidity

3.3.9.1 Overall medical comorbidity in Biologic resistant study group

There was substantial and varied medical co-morbidity in this group with all but six patients with one or more medical comorbidity.

Number of Co-Morbid Conditions present	Number of patients	% of whole study group
0	6	12%
1	12	24%
2	13	26%
3	6	12%
4	10	20%
5	3	6%
Overall median number of co-morbidities	2 (range 0-5)	

Table 3-53 Medical comorbidities in biologic resistant group

I have chosen to address the two specific and significant instances of cardiovascular risk and mood disturbance in this group.

3.3.9.2 Vascular Disease and Cardiovascular Risk

3.3.9.2.1 Overall Vascular Risk Factors

The ATP III guideline suggests those 'major' risk factors of most importance may be smoking, hypertension (systolic BP >130mmHg or diastolic BP >85mmHg OR pre-existing diagnosis/on therapy for hypertension), HDL cholesterol (target <1.03mmol/l), premature IHD in family and age (male >45yrs, female >55yrs). These values were used as framework to determine 'risk factors'. Smoking status has been shown in Table 3-27. In this way the presence of multiple risk factors can be seen.

	% of whole group	Numerical representation, cohort n=50
One CV risk factor	16%	8/50
Two CV risk factor	40%	20/50
Three CV risk factor	30%	15/50

Table 3-54 Number of cardiovascular (CV) risk factors at baseline visit in biologic resistant study group

Clinically evident vascular disease was however noted infrequently in this cohort. At baseline visit two patients had confirmed ischaemic heart disease and previous

myocardial events. 52% (26/50) reported a family history of cardiovascular disease at any age.
Two additional patients developed a new diagnosis of left ventricular dysfunction (silent) and one patient a myocardial infarct complicated by ventricular dysfunction, in the six-month follow up period necessitating secondary prevention.

3.3.9.2.2 Lipids

Lipid profile of the group at baseline is shown in table 3-55. Nine patients were treated with a statin at the time of baseline study visit, two further patients started statin therapy by second visit and a twelfth patient by the third visit (having had a myocardial infarct between visits).

	Normal range	Mean value	Range (min-max)
Total Cholesterol (mmol/L)	<5.00	5.10	2.80-8.60
Triglycerides (mmol/L)	<2.30	1.35	0.50-2.84
HDL (mmol/L)	>1.00	1.43	0.79-2.48
Ratio total Cholesterol/HDL	<4.0	3.70	2.10-6.00

Table 3-55 Lipid profile of biologic resistant study group (n=44) Normal range; Greater Glasgow Biochemistry Laboratory

3.3.9.2.3 Blood Pressure

Nineteen patients (38%) had a diagnosis of hypertension made prior to study inclusion and were treated with anti-hypertensives.

Whole group		
SBP (median, mmHg)	129.5	Range 88-173
DBP (median, mmHg)	76.5	Range 43-94
'Hypertensive' (≥140mmHg) (Peters et al. 2010)	39% (18/46)	
BHS 2004 Guidance (Williams et al. 2004)		
Normal	50% (23/46)	
High normal	4.3% (2/46)	
Mild hypertension	2.2% (1/46)	
Moderate hypertension	4.3% (2/46)	
Severe hypertension	0% (0/46)	
Grade 1 ISH	30.4% (14/46)	
Grade 2 ISH	8.7% (4/46)	
SBP target <130mmHg	52% (18/46)	

Table 3-56 Blood pressure findings of biologic resistant group at baseline visit (n=46)

Interpretation of the BHS guideline required *both* systolic and diastolic BP above their target thresholds. However, isolated systolic hypertension was common within the group and using the EULAR guideline as reference over a third of the group was hypertensive. BP was only measured on one occasion but only one patient had modification of their anti-hypertensive regime made during follow up.
Of the thirty patients on a regular NSAID, fourteen have a prior diagnosis of hypertension requiring monitoring. This group had a mean blood pressure of 131/74mmHg.
No correlation between systolic blood pressure and either inflammatory markers (ESR r=-0.066, p=0.667, CRP r=-0.096, p=0.427) or the DAS28 (r=-0.0.89, p=0.694) was observed.

3.3.9.2.4 Cerebrovascular Disease

Two patients had an incidental finding of asymptomatic small vessel infarcts on CT brain and 22% (11/50) reported a family history of stroke.

3.3.9.2.5 Features of the Metabolic Syndrome

Numbers with constituent variables of the metabolic syndrome (MetS) were calculated using combined thresholds from the ATP III, SIGN guidance and WHO guideline. The presence of three or more features is suggested. Neither serum glucose nor evaluation of proteinuria were examined and this scoring incomplete for the WHO and NCEP guidance.

Variable	Mean value (range)	Target Value (source/reference)		% Above target
BMI	28 (16-50)	≤25 (SIGN 2007) >30 (Nishida et al. 2010)		53% (26/49) 59% (29/49)
Waist (cm, male) N=8	65.7 (35-108)	102cm (1, (SIGN 2007), (Nishida et al. 2010)		25% (2/8)
Waist (cm, female) N=24	50.5 (28-116)	88cm (1, (SIGN 2007)		21% (5/24)
Waist:hip ratio n=32	1.04 (0.87-1.21)	≥0.90 cm (M) ≥0.85 cm (F) (Nishida et al. 2010)		M 100% (8/8) F 100% (24/24)
Triglycerides (mmol/l) n=43	1.35 (0.5-2.84)	<1.69 (1) <1.70 (Nishida et al. 2010)		25% (11/44) 25% (11/44)
HDL (mmol/l) n=43	1.4 (0.79-2.48)	Males	>0.9 (Nishida et al. 2010) >1.04 (1)	12.5% (1/8) 25% (2/8)
		Females	>1.0 (Nishida et al. 2010) >1.3 (1)	17% (6/35) 34% (12/35)
BP systolic (mmHg) n=46	134 (88-173)	< 140mmHg (EULAR (Peters et al. 2010))		43% (20/46)

Table 3-57 Combined features of the MetS in the biologic resistant study group

1=(Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2001)

Significant numbers of patients displayed values outwith individual target ranges and three patients met the criteria for MetS before formal glucose or microalbuminuria assessment. Fewer waist measurements were available (mainly for reasons of patient preference) but all those recorded were above desirable levels.

3.3.9.2.6 Rose Angina Questionnaire

Negative		72% (36)
Rose positive	'Possible' angina	8% (4)
	'Atypical chest pain'	18% (9)

Table 3-58 Results of Rose angina question in biologic resistant study group

Of those thirteen with a positive Rose several significant findings were made. None of these patients had a documented history of symptomatic heart disease or an abnormal ECG. Three patients had hypertension (prior diagnosis) and four were smokers. 85% (11/13) had a family history of IHD. Two were obese, four overweight, six in normal BMI, one underweight. HAQ and EQ5D mobility values were not significantly different from whole group.
Significantly, one of those with a positive Rose questionnaire subsequently developed symptomatic peripheral vascular disease (PVD) at 3 month follow up, and one suffered a myocardial infarction complicated by left ventricular systolic dysfunction (LVSD) by six-month visit.

3.3.9.2.7 ECG results

14% (5/37 had an abnormal ECG. Of these four had ischaemic ECG changes, one demonstrated atrial fibrillation (previously known). One patient was known to have heart failure and one a prior myocardial infarct.

3.3.9.2.8 NSAIDs

60% (30/50) were prescribed and taking a NSAID regularly. Of those that were smokers, 50% (4/8) took a regular NSAID. The NSAIDs prescribed were mixed; most commonly used was etodolac 600mg (13/30), diclofenac (5/30), naproxen (3/30) etoricoxib, ibuprofen and indomethacin (2/30 respectively), aceclofenac and celecoxib and nabumetone (1/30 respectively).
Of note, the two patients who developed LVSD by 6 months were both on Diclofenac prior to their new symptoms developing.

3.3.9.2.9 Family history

52% (26/50) had a family history of CV disease. This was a family history occurring at any age.

3.3.9.2.10 Estimate of risk of future cardiovascular events

3.3.9.2.10.1 SCORE risk calculator

50% (20/40) of asymptomatic patients have at least a ≥5% risk of first fatal atherosclerotic event. Their calculated risk score is shown in Table 3-60;

SCORE (10-year risk of fatal CV disease)	Number identified	Comments/Interpretation
≥5% -9%	16	Of these, the number with either BP >140mmHg and/or total Chol >/=4.5= nine (five already on a statin). These **nine** patients would mainly benefit from more aggressive BP treatment
≥10%	4	Number with either BP >140mmHg and/or total Chol >/=4.5= all 4. Therefore **all four** would merit statin and BP lowering therapy. At the time of assessment, one was treated with a statin and three would merit statin therapy. One was treated with an anti-hypertensive and the other three merit treatment.

Table 3-59 SCORE cardiovascular risk estimation of biologic resistant study group (n=40)

3.3.9.2.10.2 Framingham risk score

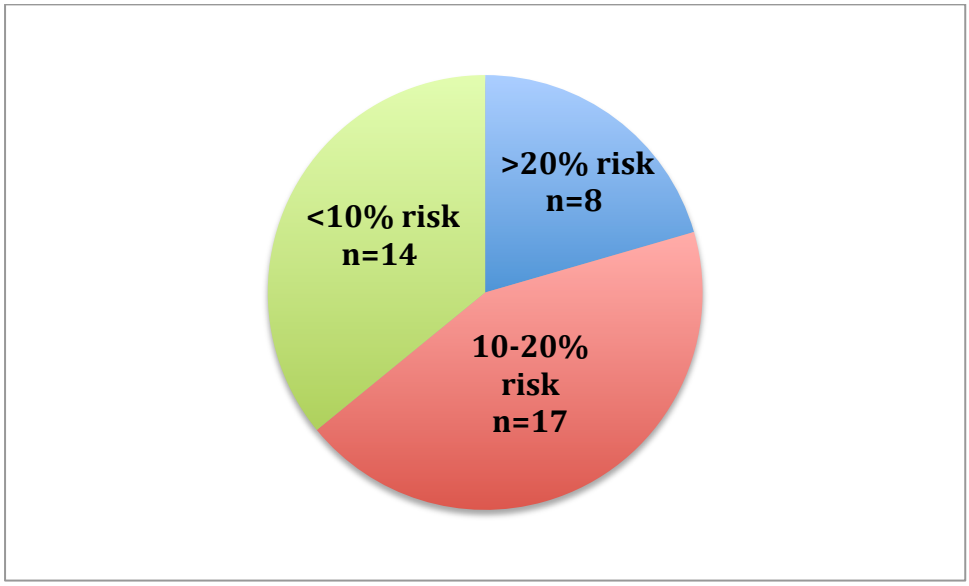

Figure 3-12 Quantification of CV risk as assessed by Framingham risk calculator

3.3.9.2.11 Discussion- Cardiovascular risk factors

The group demonstrate a number of unfavourable CV risk factors yet few instances of clinically evident vascular disease. Even in the short duration of follow up, new instances of vascular events occurred. Significant numbers with CV risk parameters observed outwith suggested target values might explain this. It may be postulated that this population have yet to display much of their CV morbidity.

Targeted lifestyle and drug treatment is necessary for those with increased risk. The SCORE calculator highlights a small number of individuals with multiple risk factors that would merit targeted intensified intervention. Isolated systolic hypertension (ISH) was a common finding. ISH is related to arterial stiffness, a finding found in RA (and may improve with TNFi therapy) (Angel et al. 2012) may explain this observation. The prescription of a regular NSAID requires regular review owing to possible increased CV events (Peters et al. 2010). Withdrawal of NSAID is usually well tolerated (McKellar et al. 2011). The Rose questionnaire is a useful screening tool but may be limited in such populations owing to reduced exercise capacity (although the disability scores would suggest not in this cohort) and possible musculoskeletal chest wall pain.

In reality, calculated risk estimates may remain an underestimate. Inclusion to this study necessitated inflammatory disease activity and, as previously outlined, this may lower the already adverse lipid profile. We have not undertaken a formal estimate of glucose tolerance and taken with the high rate of central obesity in this cohort, glucose intolerance could be anticipated. Finally, the presence of obesity is a contributory CV risk factor that is not accounted for by all CV risk calculators.

Several positive suggestions can be made from these findings. Current CV risk estimators are imperfect; the ideal *future* RA risk calculator would include both an inflammatory biomarker but also one that is expressed as a function of time (eg time spent inflamed and degree of inflammation). Secondly, estimation of CV risk does not include use of NSAIDs (which were used regularly in this cohort) and thus a comprehensive evaluation of all modifiable CV risk is necessary. Ultimately risk modification should be individualised.

3.3.9.3 Mood Disturbance- HADS Questionnaire Results

At baseline, three patients had depression listed as an active diagnosis and two patients were treated with antidepressants. One additional patient commenced an anti depressant prior to study completion. Responses and descriptive statistics of the HADS questionnaire are shown.

		HADS-A baseline	HADS-D baseline	HADS-A 6 month	HADS-D 6 month
N	Valid	46	46	43	43
	Missing	4	4	7	7
Mean		7.2	7.4	7.4	7.1
Median		6.5	7.5	7.0	6.0
Minimum		0	1	0	1
Maximum		20	15	19	17

Table 3-60 Overall HADS questionnaire results biologic resistant group

A score of between 8 and 10 may represent a 'possible' clinical state and a score of greater than or equal to 11 a 'probable' clinical state. These values provide good sensitivity and specificity (Bjelland et al. 2002). When applied to the cohort, significant numbers meet these cut off values.

	HADS-A	HADS-D
n=	43	43
Mean (+SD)	7.3 (5.2)	7.1 (4.0)
Median (IQR)	7 (9)	6 (8)

Table 3-61 Overall HADS anxiety and depression scores at baseline study visit

	Score ≥ 8 Baseline and six months		Score ≥ 11 Baseline and six months	
HADS-D	23 (50%)	18 (42%)	12 (26%)	12 (28%)
HADS-A	20 (43%)	20 (47%)	11 (24%)	10 (23%)
Both	14 (30%)	15 (35%)	8 (17%)	9 (21%)

Table 3-62 Proportion of biologic resistant group with 'possible' and 'probable' mood disturbance at baseline and six months

Between 42-50% of patients have a possible clinical state of anxiety or depression and 10-12% a probable clinical state. Both states would merit further evaluation. Of those scoring ≥11, at both baseline and six months two patients had depression as a diagnosis previously made. One patient, with prior depression, scored >8. One of the twelve patients scoring ≥11 started antidepressant therapy between assessments. HADS anxiety and depression scores were consistent over follow up.

Depression and anxiety are known to co-exist despite treatment strategies differing. Fourteen and fifteen patients respectively scored sufficiently to merit further evaluation and eight and nine respectively display scores satisfying both mood states at baseline and six months respectively.

3.3.9.3.1 Relationship with other patient related measurements- disease duration

In view of the findings of Isik et al, the relationship between disease duration and mood was examined. The authors found those that were anxious had shorter disease duration and correlation between disease duration and anxiety (negative) and depression (positive) (Isik et al. 2006).

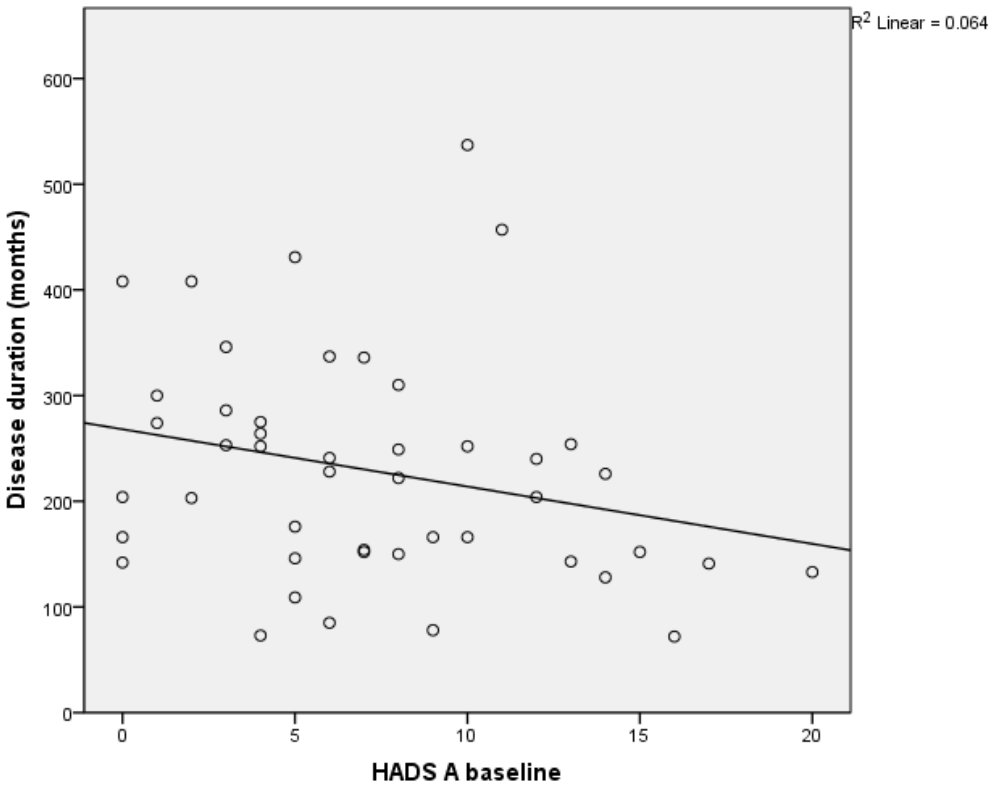

Figure 3-13 Scatterplot of disease duration and HADS-A (anxiety) at baseline

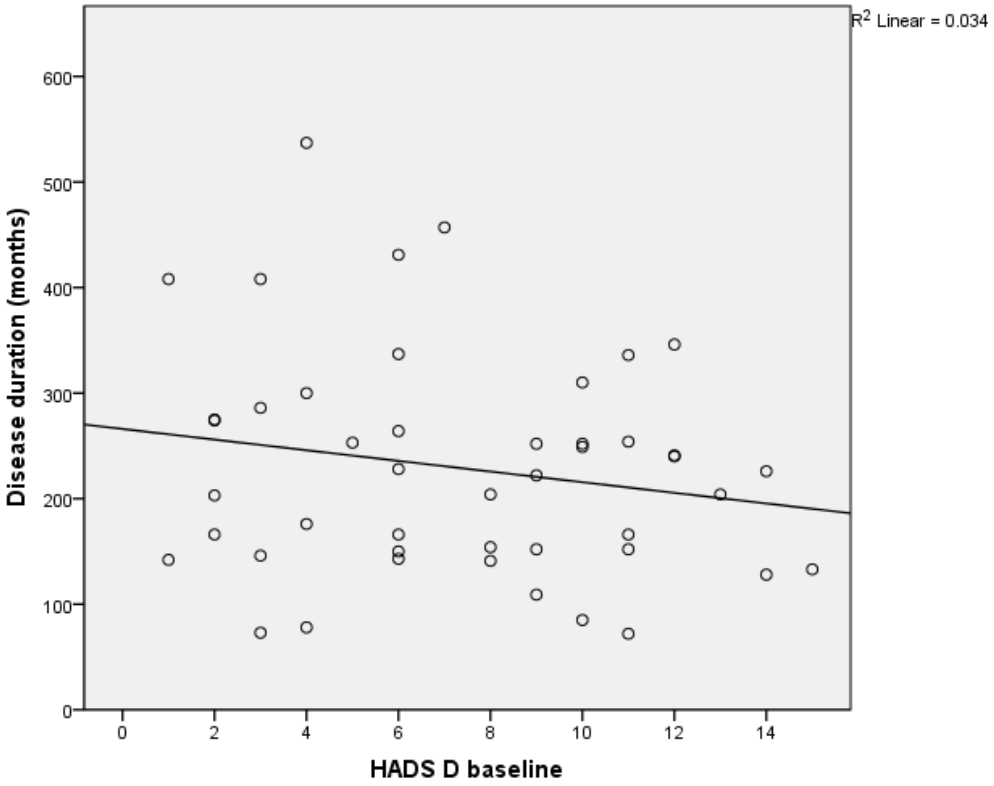

Figure 3-14 Scatterplot of disease duration and HADS-D (depression) at baseline

In this cohort, depression did not correlate with disease duration (HADS-D r=-0.151, p=0.315 Spearman's test) although anxiety did show a negative correlation (HADS-A r=-0.309, p=0.037) at baseline study visit. The plotted values would suggest there might be a weak observation of less depression and anxiety with increasing disease duration.

3.3.9.3.2 Fatigue (FACIT)

The relationship between fatigue and mood suggests moderate correlation. In keeping, higher depression and or anxiety scores correlated with higher fatigue levels.

FACIT-F Baseline	HADS-A	r=-0.435, p=0.002
	HADS-D	r=-0.513, p=<0.0001
FACIT-F six months	HADS-A	r=-0.533, p=<0.0001
	HADS-D	r=-0.485, p=<0.0001

Table 3-63 Correlation between fatigue and mood
(Spearman's correlation coefficient, significance assumed where p<0.05))

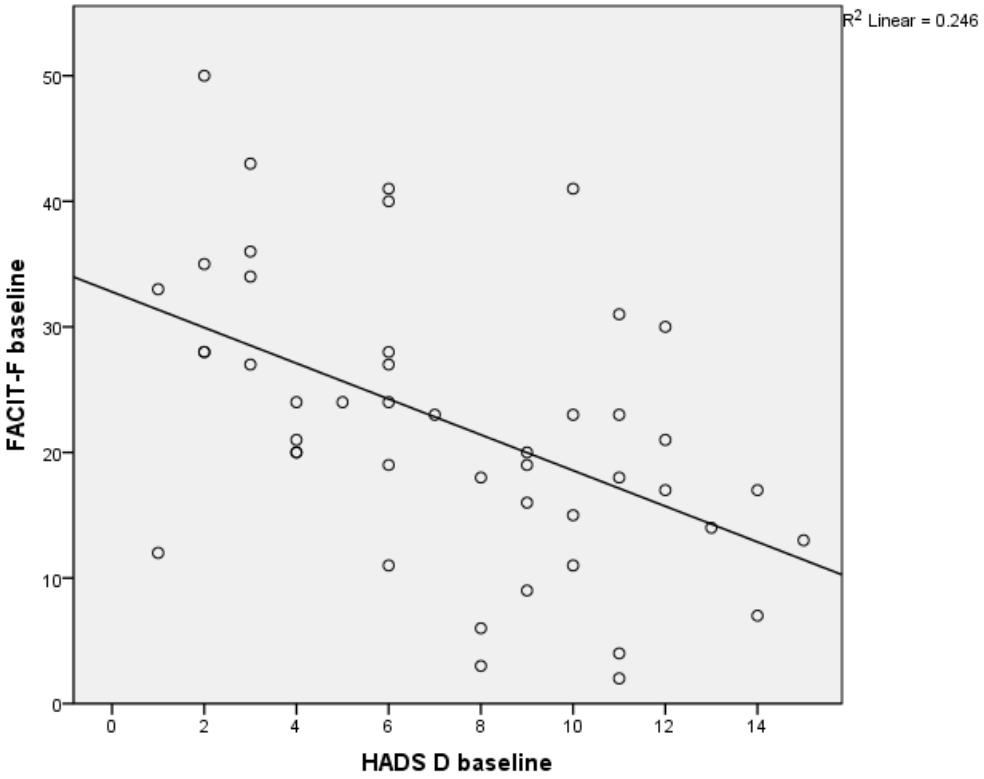

Figure 3-15 Scatterplot of fatigue and depression at baseline

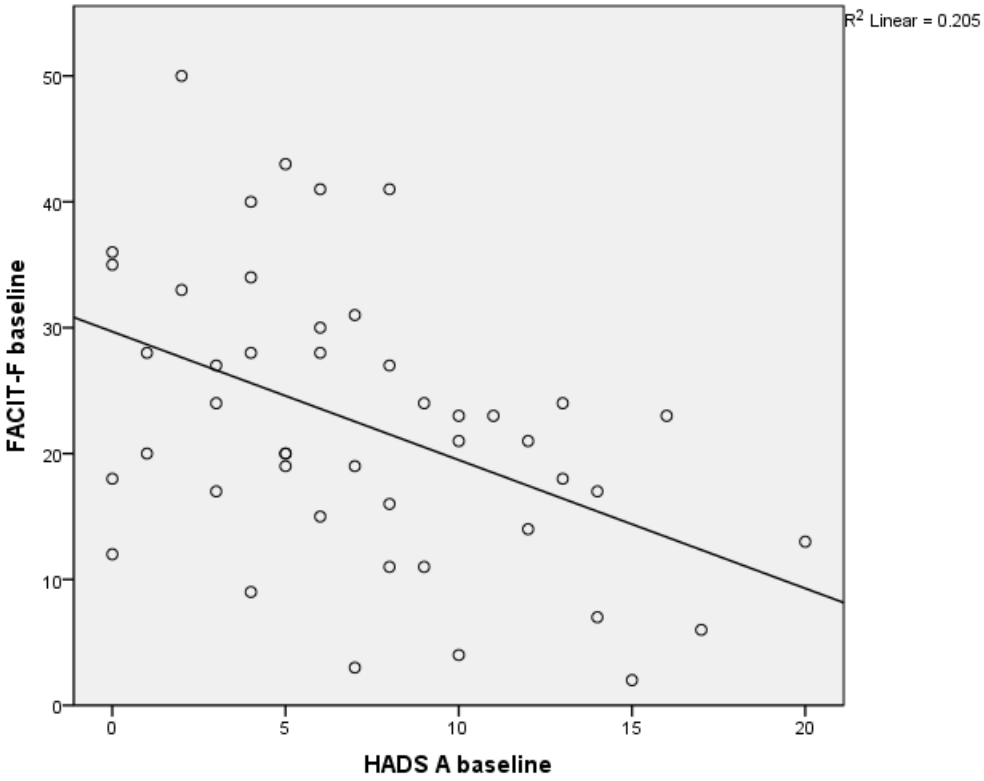

Figure 3-16 Figure scatterplot of fatigue and anxiety at baseline

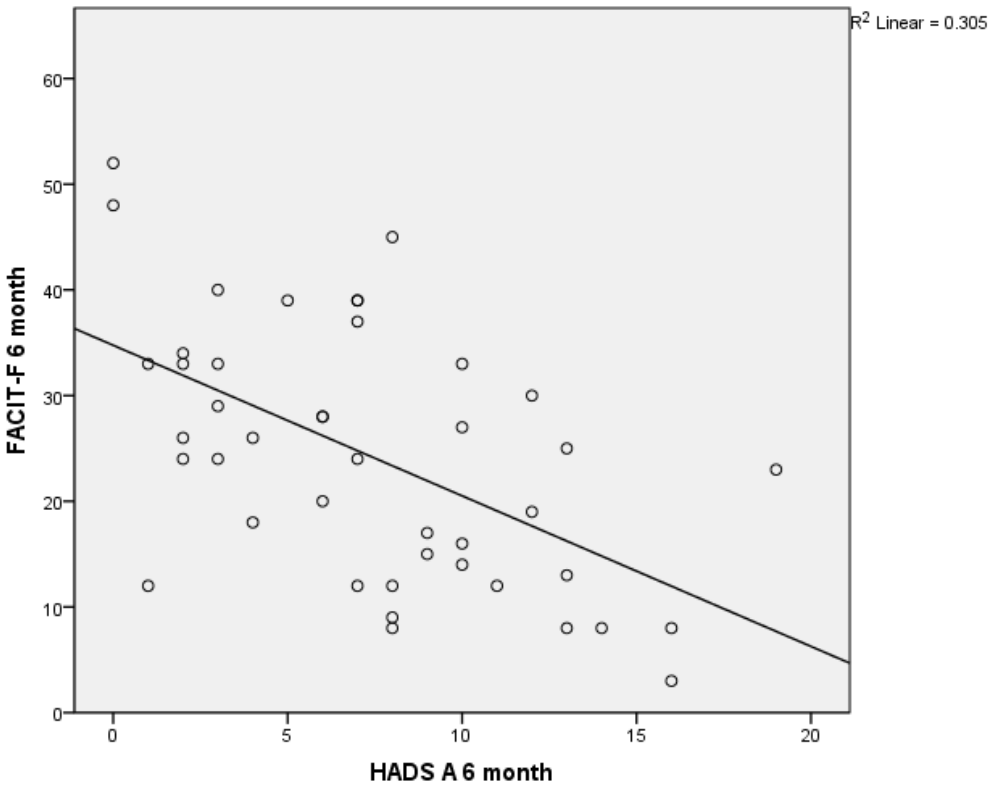

Figure 3-17 Scatterplot of fatigue and anxiety at six months

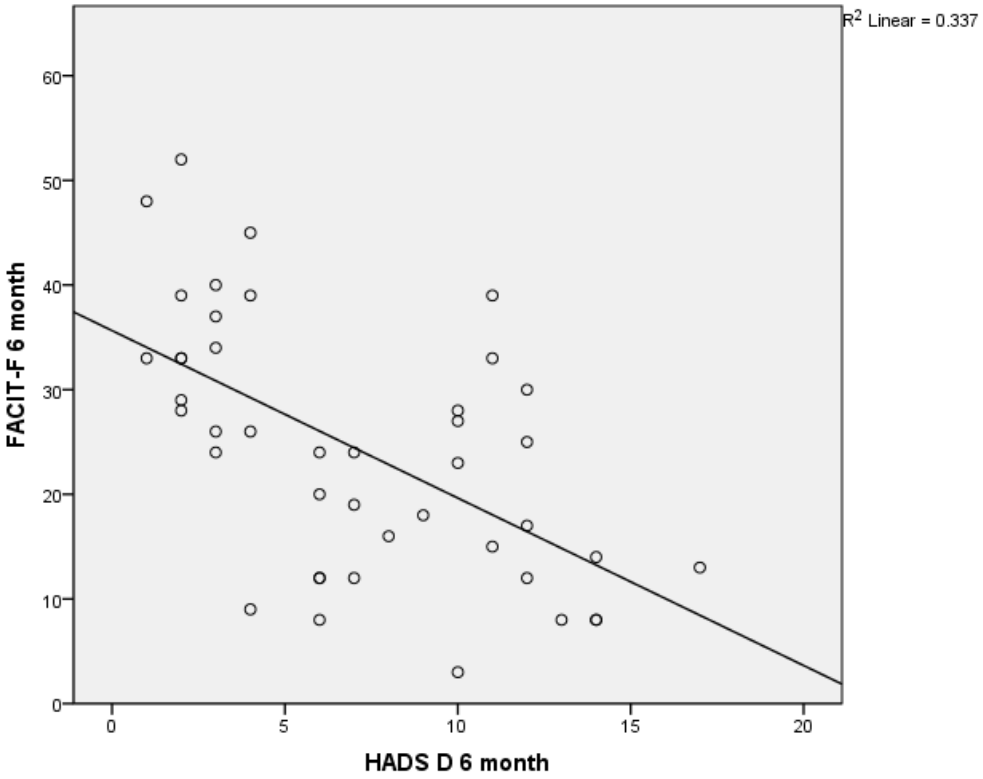

Figure 3-18 Scatterplot of fatigue and depression at six-month visit

3.3.9.3.3 Physical function (HAQ)

	HAQ baseline
HADS-A baseline	r=0.202, p=0.188
HADS-D baseline	r=0.047, p=0.761
	HAQ six months
HADS-A six month	r=0.253, p=0.106
HADS-D six month	r=0.201, p=0.203

Table 3-64 Correlation between mood and disability (HAQ)
(Spearman's correlation coefficient, significance assumed where p<0.05))

There is a lack of correlation between mood and physical function (disability) suggested by the HAQ-DI results. A lack of correlation with the SF-36 physical component score confirms this observation.

	PCS baseline	MCS baseline
HADS-A baseline	r=0.090, p=0.555	r=-0.718, p=<0.0001
HADS-D baseline	r=-0.051, p=0.737	r=-0.688, p=<0.0001
	PCS six month	MCS six month
HADS-A six month	r=-0.077, p=0.622	r=-0.877, p=<0.0001
HADS-D six month	r=-0.158,p=0.311	r=-0.805, p=<0.0001

Table 3-65 Correlation between mood (HADS score) and SF-36 physical and mental component scores
(Spearman's correlation coefficient, significance assumed where p<0.05))

Correlation with the mental component scores of the SF-36 suggests good consistency between the different methods of mood assessment.

3.3.9.3.4 Influence of clinical assessments and laboratory variables and mood

Clinical variable	HADS-A Baseline	HADS-D Baseline	HADS-A Six months	HADS-D Six months
EMS	0.143 p=0.350	0.234 p=0.122	0.305 p=0.049	**0.485★** **p=0.001**
SJC	-0.242 p=0.105	0.217 p=0.147	0.332 p=0.030	0.351 p=0.021
TJC	0.331 p=0.025	0.333 p=0.097	0.269 p=0.164	0.269 p=0.081
Pain	**0.457★** **p=0.001**	0.373 p=0.011	**0.390★** **p=<0.0001**	0.390 p=0.010
Patient Global	**0.500★** **p=<0.0001**	**0.415★** **p=0.004**	**0.541★** **p=<0.0001**	**0.501★** **p=0.001**
ESR	-0.025 p=0.870	-0.005 p=0.972	0.092 p=0.557	0.029 p=0.852
CRP	-0.046 p=0.767	0.09 p=0.524	0.028 p=0.871	0.031 p=0.857
DAS28	0.360 p=0.014	0.272 p=0.067	0.360 p=0.018	0.340 p=0.026

Table 3-66 Correlation between mood and clinical variables/inflammatory indices
(R value, Spearman's correlation coefficient, significance assumed p<0.0063★)

The correlation between individual components of the DAS and mood are shown. The relationship between mood and tender joint count, pain and patient global assessment of disease activity is less significant when correction for multiple variables applied. As a composite score the DAS-28 also shows some correlation with mood.

There is no correlation between laboratory variables/markers of inflammation and mood.

3.3.9.3.5 Discussion-mood disturbance

The prevalence of depression in this cohort with long standing disease is 42-50%, of anxiety 43-47% and of mixed disorder 30-35% using the HADS questionnaire. Ware et al propose a SF-36 MH score of 42 or below to have a sensitivity of 74% and specificity of 81% in detecting depression (Ware et al, 1994). In this cohort at baseline, 59% (27/46) and 56% (24/43) at six months later scored lower than this value. These results are consistent and may be useful as a first stage screen for depression (ie not diagnostic). Reduced vitality (VT) and social functioning (SF) reflect the impact of this disease in daily life but in domains not typically enquired in a clinic setting. However, the EQ5D would suggest that although patients recognise both pain and problems with their mobility, relatively fewer patients recognise anxiety or depression as contributing to their 'health state'. Whereas pain is easier to reflect upon and relate to arthritis, anxiety/depression may not be appreciated as contributory until severe. Only one patient at baseline and two at visit three responded as experiencing 'significant problems using the EQ5D. The HADS questionnaire responses for all three patients was >11 in both anxiety and depression scores.

The low pre-established diagnosis of both depression and anxiety from primary care and hospital records suggests a burden of unmet clinical need in this area. This may reflect lack of reporting or that it is not being sought.
No relationship between inflammation and depression was observed but mood does alter the subjective components of the DAS28. This is further explored in Chapter 6. Long-standing mood disturbance could be expected as a purely reactive/situational response to the reduction in quality of life and disability but there would not appear to be an inflammatory component on the basis of this data.

The lack of correlation between mood and function is of interest. It could be postulated that coping mechanisms are in place and that low mood is not driven by disability. In keeping with this supposition is the observed reduction in anxiety and depression with disease duration. This is further explored in Chapter 6.

In addition to scoring highly on measures of fatigue and mood (perceived as severe), correlations between fatigue and mood would also suggest that both measures are often present in such patients.

3.3.9.4 Medical Co-Morbidities

3.3.9.4.1 Dyspepsia

Eight patients had this active diagnosis. It is of note then that thirty patients regularly took NSAIDs. Data was not collected on use of gastric protection with proton pump inhibitors or H2 antagonists.

3.3.9.4.2 Anaemia

Nine patients had a listed diagnosis of anaemia of whom five were ascertained to be anaemia of chronic disease and thus related to RA.

3.4 DMARD Resistant Group

Twenty-six patients recruited to the ORBIT study provided matched samples for this study. Twenty-one samples were also collected at three-month visit. Twenty-four were made available at six-month visit.

3.4.1 Demographics and Inflammatory markers

Gender	77% female
Age (median)	57 yrs (range 38-80yrs)
Ethnicity	All patients were white Caucasian
Smoking status	38% patients were current smokers 31% ex-smokers for >one year 31% had never smoked Smoking status remained unchanged through the three study visits.
BMI (median)	28 (range 20-40)
Disease duration Months)	Median 113 Mean 141 (range 14-372)
Previous number of DMARDs (median)	3 (range 2-6)
ESR (mm/hour)	Median 25 (2-98) Mean 33
CRP (mg/dl)	Median 10 (2.4-146) Mean 23

Table 3-67 Demographics and disease characteristics of DMARD resistant group at baseline

3.4.2 Immunology

Recruitment to the ORBIT study was made on the basis of being *either* RF or CCP positive. If documented status was clear in the case notes then this was not retested and for this reason titre not available. In some cases autoantibody status had not been checked for over five years and was a historical assumption. In this way complete data is not available across the group. Where known, immunology profile is shown in Table 3.68.

RF	Number (n=26)	CCP	Number (n=26)
Negative	2	Positive	11
Equivocal	1	Negative	0
Positive	11	Unknown	15
Strong positive	12		

Table 3-68 Autoantibody status of DMARD resistant study patients

3.4.3 Prior and current therapy

Median previous number of DMARDs was 3 (range 2-6). At the time of study entry a wide range of therapy was in place from none, combination therapy with Methotrexate+Sulphasalazine+Hydroxychloroquine and variations therein, monotherapy with Gold, Penicillamine and Leflunomide.
After randomisation, twelve were treated with TNFa therapy and fourteen with Rituximab from baseline.

3.4.4 Disease Activity

	Baseline		Three month		Six month	
Clinical parameter	*Median*	*Range (min-max)*	*Median*	*Range*	*Median*	*Range*
TJC	14	2-28	5.5	0-23	5.5	0-22
SJC	10	0-25	4	0-20	2	0-14
Pain	63.5	1-100	n/a	n/a	24.5	8-80
Patient Global	67	14-100	n/a	n/a	29	0-81
DAS 28 ESR	6.04	4.05-8.41	4.13	1.97-7.02	3.89	1.02-6.86
DAS 28 CRP	5.72	3.89-7.95				
CDAI	94.5	17.6-154	n/a	n/a	58	N=13
SDAI	100.7	19.2-164	n/a	n/a	86	N=13

Table 3-69 Disease activity of DMARD resistant study patients at baseline
(n/a; no assessment at this study visit)

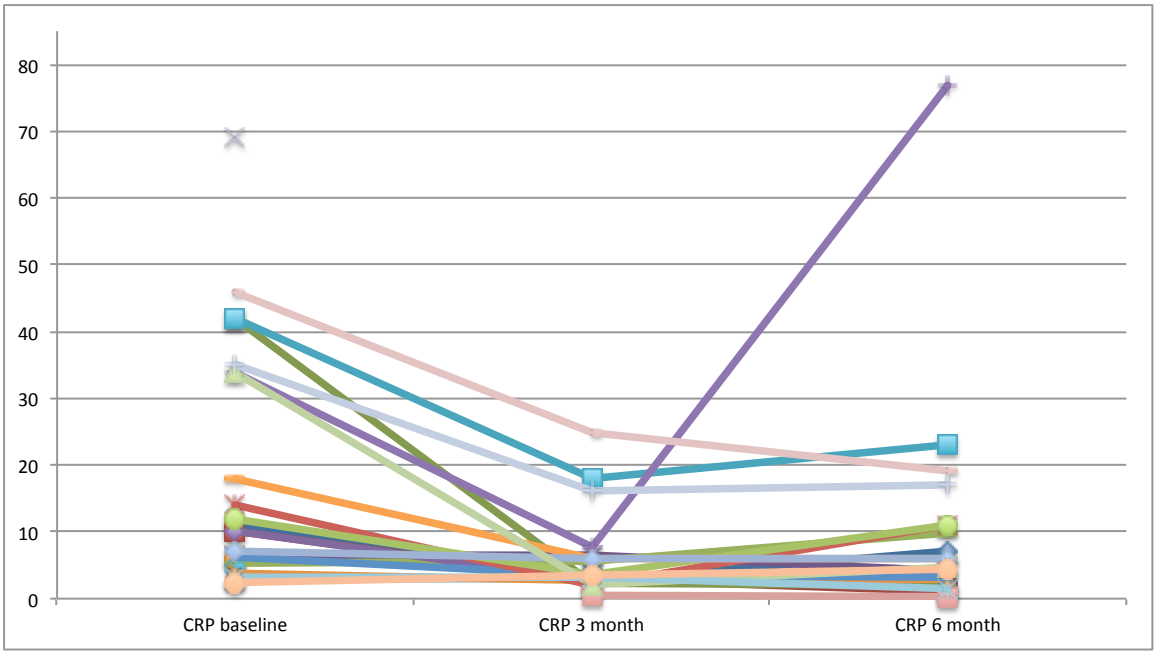

Figure 3-19 Representative changes in CRP between study visits of DMARD resistant study patients

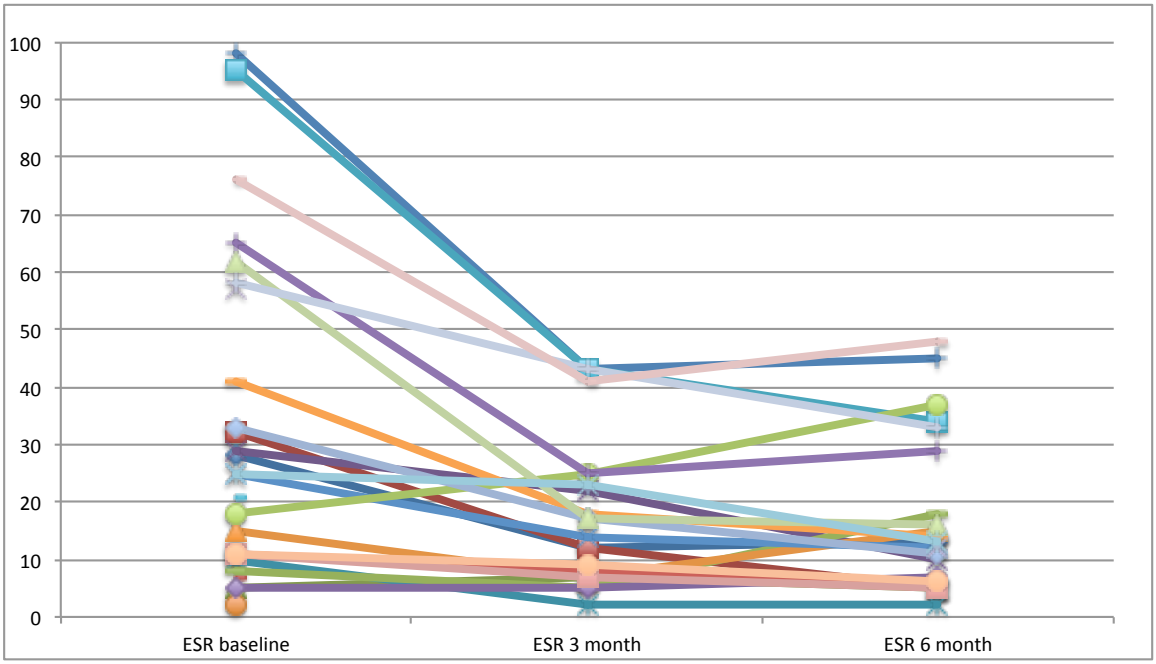

Figure 3-20 Representative changes in ESR between study visits of DMARD resistant study patients

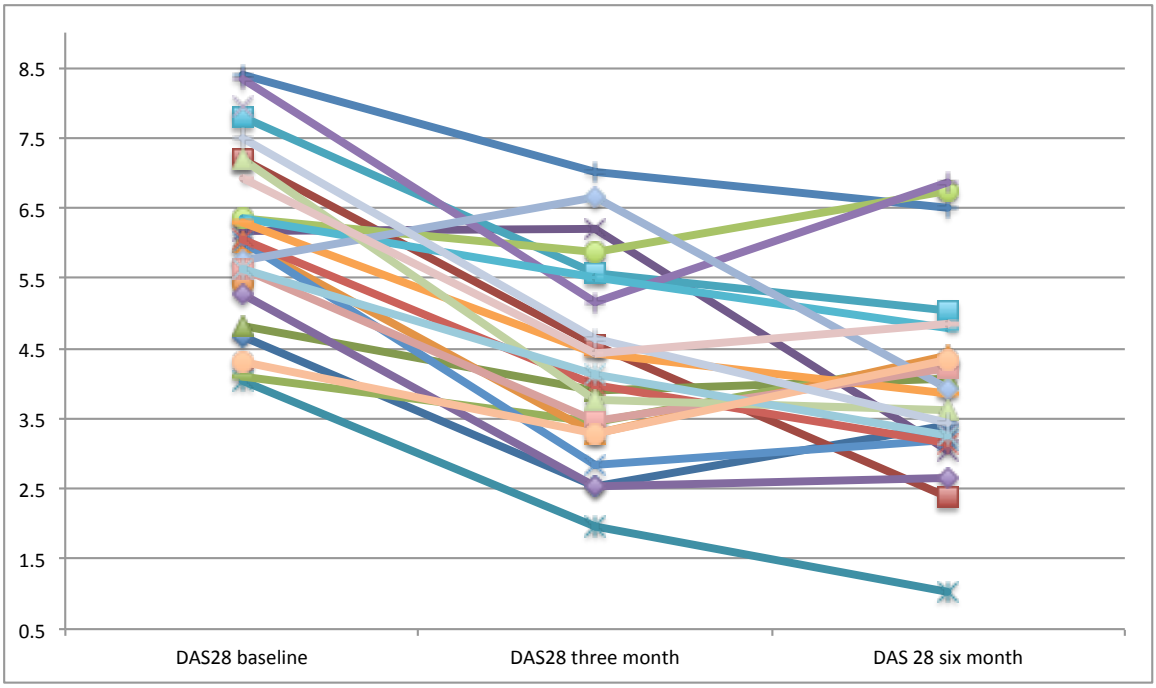

Figure 3-21 Representative changes in DAS-28 ESR between study visits of DMARD resistant study patients

According to protocol, biologic therapy was administered as per Table 3-70. Joint injections and steroid could be administered between study visits according to physician's preference.

Baseline	3 months	EULAR response 0-3 months	6month	EULAR response 3-6 months
Rituximab	Rituximab	Good	Rituximab	moderate
Rituximab	Rituximab	Good	Rituximab	good
tnfa	tnfa	non responder	tnfa	non responder
Rituximab	Rituximab	non responder	tnfa	good
Rituximab	Rituximab	Good	Rituximab	good
Rituximab				
tnfa	tnfa	non responder	Rituximab	non responder
tnfa				
tnfa	tnfa	non responder	tnfa	non responder
Rituximab	Rituximab	Good	Rituximab	good
tnfa	tnfa	non responder	tnfa	moderate
Rituximab	Rituximab	moderate	Rituximab	moderate
Rituximab	Rituximab	Good	Rituximab	good
tnfa	tnfa	moderate	tnfa	good
tnfa	tnfa	non responder	Rituximab	non responder
tnfa	tnfa	non responder	tnfa	non responder
tnfa	Rituximab	non responder	tnfa	moderate
tnfa	tnfa	moderate	tnfa	moderate
Rituximab	Rituximab	non responder	Rituximab	moderate
Rituximab	Rituximab	moderate	Rituximab	moderate
Rituximab	Rituximab	moderate	Rituximab	moderate
tnfa	none		Rituximab	moderate
Rituximab	Rituximab	moderate	Rituximab	moderate
Rituximab	Rituximab	non responder	Rituximab	non responder
Rituximab	Rituximab	moderate	Rituximab	moderate
tnfa	tnfa	moderate	tnfa	moderate

Table 3-70 DMARD resistant patients; treatment between study visits with overall EULAR response at time of sample collection

3.4.5 Medical comorbidity of DMARD resistant group

Number of comorbid medical conditions present	% of whole group (n=26)	Selected condition of note	% with condition (n=26)
0	27%	Vascular disease	15%
1	23%	Presence of vascular risk factor (>/=1)	54%
2	31%	Mood disturbance	12%
3	15%	Dyspepsia	31%
4	4%		

Table 3-71 Medical comorbidity in DMARD resistant study patients

3.5 DMARD Good responder group

Twenty-one patients were recruited to this group. One patient had a DAS28 of >3.2 after calculation and one patient that was originally included was later excluded (although disease control had been excellent for a number of years, disease had become active in the period just prior to study inclusion). Nineteen patients had data available for analysis.

3.5.1 Demographics

	Mean (+SD)	Median (range)
Mean age (years)	63 (11)	59 (45-81)
Disease duration (months)	236 months (133)	222 (78-655)
Previous DMARDs	2 (1)	2 (1-4)
Female	63%	
Smoking status	Current smoker	21%
	Ex-smokers of over a year	21%
	Never smoked	58%
Height (metres)	1.64 (0.11)	1.63 (1.49-1.82)
Weight (kg)	75 (24)	71 (38-138)
BMI	28 (8)	27 (16-52)

Table 3-72 Demographics and disease characteristics of DMARD good responder study patients

3.5.2 Comorbidity

Number of medical comorbidities	% patients
None	10%
One	20%
Two	50%
Three	0%
Four	20%

Table 3-73 Numbers of medical comorbidities in DMARD good responder study group

In this cohort, nine had identified vascular risk factors not including smoking. Two patients had had a previous myocardial infarct.

3.5.3 Disease Activity and Inflammatory markers

Disease Activity	Mean (+SD)	Median (range)
TJC	1 (1)	0 (0-3)
SJC	2 (1)	1 (0-5)
Pain	34 (23)	31 (0-71)
Patient Global	29 (24)	25 (0-97)
Physician Global	2 (1)	2 (1-3)
EMS	45 (65)	15 (0-240)
DAS-28 (ESR)	2.97 (0.54)	3.15 (1.54-3.58)
DAS-28 (CRP)	2.84 (0.60)	2.81 (1.37-4.36)
CDAI	33 (26)	29 (1-106)
SDAI	44 (27)	41 (3-110)
Inflammatory Markers		
ESR	21 (20)	13 (2-92)
CRP	11 (10)	8 (0-41)

Table 3-74 Clinical assessments, inflammatory markers and composite disease activity scores of DMARD good responder study group

3.5.4 Immunology

Autoantibody tested	Interpretation	Number (n=19)
RF	Negative	26%
	Equivocal	11%
	Positive	42%
	Strong positive	21%
CCP	Negative	26%
	Equivocal	0
	Positive	74%
ANA	Negative	63%
	Very weak positive	11%
	Weak positive	21%
	Moderate positive	5%
	Strong positive	0%

Table 3-75 Autoantibody status of the DMARD good responder study patients

3.6 Healthy Controls

Twenty-seven patients were recruited. Two patients were later excluded after assessments; one asymptomatic patient tested strongly CCP positive. The second was found to have a paraprotein and excluded in view of the link between haematological malignancy and microRNA expression.

3.6.1 Demographics, Clinical findings and inflammatory markers

Gender	84% female	
Age (years median and range)	48 (31-61)	
Ethnicity	All patients white Caucasian	
Smoking status	20% current smokers 12% ex-smokers (greater than one year) 68% never smoked.	
Tender and swollen joint count	Zero	
BMI (mean)	27 (range 21-39)	
Inflammatory markers		
	Median (range)	Mean
CRP (mg/dl)	1.4 (0.3-16)	3.1
ESR (mm/hr)	8 (0.4-30mm/hr)	9.4

Table 3-76 Summary descriptive findings of the healthy control study group

3.6.2 Immunology

In keeping with the inclusion criteria, all patients were RF and CCP negative. One patient had a weak positive ANA of 1/160.

3.7 Discussion; Analysis between groups at baseline study visit

BASELINE (median values)	Biologic Resistant Group	DMARD Resistant	DMARD good responder	Healthy Controls
Age (years, range)	59 (36-78)	57 (38-80)	59 (45-81)	48* (31-61)
Gender (% Female)	84	77	74	84
Smoking status, %)	14	38*	21	20
Disease duration (months) range	213 (72-537)	113.5* (14-372)	222 (78-655)	n/a
Previous DMARD (range)	6 (2-9)*	3 (2-6)	2 (1-4)	n/a
BMI (mean)	28	28	28	27
RF + (% positive)	48%	42%	42%	0
CCP + (% positive)	88%	42%*	74%	0
TJC	8	14*	0*	
SJC	10	10	1*	
DAS28 CRP	5.17	5.72	2.81*	n/a
DAS28 ESR	5.36	6.04	3.15*	n/a
SDAI	45.4	100.7*	40.6	n/a
CDAI	70.8	94.5	29.0	n/a
ESR	24	25	13	8*
CRP	11	10	8	1.4*

Table 3-77 Summary of clinical and biochemical results between study and control groups (* statistically significant p<0.01) difference between groups)

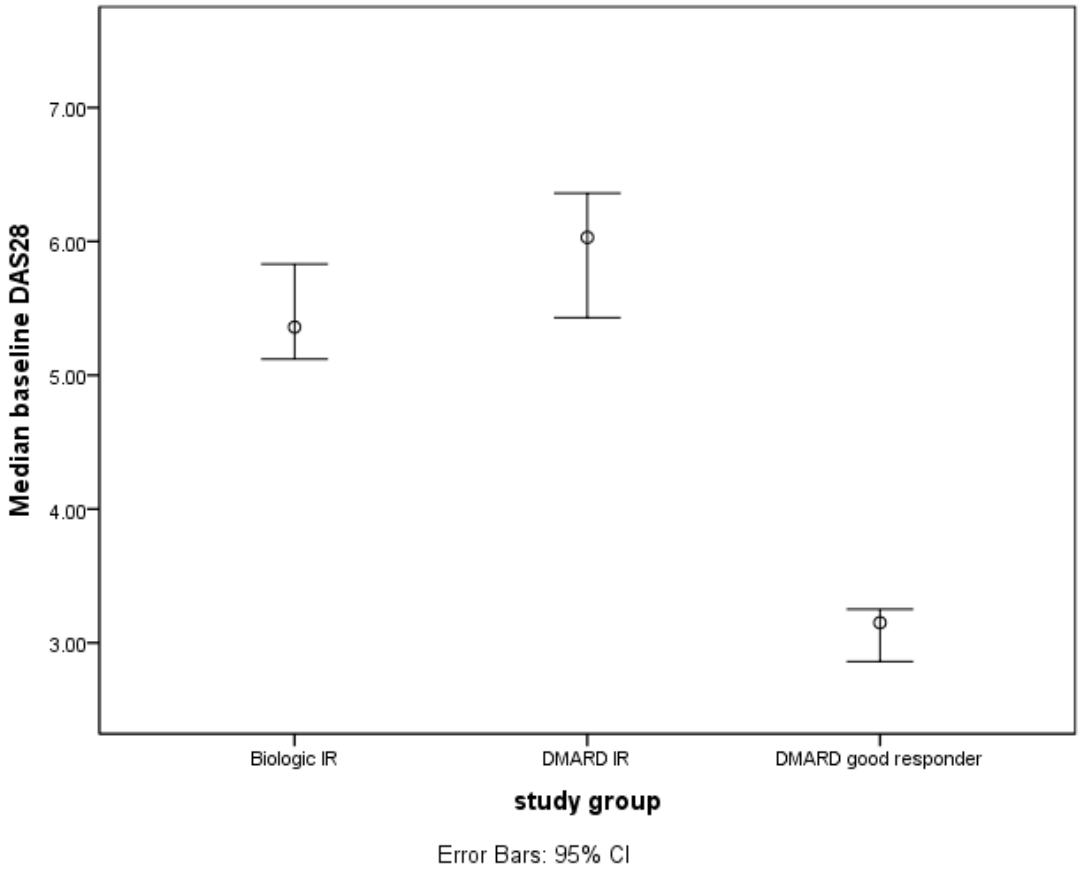

Figure 3-22 DAS28 ESR median values between study groups
(error bars represent 95% CI)

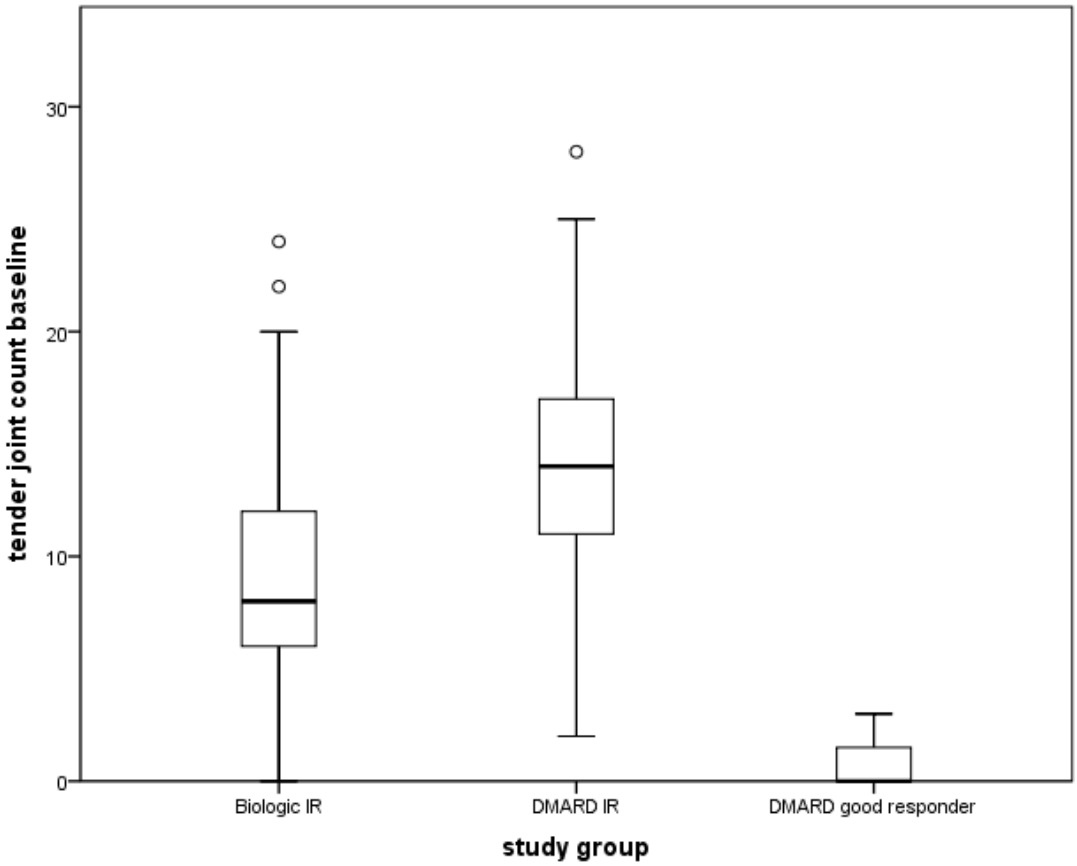

Figure 3-23 Tender joint count box and whisker plots
(median values between study groups (error bars represent 95% CI)

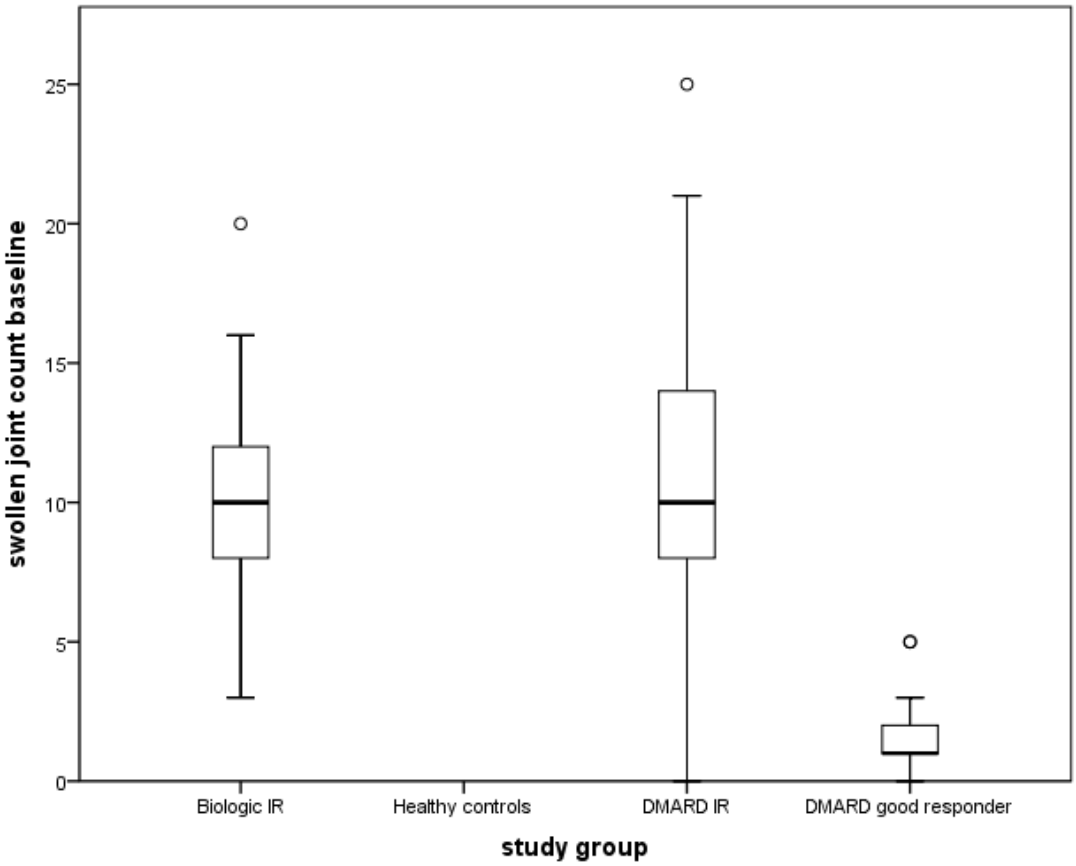

Figure 3-24 Swollen joint count box and whisker plots between study groups
(Median values between study groups (error bars represent 95% CI)

Some observations can be made between the study groups described in Table 3-77 and Figures 3-25 to 3-27;

- The aim of the DMARD resistant study group was to examine RA patients with active disease but prior to biologic treatment. In this way it was assumed that disease duration would be shorter. Disease duration was found to be shorter than both the biologic resistant group and DMARD good responder groups. Nonetheless, these patients have considerable disease duration.
- The DMARD resistant group has proportionally more smokers
- The DAS28 and SDAI are numerically higher in the DMARD resistant group than the biologic therapy resistant group although not achieving statistical significance ($p<0.05$). This is driven mainly by clinical assessments including higher tender and swollen joint counts (tender joint counts were significantly higher in the DMARD resistant group)
- ESR did not differ from the DMARD good responder group although CRP did
- The healthy volunteer group are younger than the other three patient groups. This reflected those individuals available to donate blood.

- Proportionally fewer patients in the DMARD resistant group are CCP positive but this reflects the absence of testing in small numbers; inclusion to the ORBIT study was the presence of *either* antibody and serological retesting was not undertaken.
- The DMARD resistant group has a wider range of clinical variable (tender and swollen joint counts). This could be explained by the study assessments being performed by different research staff at each study centre and introducing inconsistency. It cannot be determined if there was more chronic pain/depression/fibromyalgia driving on high tender joint counts or more destructive radiological damage.

Despite differences in disease duration and similar age, the number of medical comorbidities is similar between the biologic and DMARD resistant groups.

3.8 Chapter Conclusions and Discussion

In this chapter the clinical results of this study are presented.

The biologic resistant group comprise the main focus and as such most conclusions are drawn in order to meet the aims of this chapter. Not only is the substantial burden of co-morbidity evident but also the opportunities to address these conditions apparent in cardiovascular risk modification and mood disturbance.

When considering those elements that comprise 'severe' RA then disease duration, autoantibody status, deprivation, disease activity, quality of life measures and disability need considered. In addition to meriting separate case finding and treatment, the presence of substantial medical comorbidity influences the assessment of those measures of severity.

Employing patient reported outcome measures in the form of questionnaires is becoming increasingly common but remains challenging within the time limited clinic environment. However, this study would suggest they add an additional dimension to disease assessment. Disability and quality of life are not easy to assess in a short consultation. Therefore an understanding of how clinical and biochemical markers influence these domains is important.

From the biologic resistant patients, several notable observations that may be integrated into clinical care have been made

- Inflammatory disease remains responsive within this group but disability may be more refractory
- Mood influences QoL but not function (disability)
- Fatigue is substantial. It is closely related to mood and influences assessments using composite disease activity
- There is not a relationship between mood and inflammation
- Mood also strongly influences subjective composite disease activity measures
- Social factors (deprivation) may influence reported pain and fatigue levels and influence anxiety/depression

Several findings were notable in terms of clinical differences between the groups at baseline. Longer disease duration and greater smoking history, with relatively greater number of DMARDs, was observed in the biologic resistant group. Both groups had comparably active disease. The DMARD good responder group had comparable demographics but low disease activity. These observations are important in the context of the Chapters 4 and 5.

4.1 Chapter 4- MicroRNA profiling

4.1.1 Introduction/chapter description

The background to microRNA and their studies in RA has been extensively outlined in Chapter 1. MicroRNA are stable and accessible but have demonstrated inconsistent associations with clinical measures of disease activity. There are several specific published examples that set the context for this chapter.

(Murata et al. 2010) examined the plasma and synovial fluid profiles of RA and OA samples (specifically microRNA-16, -32, -146a, -155, and -223 and -39 only). They studied a heterogeneous RA cohort (n=30) in terms of disease duration (mean 10yrs), age (mean 60yrs) and treatment (proportionally higher use of oral steroid than a UK population). Plasma and synovial fluid microRNA profiles differed but it was of interest there was no correlation between synovial fluid microRNA and clinical variables. Synovial tissue and synovial fluid had comparable profiles suggesting the microRNA are secreted locally and may explain the disconnection between peripheral blood microRNA profiles and clinical variables. Plasma microRNAs did not correlate with CRP or ESR. MicroRNA-16, -146a, -155, and -223 inversely correlated with TJC and microRNA-16 inversely correlated with DAS28.

(Murata et al. 2013) built on their earlier cohort but with the aim of establishing a plasma profile of RA. From an initial exploratory microarray of three RA patients, those microRNA consistently and greater than fourfold differentially expressed were validated in over a hundred RA patients and healthy controls. The authors validated these results against a smaller number of SLE and OA patents. Mean DAS28-CRP was 3.42 and a third of their patients had high disease activity as judged by a DAS28 >4.1. No previous or current biologic therapy was in use. MicroRNA- 24, -26a and 125a-5p were confirmed as diagnostic biomarkers of RA and novel findings. Additionally microRNA-24 correlated with CRP and the DAS28 scores suggesting utility as a biomarker.

(Filková et al. 2013) examined selected microRNA profiles (those discussed in Chapter 1 such as -146a, -155, -223, -16, -124a, -16, -203 and -132) in early arthritis patients and followed their change with treatment over a year. Control groups were healthy controls and established RA (mean disease duration 9.28yrs and 19/26 treated with biologic therapies. Both microRNA-223 and -146a were reduced in early disease versus established and microRNA-16 showed some correlation with treatment response but no association with disease activity in established disease. The authors suggest these microRNA may be useful in determining response but this study also confirms the microRNA pattern is likely to change with duration of disease and treatment response.

Within our research group, experience of working with microRNA is established. Several microRNA of interest have been identified from microarrays and studied using the characterised cohorts described above. MicroRNA-34a, for example, was previously identified in a small series of RA synovial fluid and CD14 cells and then examined further

in these cohorts. Where different methodologies to those described in 'Methods' were applied these are referred to and all work and interpretations similarly defined.

Those microRNA referred to in Chapter 1, and having been examined in depth elsewhere, were not further studied between groups. As part of the microarray performed to seek a biologic resistant microRNA signal, primers for those microRNA referred in Tables 1-14 to 1-17 were used however.

MicroRNA-34a, -27b and -125a were first examined in circulating CD14+ cells with a view to determining profiles between groups (and thus any signal the biologic resistant group differ) and any correlation with clinical values sought in order that they might act as a biomarker of treatment resistance.

4.2 Aims

There are two broad aims to this chapter. Firstly, the study of specific candidate microRNA profiles from within the research group and secondly to seek novel candidate microRNA that are differentially regulated in the biologic resistant group from an exploratory microarray screen. The profiling of microRNA in general and how this may be approached is shown in Figure 4-28.

4.3 Methods

Two broad techniques were employed to profile microRNA but there are many variations on this theme to extract and quantify microRNA (RAYMOND 2005; Schmittgen et al. 2004; Kroh et al. 2010). This can also be considered a weakness in view of the lack of standardized methodology. 'Microarray detection' which is sensitive, specific and high throughput but can be prohibitively costly and results may be skewed by batch-to-batch variability. Kits with multiple known microRNA sequences can be pre-loaded and run in parallel. The second is quantitative, real-time PCR. This has proven useful as a validating tool. Both provide a 'snapshot' of the microRNA profile.

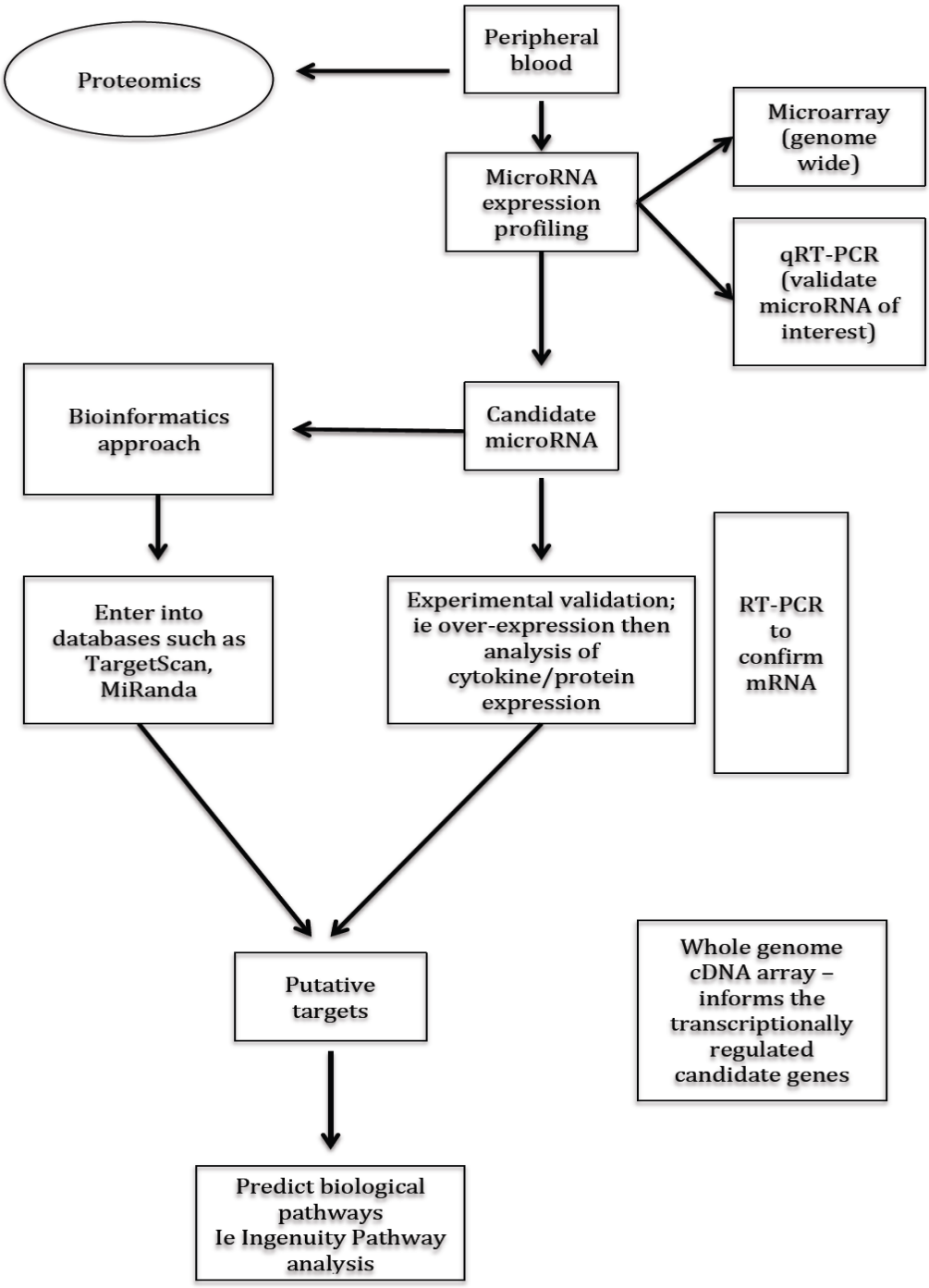

Figure 4-25 Representation of principles microRNA methodology (adapted from (Recchiuti et al. 2011)

There are challenges with microRNA measurement. These include the choice of tissue and blood (tissue lysis and disruption requirement), precursor and mature forms, reference value normalization (we have studied disease and healthy patients with housekeeping genes but even the norm dataset by age, gender or ethnicity has not been

defined) and reproducing results for validation purposes when different protocols and platforms are used.

4.3.1 Sample processing; all samples

Venepuncture was performed as per Appendix 2. Blood was drawn as per protocol and the same manufacturer's blood tube used. The effect of needle size, or time of day that blood is drawn, is unknown (Kroh et al. 2010). This was transported at room temperature to the Glasgow Biomedical Research Centre where Lynn Crawford, Laboratory Technician, carried out analyses. The GBRC laboratory has built local expertise in examining microRNA in an accurate and reliable fashion. This local knowledge fashioned Appendix 3 to be created. LC carried out all processing to avoid introducing error and following Appendix 3 outline strictly. In general, one to two patient samples only were analyzed in any single day.

4.3.2 Cell separation

Blood was separated into CD14 and CD4 cell populations as illustrated in Appendix 3. The addition of Histopaque-1077 (Sigma Aldrich, polysucrose and sodium diatrizoate adjusted to a density of 1.077 ± 0.001 g/ml) created an aqueous and organic phase layers after centrifugation. Mononuclear cells remain in the upper aqueous phase and aggregates of red cells and granulocytes are most dense gathering at the base. The mononuclear cell layer was drawn off by pipette. Bead/column magnetic technology (CD14 labeling and beads Miltenyi Biotec, AutoMACS cell separator) sorted the cells accordingly. All samples were checked for cell count purity within 24 hours using the FACSCalibur analyser (BD Biosciences) Cell line storage (archiving) occurred at this stage as per Appendix 3.

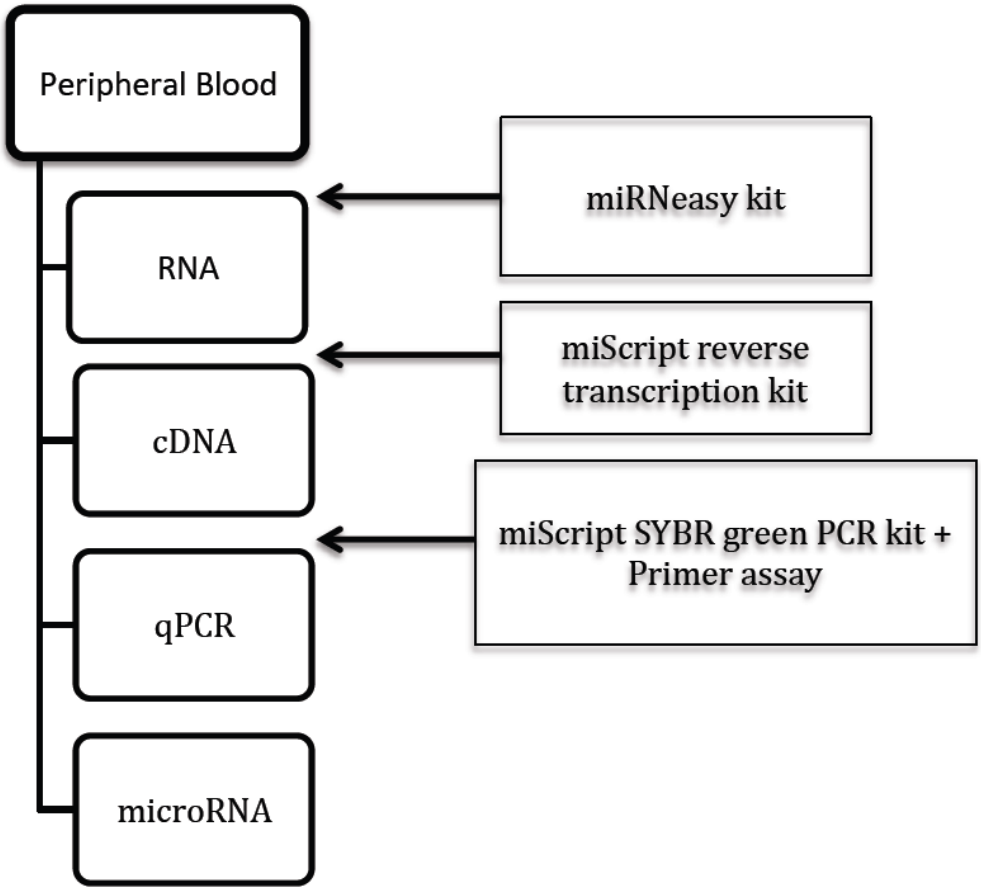

Figure 4-26 Flowchart of microRNA extraction and kits used

4.3.3 RNA extraction

Later, extraction of total RNA (including microRNA) was carried out. Total RNA was extracted using the miRNeasy mini kit (Qiagen, Cat no 217004, "quick start protocol" Jan 2011). The principle uses QIAzol lysis reagent to both inhibit RNA-ases and remove DNA/organic proteins having lysed cells. Chloroform permits the RNA to separate to an upper layer for capture with 'mini columns' following centrifugation. RNA yield and purity was determined using a Nanodrop ND-100 spectrophotometer with further RNA Integrity Number (RIN) measured using the Agilent Bioanalyser 2100 as shown in Figure 4.30.

4.3.4 cDNA formation and qPCR

cDNA formation was performed with the miScript II RT Kit (Qiagen Cat no 218161). This contains miScript reverse transcriptase buffer (oligodT primers and contents to enhance enzymatic function) with RNase free water and reverse transcriptase mix (polyA polymerase and reverse transcriptase). The miScript SYBR green PCR kit (Qiagen, Cat no

218073) contains DNA polymerase, SYBR green and Universal primer. In the first cycle, poly (A) polymerase adds a polyA tail to the microRNA. OligodT primers recognize this tail allowing reverse transcription and the addition of a universal tag (common tail sequence). Subsequently qPCR was performed with the miScript SYBR green PCR kit (Qiagen, Cat no 218073) that contains DNA polymerase, SYBR green, Universal primer complementary to the universal tag, and microRNA specific primer (miScript Primer assays). SYBR green binds to amplified double stranded DNA and fluoresces. The degree of fluorescence is proportional to the amount of product sought and the miScript primer allows amplification of the microRNA being sought.
Reactions were carried out in triplicate using ABI Prism 7900HT Sequence Detection System (Applied Biosystems), software SDS v2.4. The manufacturer's protocol for cycling conditions was followed.

4.3.5 Storage

Samples were stored in Qiazol (Qiagen, ultimately for microRNA estimation), Trizol (Invitrogen, for later transcriptomic examination) in a minus 80 degree freezer.

At this stage for analysis and bio-banking were the following;

- Whole blood in Paxgene tubes required no further processing (RNA stabilized)
- Serum from clotted tubes for biomarkers/cytokine analysis
- DNA FTA cards
- CD14 RNA/microRNA
- CD4 RNA/microRNA
- Negative fraction RNA/microRNA

4.3.6 Shipping

Proposed detailed analysis was undertaken by Expression Analysis USA. Staggered shipments were made in December 2011 and February 2012. The second shipment included ORBIT Paxgene samples (a separate application was made to the PEAC biobank for matched PaxGene tubes, see Appendix 8). Baseline analysis only of microRNA and mRNA were undertaken before samples were returned (proposed arrangement with Roche pharmaceuticals terminated).

4.3.7 Specific instances of modifications to above protocol

Research staff accessed the stored RNA samples in the Biomedical Research facility in order to generate the data analysed and produced below. As such, different methodology may have been applied and patient samples analysed which is outlined. Final selection of those patients who have data presented was determined following qPCR quality review.

4.3.7.1 MicroRNA-34a

Claire Tange at the Glasgow Biomedical Research Centre, University of Glasgow, undertook analysis. The Applied Biosystems kit for TaqMan microRNA PCR (Cat number

4427975) was used according to the manufacture's protocol. Results were presented as copy number of microRNA-34a per 10,000 let-7a control.

4.3.7.2 MicroRNA-27b

Marina Freita at the Glasgow Biomedical Research Centre, University of Glasgow, undertook analysis. She also used the Applied Biosystems kit for TaqMan. Results were presented using relative expression levels, which were determined by the delta cycle threshold (ΔCt method). Delta Ct= (mean Ct (housekeeping gene) – mean Ct (microRNA being studied). RNU6-2, which is the recommended by Applied Biosystems was used as housekeeping gene. All experiments were performed in triplicate. Data is presented as relative values which are $2^{(-\text{delta Ct})}$). (Livak & Schmittgen 2001)

4.3.7.3 MicroRNA-125a

Ashleigh Ann Rainey at the Glasgow Biomedical Research Centre, University of Glasgow, undertook analysis. The Applied Biosystems kit for TaqMan was also used. Results were presented using relative expression levels to RNU6, which were determined by the ΔCt method and presented as relative expression. Experiments were performed in triplicate.

4.3.7.4 MicroRNA microarray of all study cohorts

As previously stated, this was performed by Expression Analysis, USA. Total RNA, including microRNA fraction was isolated as described with the miRNesy kit according to manufacturer instruction (Qiagen). The integrity of RNA was ensured by analysis of ribosomal 18S and 28S RNA intensity using an Agilent 2100 bioanalyser (Agilent Technologies). Total RNA was then polyadenylated and labeled with Biotin-3DNA molecules with FlashTag™ Biotin HSR RNA Labeling Kit (Affymetrix). Labeled RNA was hybridized to GeneChip miRNA 3.0 Array (Affymetrix). Signal was developed by incubation of GeneChip with streptavidin-PE. Background correction and quintile normalisation for each probe set on the GeneChips was determined by the RMA algorithm (Robust Multi-array Analysis). One-way ANOVA with correction for multiple testing (Benjamini-Hochberg) followed by post-hoc tukey test was used. P value below 0.05 and fold change above 1.5 was chosen to determine differentially expressed miRs.

4.3.7.5 MicroRNA-423, -1275, -574 and -3178 qPCR validation

Expression levels of the four differentially expressed microRNAs identified by the Expression Analysis microarray were validated by quantitative real time RT-PCR using the methods referred to previously (Qiagen kits/SYBR green). RNU6-2 was used as the endogenous control to normalize the data. Rene Oliveira (RD) at the Glasgow Biomedical Research Centre, University of Glasgow, performed this. Results were presented using relative expression levels as above. All experiments were performed in triplicate. Manual review of outliers or poor amplification curves was necessary at three and six month analysis where nine and two patients were excluded.

4.4 Results

4.4.1 Purity

Both cell purity and RNA quality were examined and acceptable values obtained.

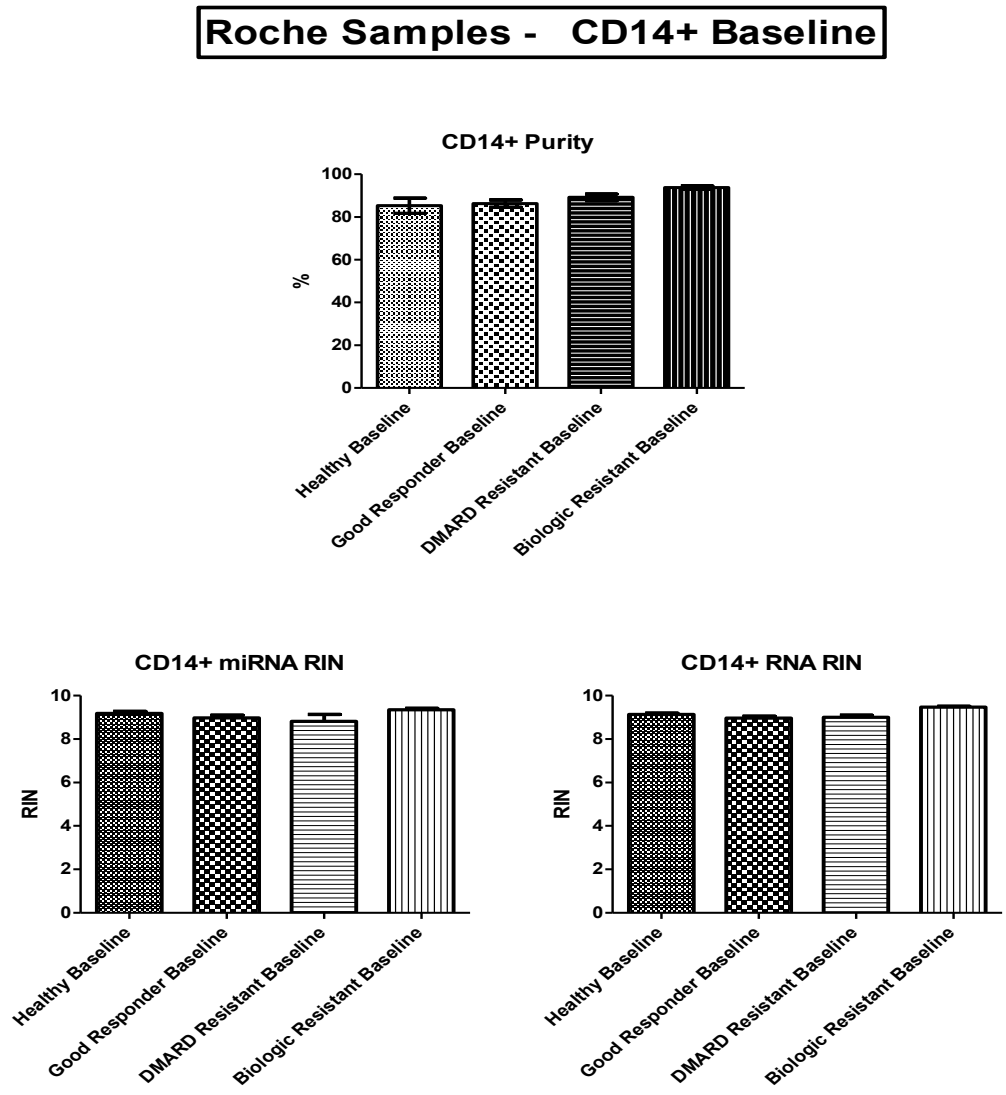

Figure 4-27 Cell purity and RNA integrity of samples undergoing analysis

4.4.2 Results- candidate approach

The microRNA expression profile of specific candidate microRNA within the study cohorts is examined below.

4.4.2.1 MicroRNA-34a

4.4.2.1.1 Clinical characteristics and findings

Median value	Biologic Resistant	Healthy Controls	DMARD IR	DMARD GR
	N=30	N=25	N=22	N=18
SJC	11	0	10	1.5
TJC	8	0	14	0.5
ESR mm/hr	23.5	7	26.5	13
CRP mg/dl	10.0	1.3	10.0	7.3
DAS28-ESR	5.44	n/a	6.11	3.15
DAS28-CRP	5.18	n/a	5.64	2.81

Table 4-78 Clinical variables between groups in microRNA-34a experiment
(where DMARD inadequate responders (DMARD IR) and DMARD GR (DMARD good responders))

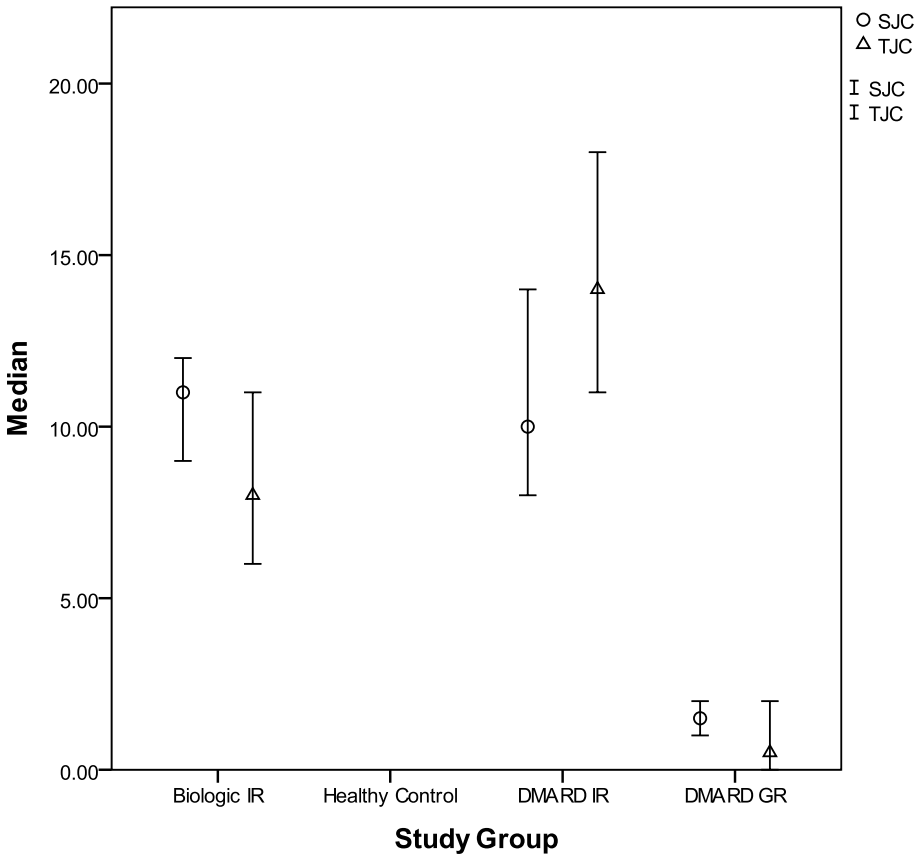

Figure 4-28 Joint counts between study groups in microRNA-34a analysis group

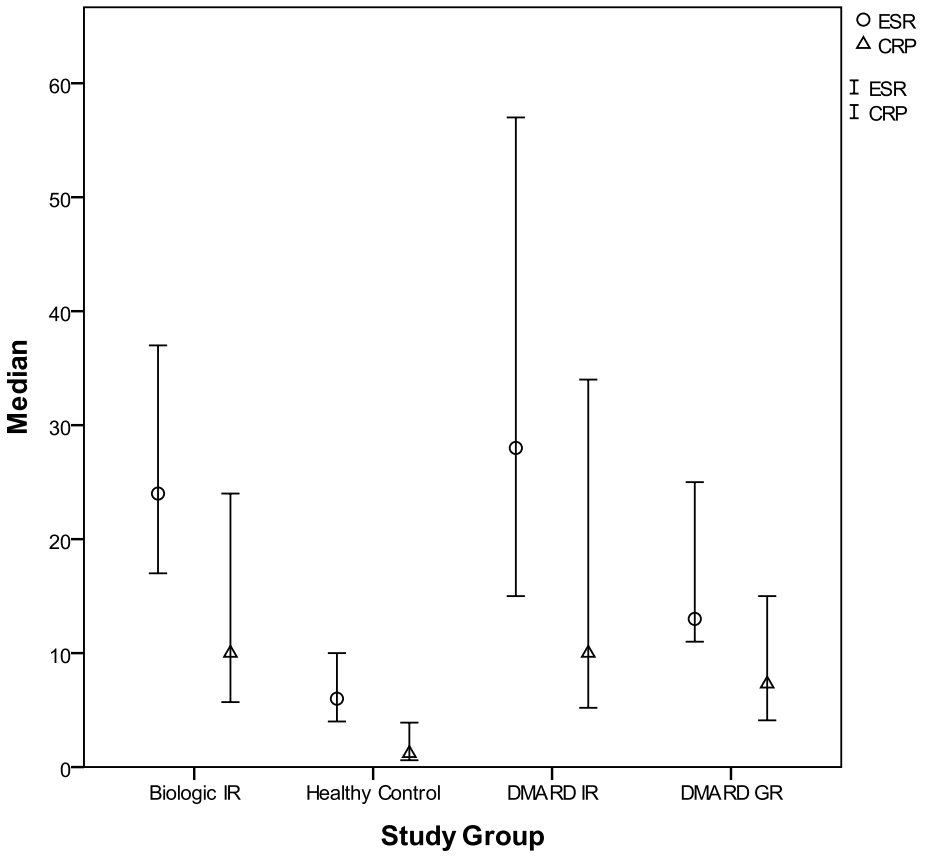

Figure 4-29 Inflammatory markers between study groups in microRNA-34a analysis group

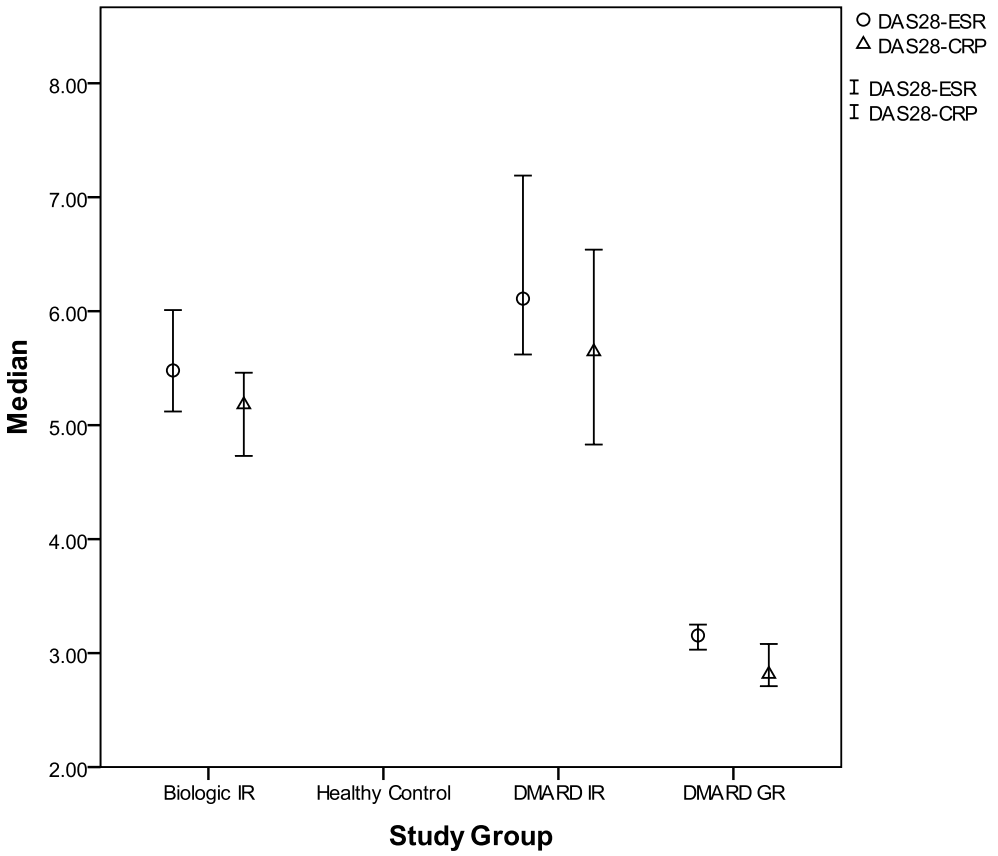

Figure 4-30 DAS28 scores between study groups in microRNA-34a analysis group

In contrast to the microRNA below, the biologic resistant and DMARD resistant group varied in some clinical variables.

Differences between the group clinical variables were present in DAS28-ESR (p=0.017) and TJC (p=0.009) but not SJC (p=0.963), ESR (p=0.683), CRP (p=0.945) and DAS-28-CRP (p=0.177). All variables were highly statistically different (p=<0.0001) between the biologic resistant group and DMARD good responder group other than ESR (p=0.183) and CRP (p=0.274). A similar observation between the DMARD resistant and DMARD good responders was made (clinical variable differing but CRP and ESR no statistical difference).

4.4.2.1.2 MicroRNA-34a cross sectional analysis

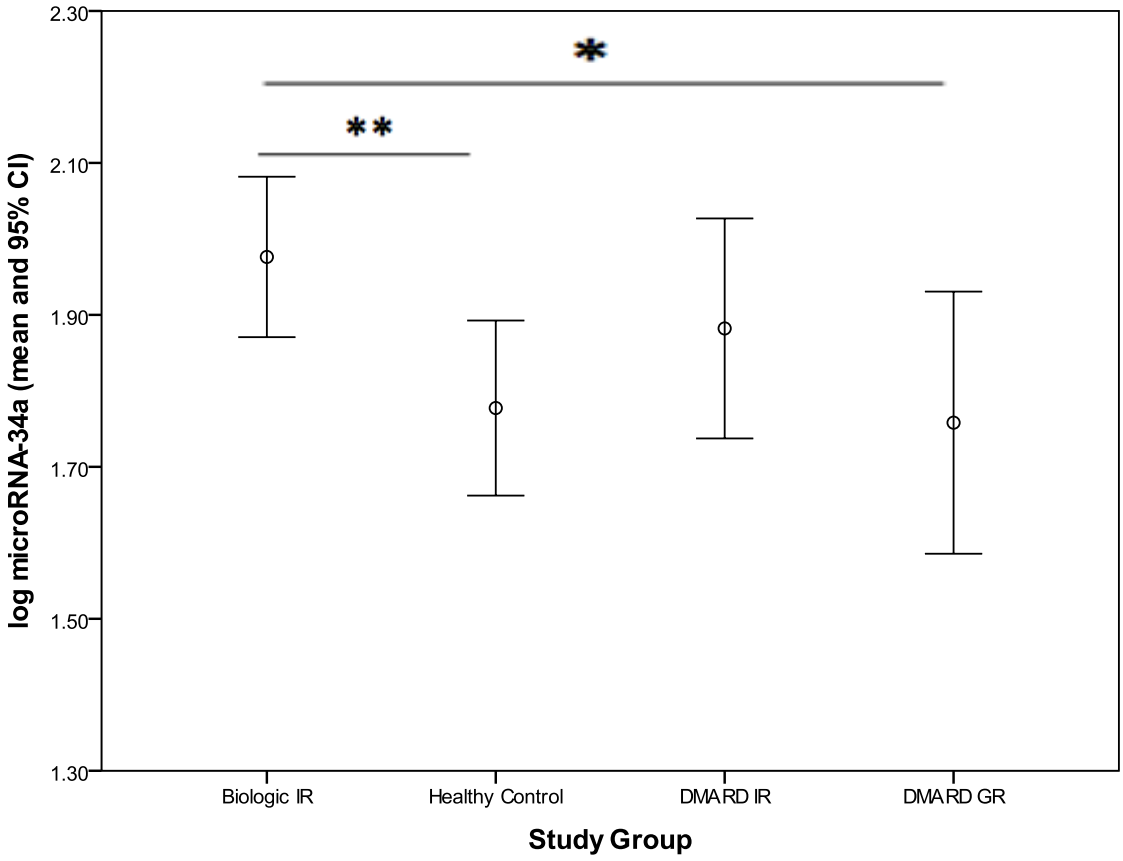

Figure 4-31 MicroRNA-34a between study groups at baseline visit
(mean and 95% error bars (where *=p<0.05 and **=p<0.01))

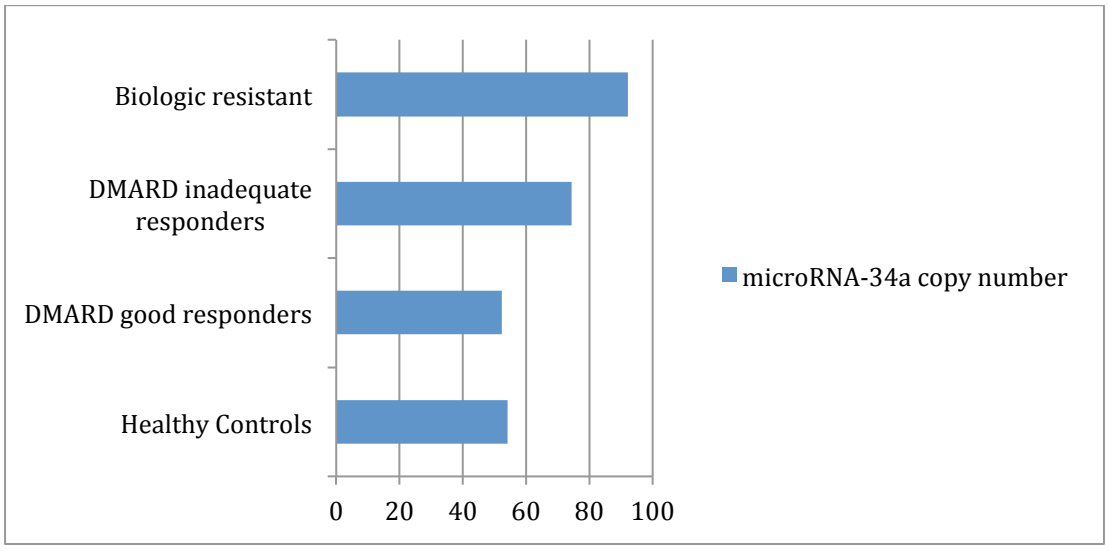

Figure 4-32 MicroRNA-34a copy number between study groups at baseline visit

Cross sectional differences in microRNA-34a copy number were observed. These were highly significant between the biologic resistant and healthy control groups (p=0.008), between the biologic resistant and DMARD responder groups (p=0.024)

4.4.2.1.3 Correlation between microRNA-34a and clinical findings

Median value	MicroRNA-34a copy number (Correlation coefficient (p value))	
	Whole group	Biologic resistant group
SJC	**0.250 (0.040)**	-0.083 (0.668)
TJC	0.149 (0.226)	-0.216 (0.260)
ESR mm/hr	0.044 (0.721)	-0.203 (0.290)
CRP mg/dl	0.230 (0.059)	-0.155 (0.422)
DAS28-ESR	0.147 (0.233)	-0.333 (0.078)
DAS28-CRP	0.151 (0.220)	-0.250 (0.192)

Table 4-79 Correlations between copy number of microRNA-34a (relative to let-7a) and clinical/biochemical variables
(Spearman's coefficient, significance assumed if p<0.05)

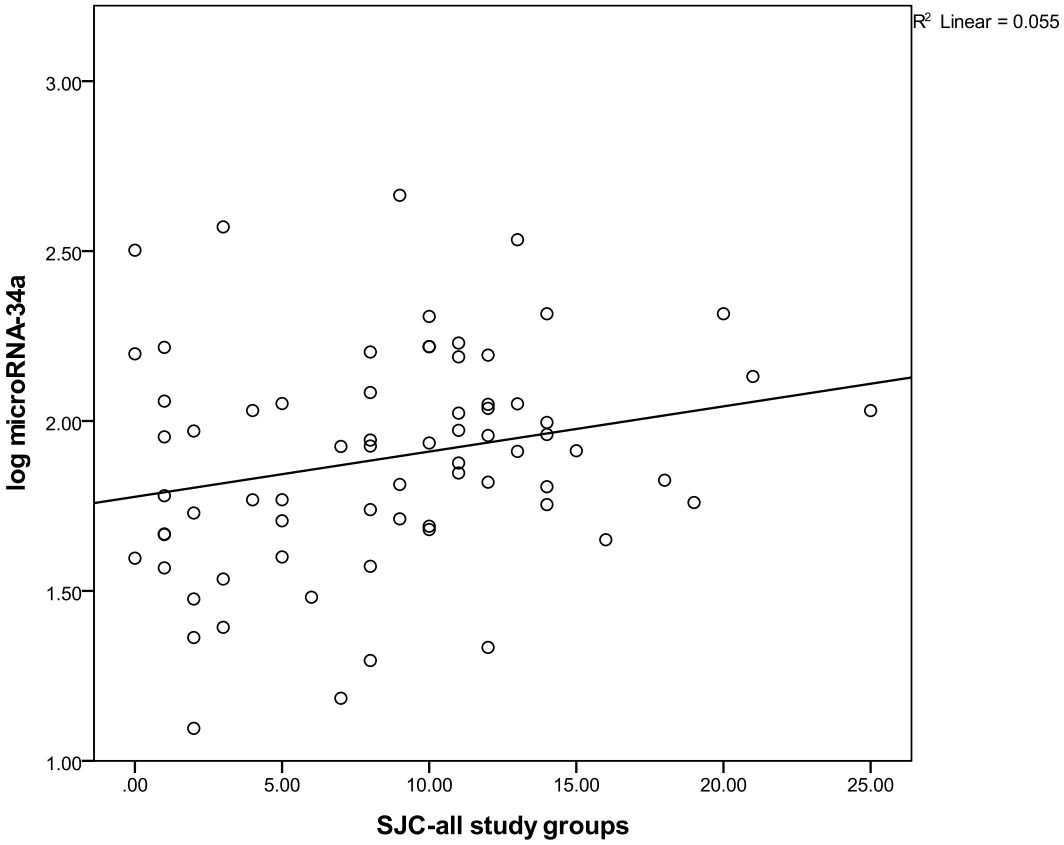

Figure 4-33 Scatterplot of log microRNA-34a and swollen joint count (all groups combined)

There was no correlation between those selected clinical characteristics sand microRNA-34a in serum CD14 cells. Correlation between whole group SJC (shown in Figure 4-36 for illustration) and microRNA-34a copy number was lost when the biologic resistant group was examined alone. Correlation between microRNA-34a copy number and SJC persisted with the DMARD good responder group ($r=-0.559$, $p=0.016$) but not the DMARD resistant group clinical variables.

In summary, there are differences in microRNA-34a copy number between the biologic resistant and healthy/DMARD good responder groups and it correlates with SJC (whole cohort) but not with systemic inflammation biomarkers such as CRP or ESR. It could therefore be hypothesised that this difference is driven by the presence of local synovial inflammation.

4.4.2.2 MicroRNA-27b

4.4.2.2.1 Clinical characteristics and findings

Median value	Biologic Resistant	Healthy Controls	DMARD IR	DMARD GR
	N=30	N=23	N=17	N=18
SJC	11	0	10	1.5
TJC	10	0	14	0.50
ESR mm/hr	23.5	5.0	29	13
CRP mg/dl	11	1.4	10.5	7.3
DAS28-ESR	5.81	n/a	6.30	3.15
DAS28-CRP	5.34	n/a	5.98	2.81

Table 4-80 Clinical variables between groups in microRNA-27b experiment
(where DMARD inadequate responders (DMARD IR) and DMARD GR (DMARD good responders))

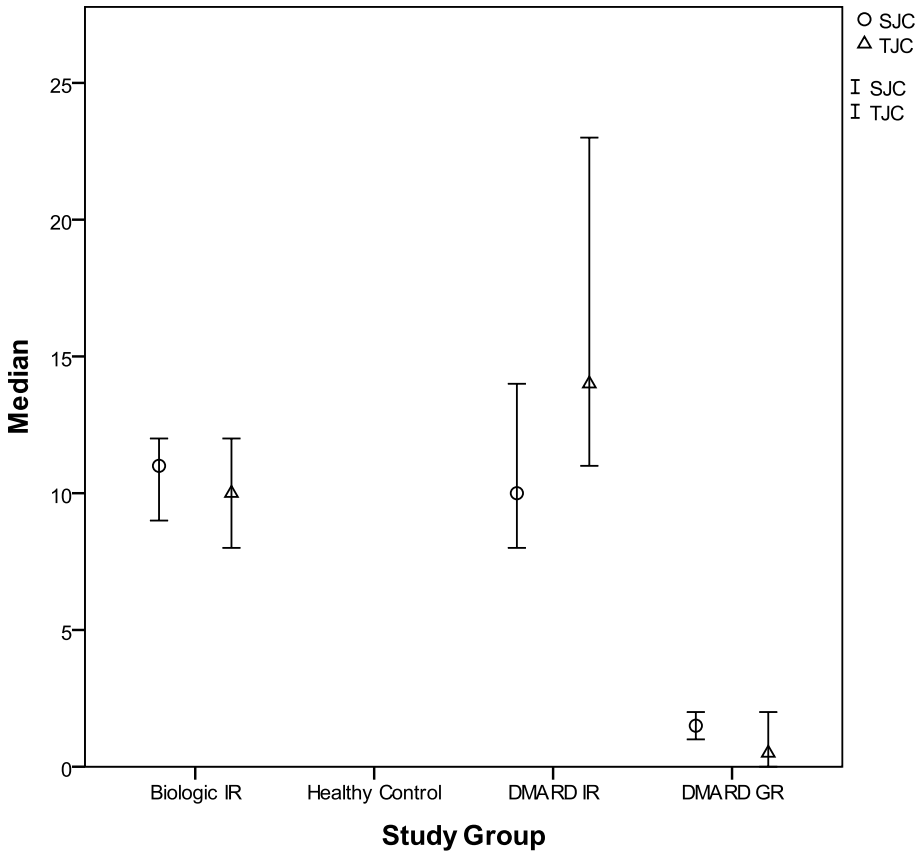

Figure 4-34 Joint counts between study groups in microRNA-27b analysis group

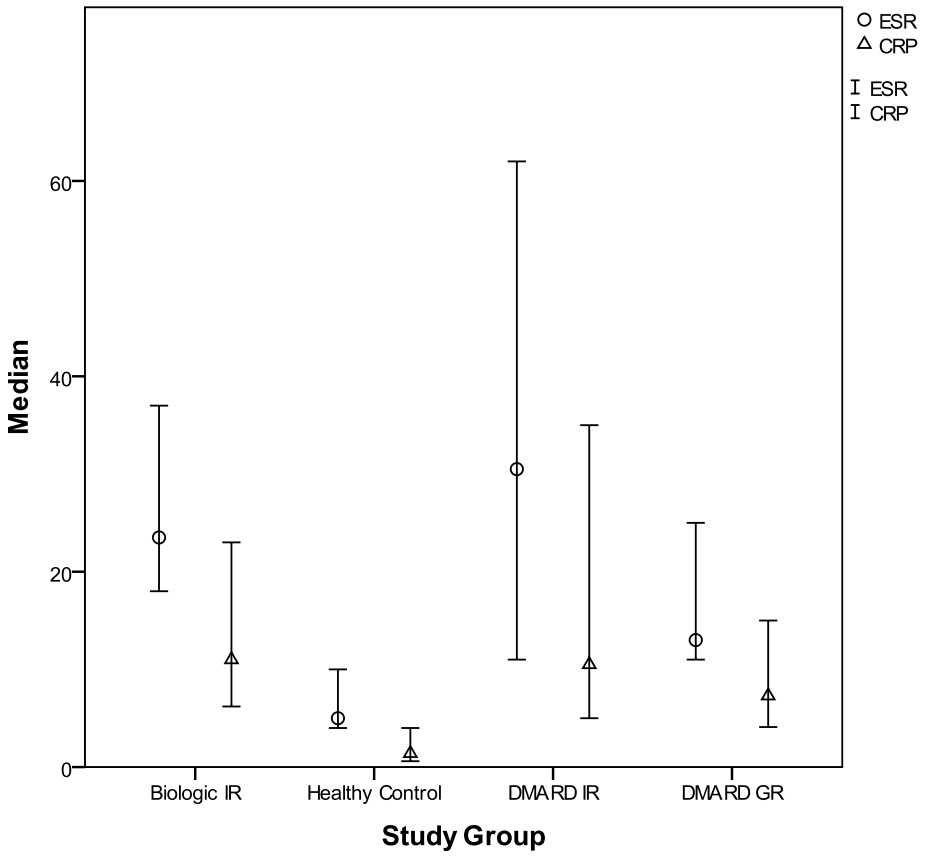

Figure 4-35 Inflammatory markers between study groups in microRNA-27b analysis group

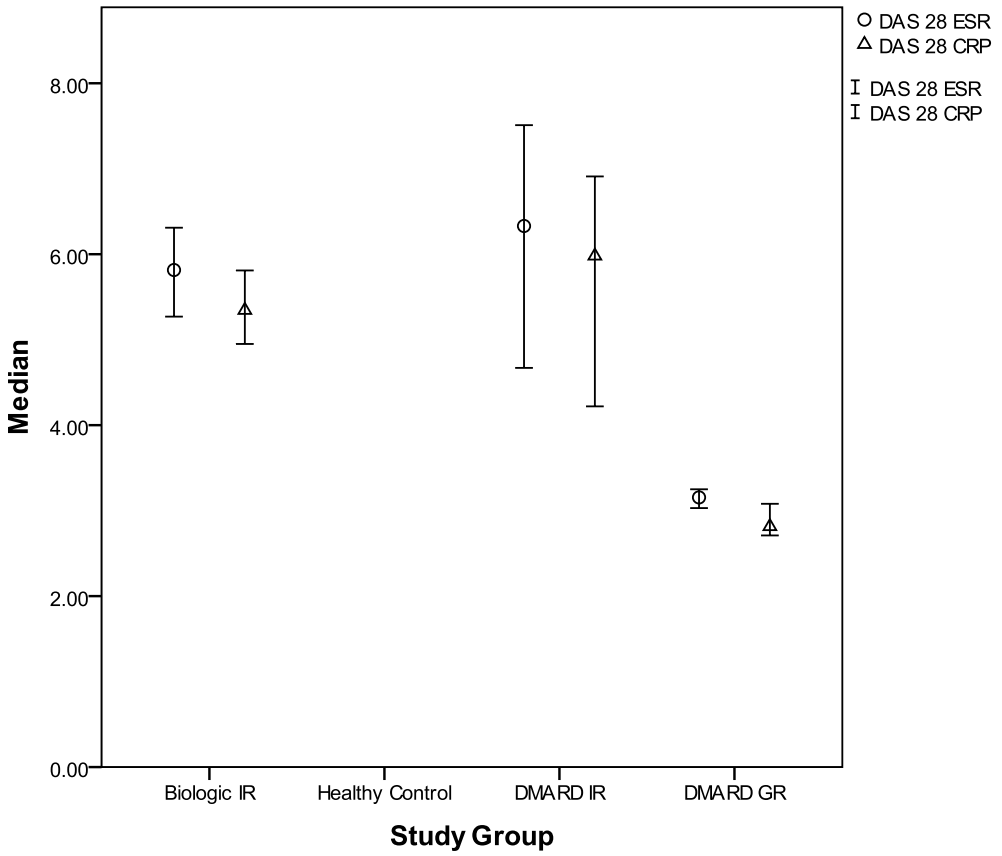

Figure 4-36 DAS28 between study groups in microRNA-27b analysis group

Confirmatory testing between the biologic resistant and DMARD resistant groups was performed. These did not reach statistical significance.

Between the biologic resistant and DMARD good responder group all variables differed (p-values were <0.0001 other than the ESR (0.058) and CRP (0.349)).

Between the DMARD resistant and DMARD good responder group a similar finding was observed (ESR (0.053) and CRP (0.255) between groups did not achieve statistical differences).

4.4.2.2.2 MicroRNA-27b cross sectional analysis

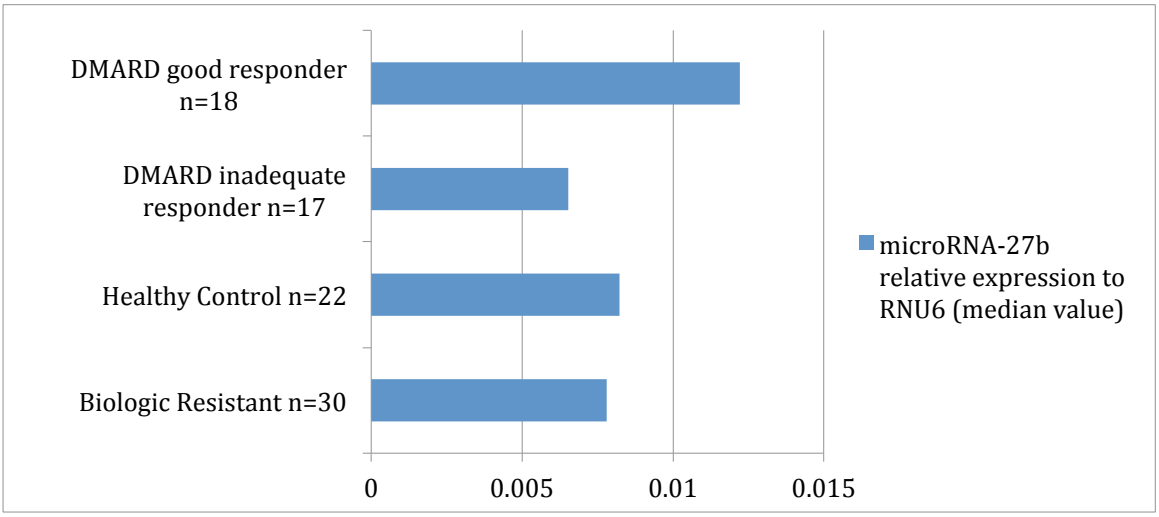

Figure 4-37 MicroRNA-27b relative expression levels between study groups at baseline

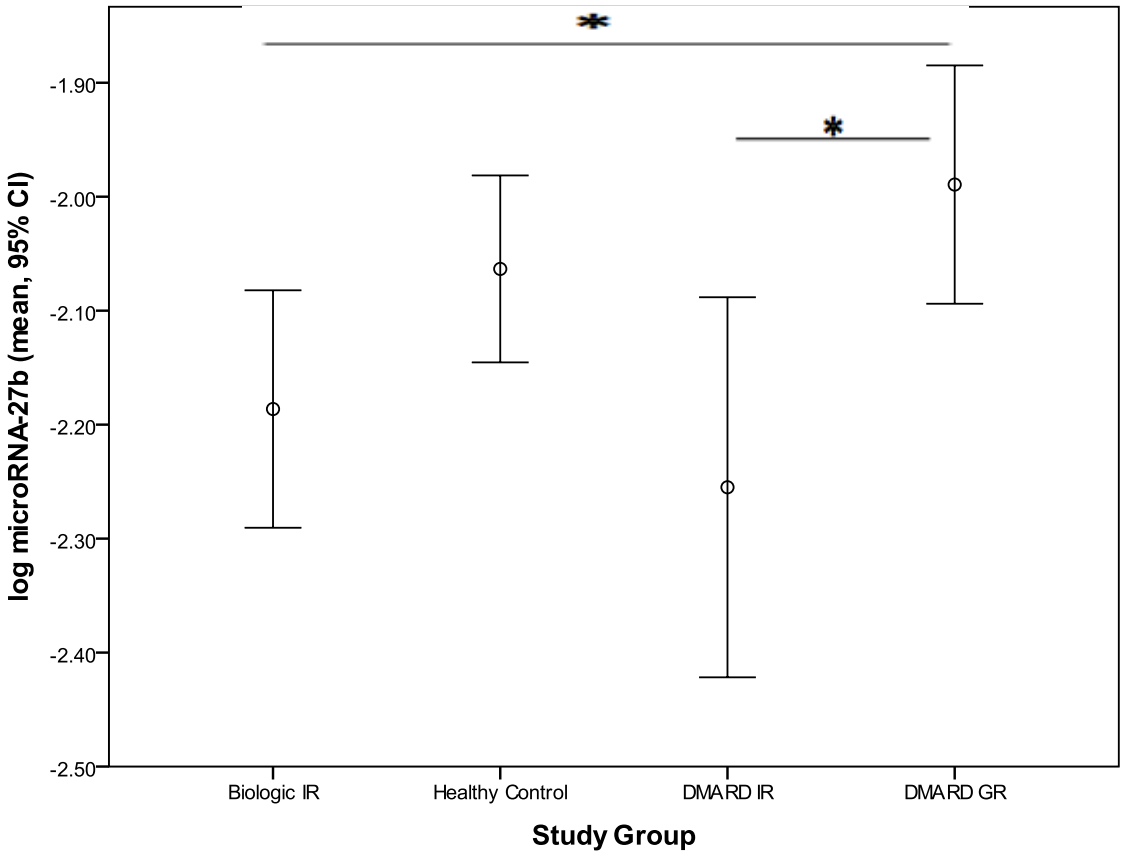

Figure 4-38 Relative expression of microRNA-27b between study groups at baseline
(where *=p<0.05 and **=p<0.01)

Clinically, the biologic resistant and DMARD IR groups were very similar and may explain the observation that microRNA-27b was down-regulated in both the biologic resistant (p=0.011) and DMARD resistant groups when compared to the DMARD good responder group (p=0.013) in PB CD14 cells. This could therefore be hypothesised as inflammation driven.

4.4.2.2.3 Correlation between microRNA-27b and clinical variables

Median value	MicroRNA-27b relative expression (Correlation coefficient (p value))	
	Whole group	Biologic resistant group
SJC	**-0.293(0.019)**	-0.215 (0.253)
TJC	-0.226 (0.072)	0.140 (0.461)
ESR mm/hr	-0.007 (0.957)	-0.044 (0.816)
CRP mg/dl	-0.084 (0.508)	-0.210(0.265)
DAS28-ESR	-0.220 (0.081)	0.047 (0.804)
DAS28-CRP	-0.240 (0.056)	-0.026 (0.890)

Table 4-81 Correlations between relative expression of microRNA-27b and clinical/biochemical variables

(significance assumed if p<0.05)

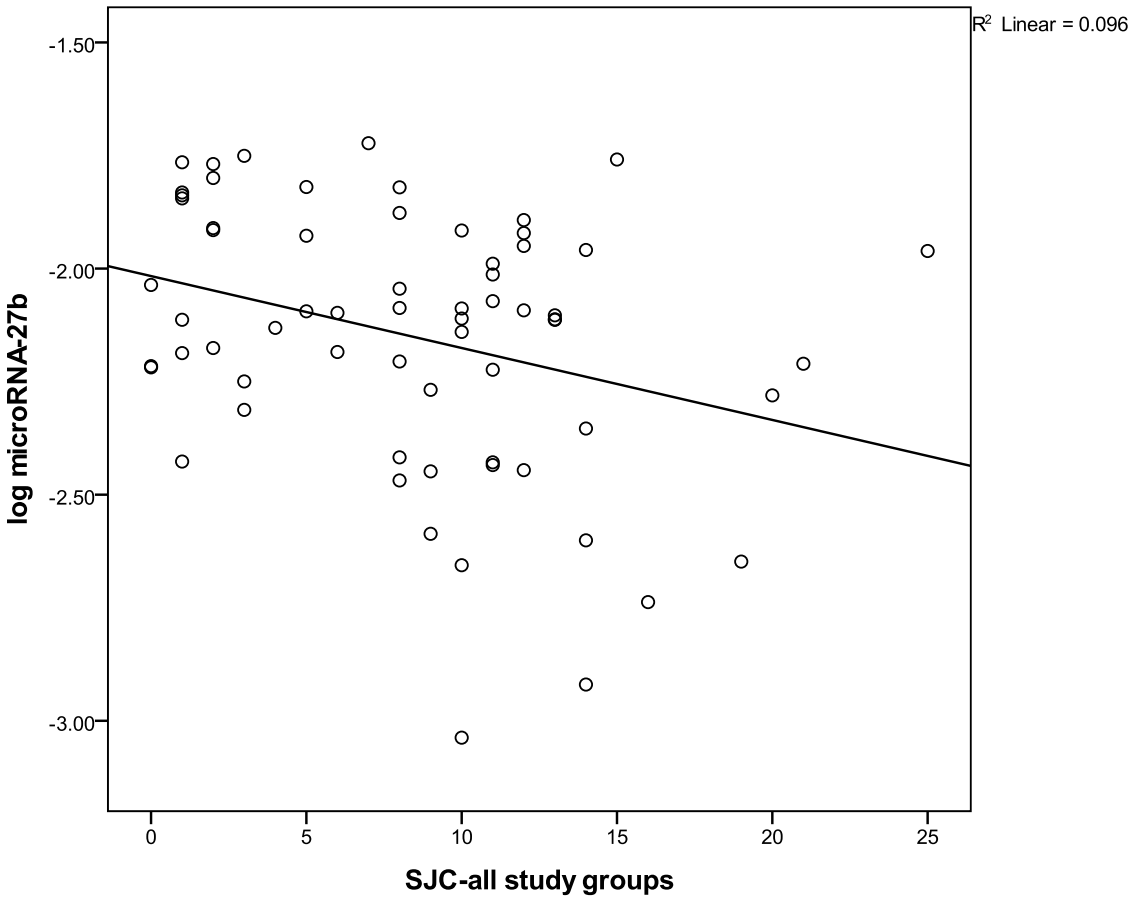

Figure 4-39 Scatterplot of relative expression levels of microRNA-27b and swollen joint group (all study groups combined)

However, once again there was a lack of correlation with selected clinical variables (e.g. CRP, DAS28) and microRNA-27b relative expression other than correlation with swollen joint count. This effect was lost when the biologic resistant group were examined in isolation. Correlation with the DMARD good responder group TJC was seen with microRNA-27b (R -0.572, p=0.013) but no correlation with the DMARD resistant group clinical variables. Thus, down-regulation of miR27b expression in biologic resistant and DMARD resistant patients could reflect local synovial inflammation rather than systemic inflammation.

4.4.2.3 MicroRNA-125a

4.4.2.3.1 Clinical characteristics and findings

Median value	Biologic Resistant	Healthy Controls	DMARD IR	DMARD GR
	n=24	n=16	n=11	n=15
SJC	11	0	10	2
TJC	10	0	17	0
ESR mm/hr	23.5	8	32	20
CRP mg/dl	12.5	1.8	22.5	12.0
DAS28-ESR	5.81	n/a	6.98	3.15
DAS28-CRP	5.45	n/a	6.39	2.81

Table 4-82 Clinical variables between groups in microRNA-125a experiment
(DMARD inadequate responders (DMARD IR) and DMARD GR (DMARD good responders))

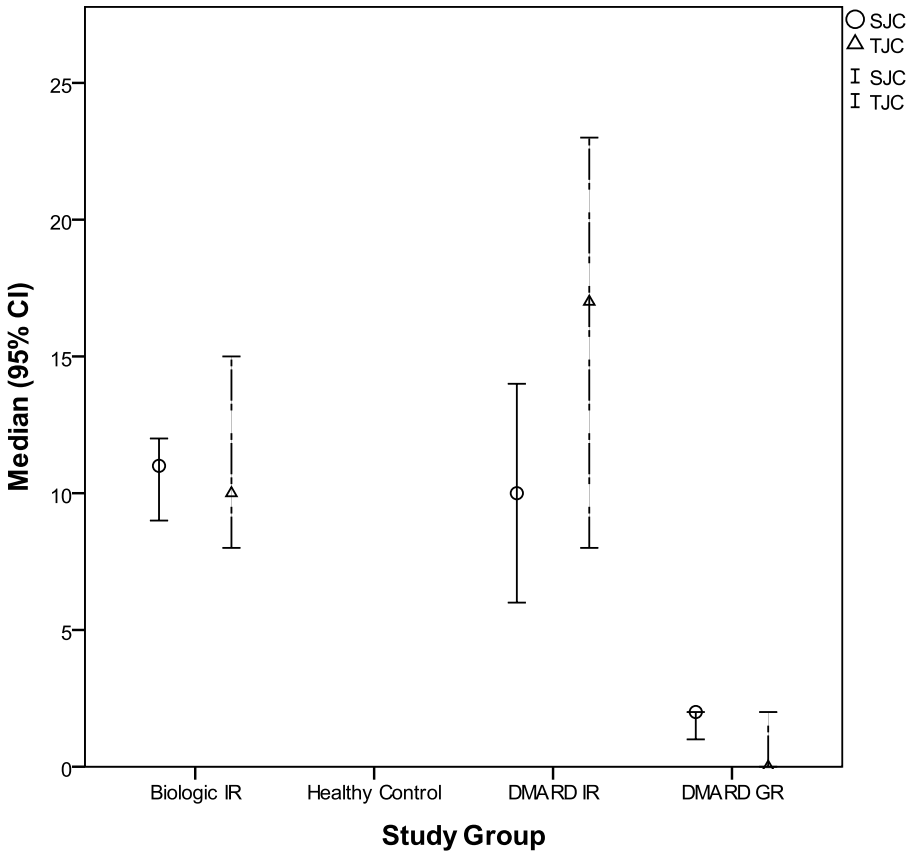

Figure 4-40 Joint counts between study groups in microRNA-125a analysis group

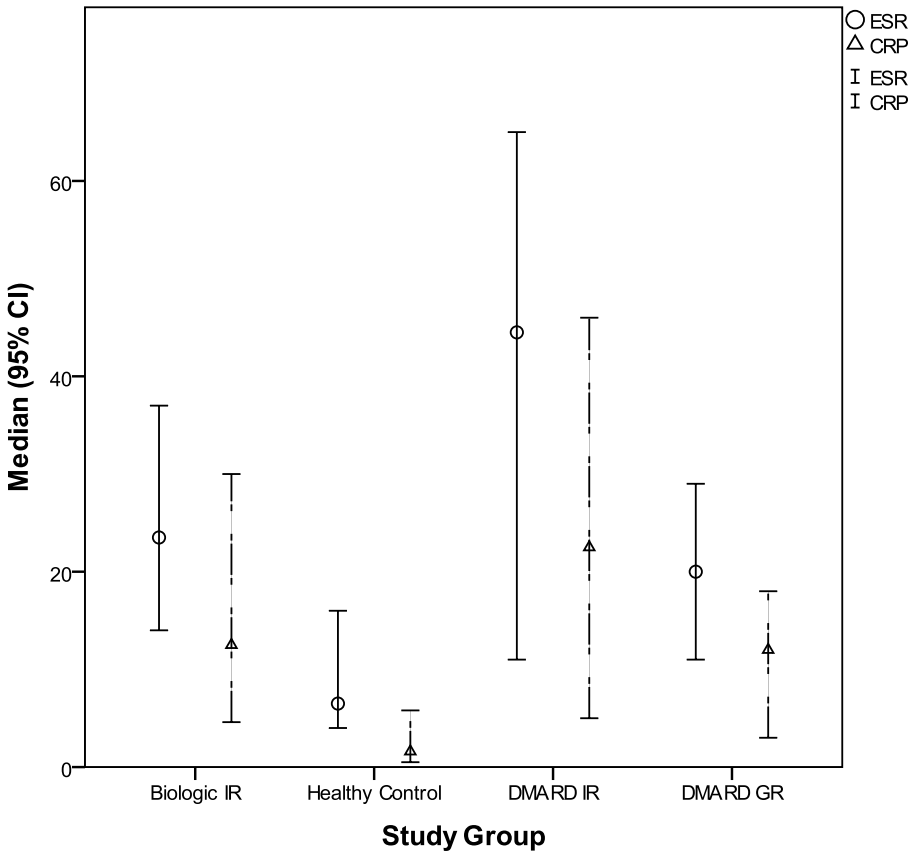

Figure 4-41 Inflammatory markers between study groups in microRNA-125a analysis group

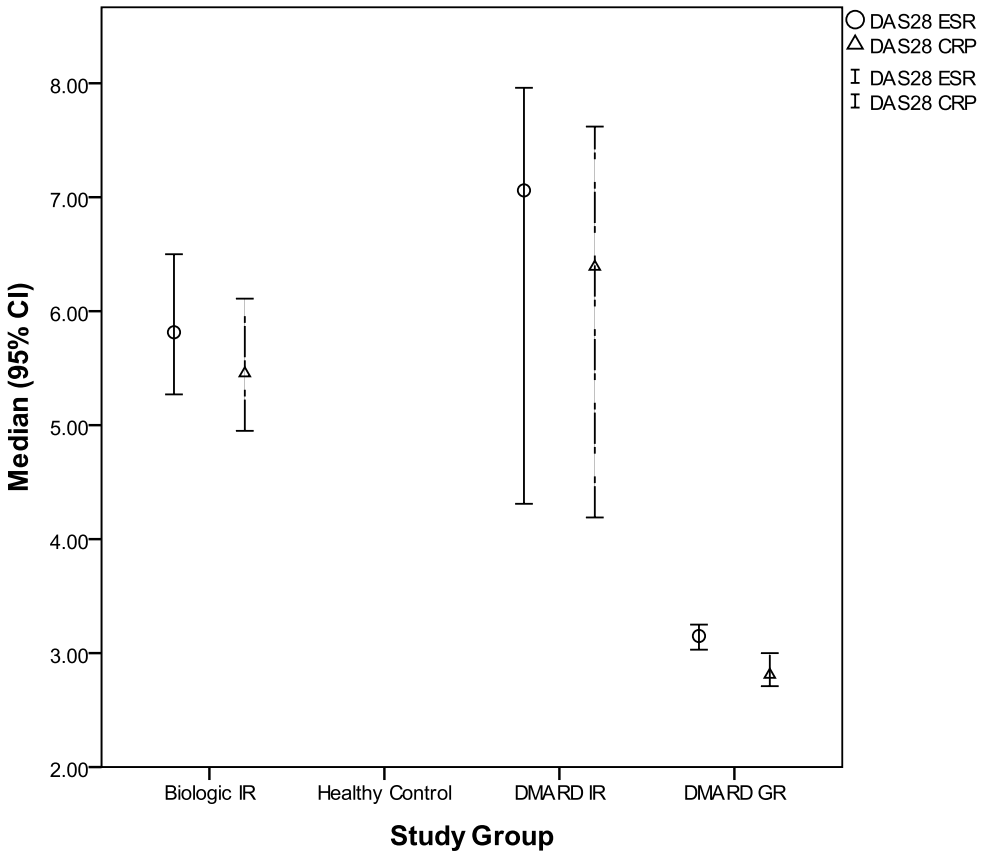

Figure 4-42 DAS28 between study groups in microRNA-125a analysis group

Disease activity was present in both the biologic resistant and DMARD resistant group when measured biochemically, clinically and using composite measures. There was no statistical difference between any of the variables (where $p<0.05$) between the biologic resistant and DMARD IR groups.

4.4.2.3.2 MicroRNA-125a cross sectional analysis

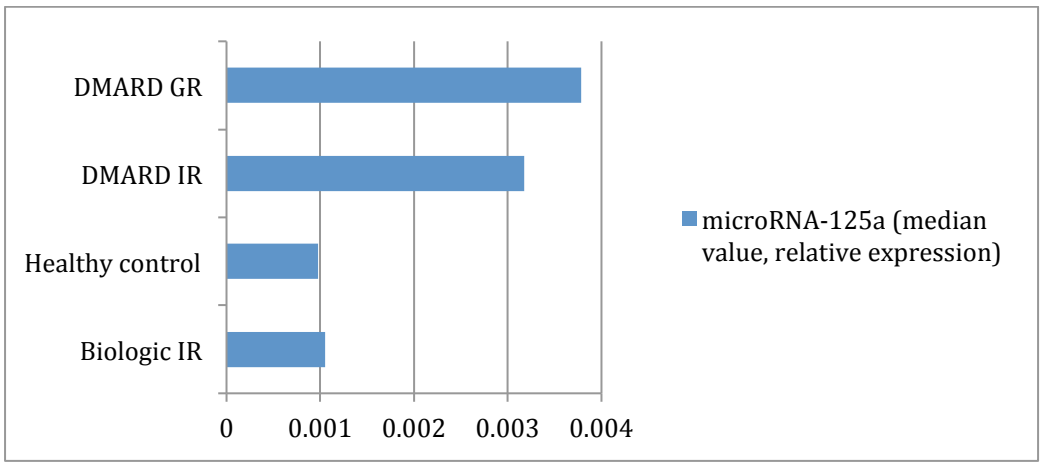

Figure 4-43 Relative expression levels of microRNA-125a between study groups at baseline

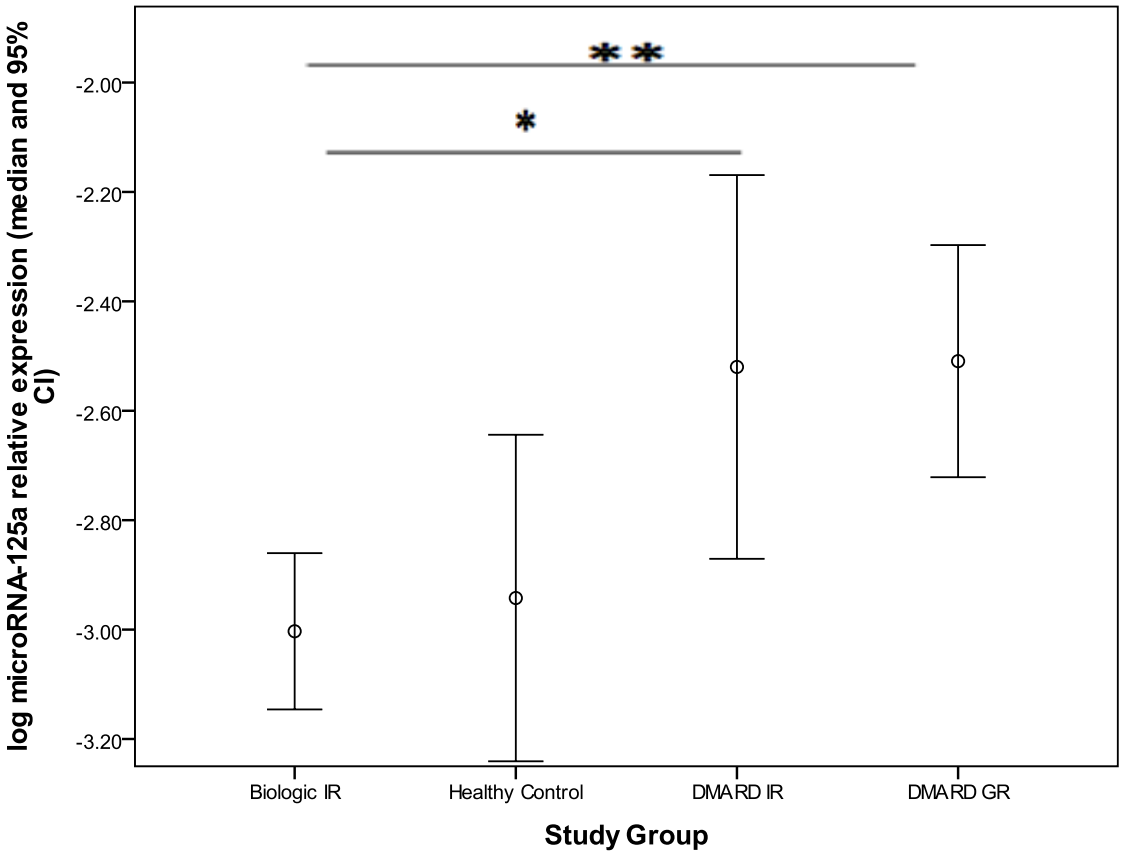

Figure 4-44 Relative expression levels of microRNA-125a between study groups at baseline visit *(where *=p<0.05 and **=p<0.01)*

MicroRNA-125a is down regulated in the biologic resistant RA PB CD14 cells compared with comparably active DMARD resistant patients (p=0.011) and DMARD good responders (p=0.001) with low clinical disease activity. Values are similar to those of healthy controls. Higher levels of miR-125a in PB CD14+ cells in both DMARD responsive and DMARDs resistant groups, as compared to other groups (healthy and biologic resistant) may suggest that miR-125a is associated with DMARD therapy (or specific anti-cytokine reduction) but not disease status.

4.4.2.3.3 Correlation between microRNA-125a and clinical variables

Median value	MicroRNA-125a relative expression (Correlation coefficient (p value))	
	Whole group	Biologic resistant group
SJC	**-0.389 (0.006)**	-0.065 (0.761)
TJC	**0.0.353 (0.013)**	-0/282 (0.181)
ESR mm/hr	0.125 (0.391)	0.202 (0.344)
CRP mg/dl	0.029 (0.845)	0.244 (0.250)
DAS28-ESR	-0.221 (0.127)	-0.002 (0.994)
DAS28-CRP	-0.277 (0.054)	-0.028 (0.896)

Table 4-83 Correlations between relative expression of microRNA-125b and clinical/biochemical variables (where significance assumed if p<0.05)

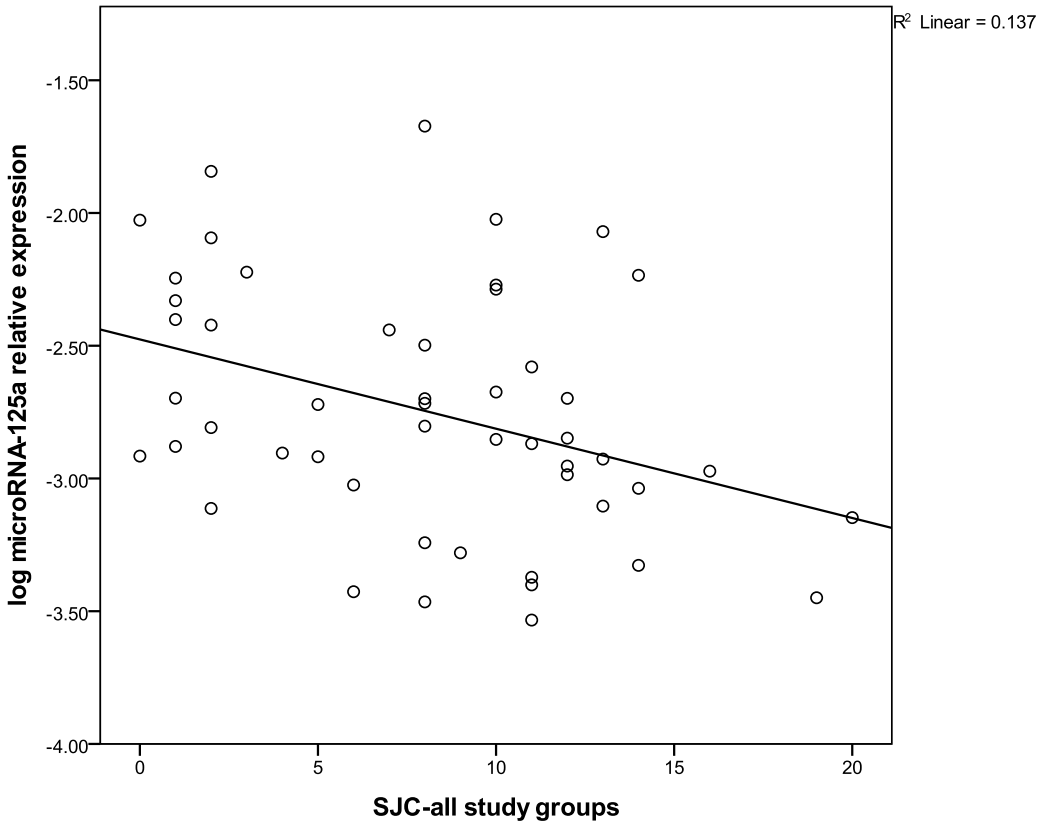

Figure 4-45 Scatterplot of relative expression of microRNA-125a at baseline and swollen joint count (all study groups combined)

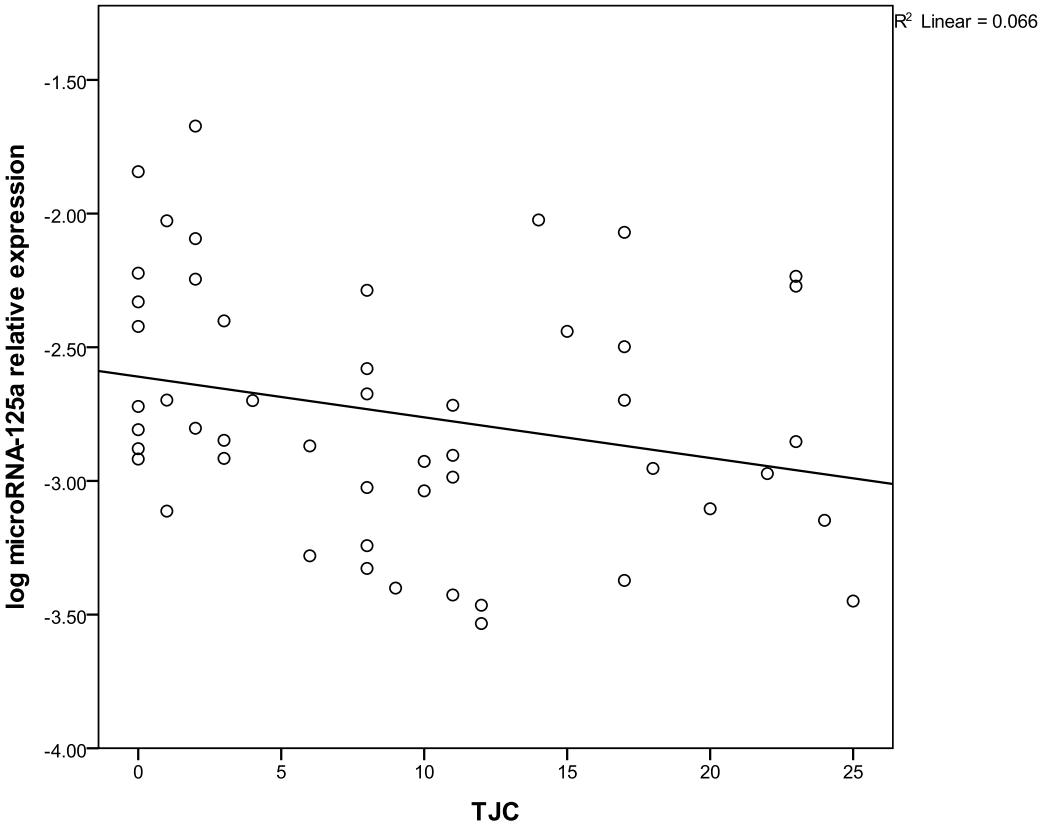

Figure 4-46 Scatterplot of relative expression of microRNA-125a at baseline and tender joint count (all study groups combined)

In a similar fashion to microRNA-27b, microRNA-125a correlated with tender and swollen joint counts but this effect was lost when the biologic resistant group were examined in isolation. Subsequently, no further correlation was seen between microRNA-125a and clinical variables in either the DMARD resistant or the DMARD good responder group.

4.4.2.4 Summary

Examination of the serum profile of several candidate microRNA in PB monocytes raises several points.

- **CD14+ cell microRNA profiles differ between the RA cohorts gathered**

This observation would suggest the aim to create distinct cohorts has been successful and a DMARD good responder group mitigated the effect of disease duration. Additionally, distinct profiles between control groups suggest pathophysiological/molecular significance.

- **There is an overall poor correlation between serum CD14+ cell microRNA-34a, -27b and -125a and clinical findings, inflammatory markers and composite disease activity scores other than swollen joint counts. This suggests that the differential expression of these candidate microRNAs may be an underlying factor contributing to response to therapies rather than simple consequence of disease activity.**

This observation is discussed in 'Chapter Conclusions and Discussion'.

4.4.3 Results- 'hypothesis free' global profiling

Global microRNA expression was first performed by Expression Analysis USA as previously described. This was performed on **all** baseline study samples as referred to in Chapter 3 and is shown in Figure 4-50 and 4.51.

Biologic IR= Biologic inadequate responders
DMARD IR= DMARD inadequate responders
DMARD GR= DMARD good responders

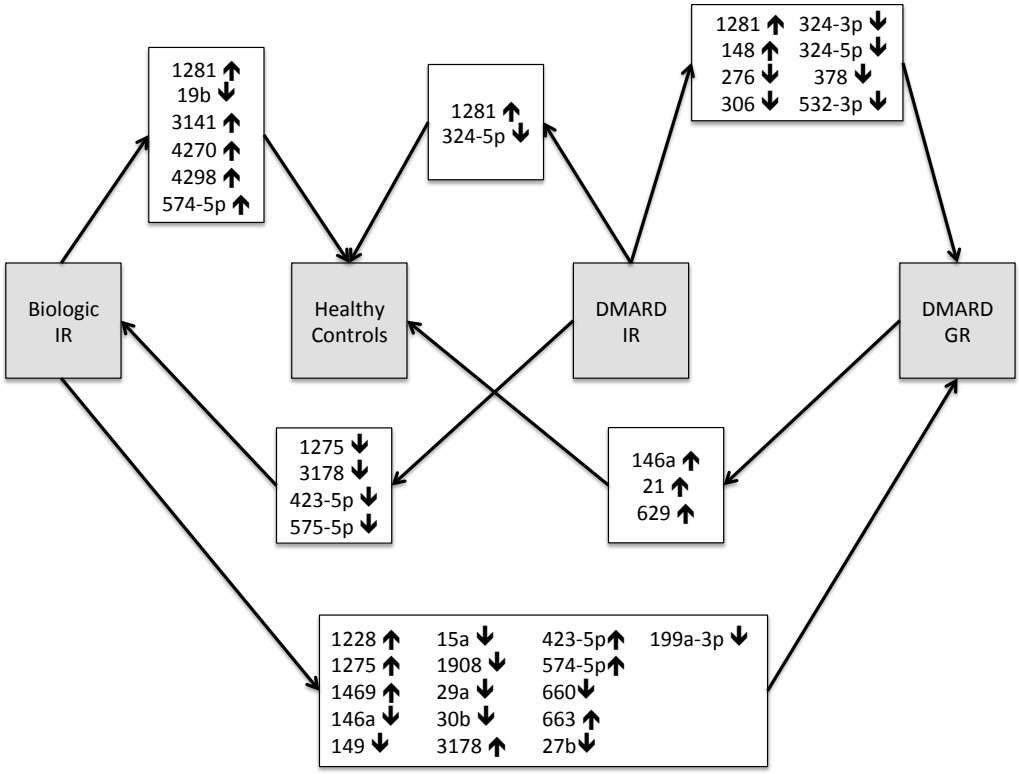

Figure 4-47 Differentially expressed microRNA between study groups

Figure 4.50 shows the relative direction of expression of microRNA between groups. For example, microRNA -1275, -3178, -423-5p and -575-5p expression levels are reduced in the DMARD IR group relation to the Biologic resistant group.

Group comparison	MicroRNA	Fold change	P value	FDR
DMARD GR v HV	146a	+1.58	0.025	0.12
	21	+1.65	<0.001	0.003
	629	+1.56	0.02	0.09
DMARD IR v HV	1281	+1.88	0.001	0.001
	324-5p	-1.56	<0.001	0.009
Biologic IR v HV	1281	+1.72	<0.001	<0.001
	19b	-1.60	<0.001	<0.001
	3141	+1.62	<0.001	<0.001
	4270	+1.50	<0.001	<0.001
	4298	+1.51	<0.001	<0.001
	574-5p	+1.97	<0.001	<0.001
DMARD IR v DMARD GR	1281	+1.59	0.017	0.055
	149	+1.58	<0.001	0.001
	27b	-1.77	<0.001	<0.001
	30b	-1.58	<0.001	0.004
	324-3p	-1.52	<0.001	<0.001
	324-5p	-1.64	<0.001	0.002
	378	-1.50	<0.001	0.002
	532-3p	-1.51	<0.001	<0.001
Biologic IR v DMARD GR	1228	+1.68	<0.001	<0.001
	1275	+1.75	<0.001	<0.001
	1469	+1.55	<0.001	<0.001
	146a	-1.66	<0.001	0.005
	149	+1.63	<0.001	<0.001
	15a	-1.51	<0.001	<0.001
	1908	+1.55	<0.001	<0.001
	199a-3p	-1.54	<0.001	<0.001
	27b	-1.53	<0.001	<0.001
	29a	-1.70	<0.001	<0.001
	30b	-1.76	<0.001	<0.001
	3178	+1.73	0.003	0.007
	423-5p	+1.64	<0.001	<0.001
	574-5p	+1.59	<0.001	<0.001
	660	-1.54	<0.001	<0.001
	663	+1.53	0.0014	0.003
DMARD IR v Biologic IR	1275	-1.55	<0.001	<0.001
	3178	-2.09	<0.001	<0.001
	423-5p	-1.57	<0.001	<0.001
	574-5p	-1.63	<0.001	<0.001

Table 4-84 Differential expression of microRNA between groups and statistical evaluation

RD, as outlined above, carried out confirmatory qPCR analysis of those four microRNA returned as differentially expressed between the **biologic resistant and DMARD resistant** groups in the four study groups. RD analysed CD14+ cells in selected baseline study visit samples using the method described above (those samples chosen represented samples with sufficient RNA quantities and quality).

4.4.3.1 'Biologic resistant' microRNA; clinical findings of patients studied in validating qPCR group

Median value	Biologic Resistant	Healthy Controls	DMARD IR	DMARD GR
	N=46	N=14	N=12	N=18
SJC	10	0	11	1.5
TJC	8	0	14	0.5
ESR mm/hr	22	1.2	18	13
CRP mg/dl	9.9	4.5	6	7.3
DAS28-ESR	5.36	n/a	5.69	3.15
DAS28-CRP	5.13	n/a	5.15	2.81
Age (yrs)	60.5	50	57	60.5
Disease duration (months)	204	n/a	116	216.5
Double antibody negative	17%	n/a	0%	6%
Male	15%	7%	17%	39%
Current smokers	15%	36%	50%	22%

Table 4-85 Clinical variables between patients studied for microRNA-423, -574, -1275 and -3178 qPCR

The clinical variables for the patients studied for the qPCR (n=46) experiment are shown in order to examine differences from the patients used in the microarray (n=50).

The biologic resistant group had comparable clinical variables, smoking status, age, gender and immune status. Disease duration was slightly lower at 204 v 213 months. Healthy controls in the qPCR group were more likely to be female (93% female vs 84%) and higher smoking rates (36% vs 20%). The DMARD good responder group in the validation group were very similar in all respects to the main study group (disease duration shorter 216.5 months vs 222 months).
The DMARD resistant group varied more notably however (n=12 vs n=26). There were fewer males (17% in the validation group vs 23% in the main study group) and proportionally more smokers (50% vs 38%). With respect to clinical variables, differences in median values were present but not statistically different (where qPCR

group vs main study group); ESR 18mm/hr vs 25 (p=0.413), CRP 6.0mg/dl vs 10mg/dl (0.110) and DAS28-ESR 5.69 vs 6.04 (p=0.470).

Therefore, the biologic and DMARD resistant group may be considered similar in clinical and biochemical measures of disease activity (except TJC p=-0.017) but differ in gender and smoking status (uncertain relevance in microRNA research).

4.4.3.2 MicroRNA-423, -1275, -574 and -3178 cross sectional analysis at baseline

4.4.3.2.1 MicroRNA-423

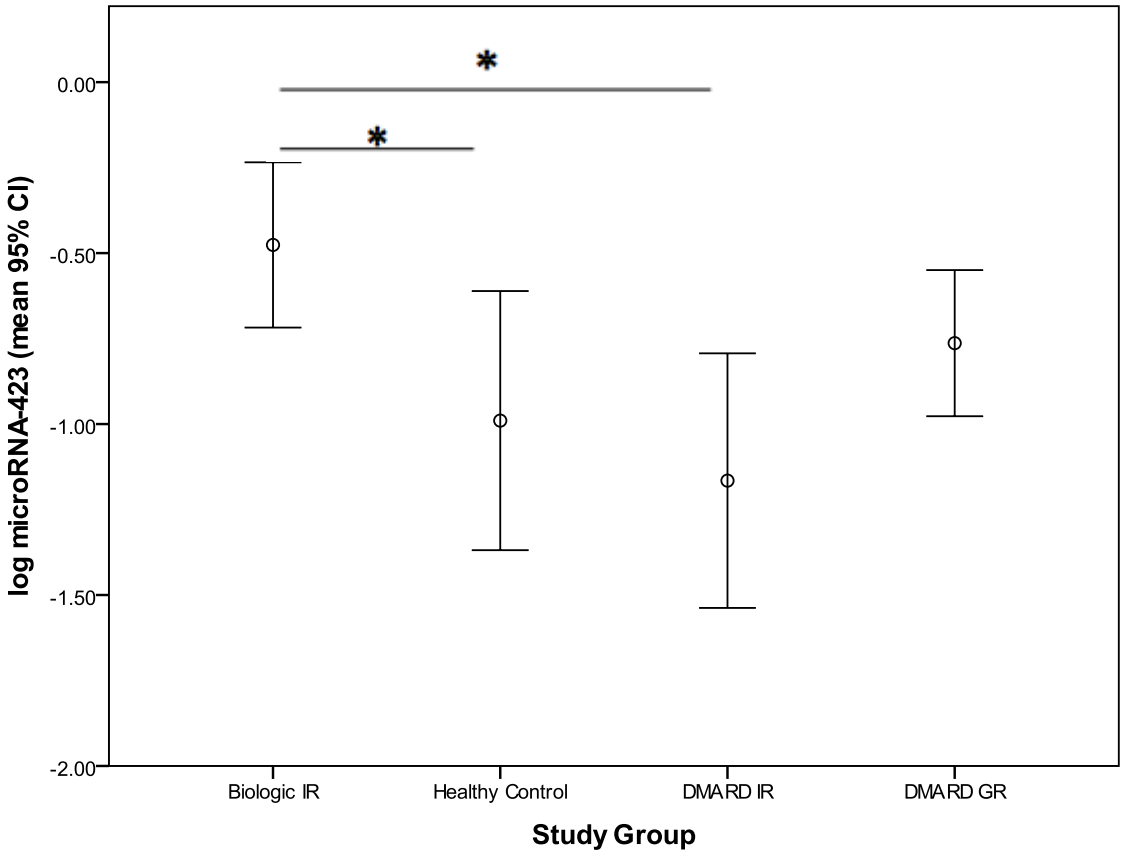

Figure 4-48 Relative expression levels of microRNA-423 between study groups at baseline study visit (where *=p<0.05 and **=p<0.01)

Significant differences were confirmed between the biologic resistant and healthy groups (p=0.028), and the DMARD resistant group (p=0.019) but not the DMARD good responder group.

4.4.3.2.2 MicroRNA-1275

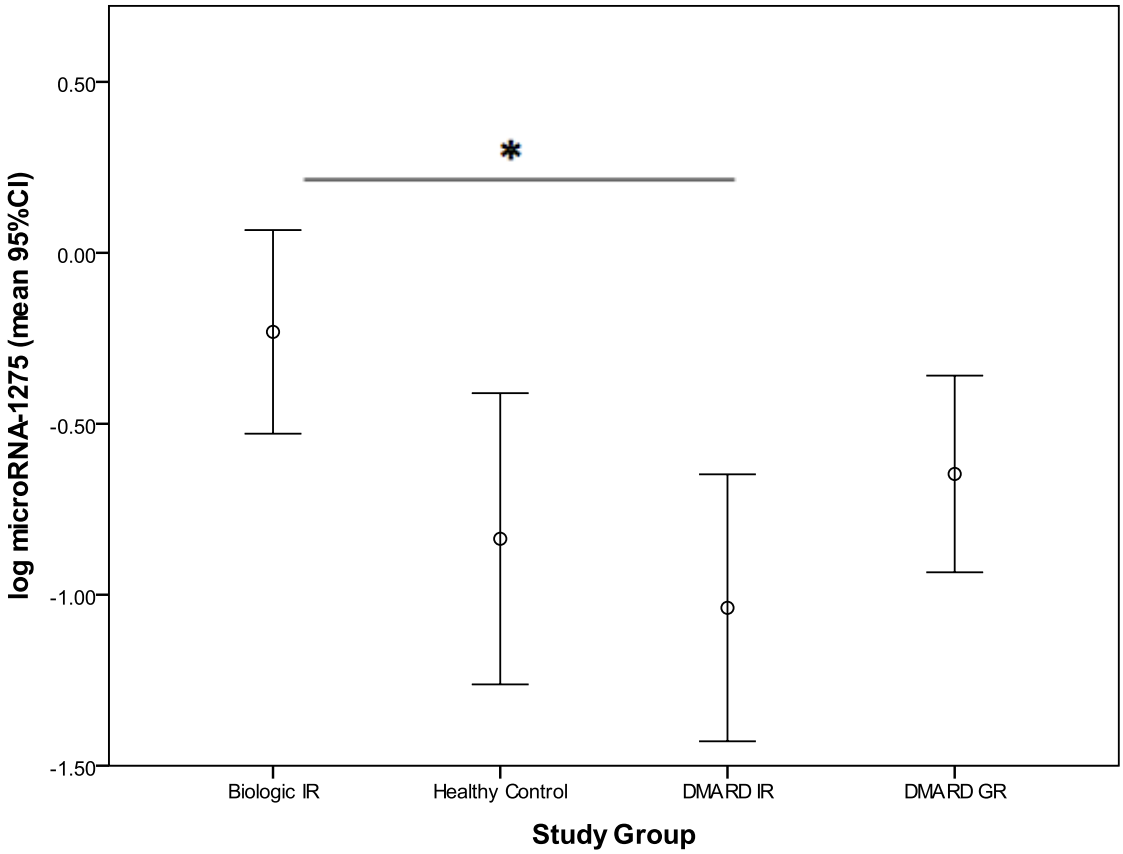

Figure 4-49 Relative expression levels of microRNA-1275 between study groups at baseline study visit (where *=p<0.05 and **=p<0.01)

Significant differences were noted between the biologic resistant and DMARD resistant groups (p=0.017) but not the healthy control group (p=0.061).

4.4.3.2.3 MicroRNA-574

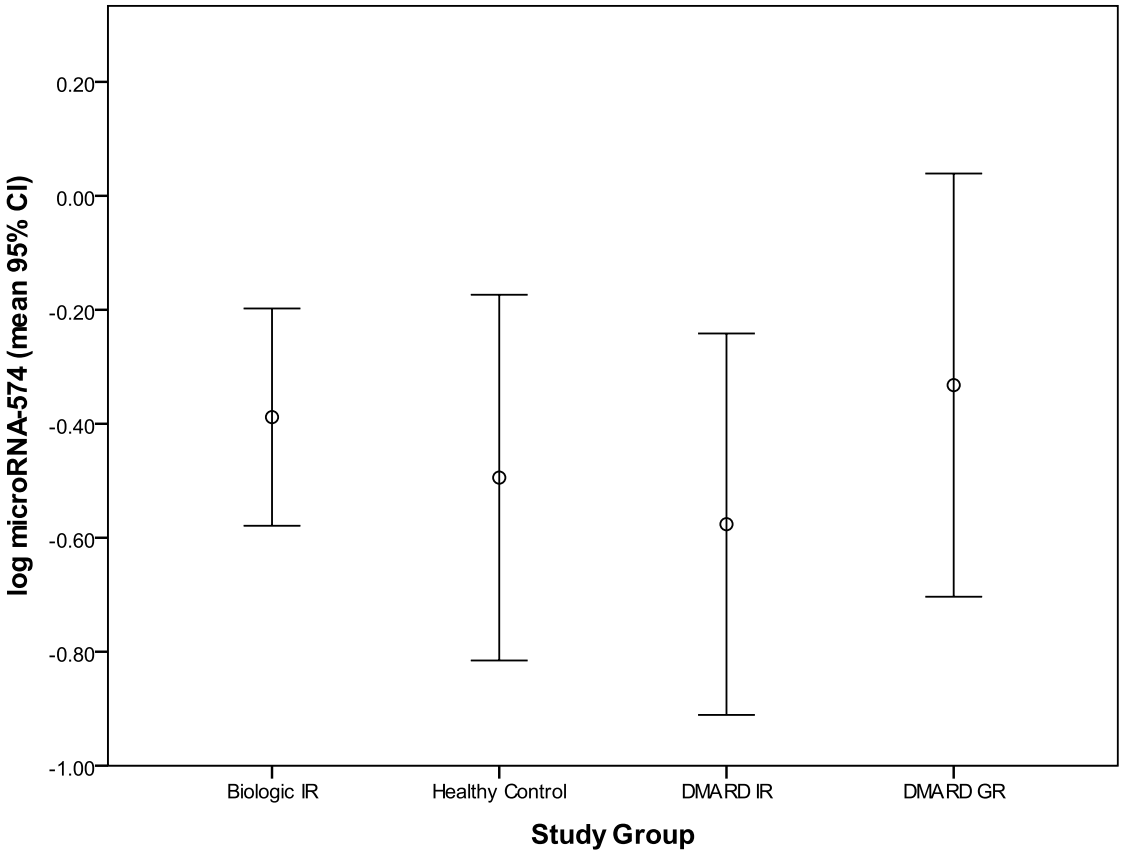

Figure 4-50 Relative expression levels of microRNA-574 between study groups at baseline study visit (where *=p<0.05 and **=p<0.01)

No significant differences were noted between the groups

4.4.3.2.4 MicroRNA-3178

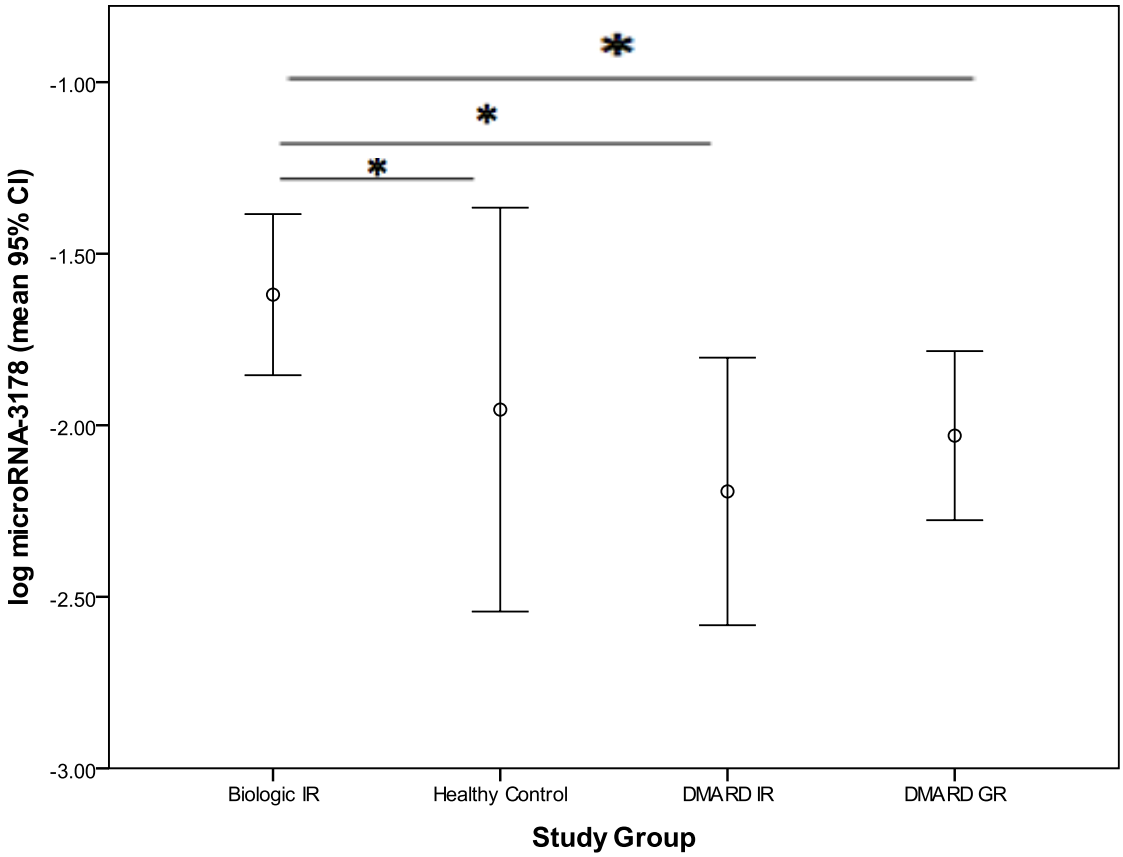

Figure 4-51 Relative expression levels of microRNA-3178 between study groups at baseline study visit (where *=p<0.05 and **=p<0.01)

Significant differences were confirmed between the biologic resistant group and the Healthy controls (p=0.039), DMARD resistant (p=0.040) and DMARD good responders (p=0.050).

The microarray results (Figure 4.50) identified a panel of four microRNA that were differentially expressed in the biologic resistant group compared to the DMARD resistant group that we sought to confirm with qPCR. We were not able to confirm microRNA-574 as being differentially expressed neither between biologic resistant and healthy controls nor between biologic resistant and DMARD good responders. However, qPCR confirmed differential expression of three other microRNA from that panel. MicroRNA-1275 was confirmed as differentially expressed between biologic and DMARD resistant patients, but not biologic resistant and DMARD good responder. MicroRNA-423 also differed from the healthy control group and DMARD resistant group. Finally, microRNA-3178 showed differential expression in the biologic resistant group compared to all other groups. This suggests that all three microRNAs, and microRNA-3178 in particular, could serve as a biomarker of resistance to biologic therapies.

4.4.3.3 Correlation between expressed 'biologic resistant' microRNA

Close correlations were noted *between* those four microRNA identified. The strongest association was between microRNA-423 and -1275

MicroRNA association tested	R^2	r-value (p value)
423:1275	0.845	0.891 (p<0.0001)
423:574	0.608	0.819 (p<0.0001)
423:3178	0.376	0.638 (p<0.0001)
574:1275	0.596	0.821 (p<0.0001)
574:3178	0.332	0.623 (p<0.0001)
1275:3178	0.491	0.736 (p<0.0001)

Table 4-86 Correlation between microRNA-423, -1275, -1275 and -3178

These correlations are represented in figures 4-55 to 4-57. This pattern raises the possibility of co-transcription or regulation.

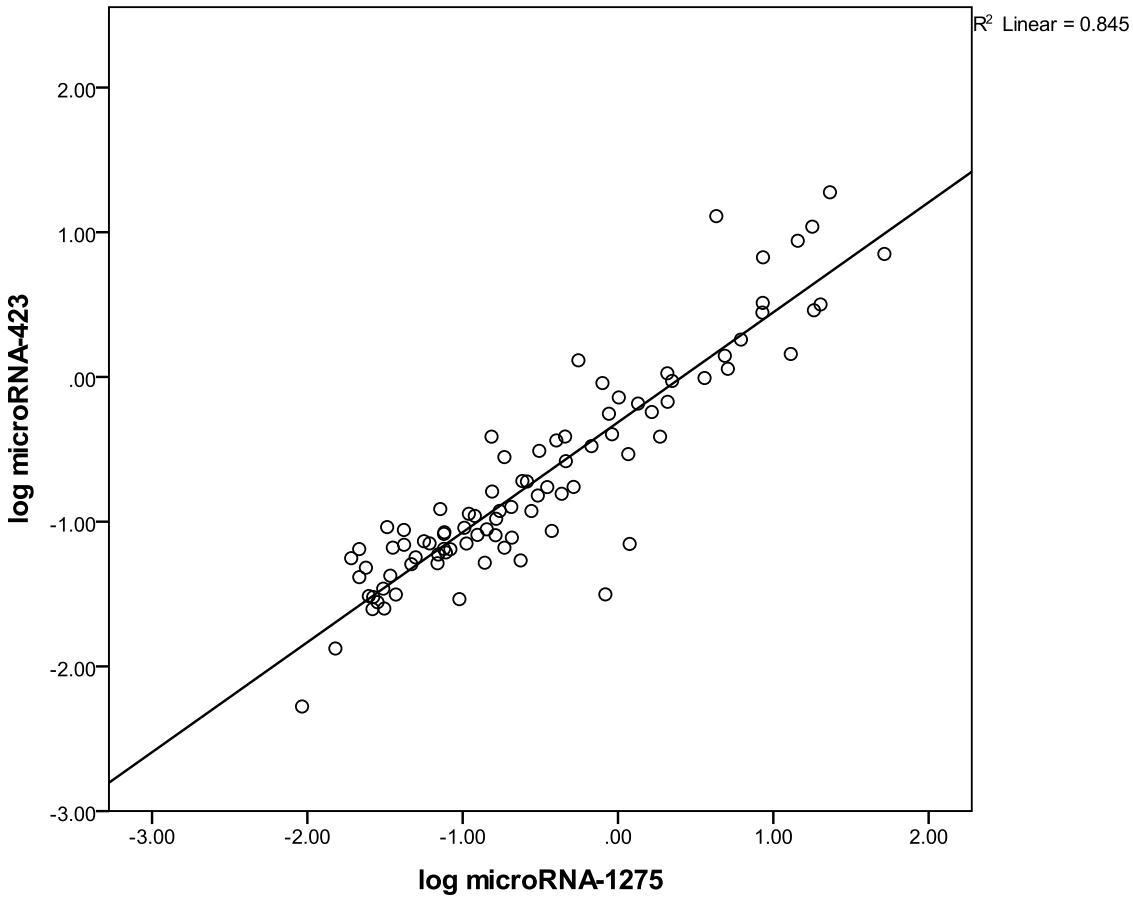

Figure 4-52 Scatterplot of relative expression of microRNA-423 and -1275

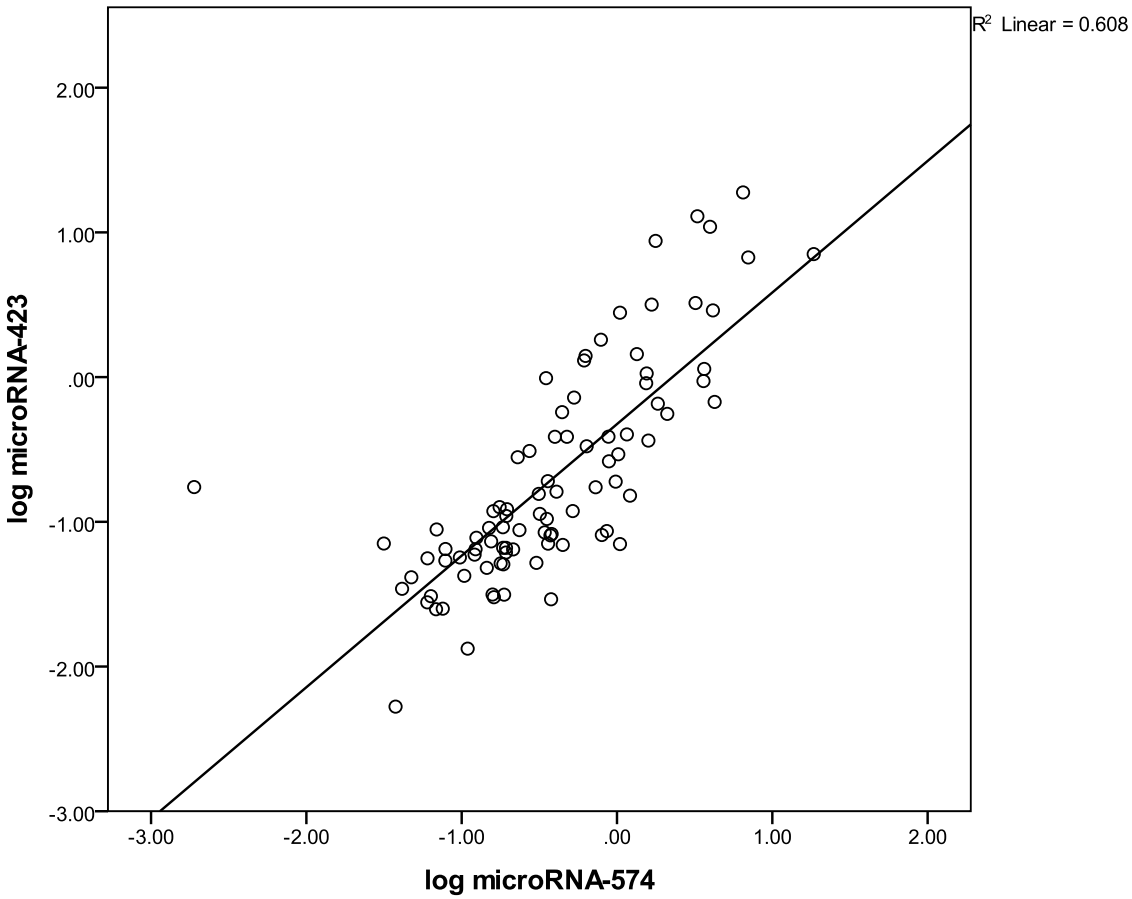

Figure 4-53 Scatterplot of relative expression of microRNA-423 and -574

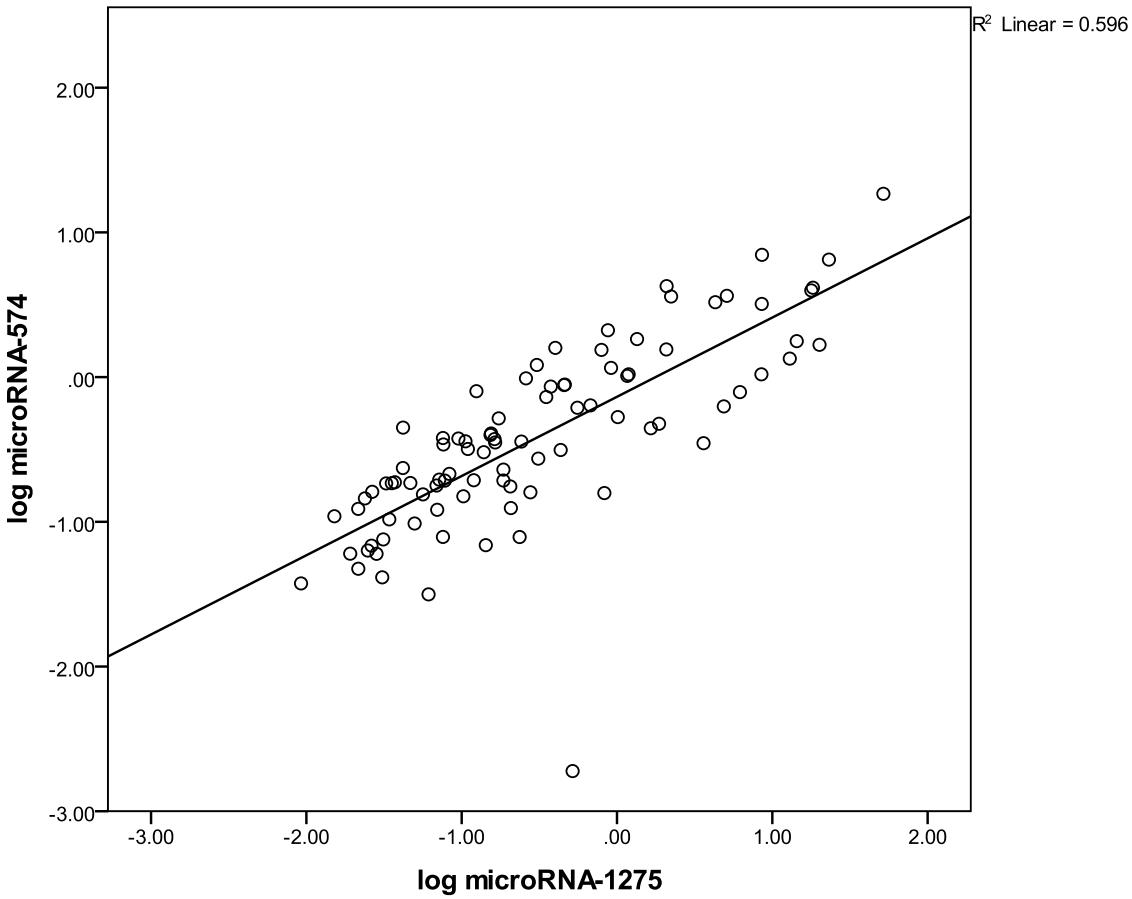

Figure 4-54 Scatterplot of relative expression of microRNA-574 and -1275

4.4.3.4 Correlation between 'biologic resistant' microRNA and clinical variables

No correlation was seen between microRNA-423, -574, -1275 and -3178 and any clinical variable including the DAS28. There was a weak correlation between microRNA-423 and ESR (r=-0.263, p=0.012) and microRNA-1275 and ESR (r=0.223, p=0.035).

Figures 4-58 and 4-59 show the correlation between ESR and baseline microRNA-423 and -1275 for illustration.

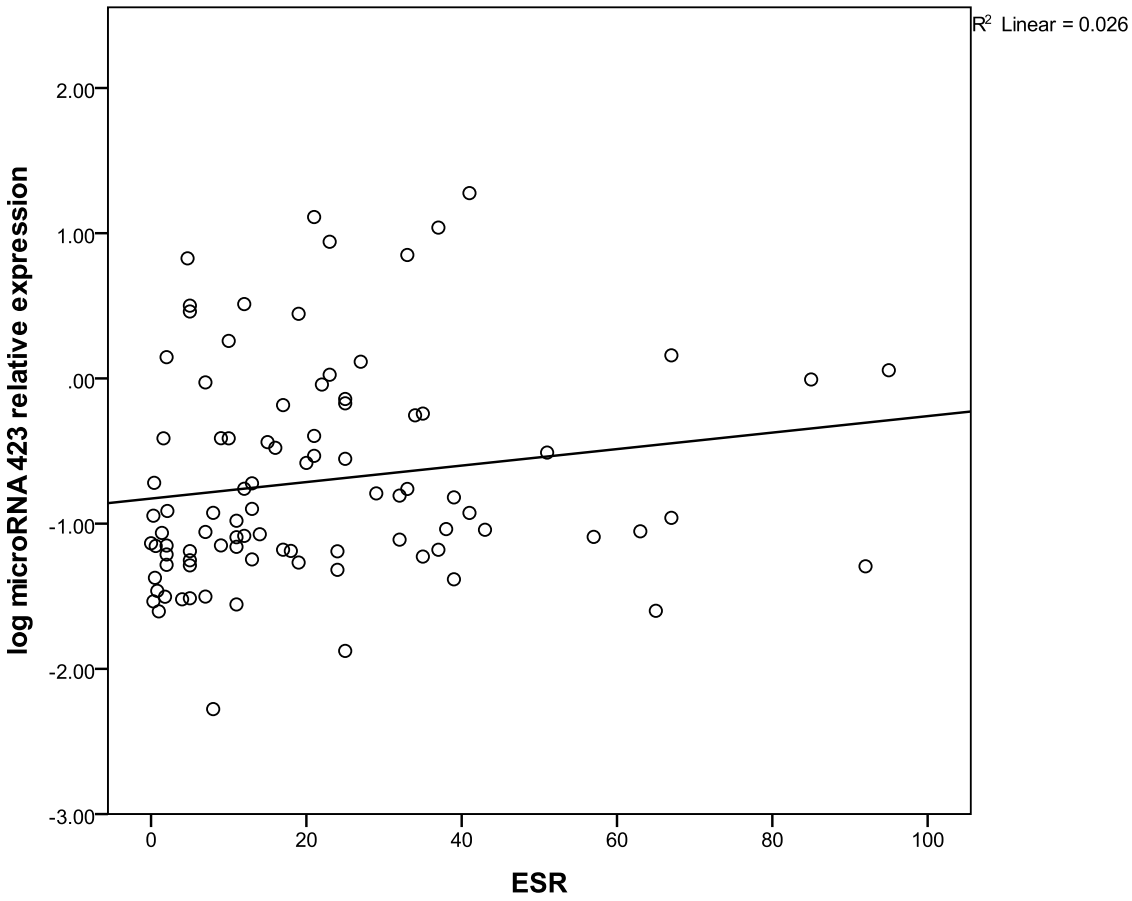

Figure 4-55 Scatterplot of relative expression of microRNA-423 and ESR at baseline

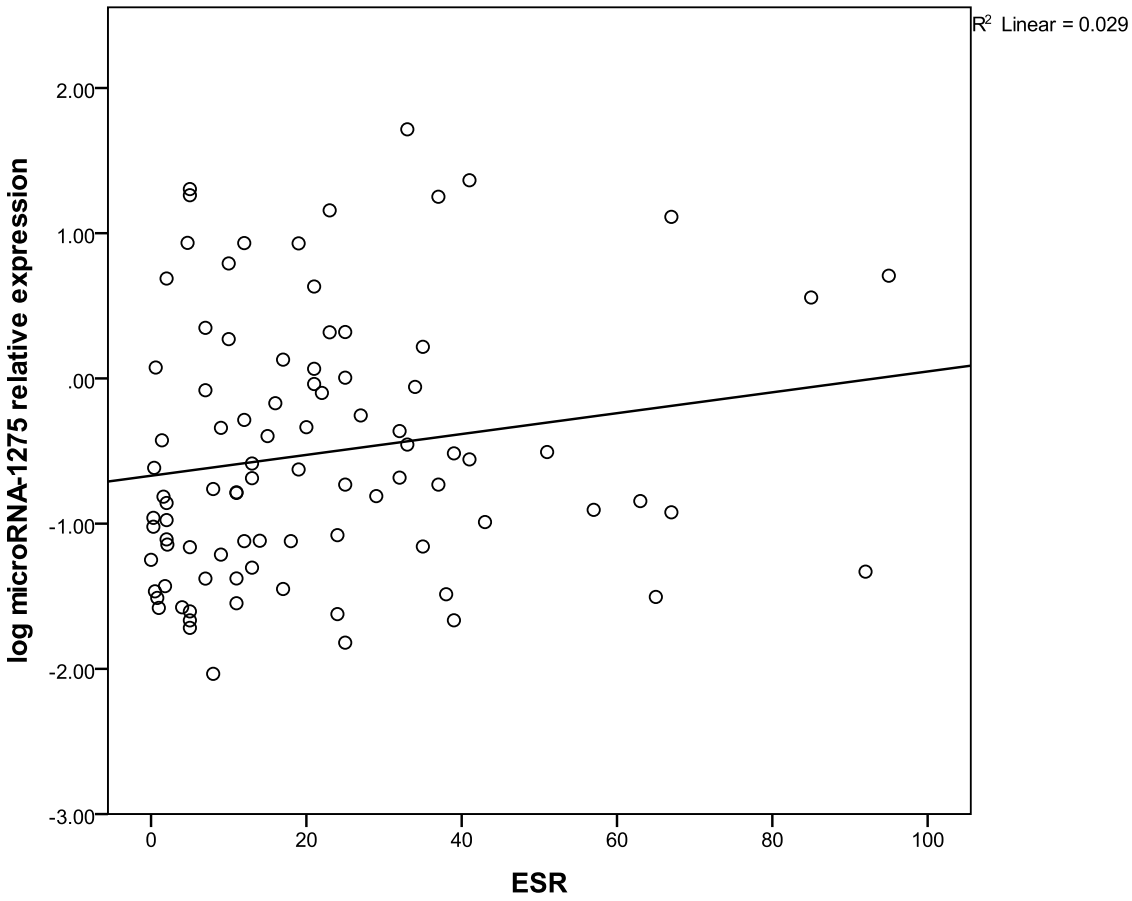

Figure 4-56 Scatterplot of relative expression of microRNA-1275 and ESR at baseline

4.4.3.5 'Biologic resistant' microRNA- longitudinal analysis

	Baseline	Three months	Six months
Biologic resistant	46	33	40

Table 4-87 Patient numbers studied at the three study visits

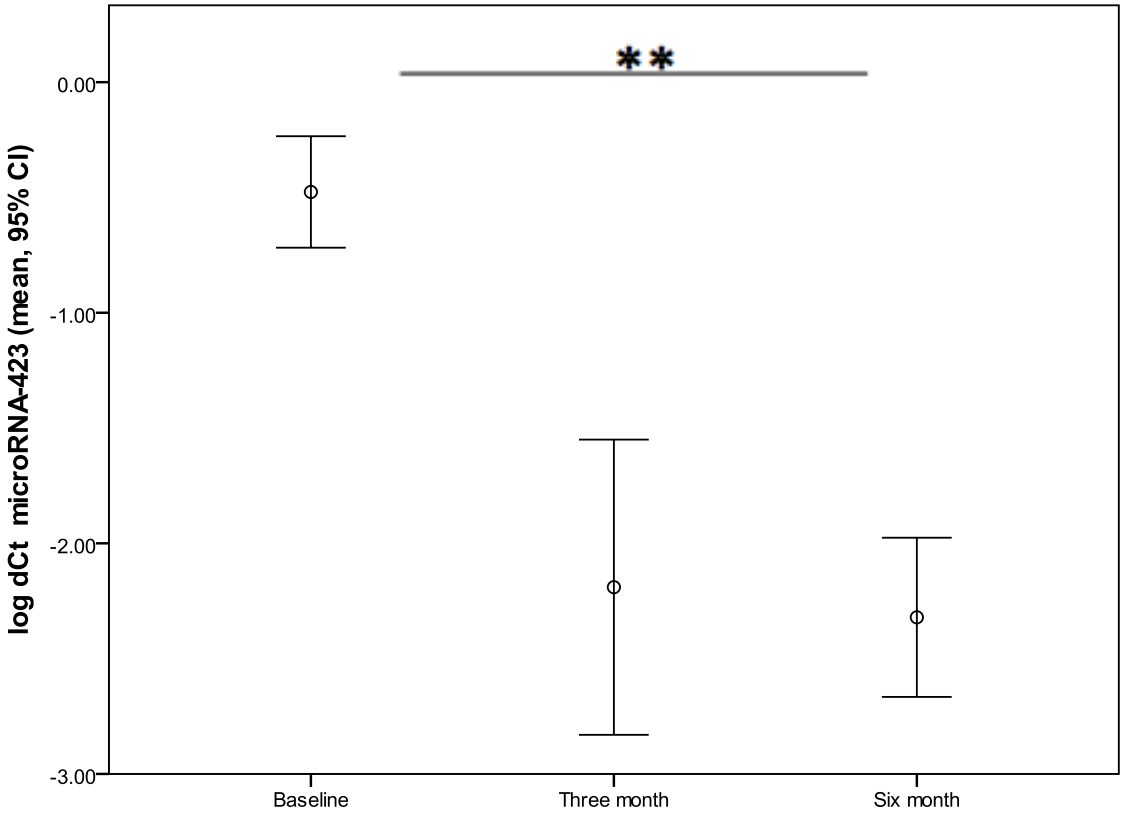

Figure 4-57 Relative expression of microRNA-423 at baseline, three and six month study visits (where *=p<0.05 and **=p<0.01)

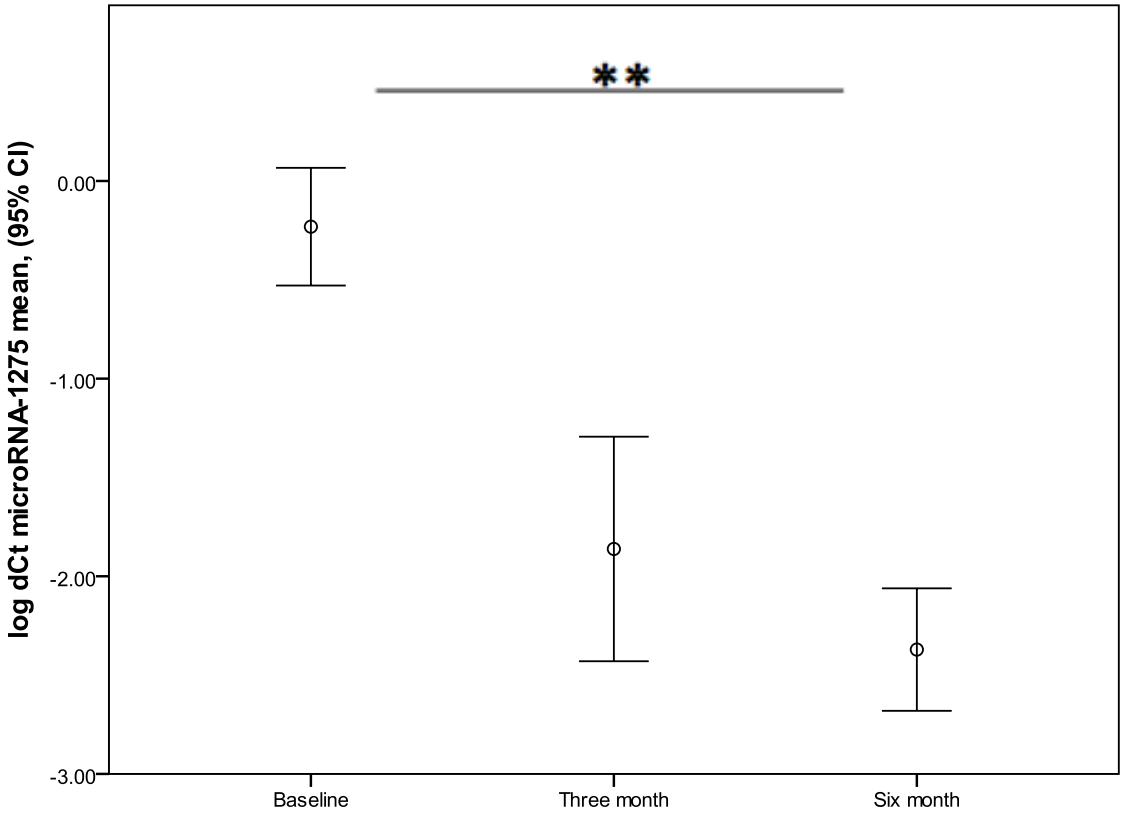

Figure 4-58 Relative expression of microRNA-423 at baseline, three and six month study visits
(where *=p<0.05 and **=p<0.01)

A highly significant reduction in both microRNA-423 and -1275 was observed in the biologic resistant study group by six months. In order to further explore this, clinical variables were examined and change to treatment examined. Figures 4-62 to 4-65 demonstrate these findings. A significant reduction in DAS28-ESR (p=0.001) was seen mainly driven by clinical assessments (TJC p=0.013) and SJC p=<0.001, CRP p=0.029 but ESR p=0.142)

Of those patients examined, between baseline and three-month visit, 11/33 had an *increase* in their treatment (such as DMARD change, steroid by intramuscular or articular route). 6/11 started a new biologic therapy between visits. Between the three and six month visit, twelve had treatment increases, of which two started biologic therapy (one new and one re-start after a suspension). Therefore appropriate escalations in therapy would account for the fall in DAS28 observed and shown in figures 4.34 to 4.37.

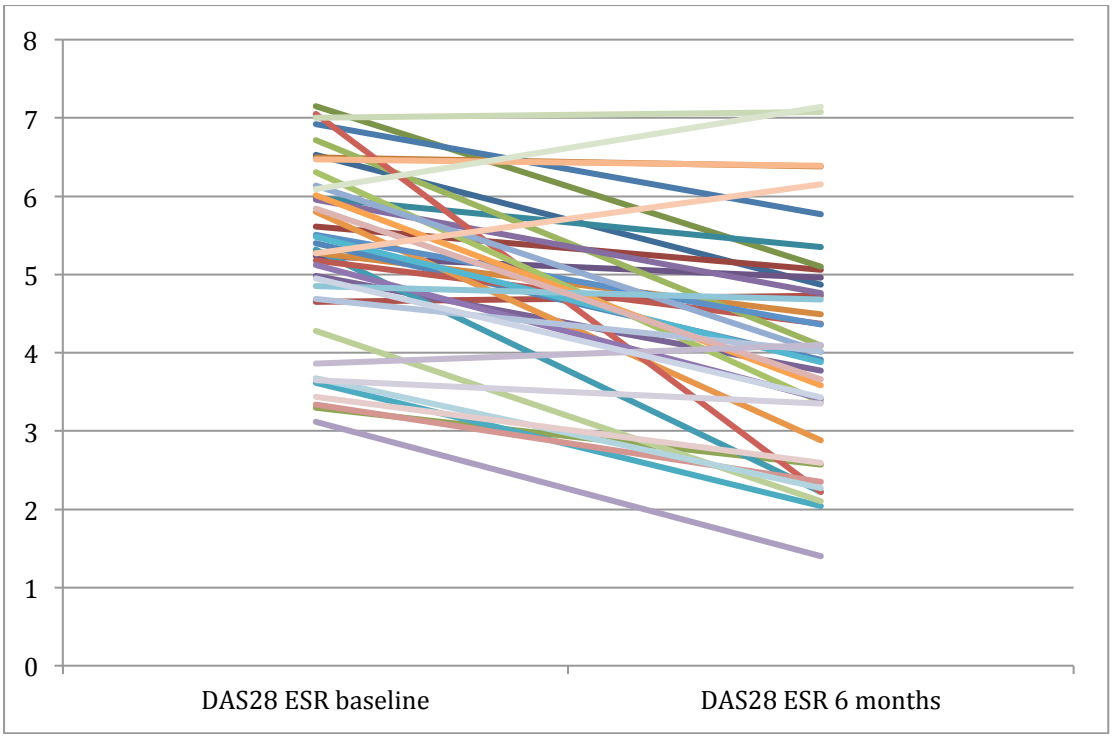

Figure 4-59 DAS change between baseline and six months of biologic resistant study group (n=40)

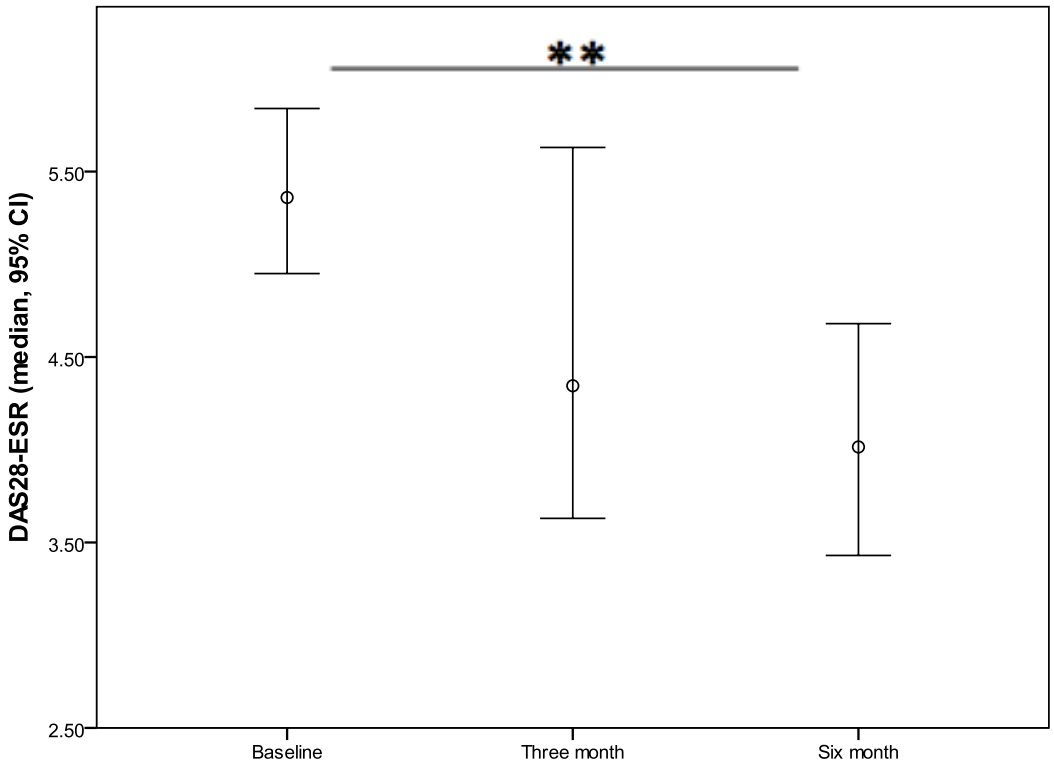

Figure 4-60 Reduction in DAS28 ESR between study visits of biologic resistant group
(where *=p<0.05 and **=p<0.01)

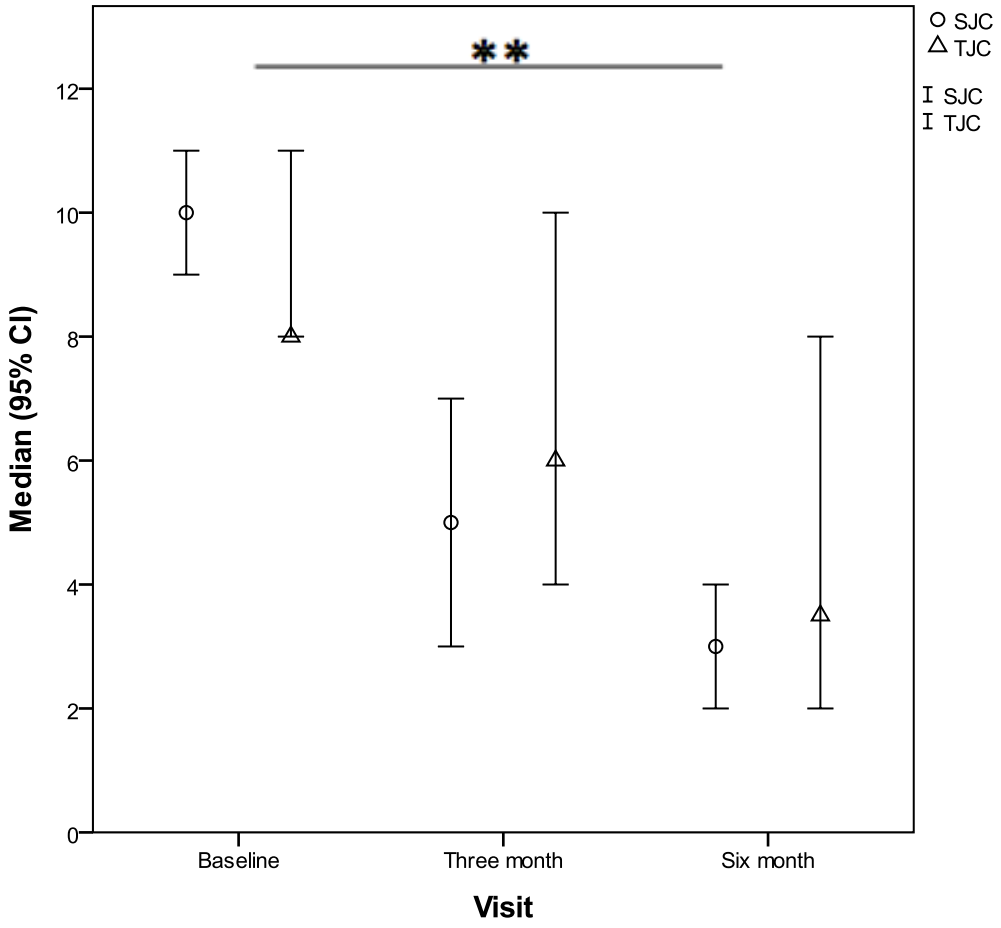

Figure 4-61 Reduction in joint counts between study visits of biologic resistant group
(where *=p<0.05 and **=p<0.01)

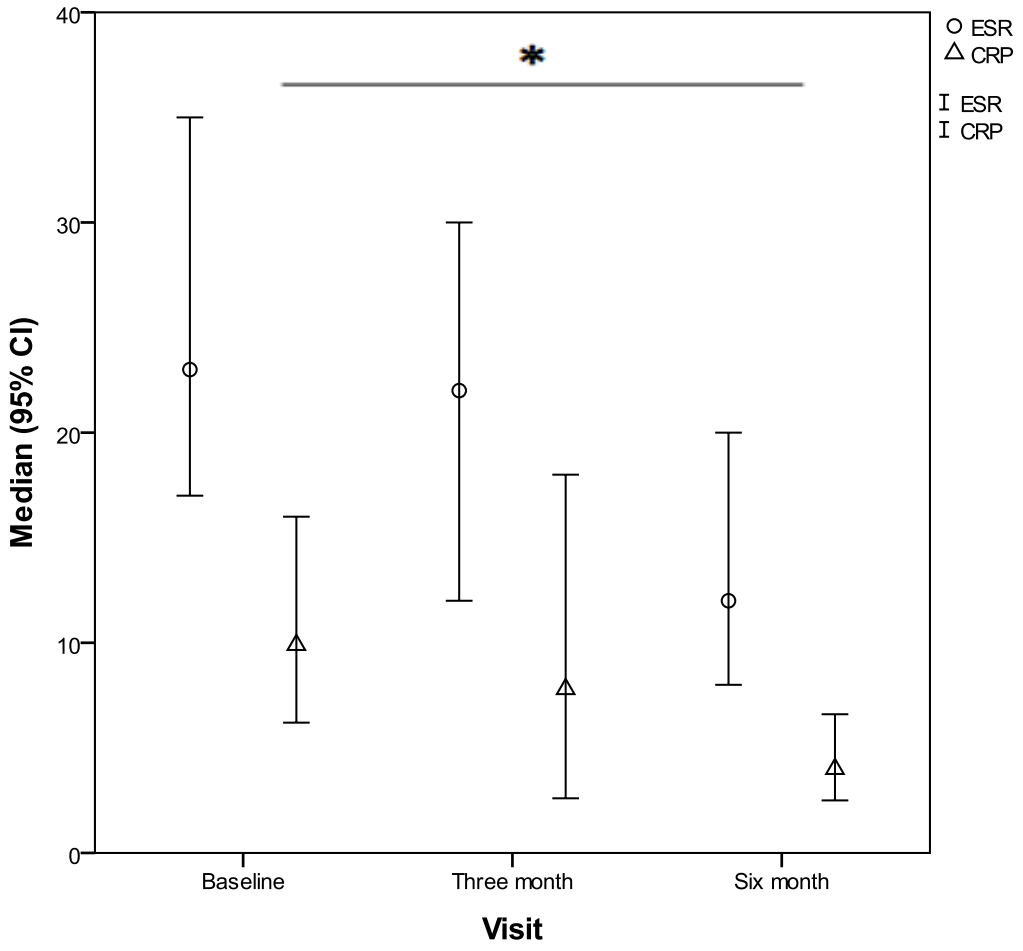

Figure 4-62 Reduction in inflammatory markers between study visits of biologic resistant group (where *=p<0.05 and **=p<0.01)

4.4.3.6 Correlation between 'biologic resistant' microRNA and change in DAS28

In order to assess the utility of microRNA-423 or -1275 at baseline and change in DAS-28, figures 4-66 and 4-67 are shown. 40 patients had valid qPCR results at baseline and six months and thus shown.

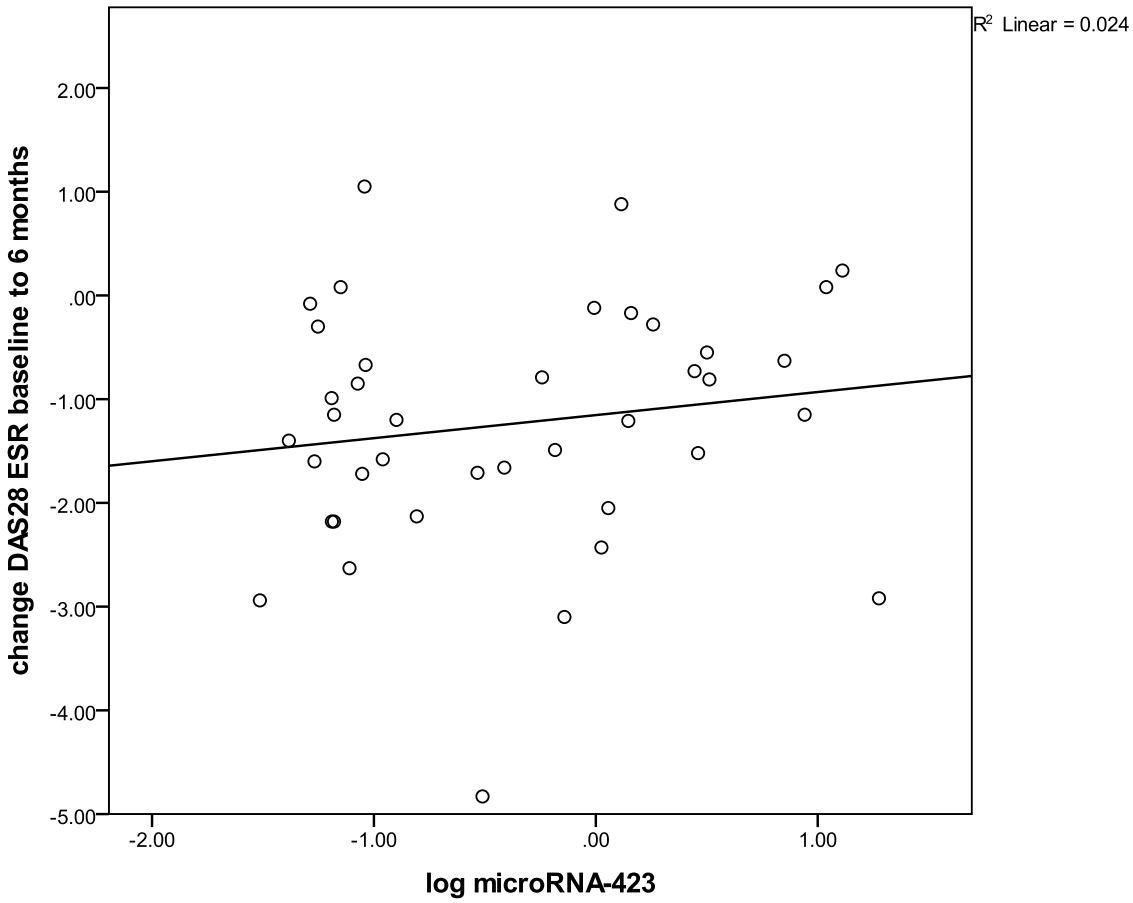

Figure 4-63 Scatterplot of change in DAS28 ESR from baseline study visit to six-month study visit against relative expression of microRNA-423

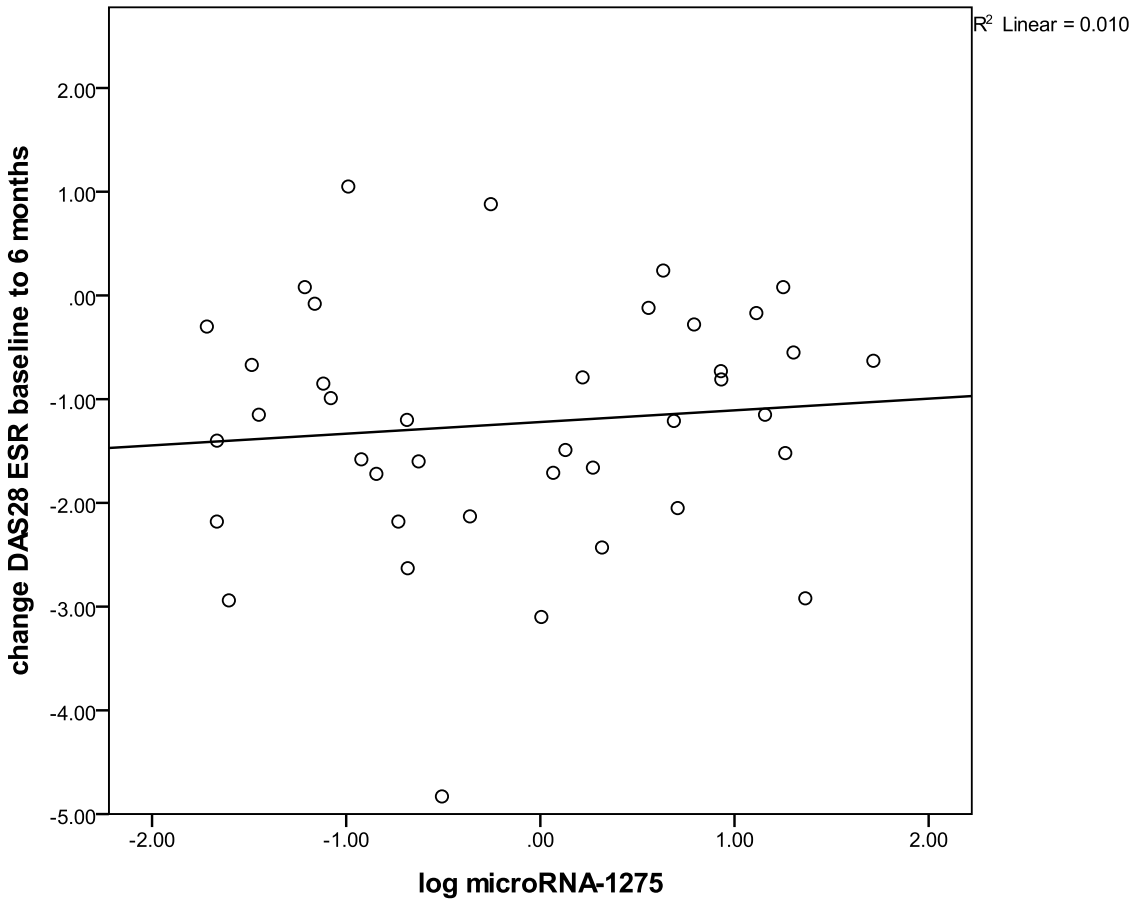

Figure 4-64 Scatterplot of change in DAS28 ESR from baseline study visit to six-month study visit against relative expression of microRNA-1275

Despite both DAS28 ESR and both microRNA-423 and -1275 falling over the six month follow up, the correlation between the two is poor.

4.4.3.7 Correlation between 'biologic resistant' microRNA and cytokines

Correlation coefficients (Pearson's) were calculated between baseline cytokines values examined in Chapter 5 and microRNA-423, -1275, -574 and -3174.

MicroRNA-423 and baseline RANKL (r=-0.333, p=0.031) and MMP-12 (r=-0.304, p=0.050) are shown in Figures 4-70 and 4-71. Additionally, microRNA-423 and -574 correlated with MCP-1 (r=-0.313, p=0.043 and r=-0.321, p=0.032 respectively).

There was no correlation between microRNA-1275 and those cytokines examined in Chapter 5.

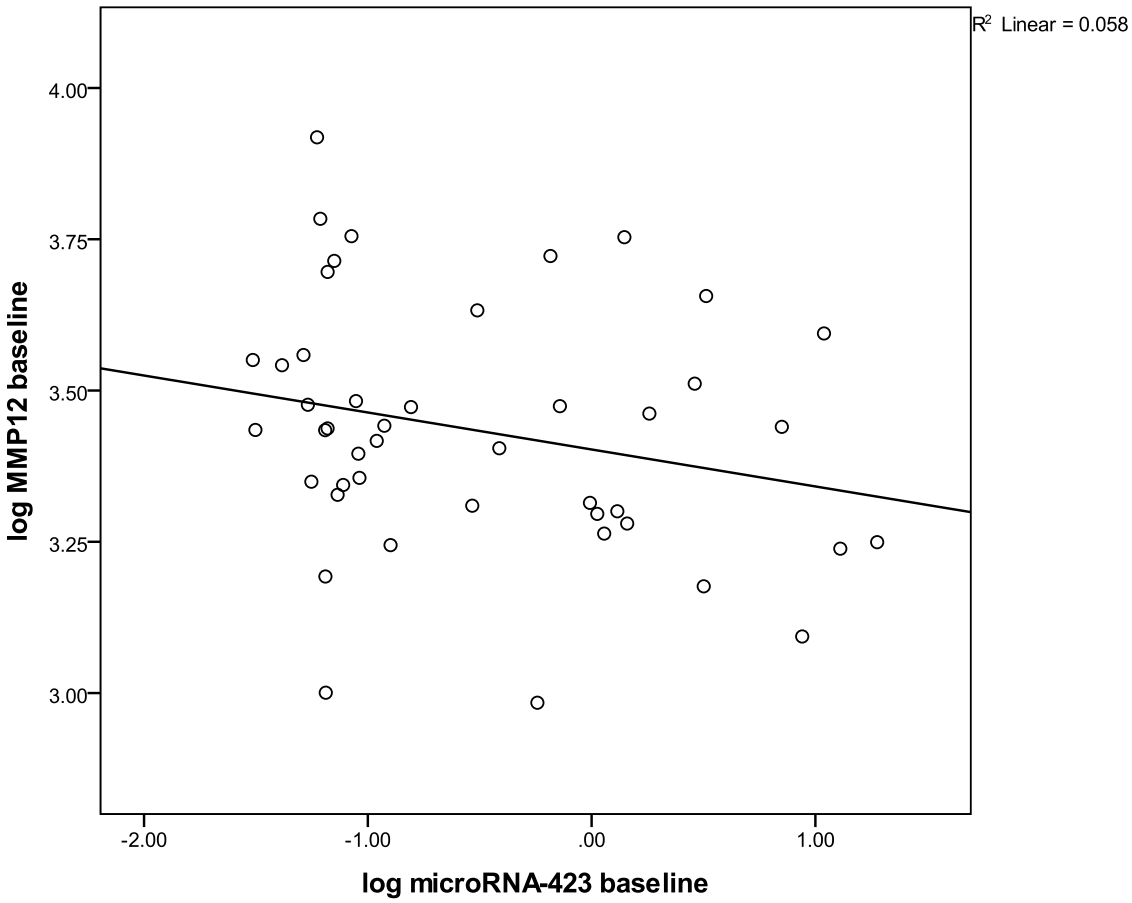

Figure 4-65 Scatterplot of MMP-12 at baseline visit and relative expression of microRNA-423

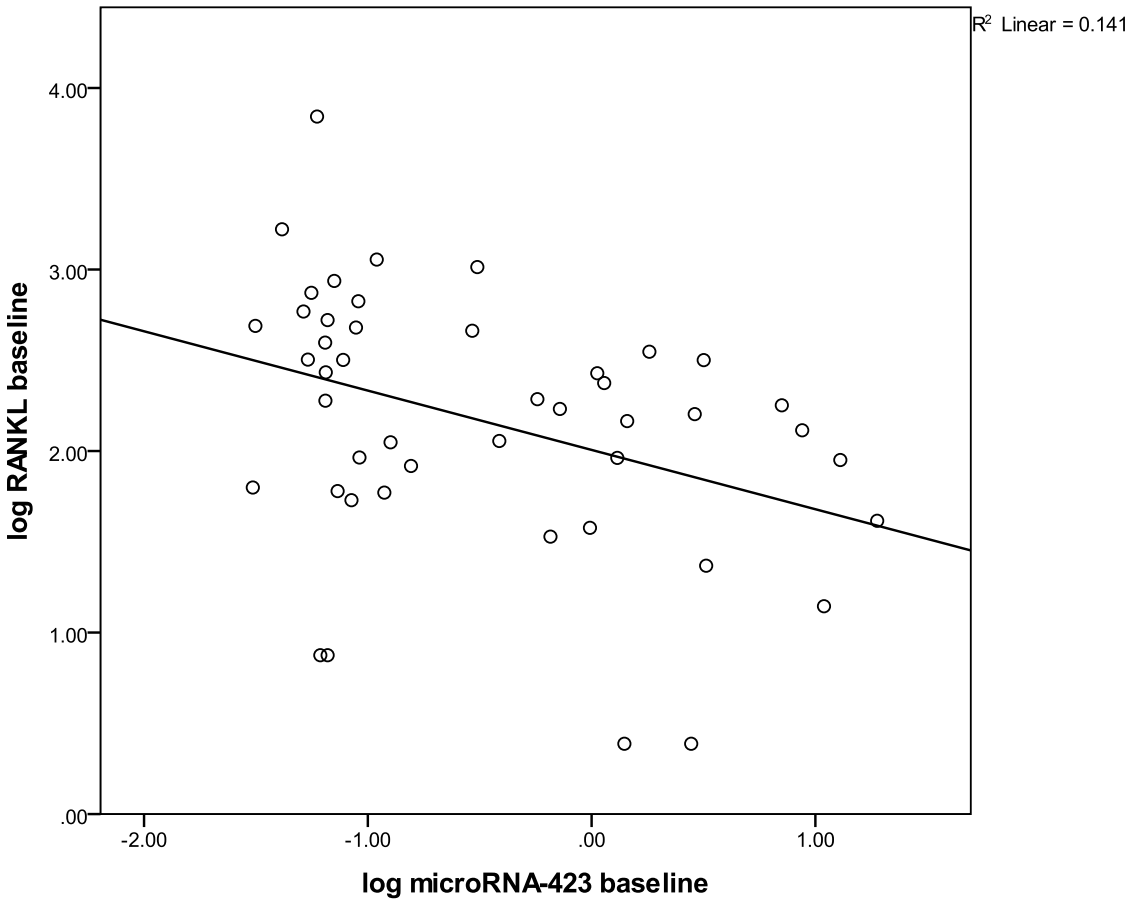

Figure 4-66 Scatterplot of RANKL at baseline visit and relative expression of microRNA-423

4.5 Chapter Conclusions and Discussion

MicroRNA represent a rapidly evolving field of study and represent important regulators of the inflammatory pathways dysregulated in RA. Previous studies have shifted focus from the presence of microRNA signatures to biological roles. Within a global microRNA PB CD14+ profile we identified several microRNA, subsequently validated by qPCR, to be differentially expressed in a biologic resistant cohort.

Previous studies have used heterogeneous groups of patients in an already highly heterogeneous condition treated with a range of treatments. It was first important to define specific cohorts on the basis of clinical factors in order to identify a specific microRNA profile unique to a biologic resistant cohort but to also remain applicable in daily clinical practice. For this reason we chose to examine the microRNA profile of CD14+ cells. We chose CD14+ cells as they contribute to systemic production of pro-inflammatory mediators and, most importantly, they are the precursors of synovial macrophage, which are the pivotal source of TNFa, IL-6 and IL-1b.

4.5.1 Candidate microRNA

The microRNA studied in this chapter have been identified in previous microarrays performed in our centre. Local interest has thus been developed. The results of the analysis herein suggest differences in relative expression are exhibited between selected RA patient groups according to their treatment response. This can be summarised as

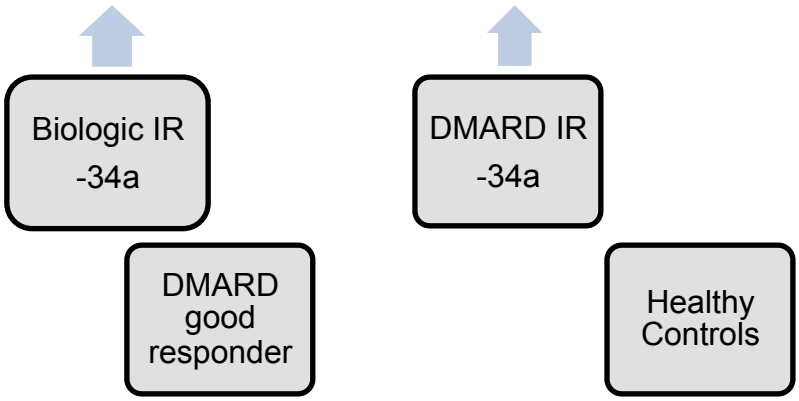

Figure 4-67 MicroRNA-34a- relative increased expression in Biologic IR group vs other group

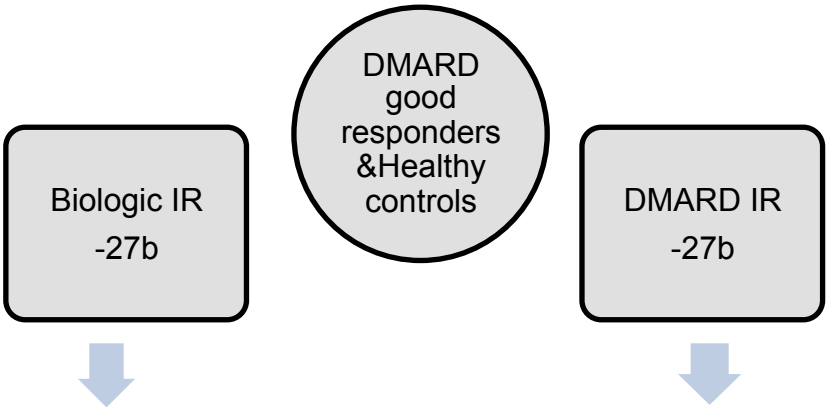

Figure 4-68 MicroRNA-27b- relative reduced expression levels in inflamed groups

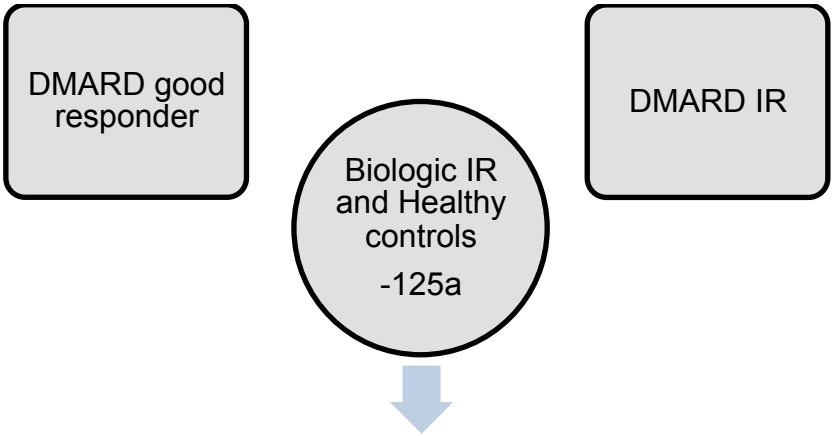

Figure 4-69 MicroRNA-125a- relative reduced expression levels in biologic IR group vs DMARD responder and inadequate responder

MicroRNA-34a and miR-27b showed reciprocal expression in CD14+ cells of RA patients with the most severe disease: up-regulation or down-regulation, respectively in both groups, biologic and DMARD resistant patients compared to other groups. MicroRNA-125a showed an interesting pattern of expression: despite comparable inflammation and clinical findings between the groups DMARDs and biologic resistance, microRNA-125a is comparatively reduced in the latter. In contrast, it has similar expression levels in both DMARDs treated groups suggesting that it may be induced by DMARD therapy.

MicroRNA-34a, -27b and -125a all correlate with the swollen joint count but not with composite measures of disease activity. It is relevant that the microRNA profiles do not correlate with ESR and CRP. Both are well-established surrogates of inflammation but the strength of the correlation with the SJC is not strong enough to overcome this and reflect in correlation with the DAS28 score.

These observations confirm the elucidation of precise molecular mechanisms will be key. The confirmation of unique microRNA profiles between treatment groups and their

response both confirms the integrity of the cohorts as fundamentally different but also the weakness of currently employed measures of disease activity.

4.5.2 'Hypothesis free' findings- candidate 'resistant microRNA'

We identified three microRNAs that are expressed in PB monocytes of RA patients that are resistant to biologic therapies. This includes miR-423, miR-1275 and miR-3178. None of the four candidate microRNA from the microarray screen have been identified in relation to RA to the best of my knowledge. There are a small number of previous publications referring to microRNA-423. (Lin et al. 2011) identified microRNA-423 as promoting cell growth and cell cycle progression in hepatocellular carcinoma. (Tijsen et al. 2010; Goren et al. 2012) have independently identified microRNA-423 as up-regulated in patients with heart failure. (Katsushima et al. 2012) identified microRNA-1275 as down regulated in gliolastoma and may be involved in dendritic cell differentiation. (B. Yang et al. 2013) identified microRNA-3178 as one of a number of up-regulated microRNA and may contribute to lymphatic metastasis of gastric cancer.

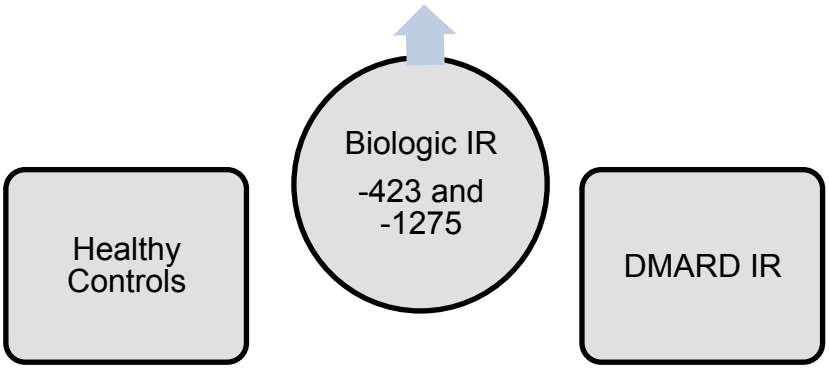

Figure 4-70 MicroRNA-423 and -1275; higher relative expression in biologic resistant vs DMARD resistant and healthy controls

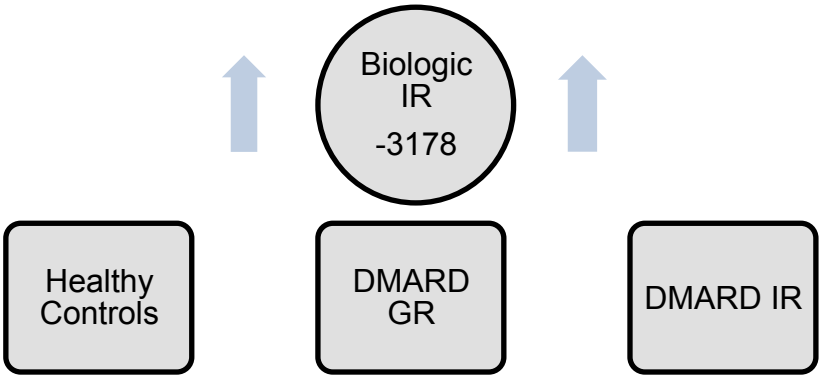

Figure 4-71 MicroRNA-3178; higher relative expression in biologic resistant group vs all control groups

It was important that the validation qPCR group were similar to the study group to allow valid comparisons. In a similar way to the observations of the candidate microRNA, there were differences between groups that persisted despite similar clinical findings and measures of inflammatory disease. The microRNA differentially expressed have minimal correlation with clinical variables. Despite comparable active inflammation, microRNA-423, -1275 and -3178 were up-regulated in biologic resistant CD14+ cells. In particular, miR-3178 seems to be a good candidate biomarker of resistance to biologic therapies as it distinguishes biologic resistant group not only from DMARDs resistant group but also from the other two groups tested.

There was a close 'co-correlation' between the four microRNAs examined and this was strongest between microRNA-423 and -1275. The same microRNA were observed to correlate with ESR. This may suggest co-transcription. Correlation was observed between microRNA and several cytokines. The correlation with MMP and RANKL is of interest raising a pathological link with joint damage and erosions. There was also correlation between microRNA-423 and -574 with MCP-1. Liou et al identified MCP-1 as a useful marker of disease activity in heir population when combined with clinical factors (Liou et al. 2013). Further examination of these links is desirable.
Over the follow up, there was a significant reduction in DAS28 reflecting treatment escalation. There was a concurrent fall in both microRNA-423 and -1275 but neither correlated with the DAS28.

Disease duration between the biologic resistant and DMARD resistant group differs. MicroRNA profiles change with both age and disease stage (Filková et al. 2013). However, it would seem unlikely that substantially different processes are at play in similar cohorts with such well-established disease. Observed differences could represent a treatment effect (concurrent DMARD, steroid use, intermittent infusions of Rituximab) rather than lack of suppression of inflammation. This however represents strength of this observation- that irrespective of treatment, several microRNA are up regulated.

It is not clear if this profile defines treatment resistance mediated by an unidentified pathophysiological process. The next step would be microRNA:target pathway analysis. Further key follow up work would include seeking these microRNA in an early arthritis cohort (blood and synovial cell lines) and the requirement to validate and replicate these findings in independent cohorts.

In conclusion, a microarray of biologic resistant and DMARD resistant patients with similar clinical characteristics has identified three microRNA not previously identified that are both differentially expressed between treatment-response groups but also associate with changes in disease activity. Further investigation of their utility as a biomarker of disease activity and biologic targets will elucidate the role of these microRNA in rheumatoid arthritis.

5.1 Chapter 5; Results- Cytokine profiling

5.2 Introduction and chapter description

The aim of this chapter is to measure the cytokine profile of the study groups with a focus on defining the biologic resistant group.

Where PROMs provide subjective representative assessments, their weakness remains that they may not reflect true biological pathway activity. There therefore remains a complementary role for clinical, PROMs and proteomic studies.

In order to perform this an exploratory cytokine panel was chosen and multiplex methodology used. The aims included

- Establishing the cytokine profile of the biologic resistant group and comparing this with the profile of the control groups (cross sectional analysis)
- Examine the biologic and DMARD resistant group at three time points to examine the stability with time of the serum cytokine profile and correlate this with the observed improvements in disease activity observed in Chapter three (longitudinal analysis)
- Correlate the clinical observations of Chapter 3 and microRNA findings of Chapter 4 with the cytokine profile and seek any candidate cytokine biomarker reflecting disease activity in the biologic resistant cohort

The role of and wide range of cytokines that have been profiled in RA are first outlined followed by a selection of previous studies in RA to put any results in context.

I have then outlined the multi-analyte panel chosen and the methodology underpinning this technology.

5.3 Background

The network of cell involved in the immune response requires intense coordination, which is mediated by cytokines. Inter-cellular communication is facilitated by small soluble or membrane bound molecules with pleiotropic roles. At the cellular level, receptor binding triggers downstream intra-cellular signalling leading to a cellular response through gene transcription. There must be a balance of pro and anti-inflammatory activity. It is this balance that is dysregulated in autoimmune conditions (Chizzolini et al. 2009). The wide heterogeneity of the RA phenotype is also underpinned by wide heterogeneity of cytokine profiles, a situation also seen in RA at different disease stage

Numerous cytokines are thus typically observed in RA produced both locally within the synovial membrane in addition to adjacent bone marrow and recruited circulating immune cells. The major pro-inflammatory cytokines reflect those local processes namely general inflammation, tissue destruction, bone damage and turnover, fibrosis and vascular (angiogenesis). Examples include TNF, IL-6 and IL-1, which have gone on to become therapeutic targets, and are mainly derived from macrophages and synoviocytes. DMARDs have an uncertain mode of action but the advent of treatments targeting specific cytokines allows their effect to be studied in detail.

Below several selected cytokines involved in RA are noteworthy;

5.3.1 Interleukin 6

The IL-6 signal transducing receptor molecule has two subunits, gp130 and IL-6R alpha that exist both soluble and membrane bound. IL-6 is secreted by monocytes, T and B cells and fibroblasts and can be found in serum and synovial fluid. Binding activates STAT3 dependent pathways and nFkB pathways. IL-6 correlates with CRP and knockout mice are resistant to CIA. The systemic effects of Il-6 are notable; with TNFa it mediates the anaemia of chronic disease often seen with active RA, acts as a mediator of fatigue through the HPA axis and a potential role in the observed accelerated vascular disease. Therapeutic blockade of the IL-r receptor with Tocilizumab has been successful.

5.3.2 TNF alpha

TNFa is one of the major pro-inflammatory mediators released predominantly by cells of the monocyte lineage but also T-cells, B cells, polymorphonuclear cells, mast cells and synovial fibroblasts (Larché et al. 2005). It exists as a homotrimer, having been cleaved by TNFa converting enzyme (TACE) from a transmembrane portion. Target cells express surface TNF receptor (TNFR), which exists in two forms. Soluble TNFa acts on TNFR1, and is responsible for the majority of the observed TNFa effects, where membrane bound TNFa is thought to act on TNFR2. Binding is thought to act through p38/JNK kinases leading to nFkB transcription. Excessive and unchecked production occurs in RA, which leads to further pro-inflammatory actions including fibroblast activation and further cytokine release, T-cell activation and antibody production by B-cells,

endothelial cell adhesion molecule expression (and thereby further T-cell recruitment) and osteoclast bone resorption.
The central role TNFa plays in human arthritis has been borne out by multiple successful trials reducing the signs and symptoms of RA for as long as treatment is continued.

5.3.3 Interleukin 1

IL-1 was the one of the first cytokines to be identified and then named. It is mainly produced by monocytes and is the classic mediator of the febrile response in infection. It belongs to the IL-1 superfamily of related molecules, the most significant being IL-alpha and IL-1beta. Therapeutic blockade of IL-1 was successful in murine models yet has been disappointing in the magnitude of effect in humans and no longer forms part of UK treatment recommendations.

5.3.4 Interleukin 18

Is a member of the IL-1 superfamily and is a potent stimulator of T cell differentiation toward a Th1 phenotype. Secretion is widespread throughout inflammatory cell lineage in addition to chondrocytes and osteoblasts. IL-18 induces TNFa and is greatly enhanced by IL-12 and 15 and inhibited by IL-10 and TGFB (Liew et al. 2003). It promotes angiogenesis through VEGF expression, increases chemokine secretion, inhibits chondrocytes and may facilitate cartilage damage (Gracie et al. 1999). It also acts to activate NK cells, macrophages and neutrophils through MyD88, IRAK/TRAF signalling. Mouse models of arthritis are worsened by the addition of IL-18. Therapeutic blockade has been effective in murine models (Plater-Zyberk et al. 2001; Liew et al. 2003).

5.3.5 Interleukin 15

Is produced primarily by macrophages, by FLS and mast cells. It has been demonstrated to be elevated in synovial fluid and synovial membrane. Levels are lower in serum with some correlation with DAS28. IL-15 is a pleiotropic cytokine– for example it acts synergistically with those cytokines above and acts to facilitate B cell differentiation, stimulate neutrophils, activate synovial fibroblasts and monocyte/macrophage activation through JAK/STAT signalling pathways. Therapeutic blockade of the IL-15 receptor has taken place in murine models and humans with some success (Asquith & McInnes 2007; McInnes et al. 2003)

5.3.6 Others

Granulocyte macrophage colony stimulating factor (GM-CSF)-produced by synovial macrophages initiates T-cell activation if appropriate antigen is present. IL-17 is produced by T-cells and can further stimulate osteoclast activation. IFNg is the primary T-cell derived cytokine with mixed pro and anti-inflammatory roles.

Chemokines are further small molecules also involved in the coordination of white cells and the inflammatory response. There are 4 families of chemokines according to their structure and share some similarities in their function in this way (Iwamoto et al. 2008). Examples of key chemokines are shown in Table 5-88.

Pro-Inflammatory		Anti-Inflammatory	Variable function
IL-1Beta	IL-15	IL-6R alpha, gp130	IFN gamma
Il-2	IL-17	IL-4	IL-10 (mainly anti-inflammatory
IL-6	IL-18	IL-13	IL-33
IL-12	IL-23	IL-25	TGF-Beta
TNF alpha	IL-1Beta		
GM-CSF			

Table 5-88 Selected cytokines grouped by postulated function (adapted from (Boissier 2011)

5.4 Selected publications examining cytokines in RA

There are numerous publications pertaining to cytokine evaluations in RA at different stages of disease and in response to therapy. In 'pre-arthritis', (Kokkonen et al. 2010) examined the cytokine profile of individuals who went on to develop RA. This has been replicated by Deane et al and shortly *after* symptoms develop where the cytokine profile associates with autoantibody status (and thus disease 'subsets') (Deane et al. 2010; Hitchon et al. 2004). Correlation with disease activity has been long recognised and several authors show the cytokine profiles improve with DMARD treatment (Tetta et al. 1990)

Wagner 2013 demonstrated two biomarkers (EGF and CD40L) of progression as defined by radiographic progression in an early arthritis cohort but results of profiling at baseline to predict response has been less clear (Wagner et al. 2013; Emery & Dörner 2011). (Lequerré et al. 2007) sought a predictive cytokine signal of Infliximab response. They could not identify a signal in the clinical, bone marker or autoantibody profiles. In contrast, den Broeder et al suggest COMP and ICAM-1 levels as a marker of cartilage turnover may predict Adalimumab response when measured at baseline (Broeder et al. 2002). (Visvanathan et al. 2010) sought differences in cytokine profile between responders and non-responders after Golimumab therapy. They demonstrated distinct significant differences in IL-6, CRP, MMP-3 and VEGF (amongst others). There was some correlation with PROMs. Despite these, there was no useful predictive profile.

More recently, (Liou et al. 2013) identified MCP-1 as a promising biomarker. There was close correlation with elements of the DAS28 in addition to inflammatory markers. A 'DAS28-MCP-1' correlated with the DAS28-CRP and performed better than ESR or CRP alone in their cohort of mixed new RA cases and those regularly treated/followed up (also one of the study weaknesses).

One of the major difficulties in studying cytokine profiles is both the marked inter-individual heterogeneity observed and that the true cytokine signal resides within the synovial compartment. In one of the earliest studies that aimed to characterise the

synovial improvements with TNFa treatment (Ulfgren et al. 2001) demonstrated cytokine changes in synovial biopsies after Infliximab (n=8). The same author group demonstrated the reduction in effector MMPs after Etanercept treatment (Catrina et al. 2002). (Lindberg, af Klint, Ulfgren, et al. 2006b) examined synovial biopsies of RA patients and highlighted the enormous inter-individual variability.

Of those microRNA papers referred to in Chapter 1, several authors attempted to examine cytokine profiles along with their microRNA studies. (Stanczyk et al. 2008) were able to demonstrate a reduction in MMP-3 by microRNA-155 but not MMP-1,-9, -13 nor IL-6. Li 2010 demonstrated correlation between microRNA and TNFa levels in both serum and synovial fluid. They also measured IL-2, -4, -6, -10 and IFNg- there was no correlation with these other cytokines nor DAS28, CRP, ESR or autoantibody status. Finally, (Murata et al. 2010) examined CRP, ESR and MMP-3. They found no correlation between the microRNA they identified and these cytokines.

(Hirata et al. 2013) examined the BeSt early arthritis cohort and correlated a panel of cytokine biomarkers with disease activity; these included serum amyloid A, VEGF A, MMP-1 and -3, leptin, VCAM, TNF-RI among others. Close correlation was found suggesting it may be possible to study early disease activity with (not instead of) clinical assessments.

(Raza et al. 2005) examined cytokine profiles in the serum of a very early arthritis cohort, some of whom went on to develop RA. Their comparator was established RA, crystal arthritis and OA. Distinct profiles could be seen in the early RA group creating the appropriate synovial environment that leads to persistent synovitis. These patients were treatment naïve.

(Hueber et al. 2007) identified distinct profiles between RA, PsA and healthy controls. There was also some correlation with global scores, disability and conventional laboratory markers of inflammation (CRP, ESR). They noted a cytokine ‘high’ group that also displayed high autoantibody titres.

5.5 Methods

The laboratory has extensive experience using the technologies described and all experiments were performed by Lynn Crawford, Senior Laboratory Technician, Glasgow Biomedical Research Centre (University of Glasgow) and in duplicate.

As described in Chapter 2, 50 biologic resistant (biologic IR (inadequate responders) were profiled at baseline, three and six months. 25 DMARD IR patients were examined at baseline and three months. 25 healthy controls and 19 DMARD good responders were examined at baseline only.

5.5.1 Analysis

Choice of analytes was pragmatically based on the both the manufacturer able to offer a broad and relevant selection of candidate cytokines to give a representative overview and on cost. The Invitrogen Human Cytokine 30-plex (catalogue number LHC6003) chosen examines those analytes shown in the table below. In addition, a MMP 3–plex kit, interleukin-21 and -23, RANK ligand, OPG and CXCL-11 were studied using Milliplex single-plex kits. These are shown in Table 5-88.
The kit employs bead technology whereby beads conjugated with protein specific antibody binds the cytokine of interest. This complex in turn binds a antibody:fluorescent molecule. The Luminex® 100 system quantifies this fluorescence and thus derives concentration of the cytokine of interest.

All assays were performed in duplicate using the manufacturers standard protocol by LC. Briefly, 50µL of serum or standard were mixed (shake on orbital shaker for two hours at room temp in the dark) in order to capture the analyte, before being washed and incubated with detector antibody (further shake for 60mins, room temp and kept in the dark). After further wash 100 µL of Streptavadin-RPE was added and mixed (shake for 30 minutes at room temperature) and washed again. The beads were suspended in 100µL of wash buffer, applied to a 96-well format and then read in the Luminex® 100. Quantification (concentrations) was performed with reference to standard manufacturer values supplied with kits and entered in the Luminex with each analyte type having a different standard range. Mean value of outputs was used. Where one value was out of range or extrapolated, the value from the other run was used. Out of range values were treated as follows; if both replicates were below the minimum standard range value, half the given range value was used. If above the maximum standard range value it was multiplied by 1.5.

Statistical methodology is referenced in Chapter 2.

Cytokine studied		Brief role	Effect
EGF	Epidermal growth factor	Role in growth and cellular differentiation thus in synovial cell proliferation. May be future therapeutic target (Killock 2012)	Pro
Eotaxin (CCL11)		Role in eosinophils and some evidence may be related to erosions (Syversen et al. 2008) and susceptibility (Chae et al. 2005)	Pro
FGF-basic	Basic fibroblast growth factor	Produced locally in the synovium leading to autocrine stimulation	Pro
G-CSF	Granulocyte colony stimulating factor	Stimulate and activate myeloid cell lines. Potential treatment target (Cornish et al. 2009)	Pro
GM-CSF	Granulocyte/macrophage colony stimulating factor	Produced by synovial macrophages. Stimulate T cell activation and TNFa, IL-1	pro
HGF	Hepatocyte growth factor	With OPG may limit osteoblast differentiation. Usually elevated and this predicts erosions. Also pro angiogenic	anti
IFN alpha	Interferon alpha	Generally produced in response to viral infections and useful immunosuppressive/anticancer therapy.	pro
IFN gamma	Interferon gamma	Produced as part of the innate immune response; CD4 cells differentiate into Th1 cells. Suppresses osteoclast formation	pro
IL-1ra	Interleukin	Decoy receptor for IL-1 and thus anti-inflammatory.	anti
IL-1 Beta	Interleukin-1 beta	Synergistic with TNFa	pro
IL-2	Interleukin 2	Produced by activated T cells and further stimulates T and B cells	pro
IL-2r	Interleukin 2 receptor	IL-2 is a growth factor for T cells; the soluble form of the receptor is measured. Correlation with disease activity has been described	pro
IL-4	Interleukin 4	Usually low titre; can inhibit TNFa and IL-6	anti
IL-5	Interleukin	Th2 cytokine that + B-cells. Main role in allergic diseases (eosinophils, anti IL-5 treatment in asthma)	pro
IL-6	Interleukin 6	Widespread pro inflammatory actions	pro
IL-7	Interleukin 7	Increased in RA in joint but variable in serum; stimulates monocytes/macrophages (Churchman & Ponchel 2008)	pro
IL-8 (CXCL8)	Interleukin 8	Action to recruit neutrophils. Previously shown to be higher in synovial fluid than blood (De Gendt et al. 1996)	pro
IL-10	Interleukin 10	Counter to inflammatory process, found elevated but not effective enough to abrogate inflammation.	anti
IL-12	Interleukin 12	T-cell development. Elevated in RA (Morita et al. 1998). Drives a Th1	pro

		response as is seen in RA	
IL-13	Interleukin 13	Increased in many autoimmune disorders but may have suppressive role (Isomäki et al. 1996).	Anti
IL-15	Interleukin 15	From macrophages induces TNFa and activates neutrophils. See text	Pro
IL-17	Interleukin 17	Lead to IL-6 production and role in osteoclast action. See text	Pro
IP-10 (CXCL 10)	Interferon gamma inducible protein	Chemokine that may have pathogenic role (Kwak et al. 2008) recruiting cells into synovium	Pro
MCP-1 (CCL2)	Monocyte chemo-attractant protein 1	Elevated in RA, chemotactic for leucocytes and some relation to DAS28 (Liou et al. 2013)	Pro
MIG (CXCL 9)	Monokine induced by IFNg	Elevated in RA	Pro
MIP-1 alpha (CCL 3)	Macrophage inflammatory protein 1a	Chemoattractant for a number of white cells	Pro
MIP-1 beta (CCL 4)	Macrophage inflammatory protein	Chemotactic for macrophages and neutrophils in the RA joint (Koch et al. 1994) (Hatano et al. 1999)	
RANTES (CCL 5)	Chemokine ligand 5	Chemo attractant; fibroblasts produce upon TNFa and IL-1 stimulation	Pro
TNFa	Tumour necrosis factor alpha	Produced by activated macrophages and T-cells; prototypical pro-inflammatory cytokine; B and T cell proliferation, adhesion molecule expression	Pro
VEGF	Vascular endothelial growth factor	Promotes proliferation and vascular permeability. Thus increased in RA and correlates with ESR and CRP (Hong et al. 2007)	Pro
IL- 21	Interleukin 21	Enhances maturation of NK cells with IL-15, Th17 cells and promotes osteoclastogenesis	Pro
IL-23	Interleukin 23	Role in Th-17 cell activation and differentiation and correlates with clinical variables (Rong et al. 2012)	Pro
MMP 3	Matrix metalloproteinase 3	Important effector molecule destroying tissue matrix	Pro
MMP 12	Matrix metalloproteinase 12		
MMP 13	Matrix metalloproteinase 13		
CXCL 11	Chemokine ligand 11	Chemotactic for activated T-cells	Pro
OPG	Osteoprotegerin	Binds to RANKL and prevents it biding to it's receptor RANK. Action to reduce number of osteoclasts maturing to effect bone damage	Anti
RANKL	Receptor activator of nuclear factor kappa-B ligand	Expressed on osteoblast. Binding to RANK on osteoclast precursors lead to their maturation.	Pro

Table 5-89 Cytokines studied in biologic and DMARD resistant study groups, brief function and role

5.6 Results

5.6.1 Cross-sectional analysis

Results for those analytes tested across the four study groups at baseline are shown and statistical differences illustrated.
Boxplots with 95% confidence error bars are shown to represent the cytokine examined and all values are pg/ml.

5.6.1.1 Pro-inflammatory cytokines

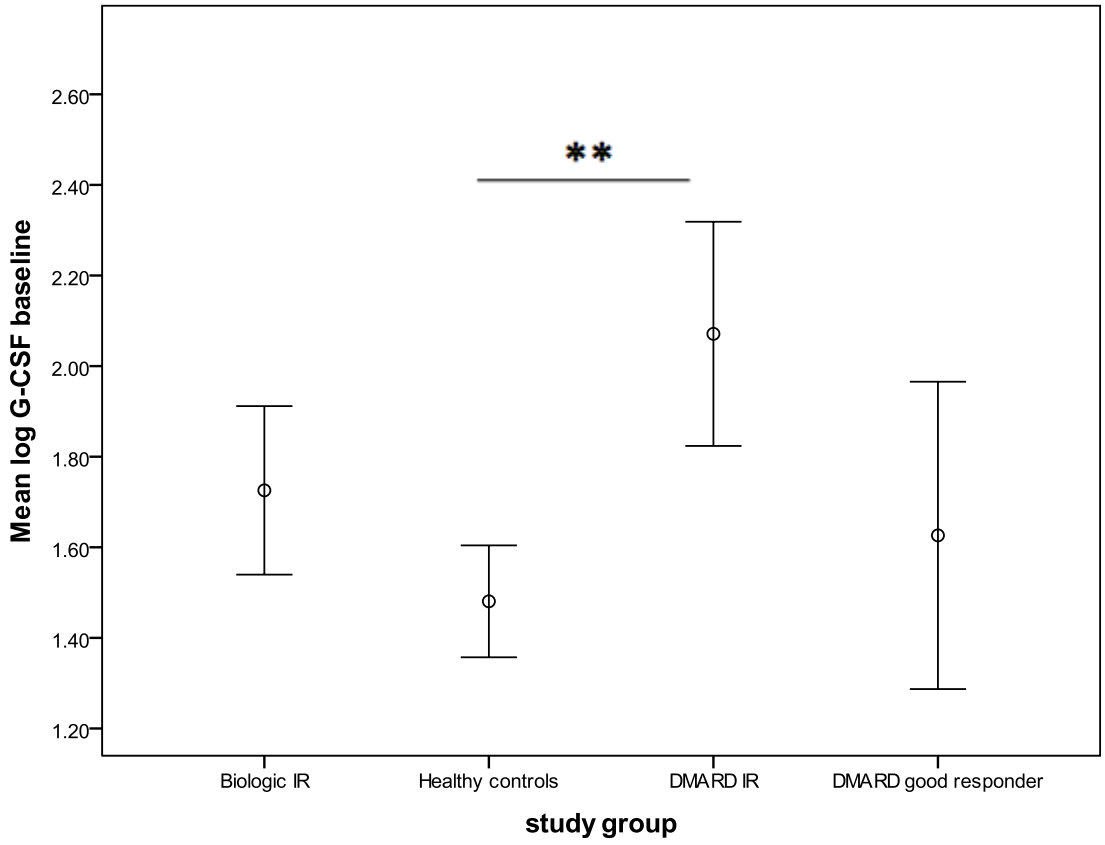

Figure 5-72 G-CSF by study group (where *=p<0.05 and **=p<0.01)

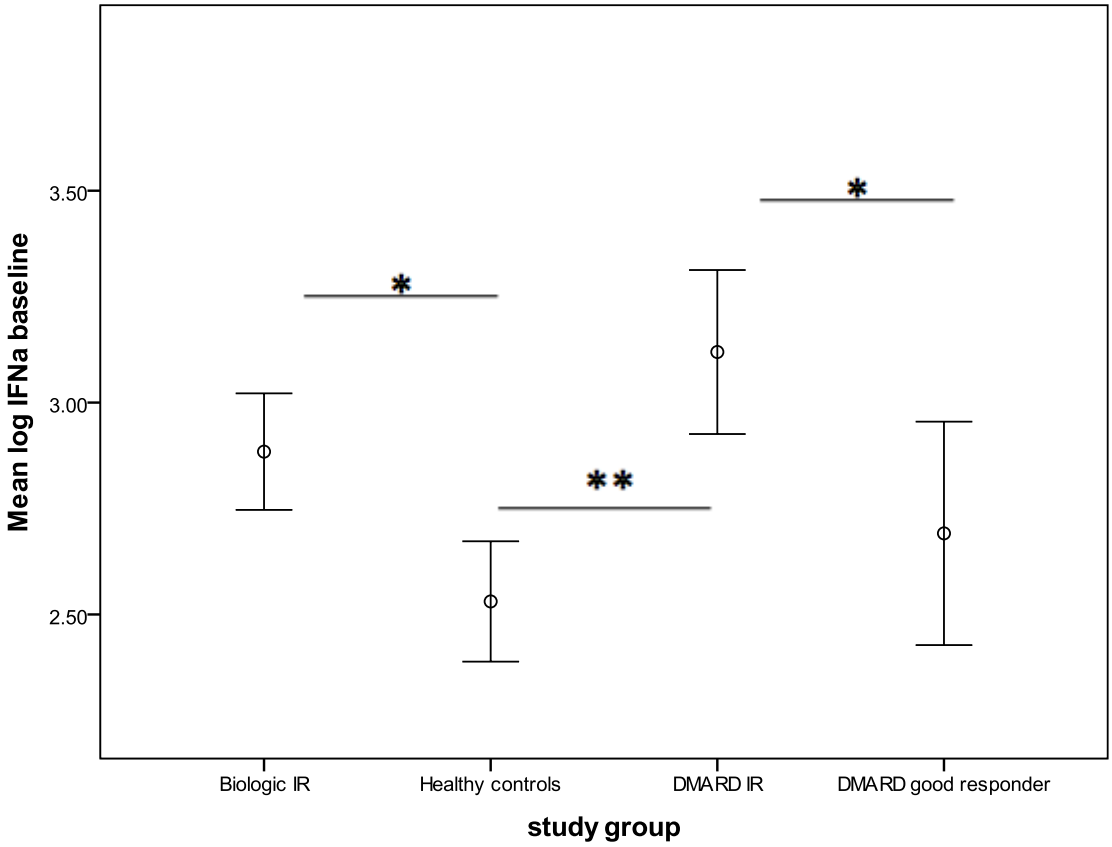

Figure 5-73 IFNa by study group (where *=p<0.05 and **=p<0.01)

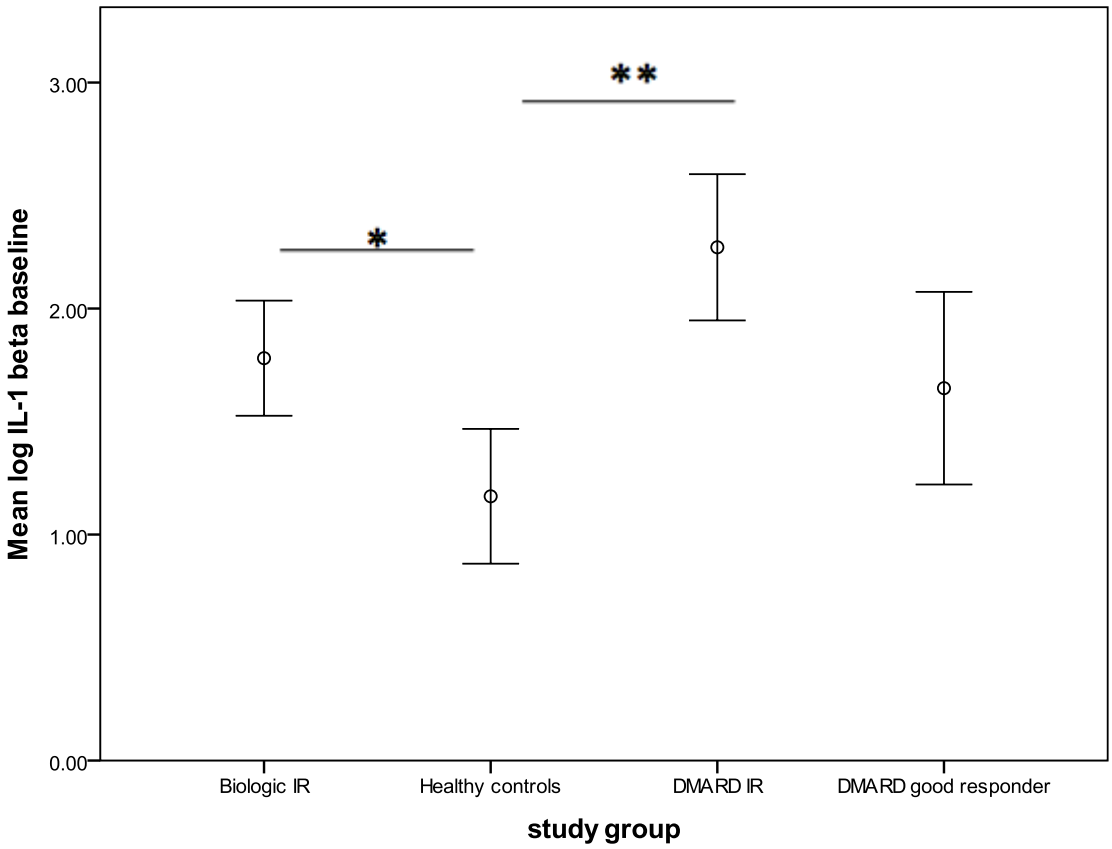

Figure 5-74 IL-1 beta by study group (where *=p<0.05 and **=p<0.01)

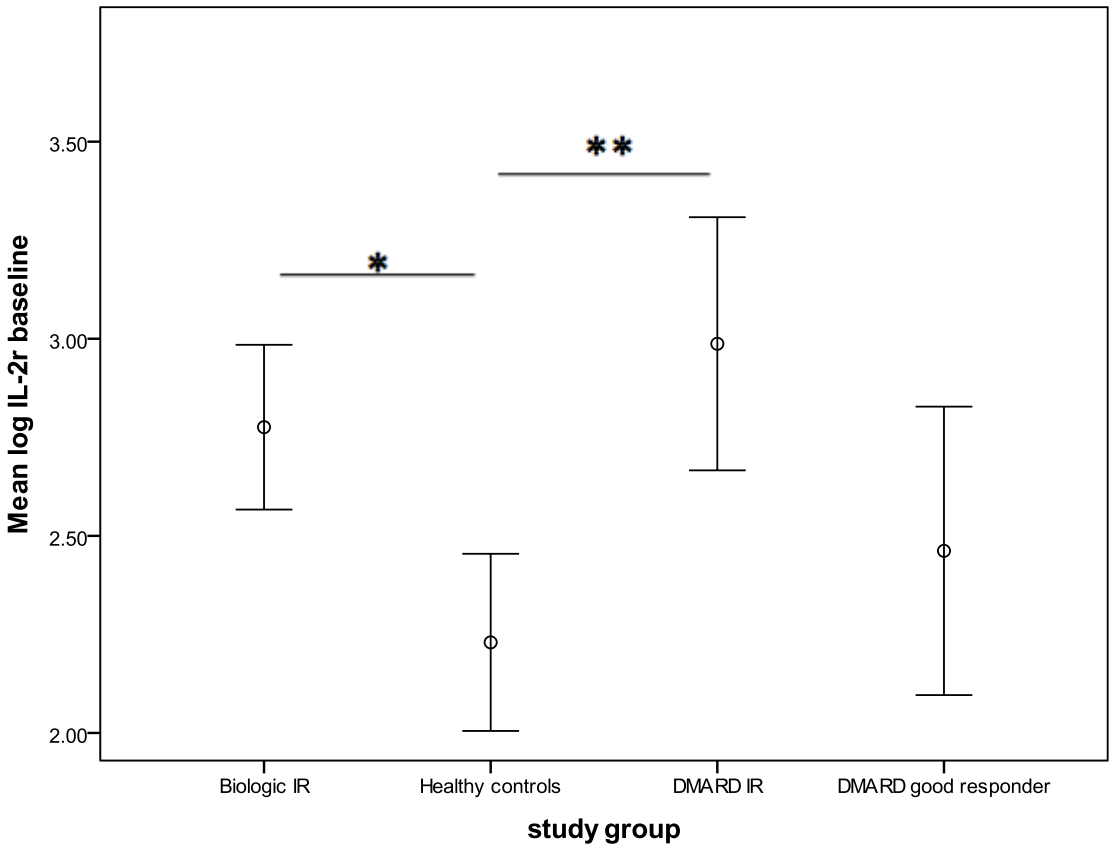

Figure 5-75 IL-2r by study group (where *=p<0.05 and **=p<0.01)

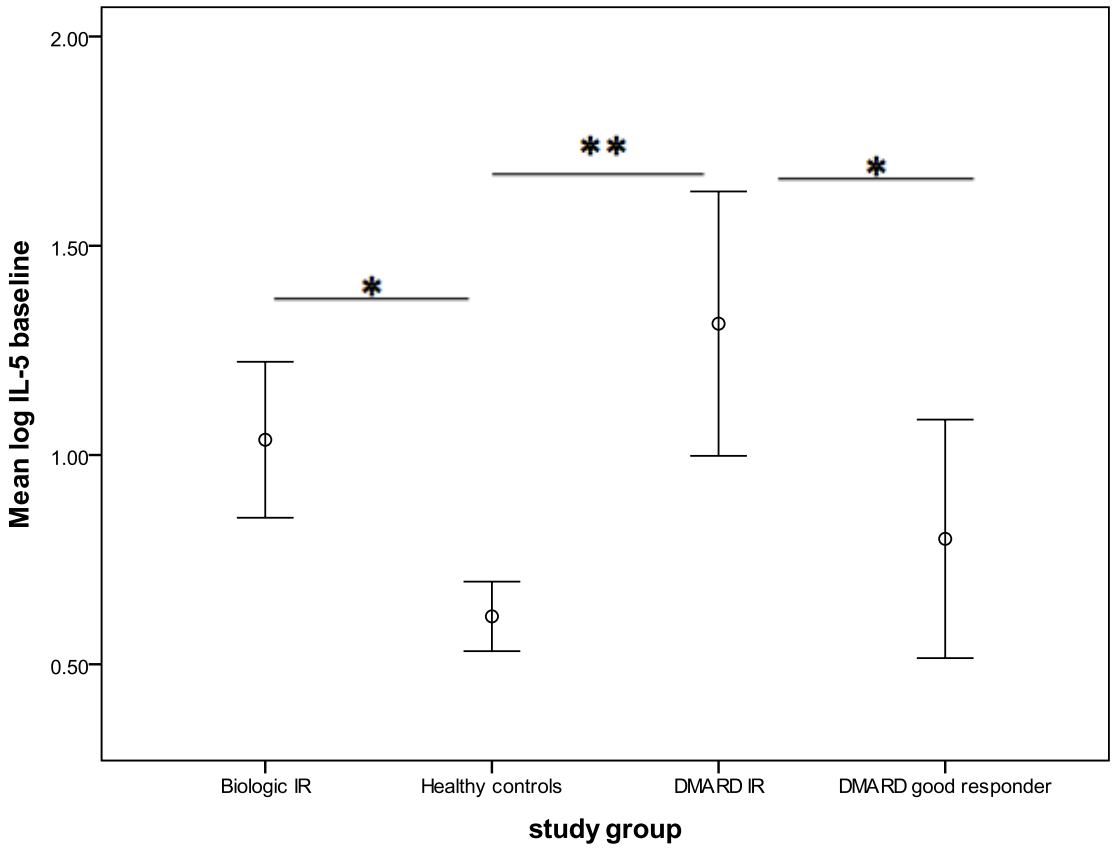

Figure 5-76 IL-5 by study group (where *=p<0.05 and **=p<0.01)

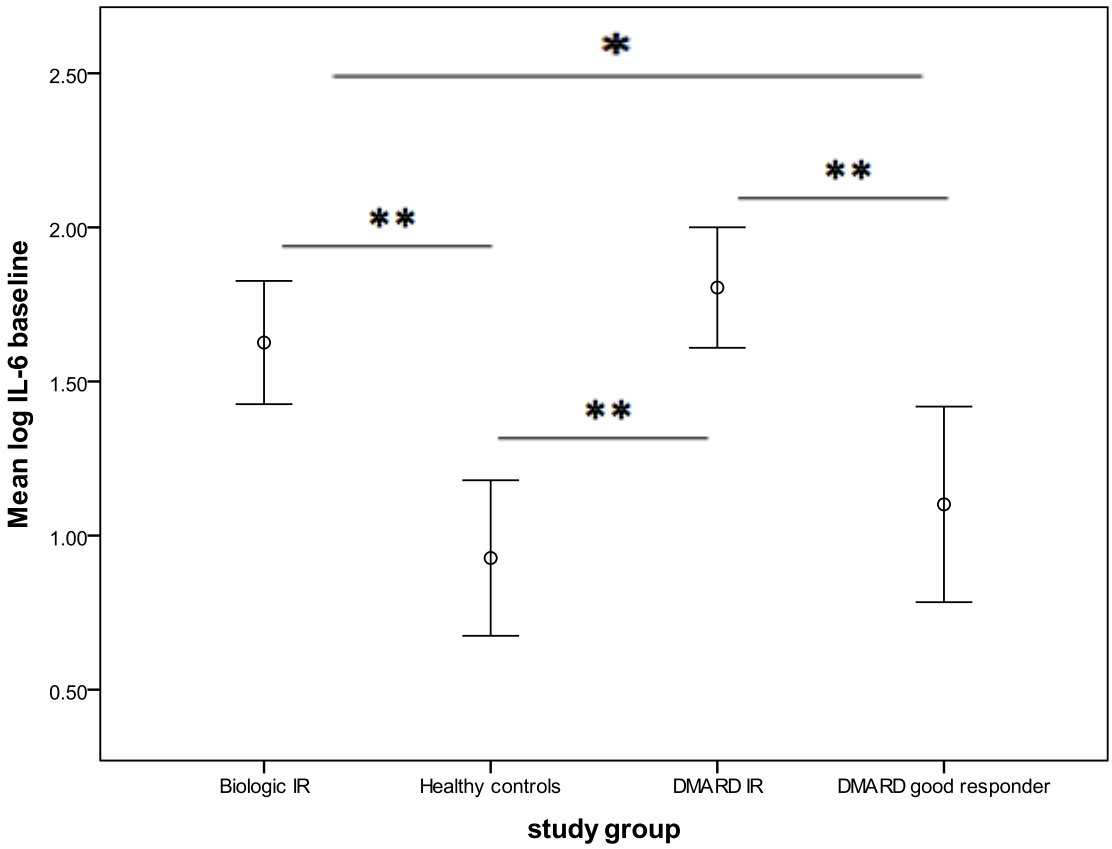

Figure 5-77 IL-6 by study group (where *=p<0.05 and **=p<0.01)

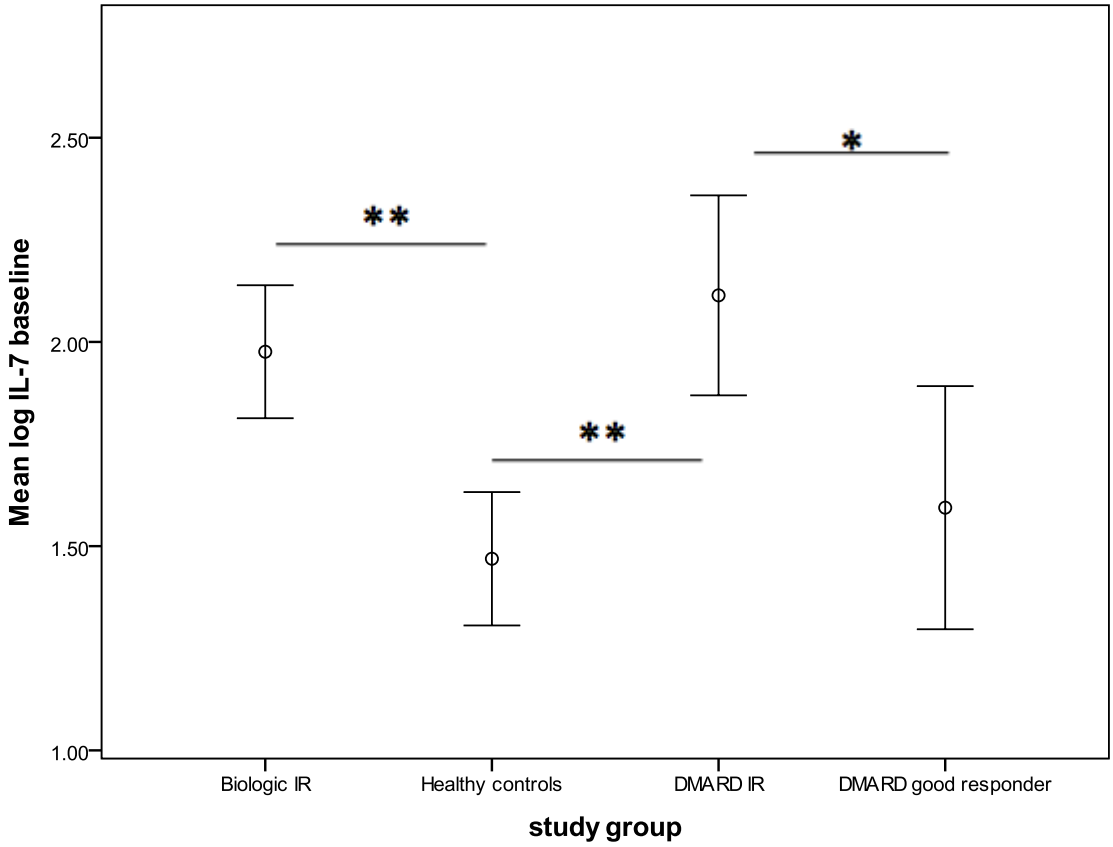

Figure 5-78 IL-7 by study group (where *=p<0.05 and **=p<0.01)

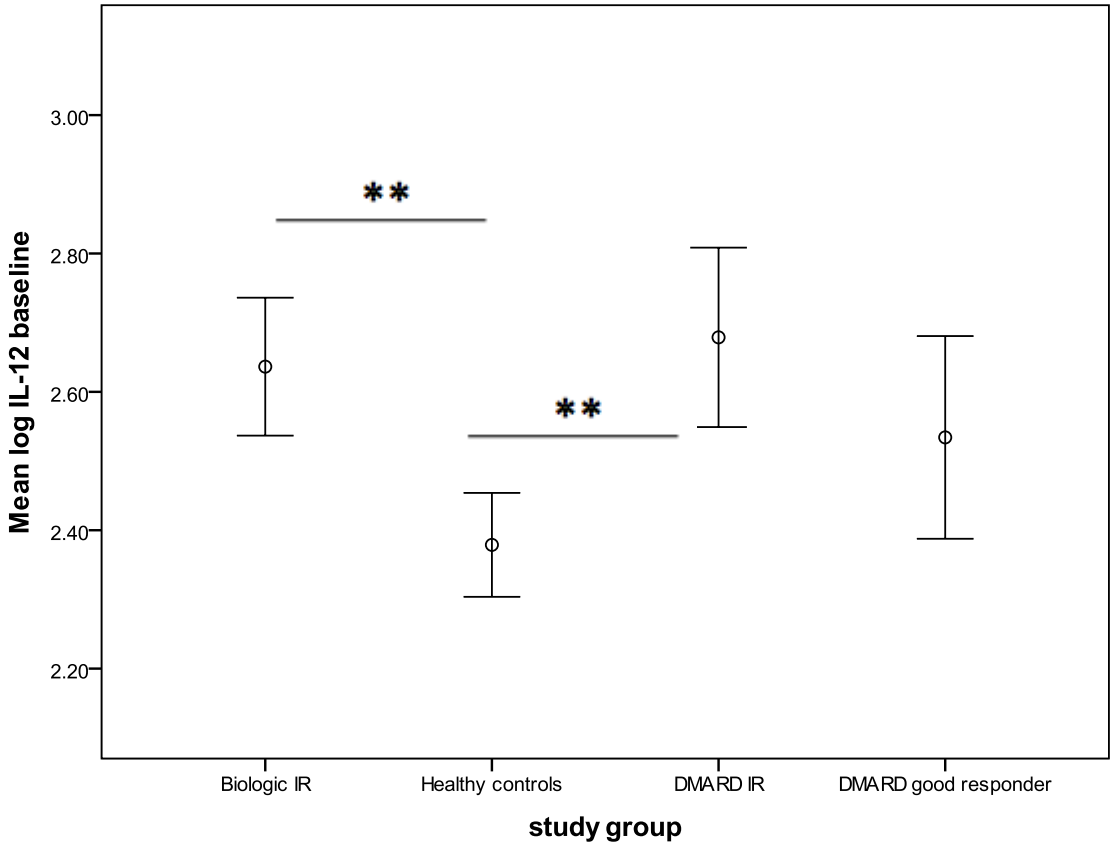

Figure 5-79 IL-12 by study group (where *=p<0.05 and **=p<0.01)

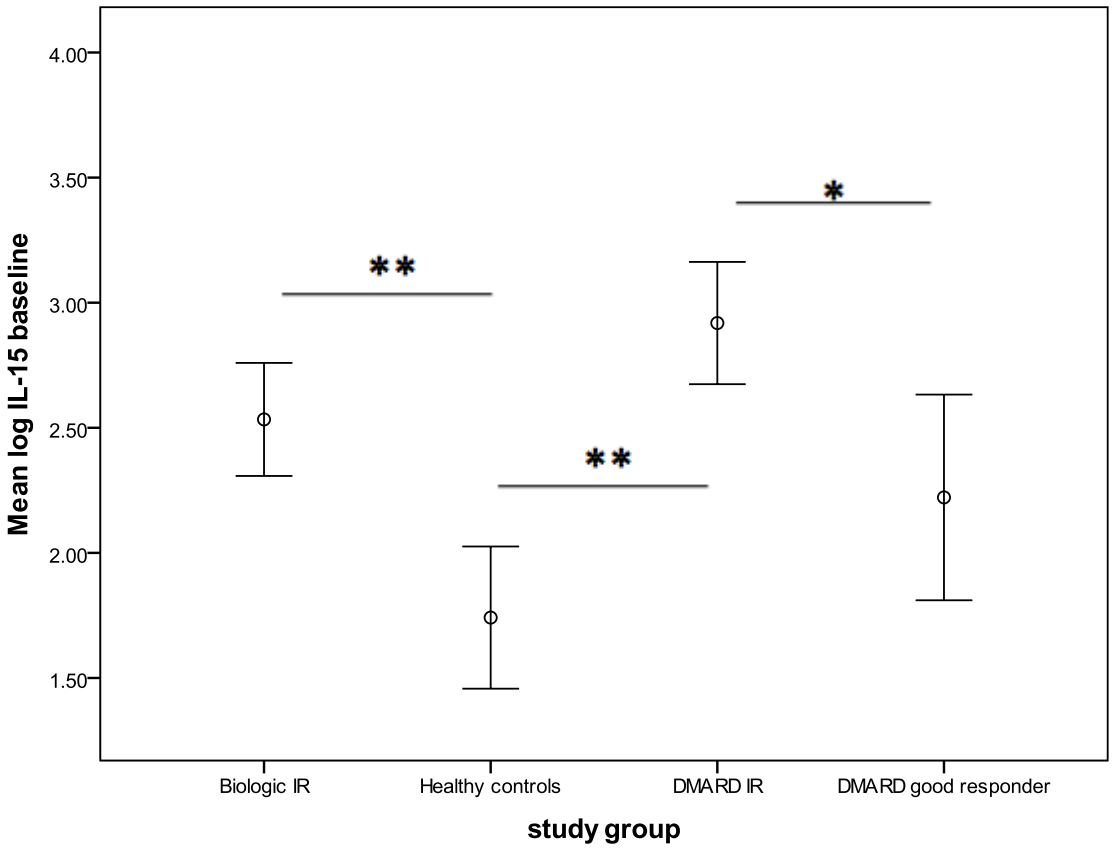

Figure 5-80 IL-15 by study group (where *=p<0.05 and **=p<0.01)

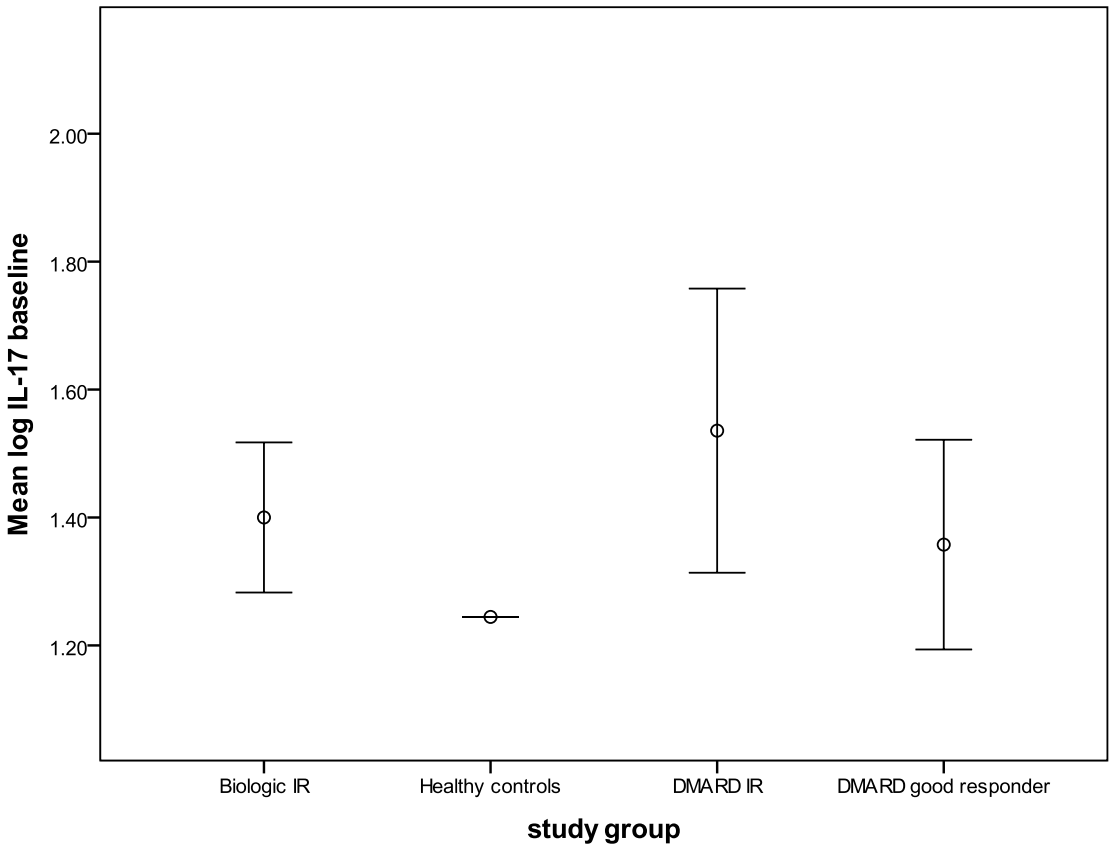

Figure 5-81 IL-17 by study group (where *=p<0.05 and **=p<0.01)

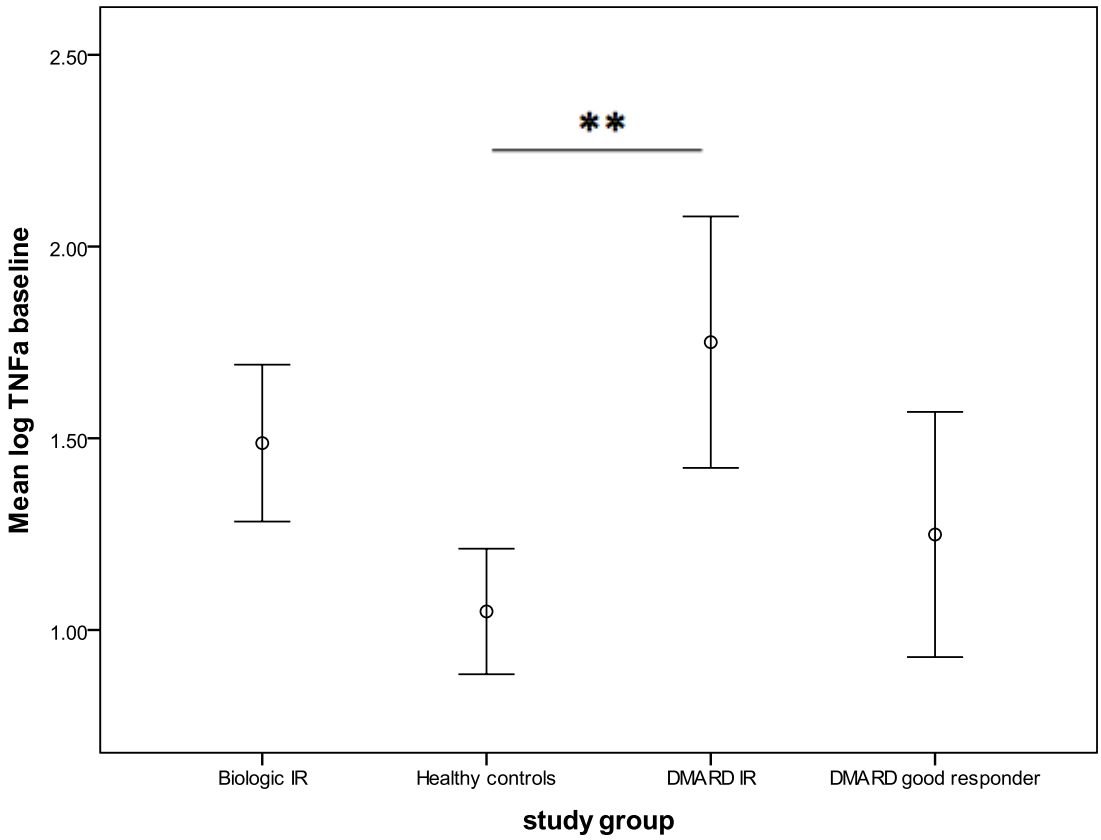

Figure 5-82 TNFa by study group (where *=p<0.05 and **=p<0.01)

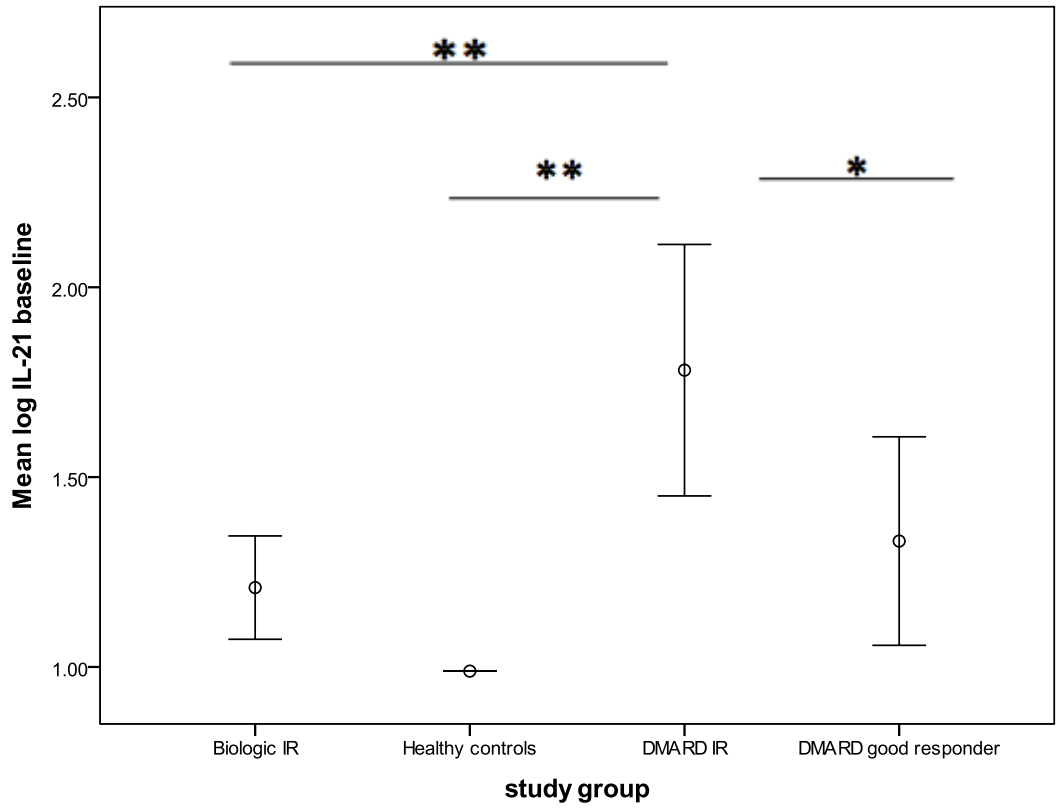

Figure 5-83 IL-21 by study group (where *=p<0.05 and **=p<0.01)

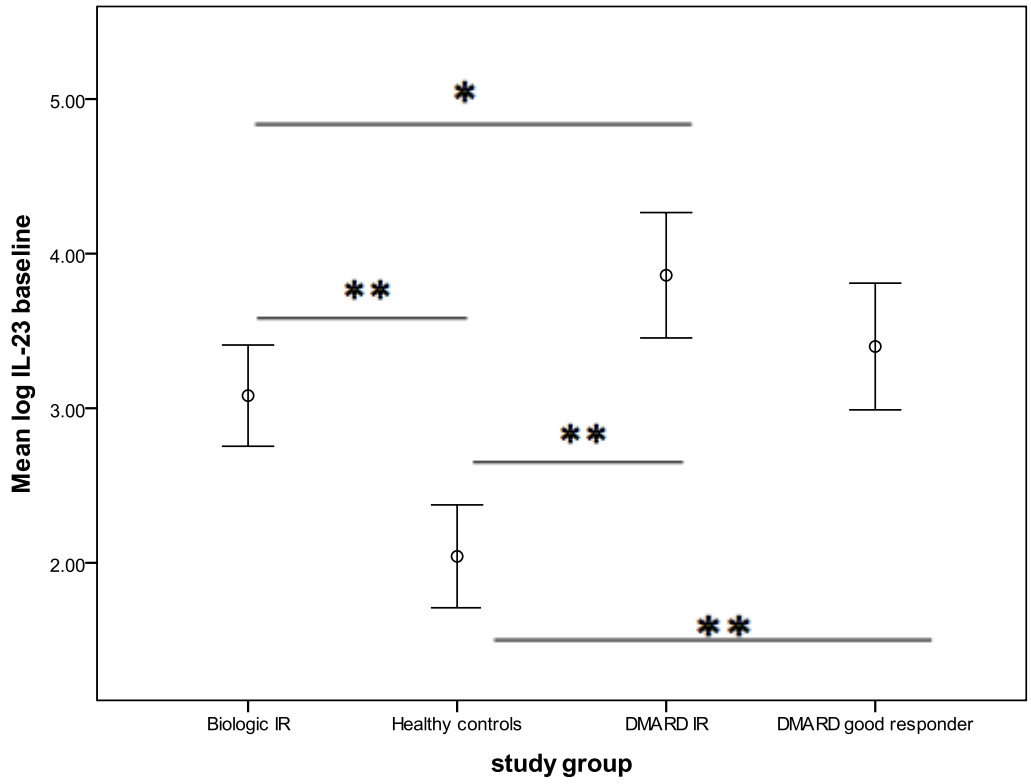

Figure 5-84 IL-23 by study group (where *=p<0.05 and **=p<0.01)

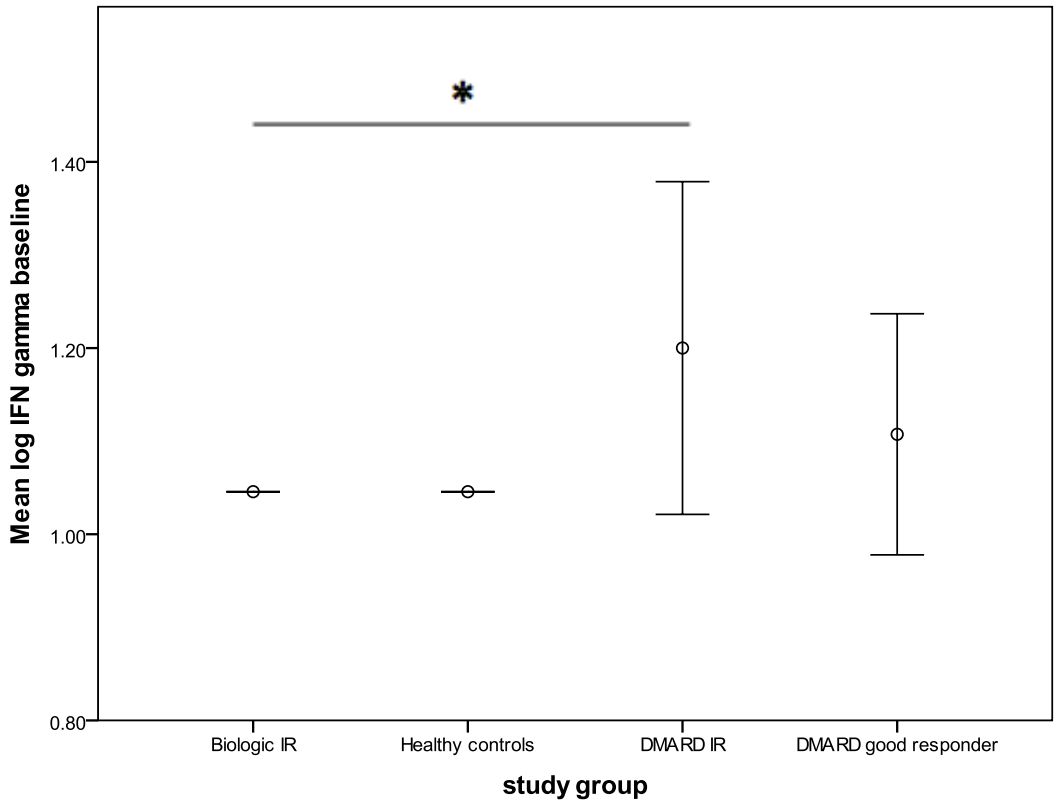

Figure 5-85 IFN gamma by study group (where *=p<0.05 and **=p<0.01)

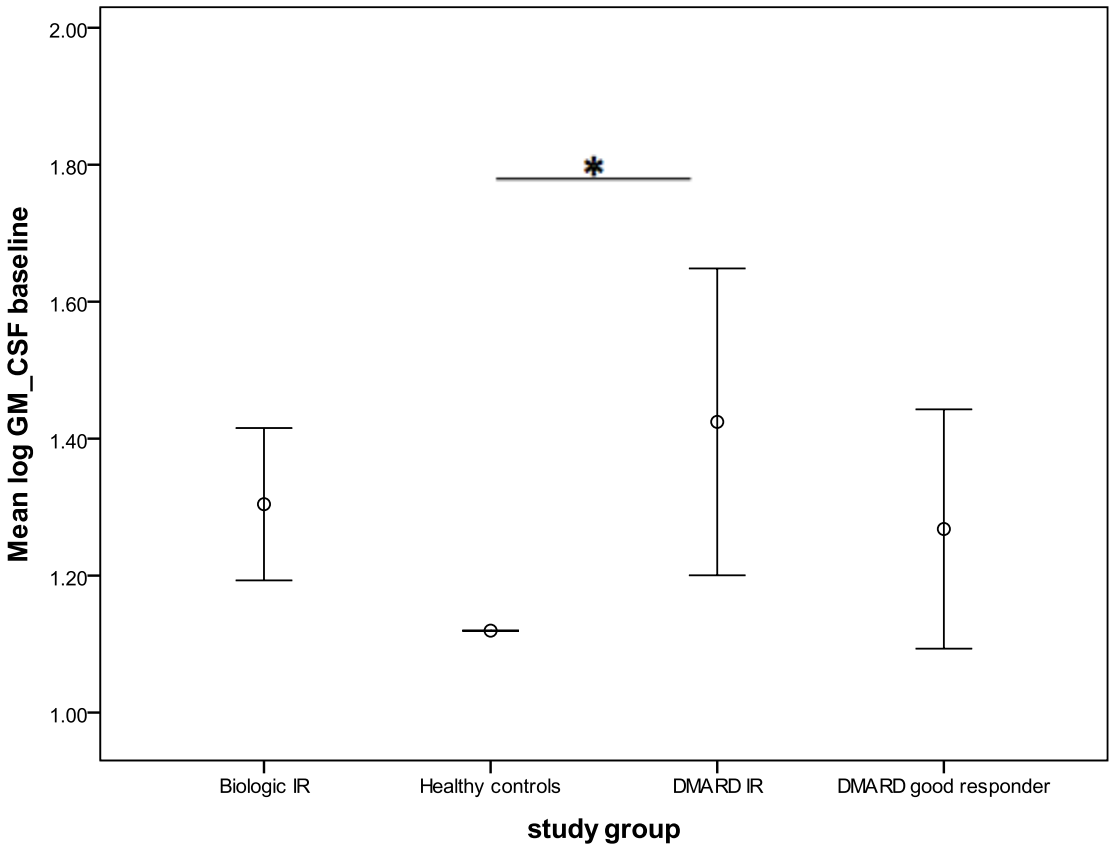

Figure 5-86 GM-CSF beta by study group (where *=p<0.05 and **=p<0.01)

5.6.1.2 'Anti-inflammatory' cytokines

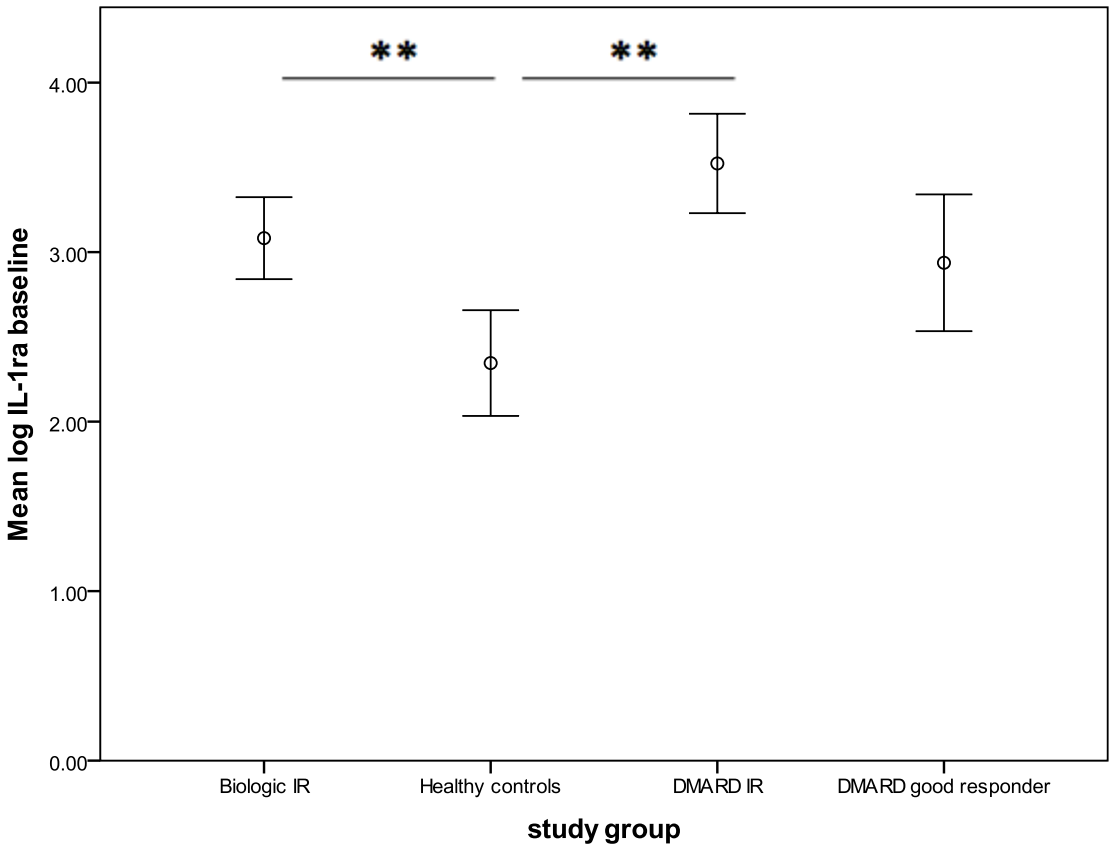

Figure 5-87 IL-1ra by study group (where *=p<0.05 and **=p<0.01)

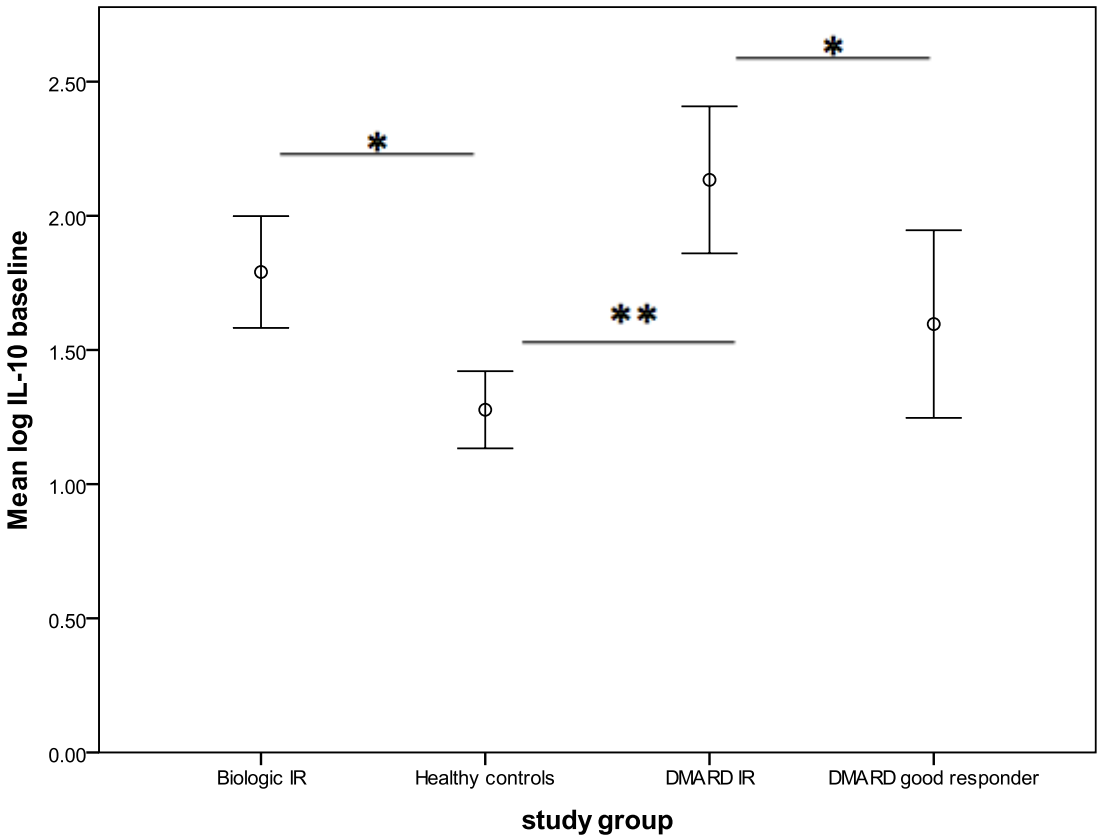

Figure 88 IL-10 by study group (where *=p<0.05 and **=p<0.01)

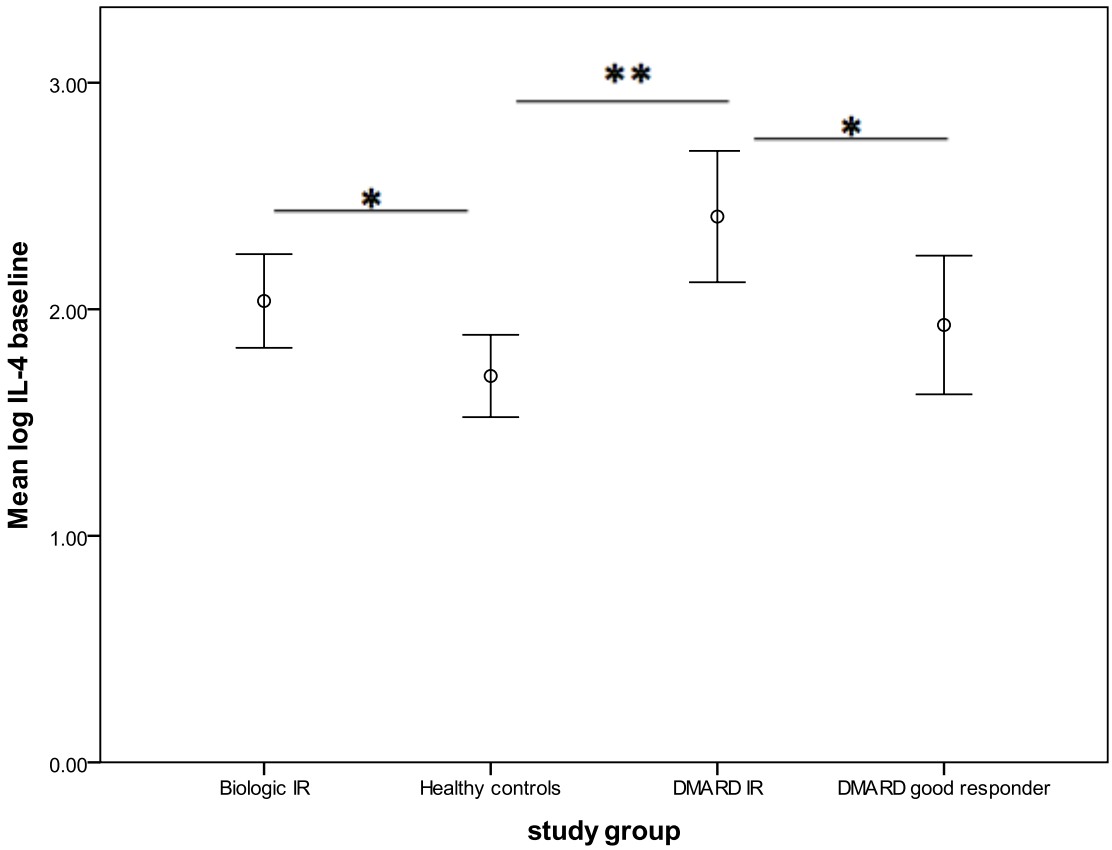

Figure 89 IL-4 by study group (where *=p<0.05 and **=p<0.01)

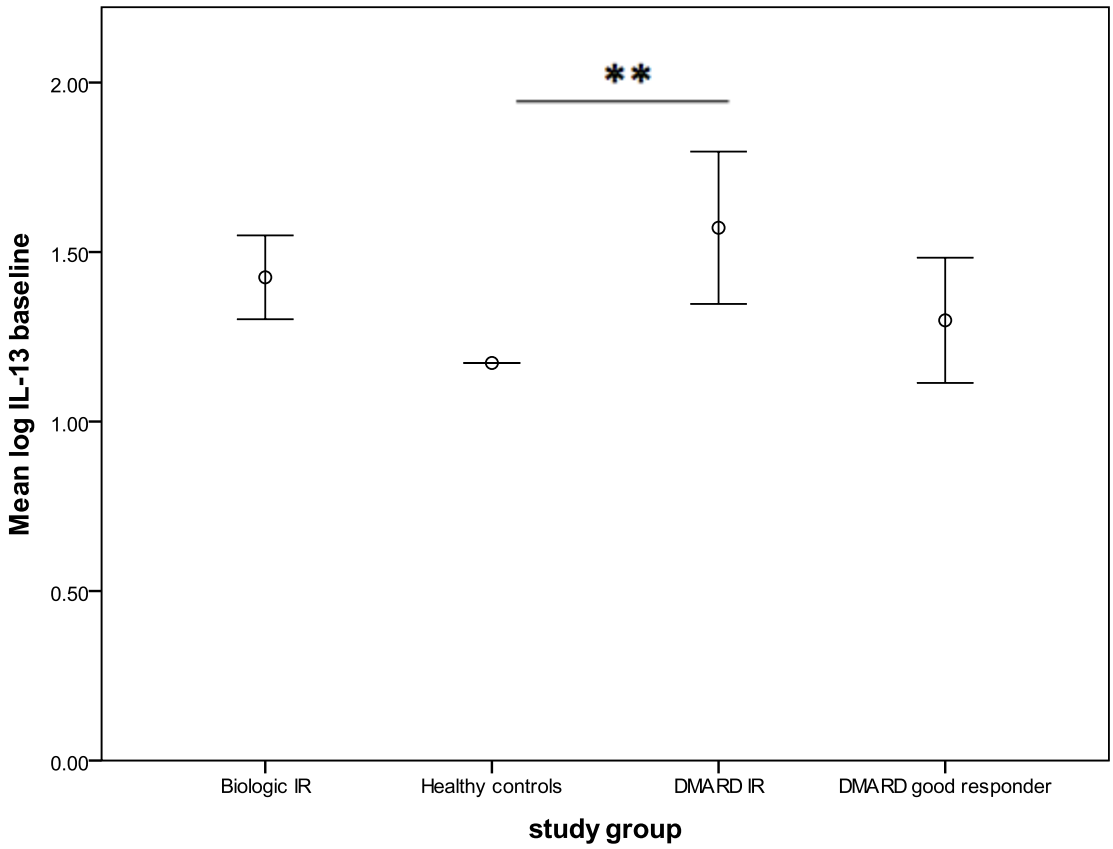

Figure 5-90 IL-13 by study group (where *=p<0.05 and **=p<0.01)

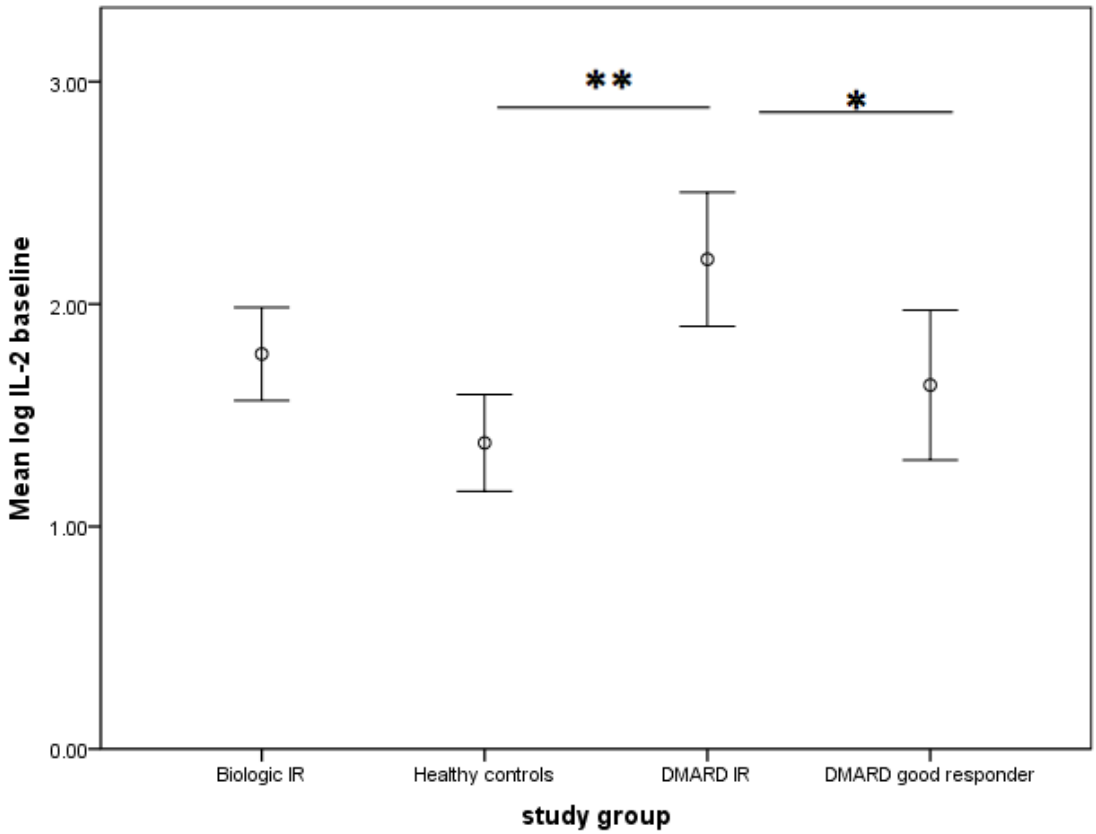

Figure 5-91 IL-2 by study group (where *=p<0.05 and **=p<0.01)

5.6.1.3 Bone cytokines and MMPs

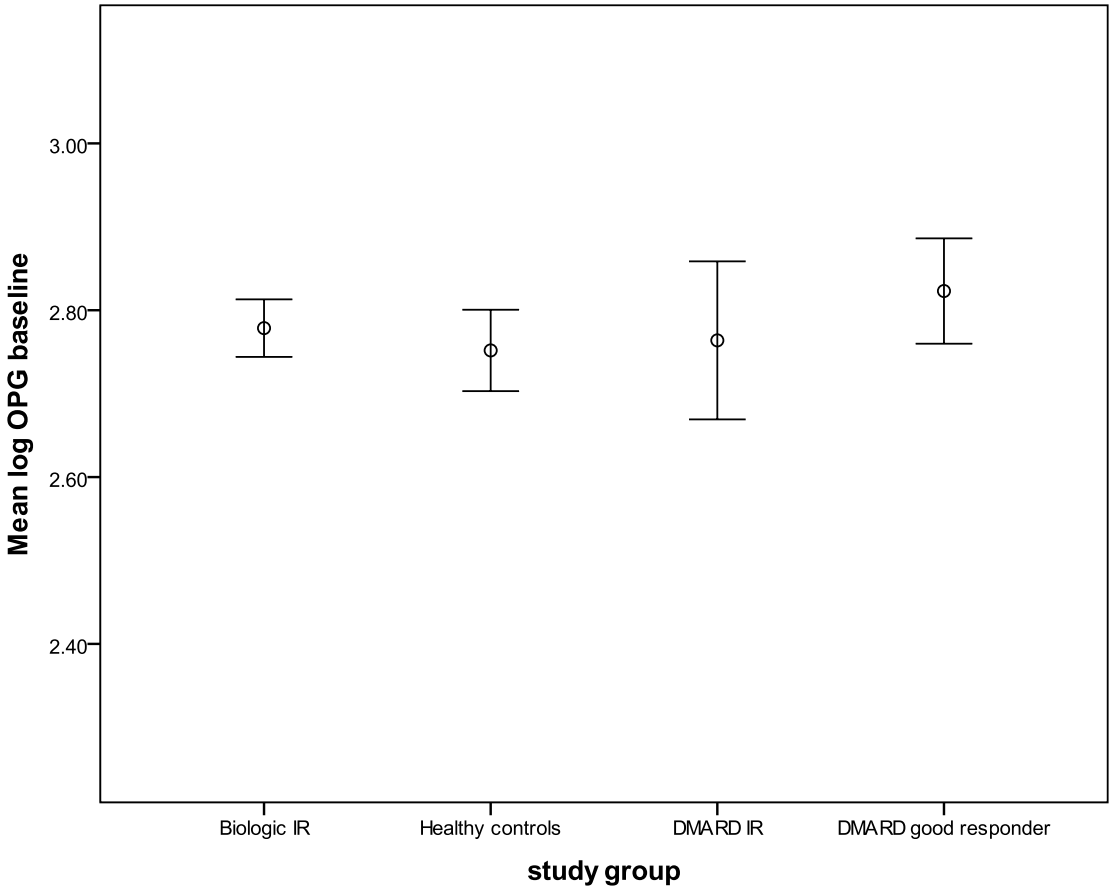

Figure 5-92 OPG by study group (where *=p<0.05 and **=p<0.01)

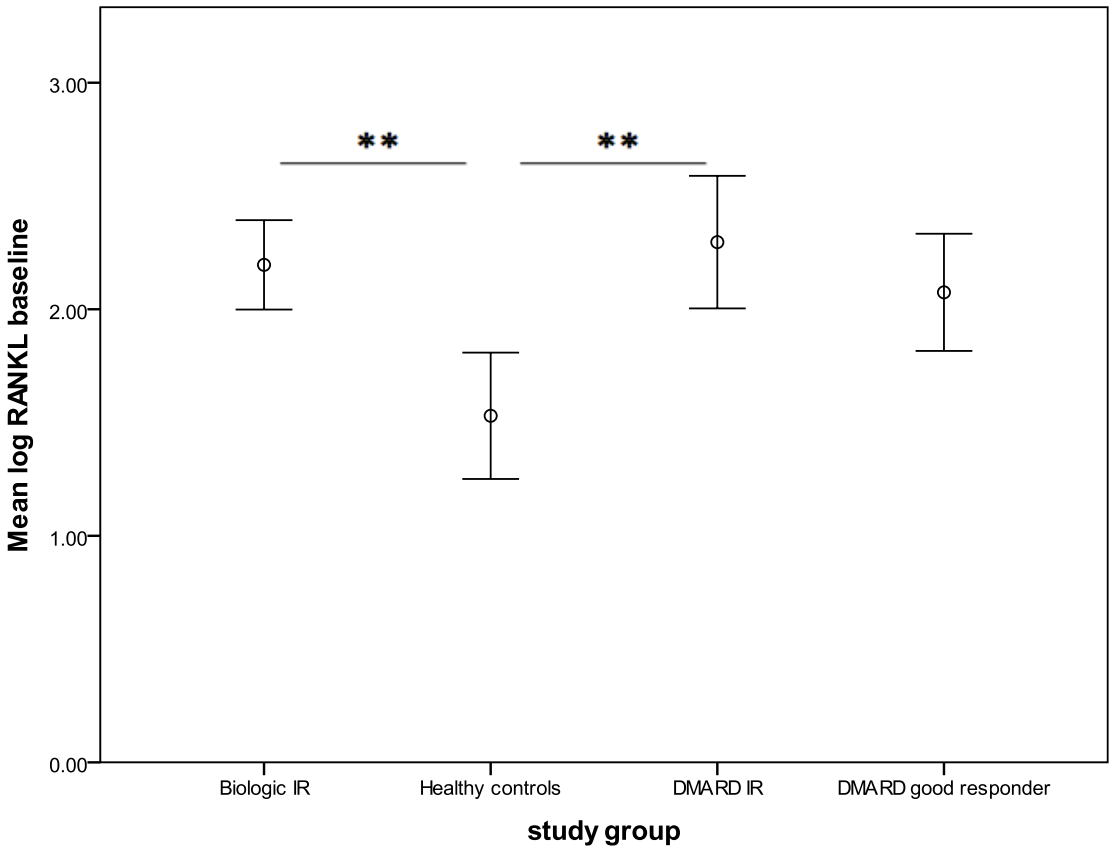

Figure 5-93 RANKL by study group (where *=p<0.05 and **=p<0.01)

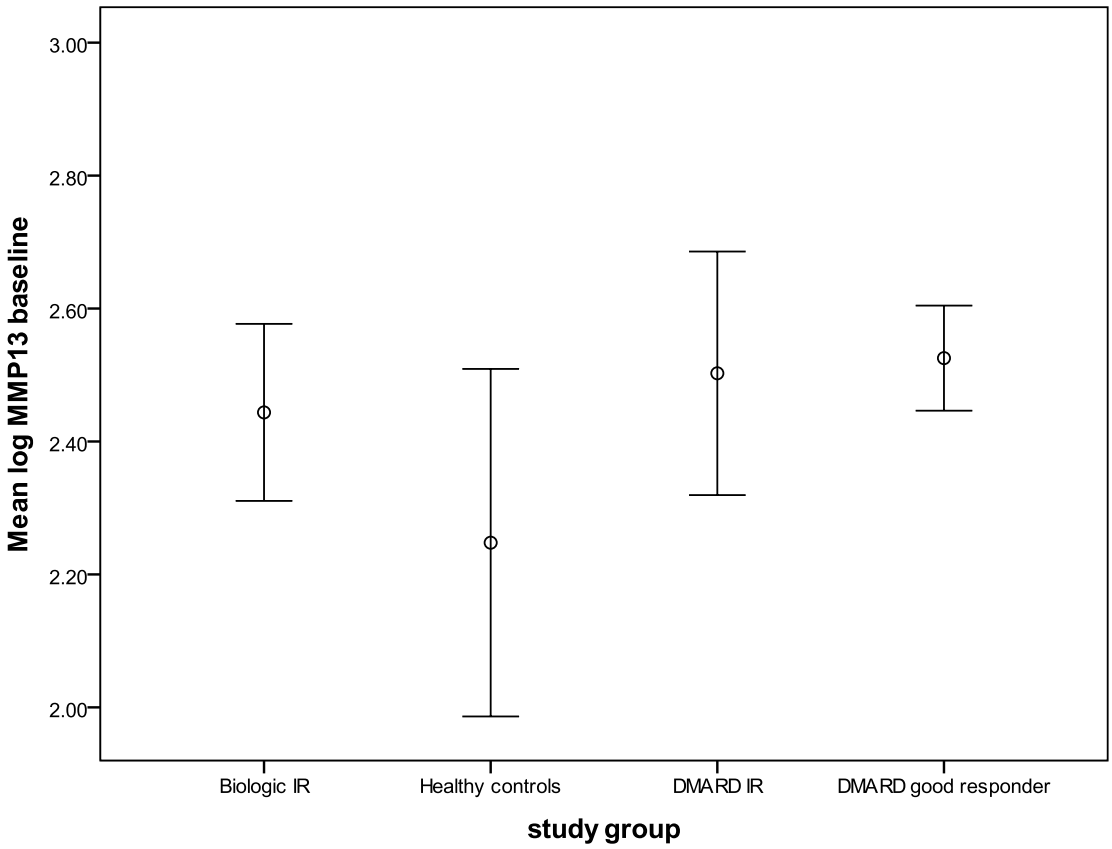

Figure 5-94 MMP-13 by study group (where *=p<0.05 and **=p<0.01)

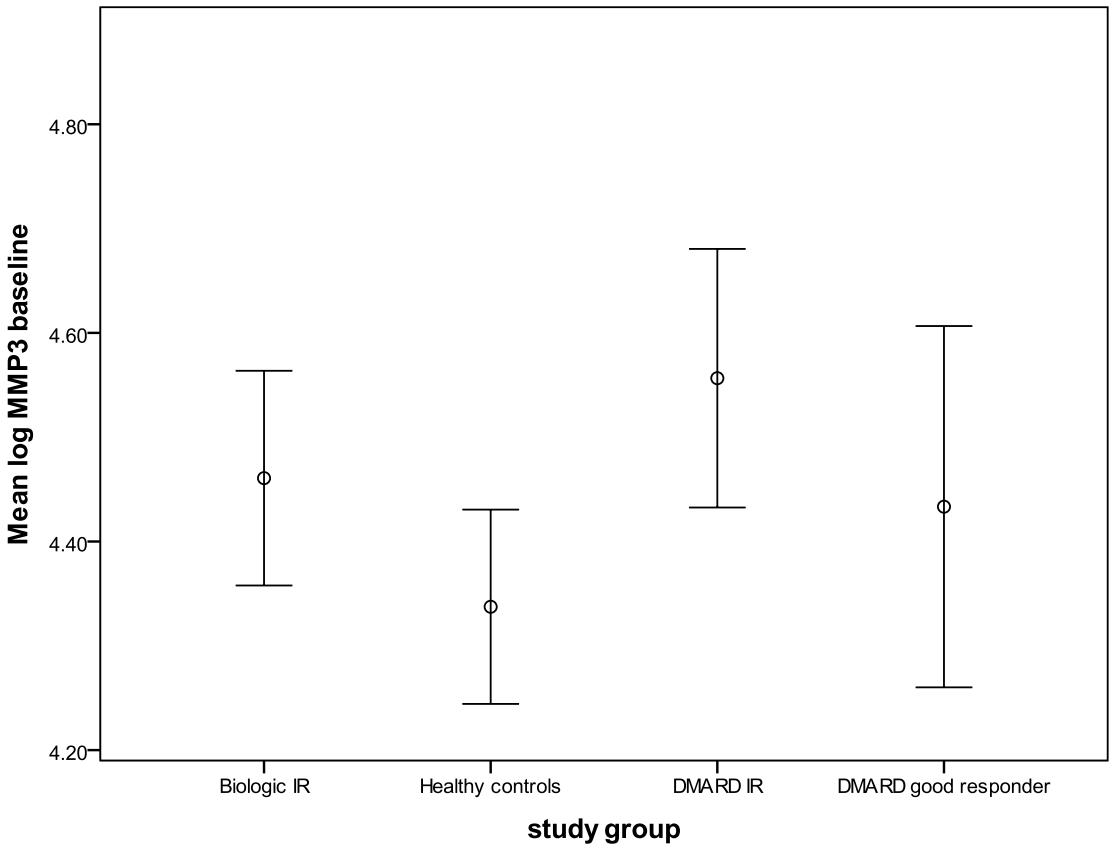

Figure 5-95 MMP-3 by study group (where *=p<0.05 and **=p<0.01)

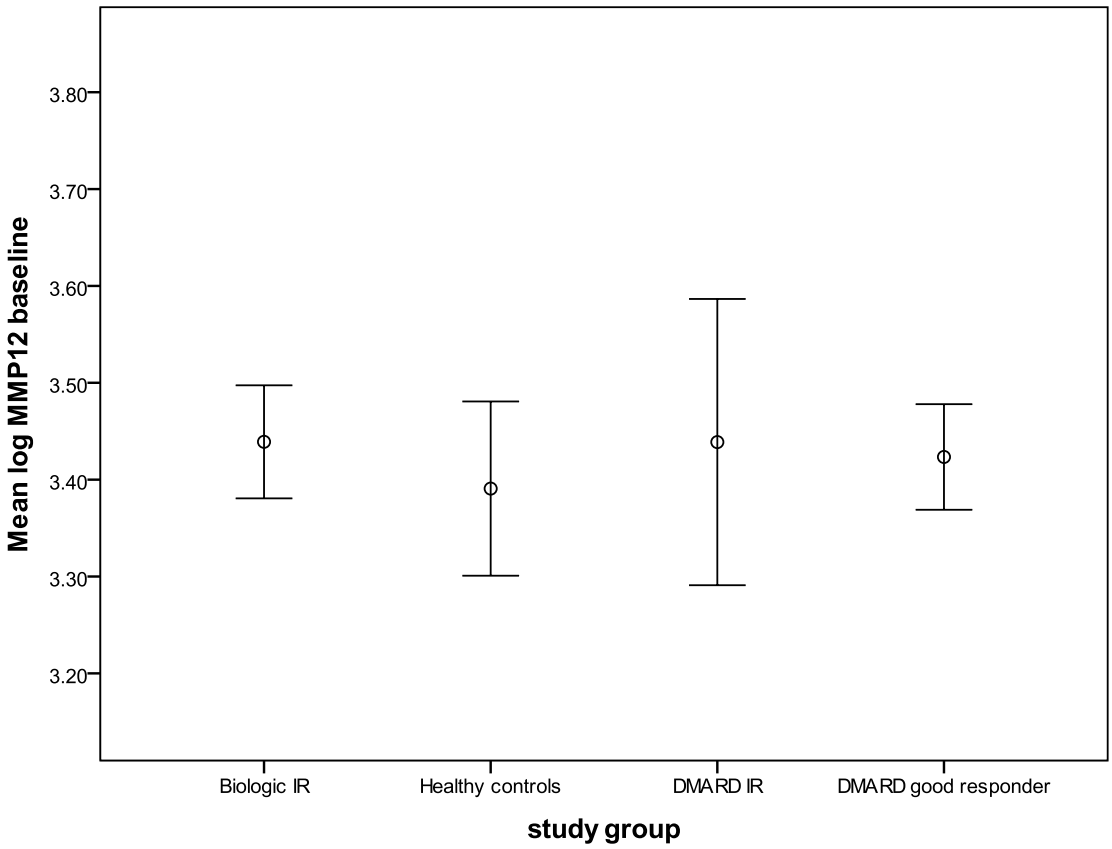

Figure 96 MMP-12 by study group (where *=p<0.05 and **=p<0.01)

5.6.1.4 Chemokines

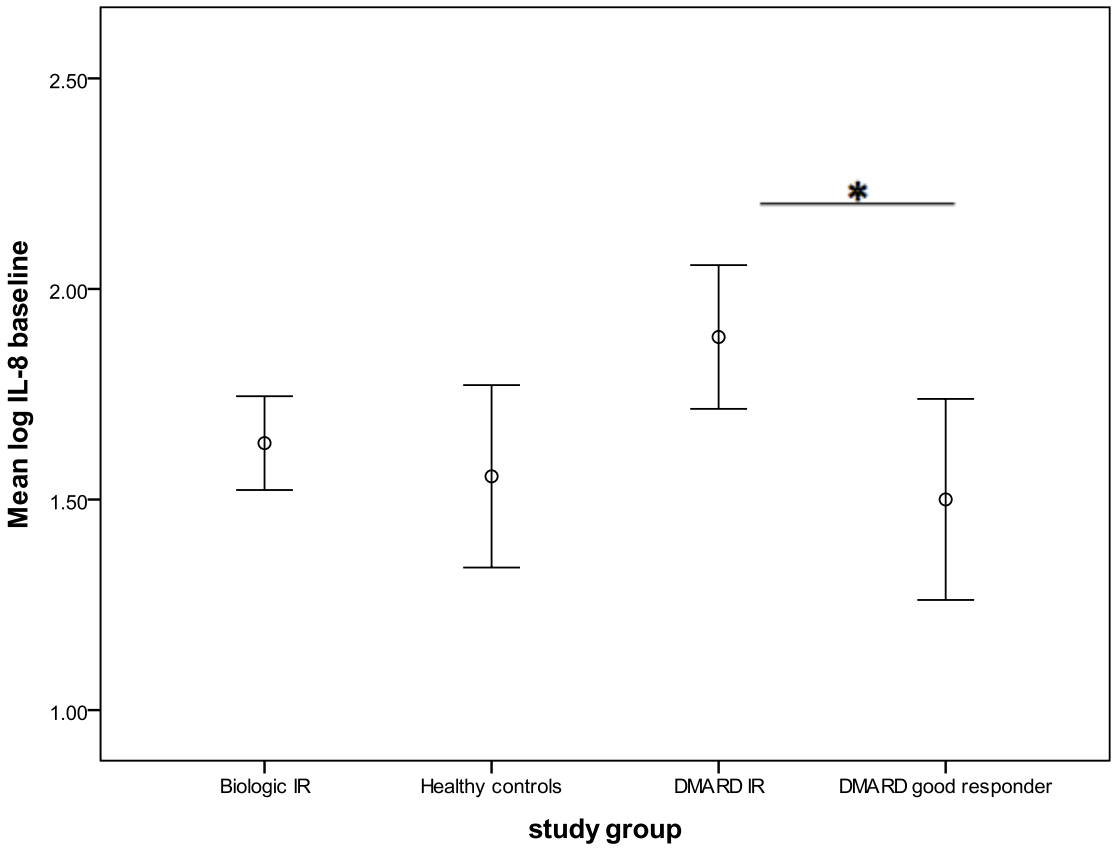

Figure 5-97 IL-8 by study group (where *=p<0.05 and **=p<0.01)

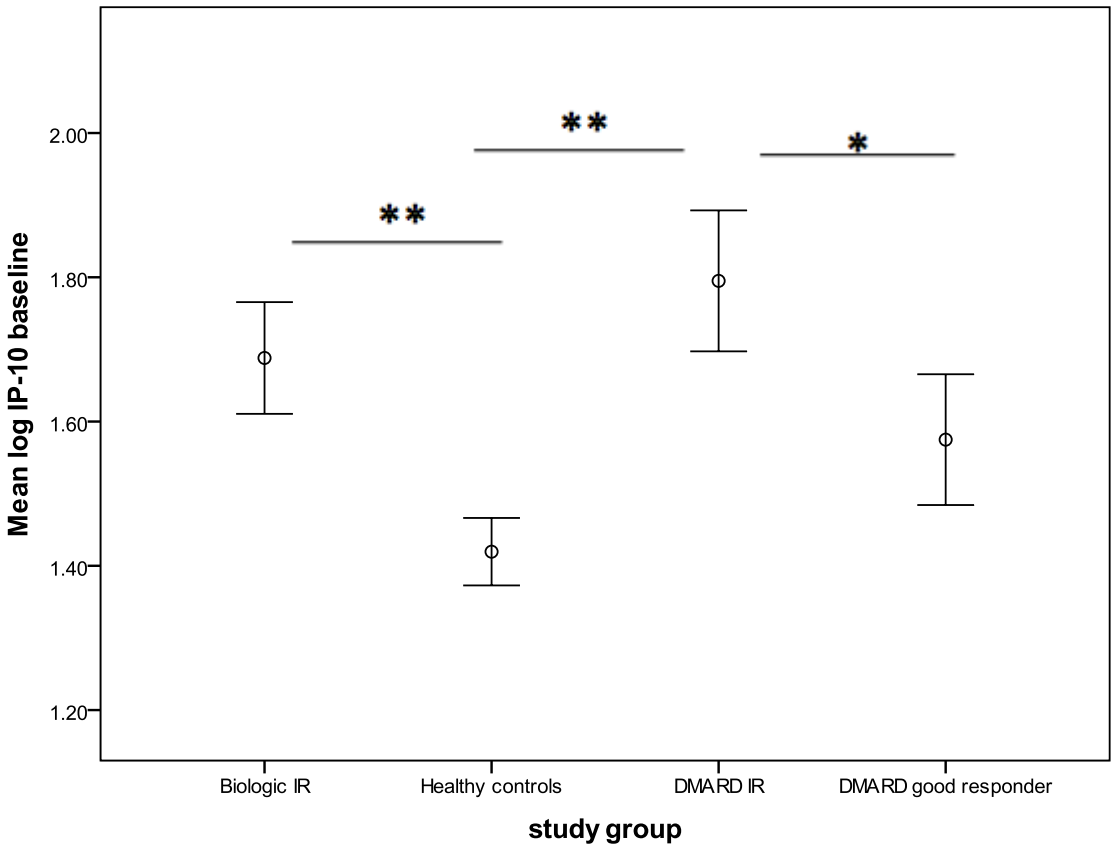

Figure 5-98 IP-10 by study group (where *=p<0.05 and **=p<0.01)

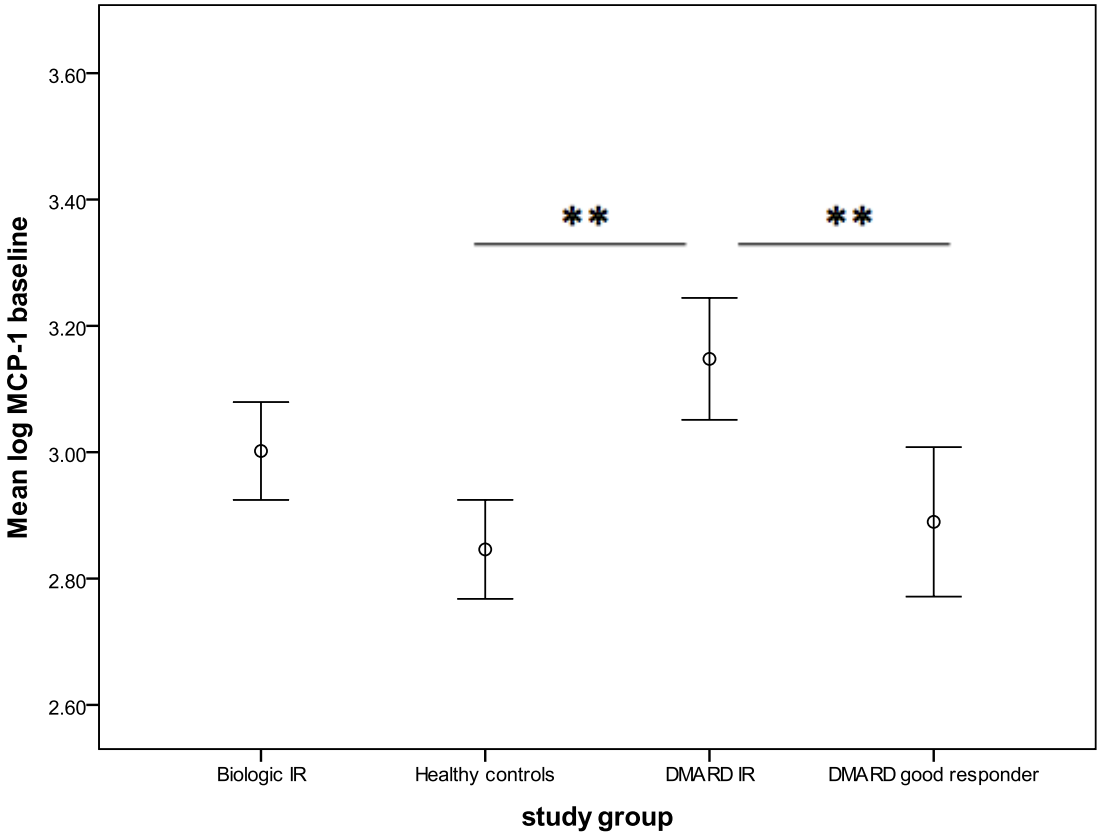

Figure 5-99 MCP-1 by study group (where *=p<0.05 and **=p<0.01)

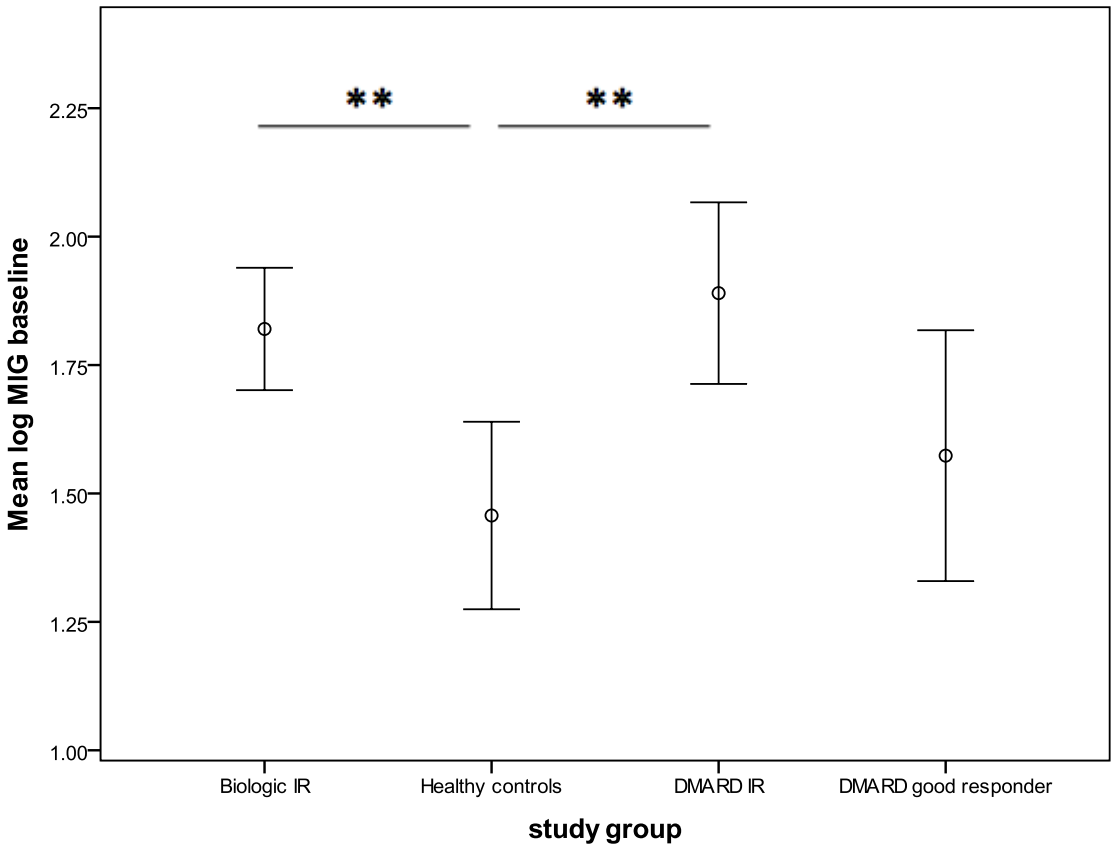

Figure 5-100 MIG by study group (where *=p<0.05 and **=p<0.01)

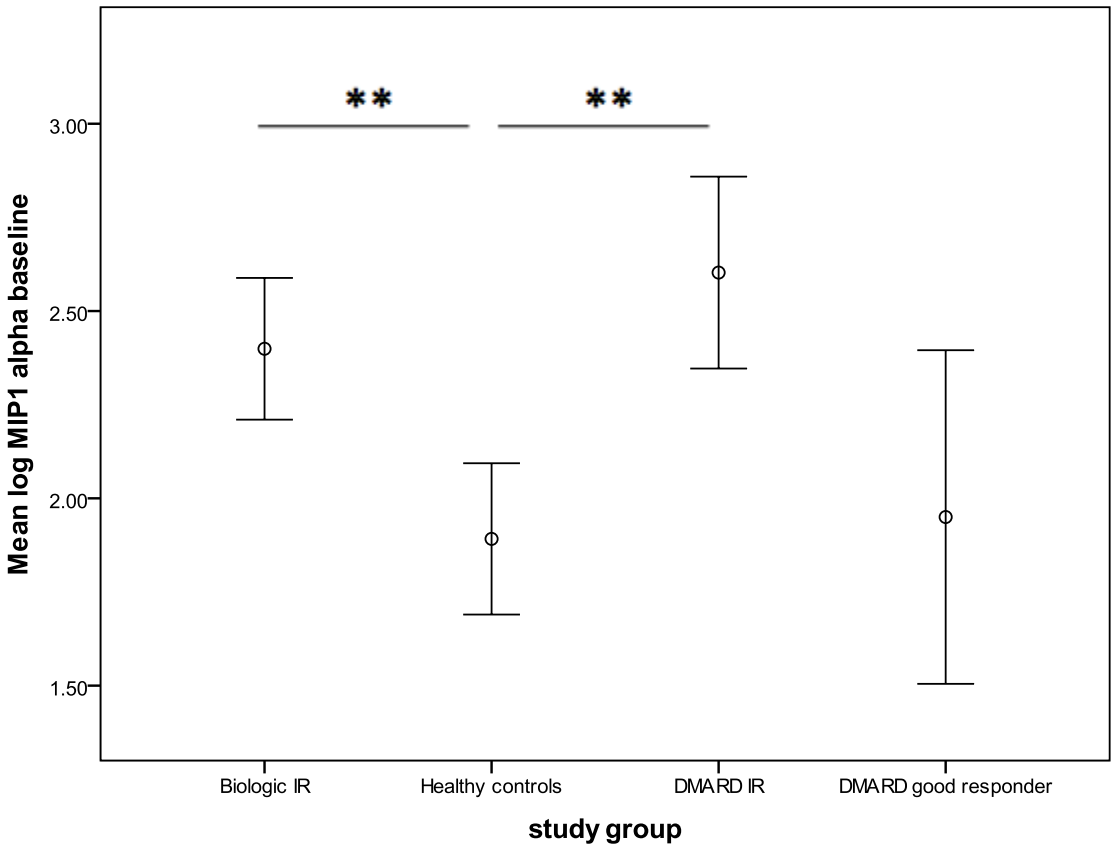

Figure 5-101 MIP1 alpha by study group (where *=p<0.05 and **=p<0.01)

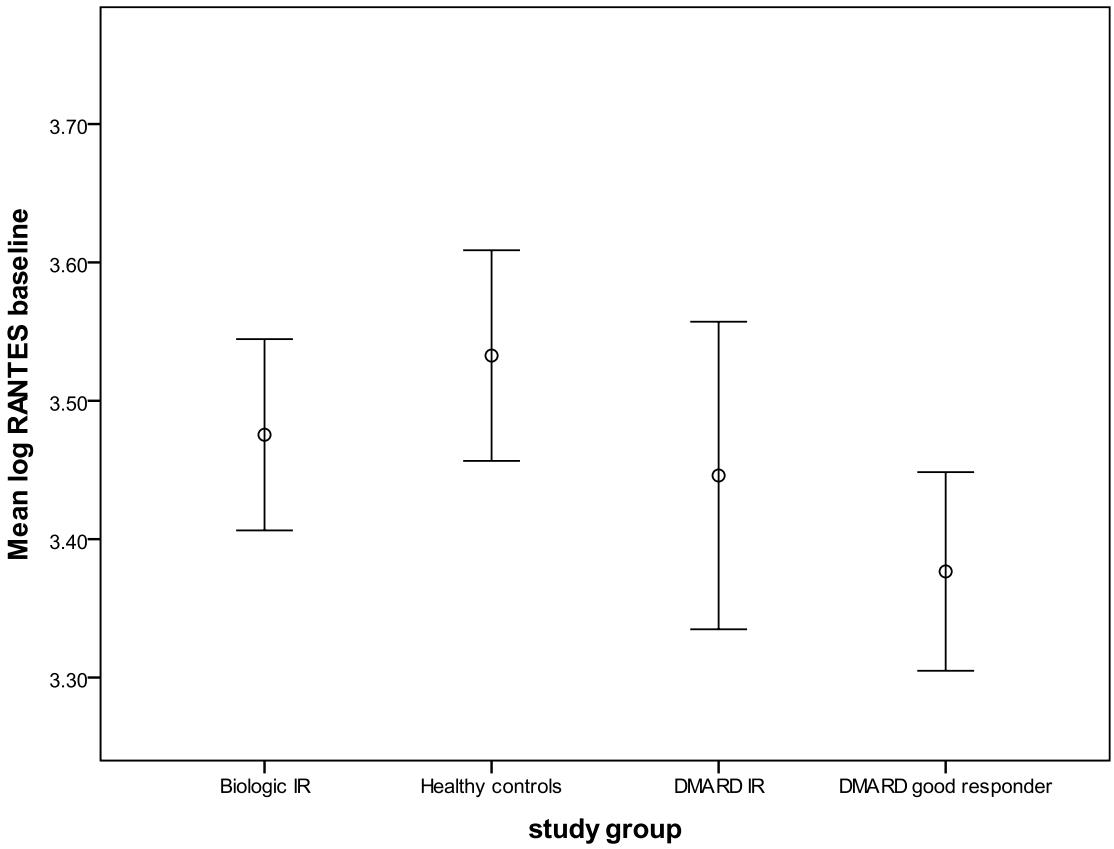

Figure 5-102 RANTES by study group (where *=p<0.05 and **=p<0.01)

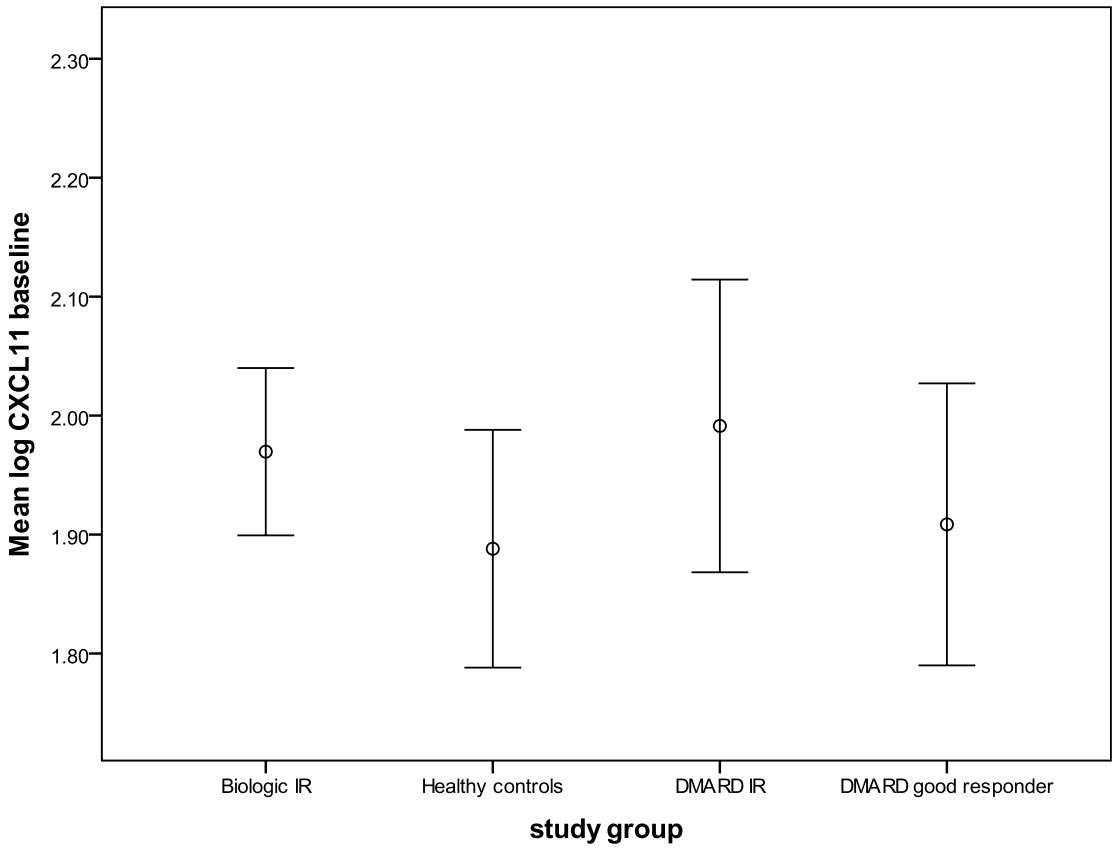

Figure 5-103 CXCL11 by study group (where *=p<0.05 and **=p<0.01)

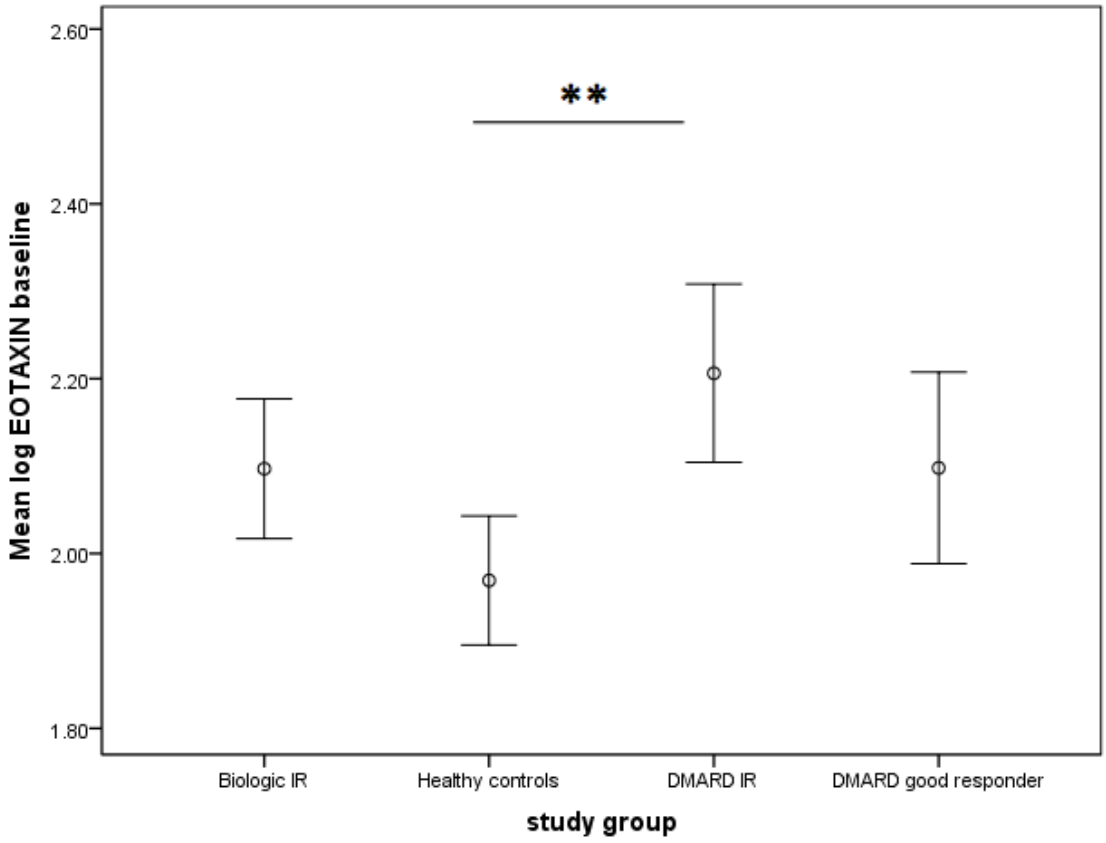

Figure 5-104 EOTAXIN by study group (where *=p<0.05 and **=p<0.01)

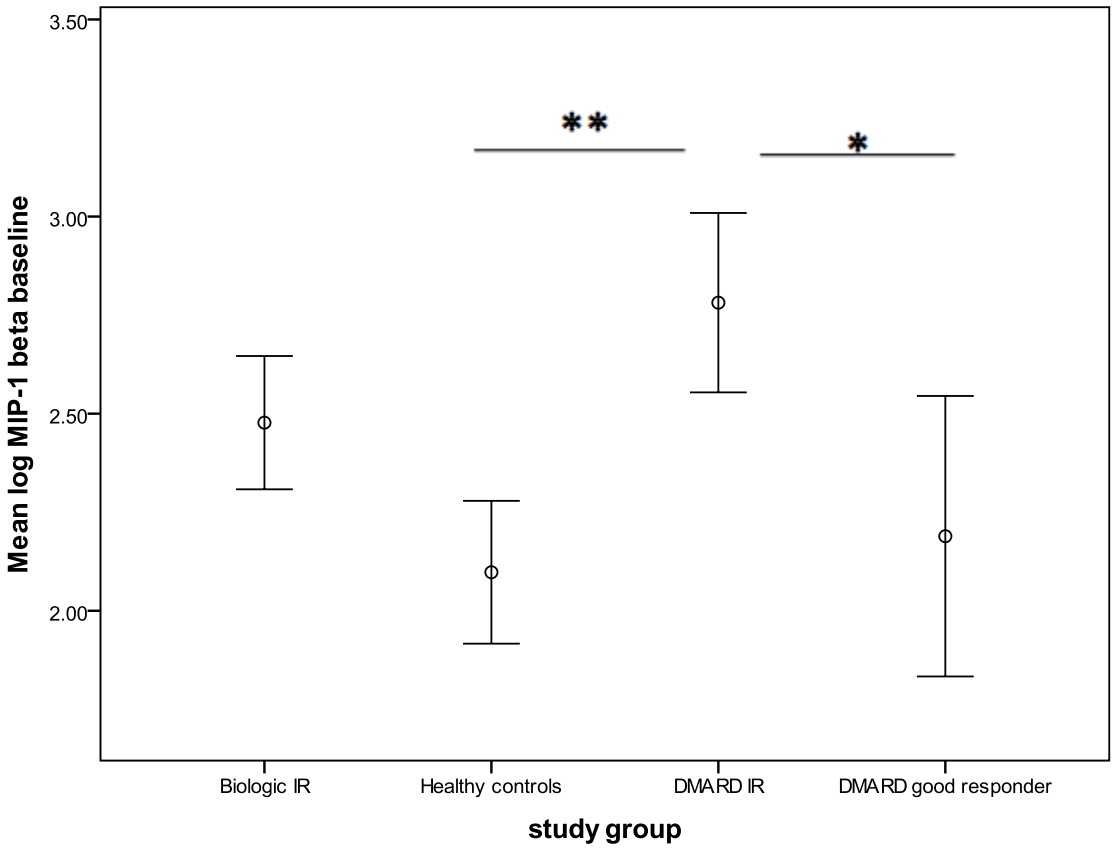

Figure 5-105 MIP-1 beta by study group (where *=p<0.05 and **=p<0.01)

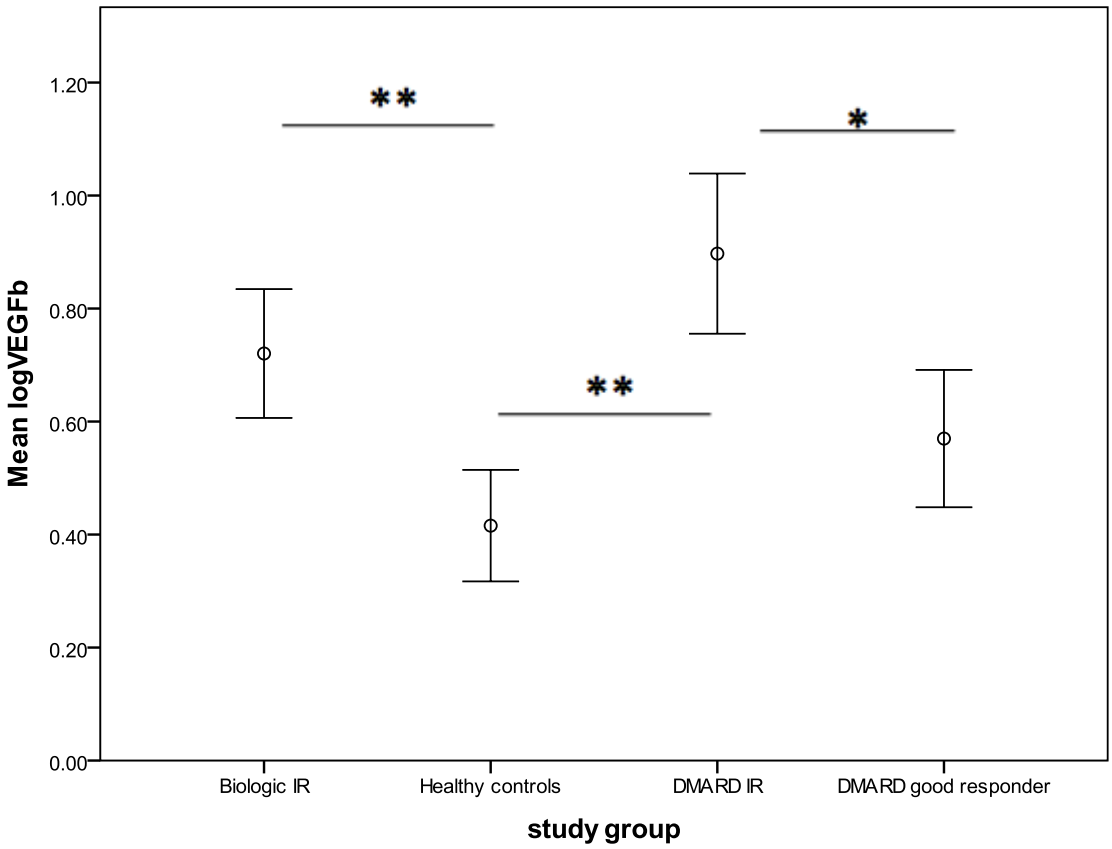

Figure 5-106 VEGFb by study group (where *=p<0.05 and **=p<0.01)

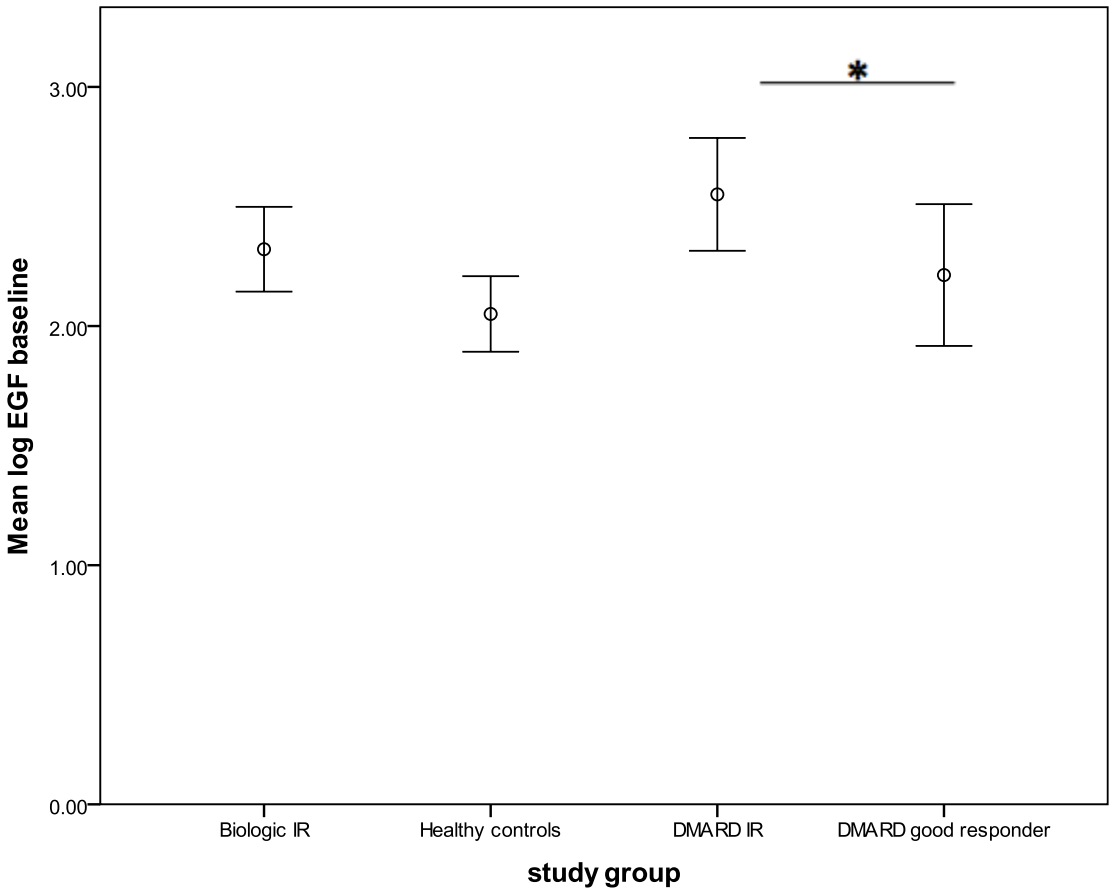

Figure 5-107 EGF by study group (where *=p<0.05 and **=p<0.01

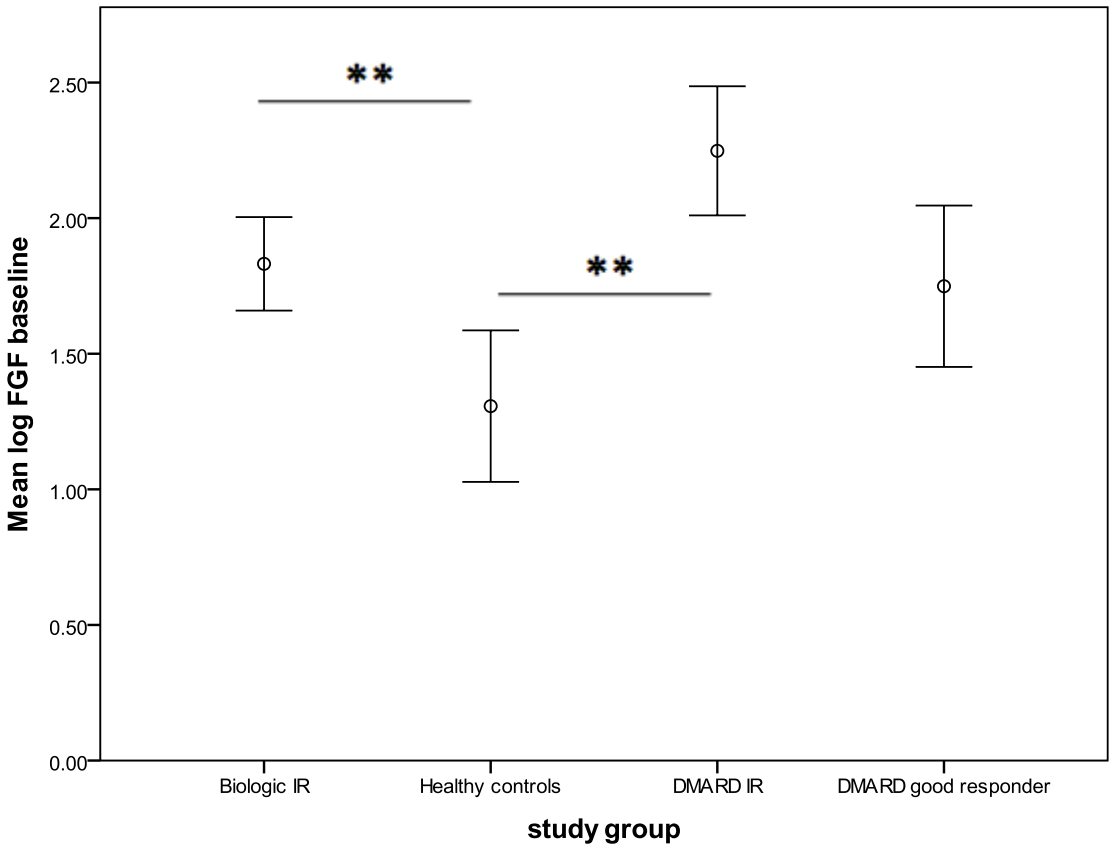

Figure 5-108 FGF by study group (where *=p<0.05 and **=p<0.01)

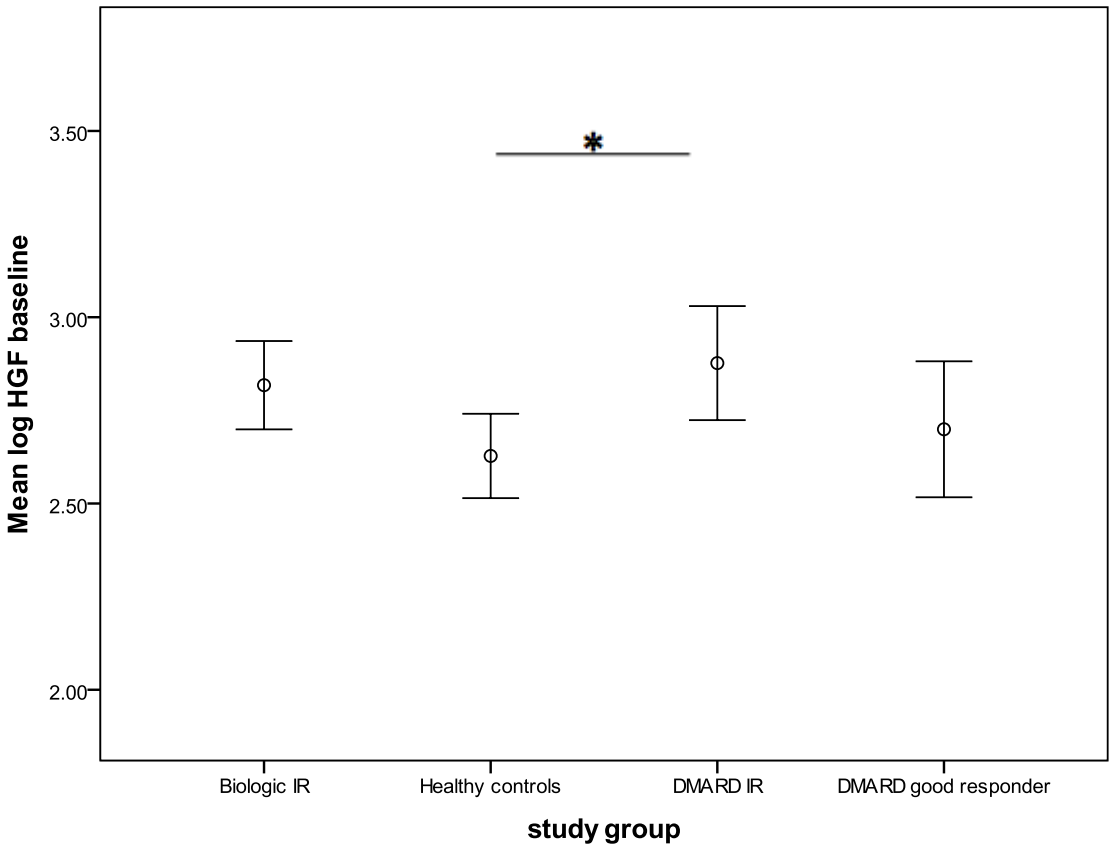

Figure 5-109 HGF by study group (where *=p<0.05 and **=p<0.01)

5.6.1.5 Summary

Of those cytokines tested, the majority demonstrated significant differences from healthy control samples as expected.

Measured cytokines between the biologic resistant and DMARD resistant group were statistically different only in those pro inflammatory cytokines IL-6, IL-21 and IL-23 and the pro-inflammatory chemokine MIP1-beta. This may be in keeping with the higher DAS28 observed in Table 3-77 (although ESR and CRP did not differ statistically). A link between IL-6, -21 and -23 has been described by Zhou et al (Zhou et al. 2007).
Together, the biologic resistant and DMARD resistant groups displayed higher pro- and anti-inflammatory cytokine levels than the DMARD good responder group in keeping with clinical and biochemical measurements previously described.
Overall, fewer differences were observed than expected between the biologic resistant and DMARD good responder groups. Few pro- or anti-inflammatory cytokines, none of the growth factors, negatively regulating cytokines, chemokines, neither MMPs nor OPG/RANKL achieved statistically significant differences. Only IL-6 measured was found to be statistically higher in the biologic resistant group than the DMARD good

responders. At baseline measurement, eight patients were treated with the IL-6 inhibitor Tocilizumab and five with TNFi therapy. It is perhaps notable that despite this mode of action, IL-6 remains statistically different between groups.

Finally, only IL-23 measured between the healthy controls and DMARD good responder group achieved statistically significant differences although the majority of cytokines displayed numerically higher levels than healthy controls. This suggests the phenotype of clinical low disease activity seen in the DMARD good responder group persists in the cytokine profile but that low-level immune activity may persist.

5.6.2 Longitudinal analysis

Longitudinal analysis was available for the biologic and DMARD resistant groups only as described. In order to seek those analytes whose concentration changed sufficiently to potentially reflect those treatment changes and change in disease activity observed, *only those analytes demonstrating significant differences between study visits are shown*. Paired t-tests were performed between study visits and statistically significant differences shown in Table 5-90. These are then shown as clustered error bars with 95% confidence intervals (figure 5-113 to 5-122).

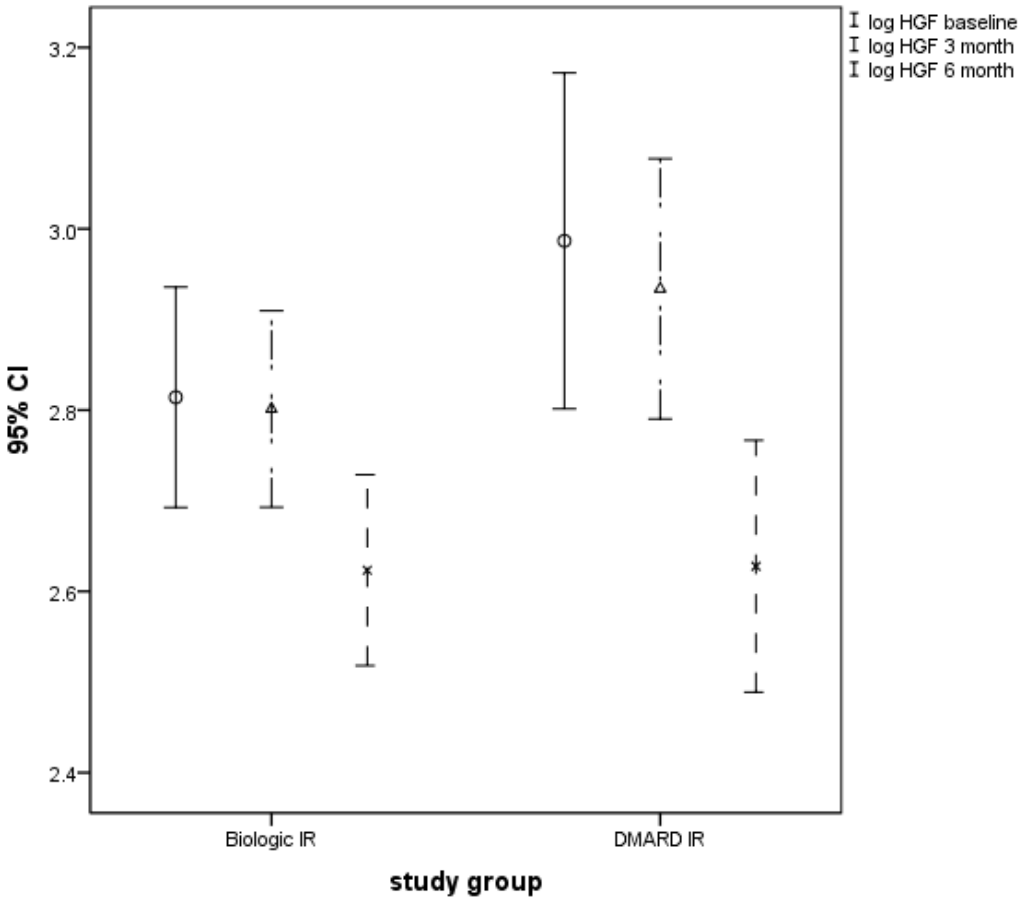

Figure 5-110 HGF in biologic and DMARD resistant study groups at baseline, three and six-month study visit

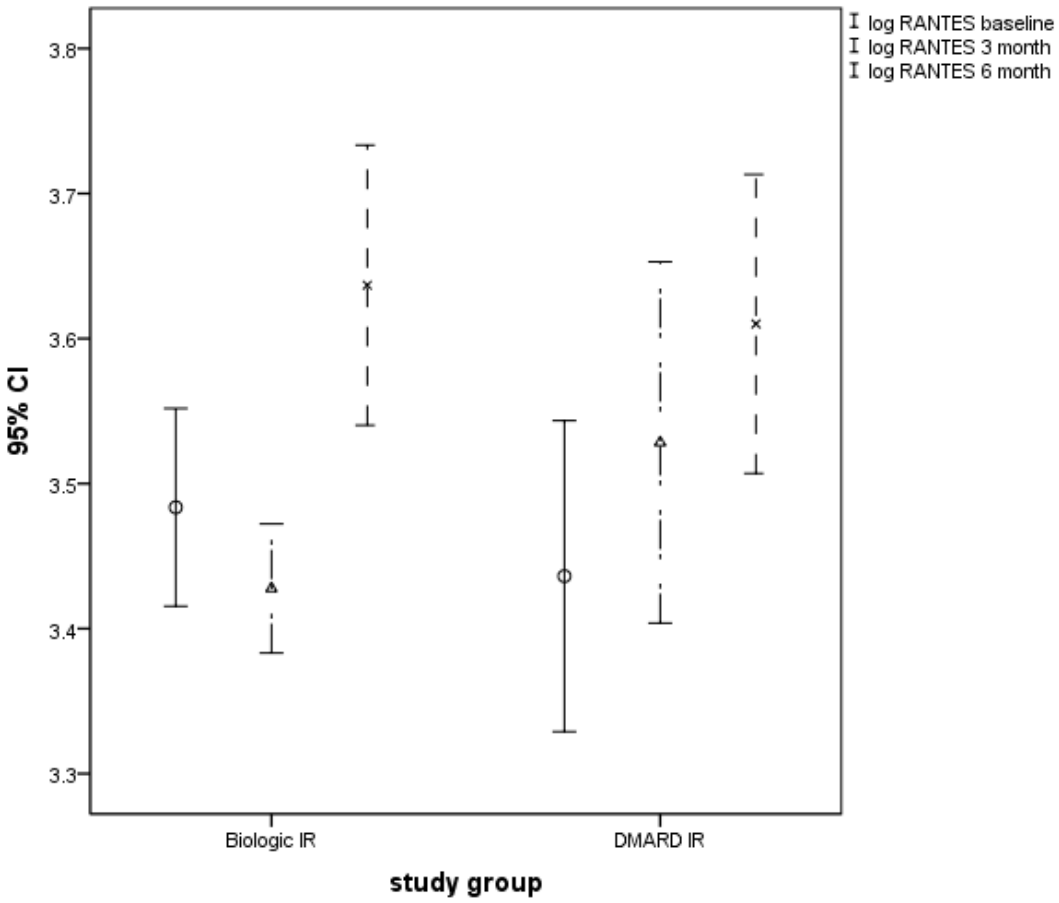

Figure 5-111 RANTES in biologic and DMARD resistant study groups at baseline, three and six-month study visit

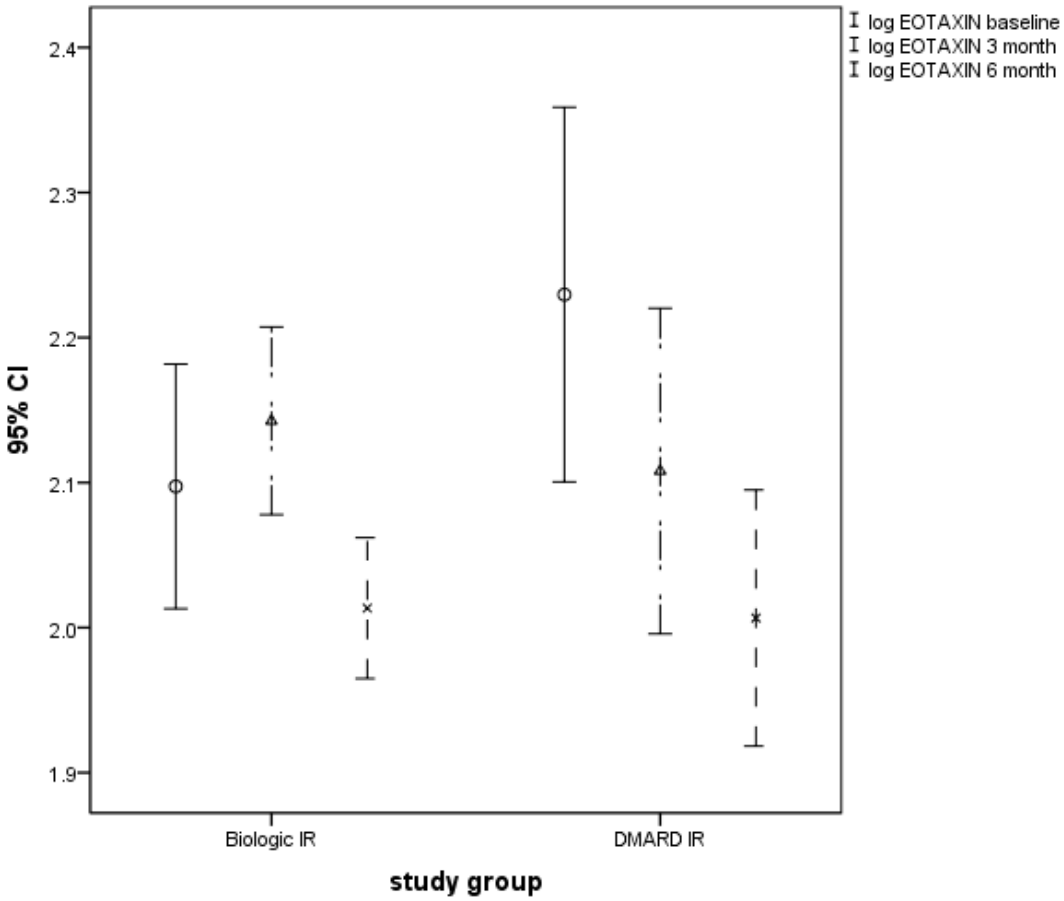

Figure 5-112 EOTAXIN in biologic and DMARD resistant study groups at baseline, three and six-month study visit

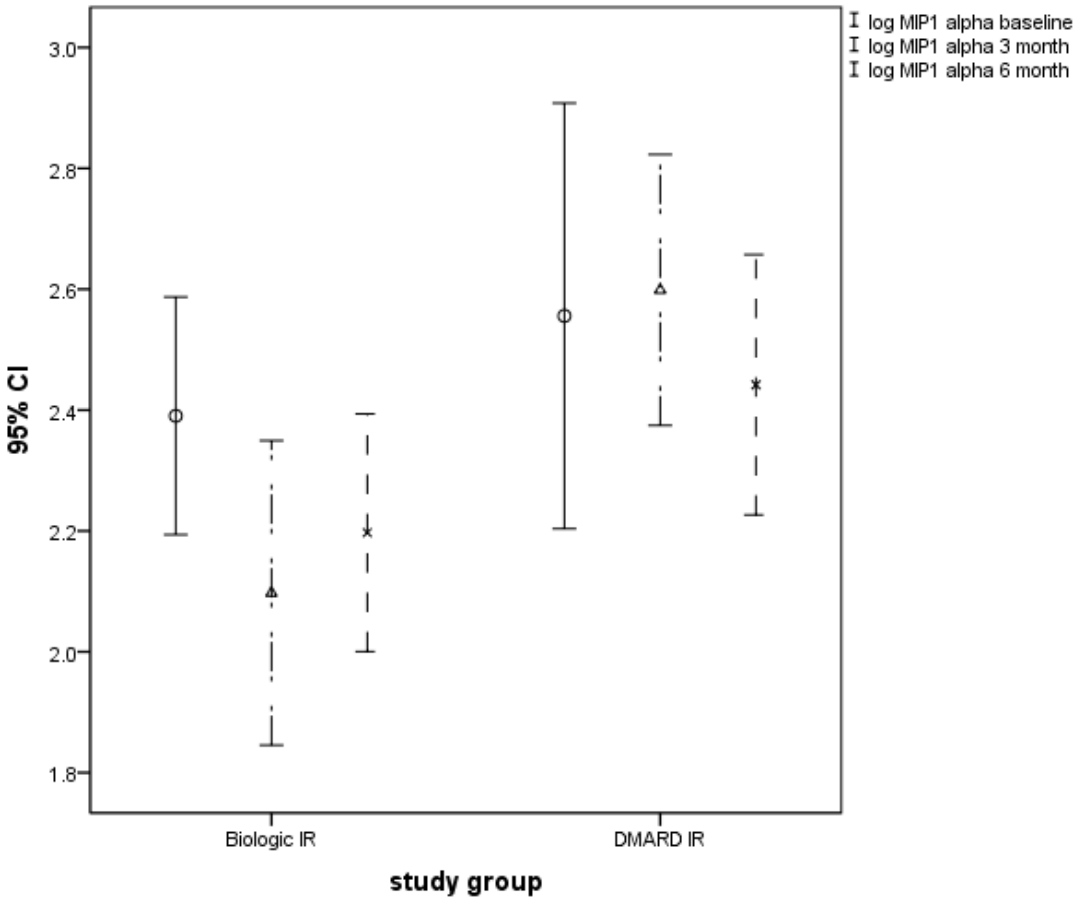

Figure 5-113 MIP1 alpha in biologic and DMARD resistant study groups at baseline, three and six-month study visit

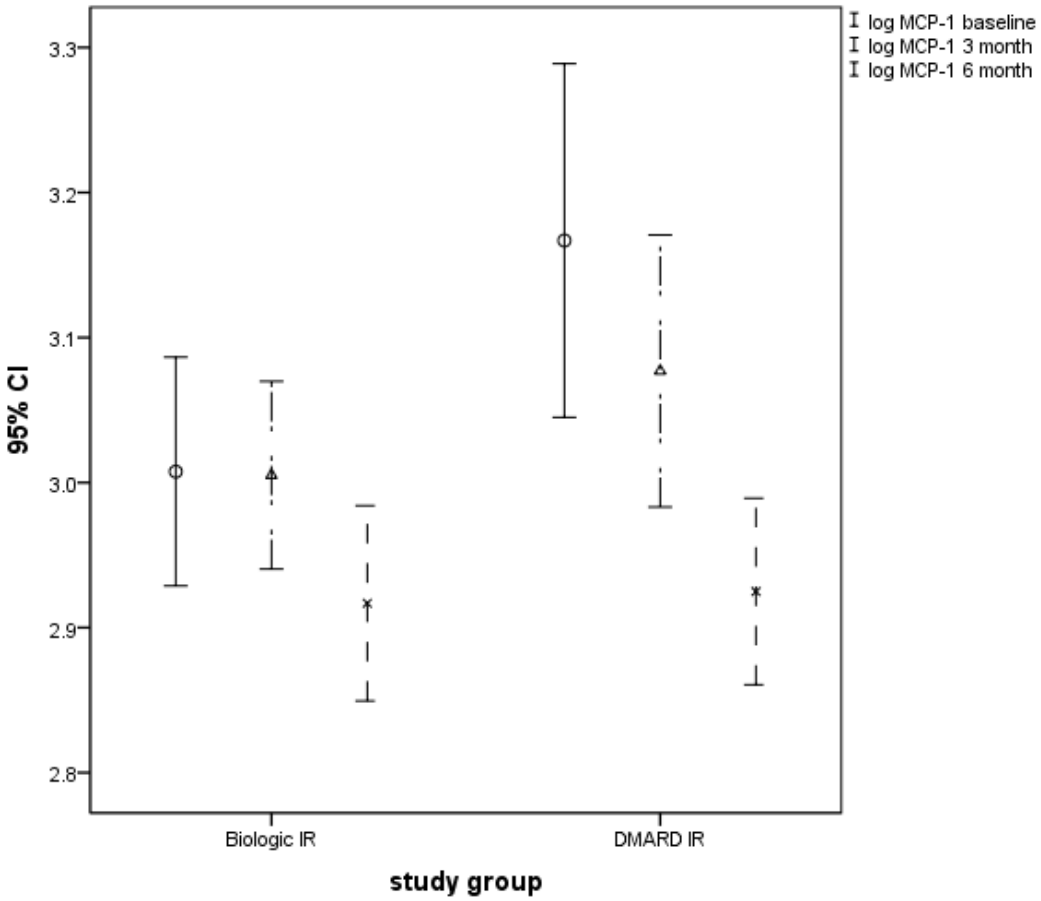

Figure 5-114 MCP-1 in biologic and DMARD resistant study groups at baseline, three and six-month study visit

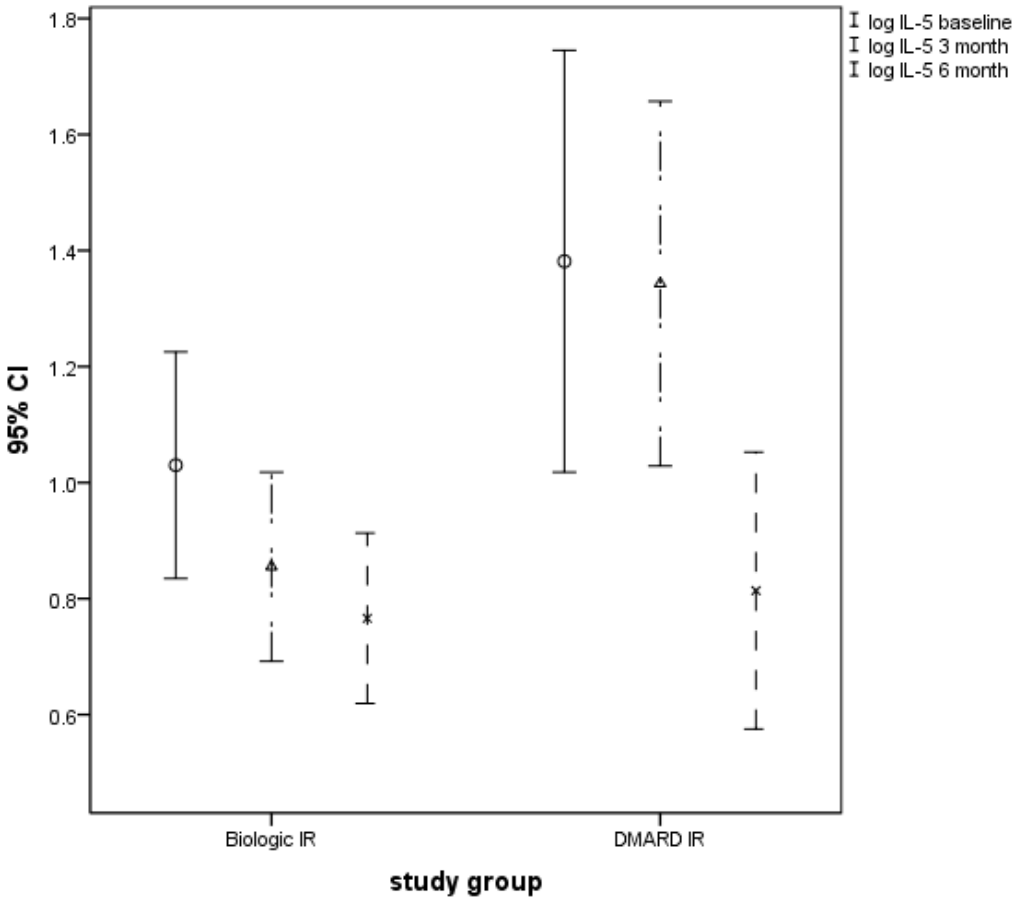

Figure 5-115 IL-5 in biologic and DMARD resistant study groups at baseline, three and six-month study visit

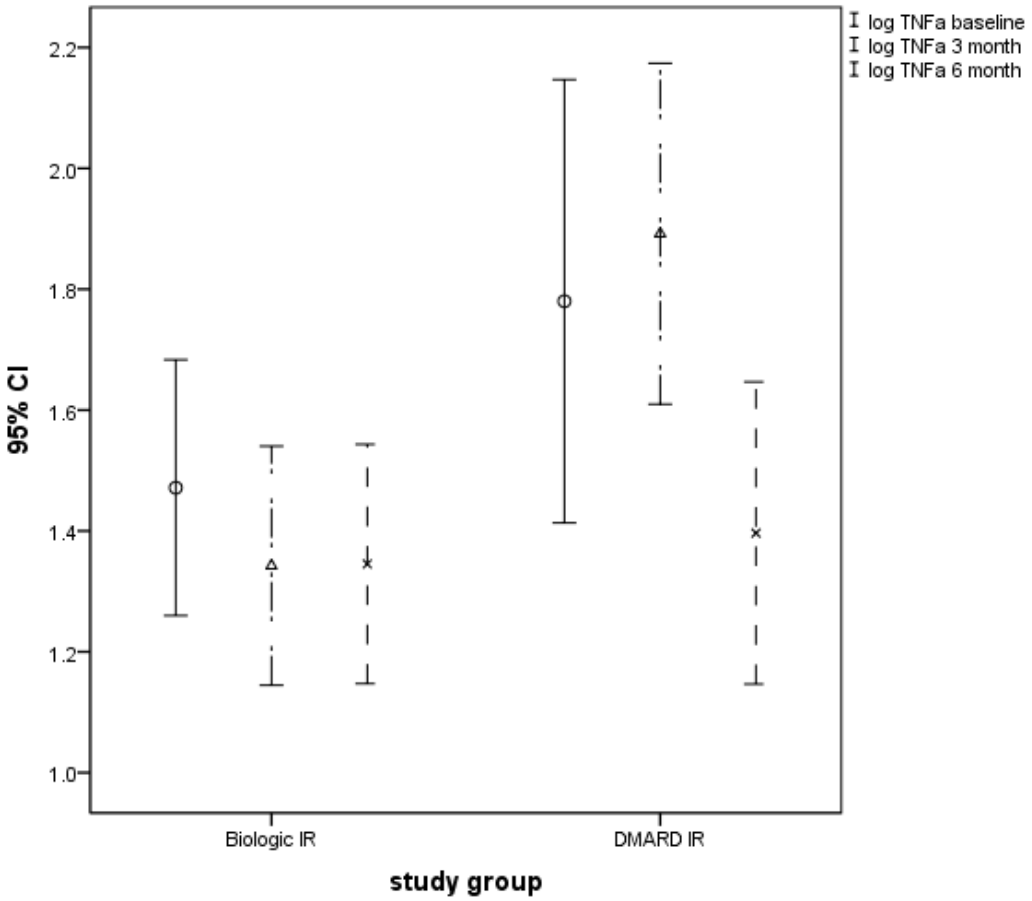

Figure 5-116 TNF alpha in biologic and DMARD resistant study groups at baseline, three and six-month study visit

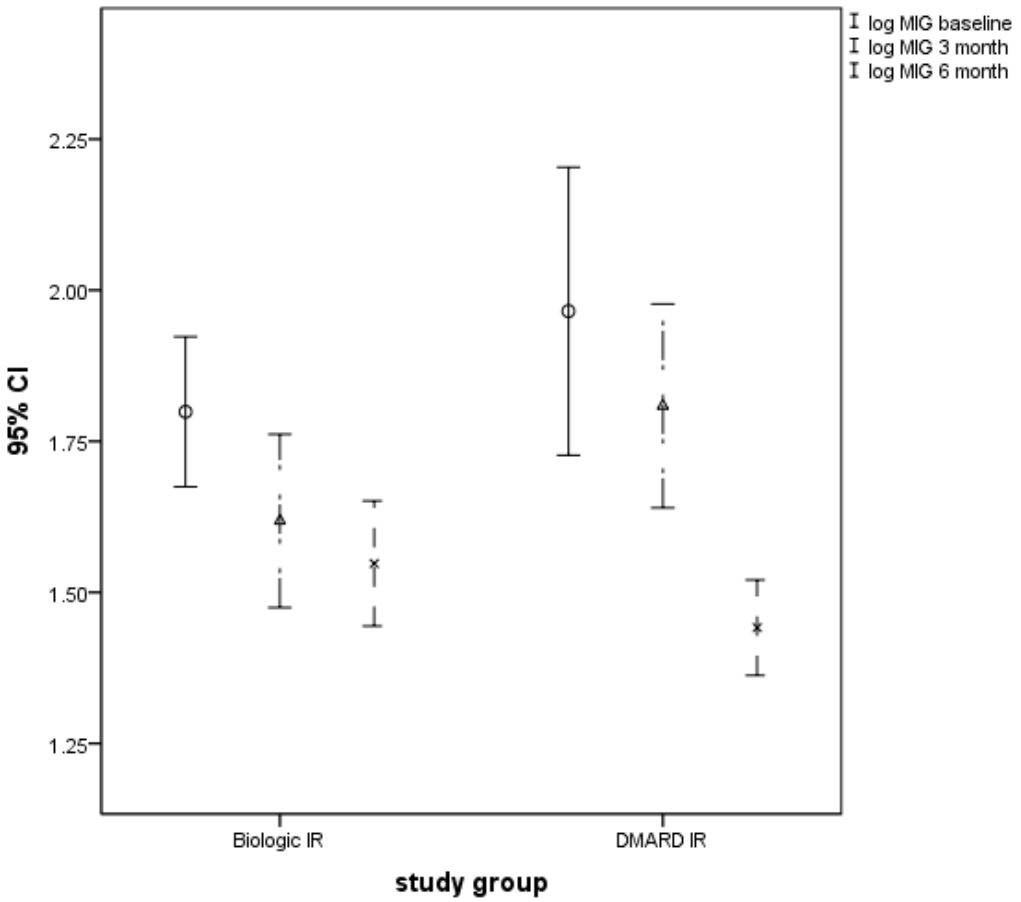

Figure 5-120 MIG in biologic and DMARD resistant study groups at baseline, three and six-month study visit

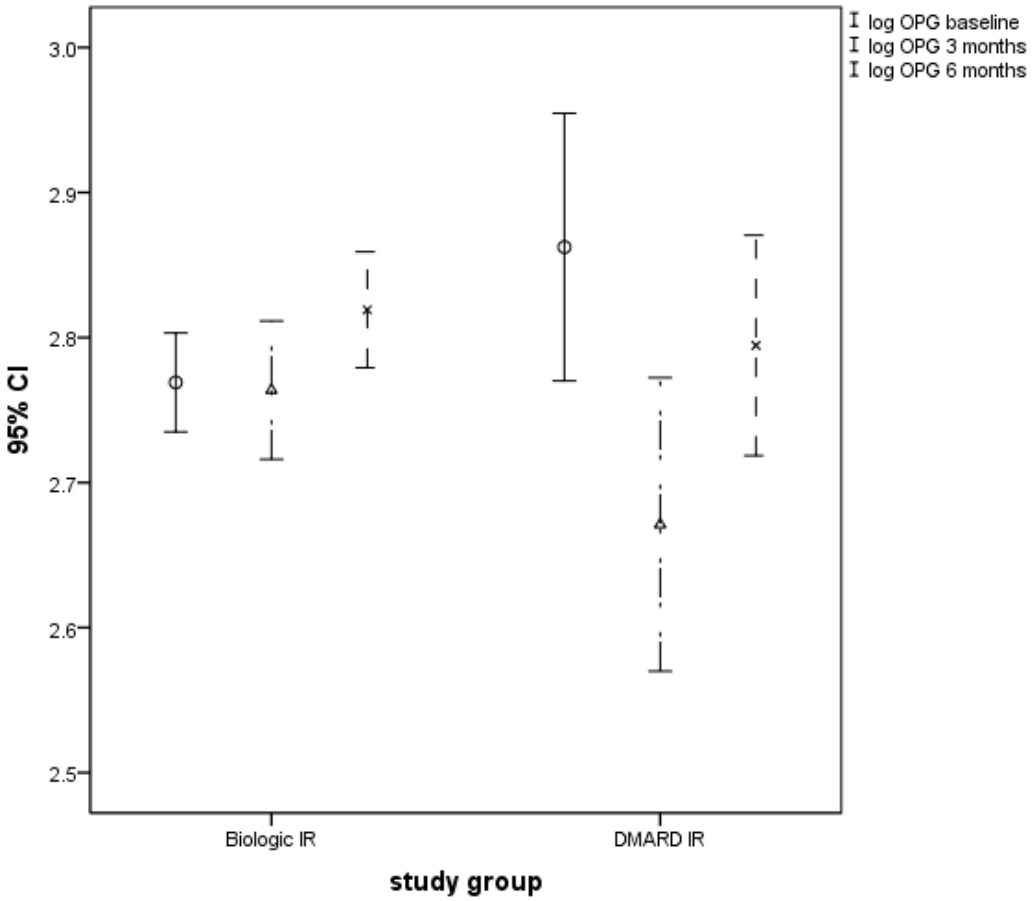

Figure 5-121 OPG in biologic and DMARD resistant study groups at baseline, three and six-month study visit

Analyte (log value)	Biologic Resistant		DMARD resistant	
	0-3 months	3-6 months	0-3 months	3-6 months
IL-1 Beta	0.239	0.383	0.239	0.383
G-CSF	0.040	0.621	0.317	0.039
EGF	0.106	0.462	0.352	0.171
IL-10	0.633	0.816	0.226	0.568
HGF	0.471	0.004	0.418	**0.008**★★
FGF-basic	0.702	0.613	0.585	0.182
IFN alpha	0.322	0.757	0.582	0.140
IL-6	0.673	0.486	0.225	0.793
Il-12	0.407	0.680	0.296	0.370
RANTES	0.292	**<0.0001**★	0.145	0.196
EOTAXIN	0.353	**<0.0001**★	0.019	0.167
IL-13	0.339	0.809	0.649	0.054
IL-15	0.191	0.719	0.901	0.384
IL-17	0.405	0.852	0.012	0.329
MIP1 alpha	0.004	0.457	0.767	0.336
GM-CSF	0.063	0.257	0.666	0.215
MIP-1 beta	0.051	0.778	0.958	0.316
MCP-1	0.687	0.018	0.119	0.017
IL-5	0.112	0.313	0.876	0.010
IFN gamma	0.161	0.147	0.577	0.213
TNFa	0.979	0.589	0.523	0.017
IL-1ra	0.728	0.004	0.839	0.371
IL-7	0.443	0.503	0.779	0.141
IP-10	0.186	0.057	0.560	0.008
IL-2r	0.993	0.003	0.578	0.229
MIG	0.281	0.325	0.196	**<0.0001**★
IL-4	0.275	0.470	0.457	0.086
IL-8	0.470	0.195	0.849	0.090
MMP-3	0.837	0.268	0.921	0.831
MMP-12	0.079	0.619	0.437	0.863
MMP-13	0.404	0.595	0.956	0.731
CXCL 11	0.722	0.070	0.022	0.338
OPG	0.565	0.025	**0.001**★	0.007
RANKL	0.829	0.834	0.341	0.827
IL-21	0.600	0.465	0.057	0.142
IL-23	0.477	0.491	0.325	0.939
IL-2	0.286	0.215	0.789	0.999
VEGF	0.285	0.301	0.548	0.625

Table 5-90 Paired sample t-test results (p value) between baseline and three months and three month and six month study visits for all cytokine analytes

(Significance assumed at the p<0.0014 after Bonferroni correction)

Those cytokines measured between visits did not demonstrate a consistent pattern of change reflecting that observed in the clinical, PROM measures or microRNA evaluated in Chapter 4 between study visits.

5.6.3 Correlation with clinical factors

Correlation between categorical clinical outcomes was first investigated. There were no differences between any cytokines examined when tested by smoking category (current, non-smoking and ex > 1 year or never smoked, therapy (on biologic therapy or not) nor fatigue (FACIT fatigued or not). In the same way, when corrected for multiple testing, neither clinical variables (SJC, TJC and DAS28) nor inflammatory markers (ESR, CRP) demonstrated any significant correlation with those cytokines measured.

There were differences in serum cytokine values when tested by autoantibody category (either autoantibody positive, RF or CCP or double antibody negative) as shown in Table 5-91.

	Autoantibody positive N=34 pg/ml		Autoantibody negative N=8 pg/ml		Statistical test; Mann Whitney test
	Median	IQR	Median	IQR	
ESR	32	23	6	13	**0.002**
CRP	14	24.8	5.8	10.6	0.078
IL-6	56.34	117.16	40.94	203.58	0.306
IL-15	489.26	1215.15	63.88	726.37	**0.001**
IL-23	2596.97	11887.37	24.4	415.03	**0.001**
IFNa	1128.80	1802.14	252.31	111.10	**0.001**
IL-7	103.08	299.10	41.48	216.23	0.139
MIG	65.74	92.11	53.04	49.37	**0.049**
G-CSF	58.4	118.6	23.95	11.11	**0.028**
MCP-1	1102.68	915.65	674.52	318.93	**0.022**
FGF basic	108.96	144.38	16.61	23.47	**0.002**

Table 5-91 Selected cytokines and inflammatory markers examined by autoantibody status

Subsequently, correlation with mood (HADS score) and fatigue (FACIT score) was performed as shown in Table 5-92 below. No correlation was observed in the important pro-inflammatory cytokines shown.

Log value cytokine		FACIT baseline	HADS-A baseline	HADS-D baseline
G-CSF Biologic IR	Pearson Correlation	-.158	.073	.236
	Sig. (2-tailed)	.289	.629	.115
IL-10 Biologic IR	Pearson Correlation	.045	.151	.159
	Sig. (2-tailed)	.762	.317	.293
FGF-basic Biologic IR	Pearson Correlation	-.096	.133	.244
	Sig. (2-tailed)	.519	.378	.102
IFNa Biologic IR	Pearson Correlation	-.173	.062	.270
	Sig. (2-tailed)	.245	.681	.070
IL-6 Biologic IR	Pearson Correlation	-.021	-.054	.113
	Sig. (2-tailed)	.889	.722	.454
IL-15 Biologic IR	Pearson Correlation	-.172	.020	.221
	Sig. (2-tailed)	.248	.894	.139
MCP-1 Biologic IR	Pearson Correlation	-.155	-.001	.220
	Sig. (2-tailed)	.303	.996	.147
TNFa Biologic IR	Pearson Correlation	-.068	.007	.124
	Sig. (2-tailed)	.650	.961	.412
IL-7 Biologic IR	Pearson Correlation	-.089	.031	.147
	Sig. (2-tailed)	.551	.838	.329
MIG Biologic IR	Pearson Correlation	-.032	.005	.108
	Sig. (2-tailed)	.832	.972	.474
IL-23 Biologic IR	Pearson Correlation	-.148	.012	.138
	Sig. (2-tailed)	.327	.937	.366
N		46	45	45

Table 5-92 Correlation between selected cytokines and mood (HADS) and fatigue
(Biologic IR- biologic resistant study group)

5.7 Chapter Conclusions and Discussion

The results of this analysis firstly confirm the wide range of results observed in the serum cytokine profile of those with RA irrespective of phenotype. The profile identified in this cohort represents longstanding yet active RA as judged by the DAS28. (Hirata et al. 2013) refer to their use of a multi-analyte panel and suggest that no single marker will define disease activity. In general, cytokine profiling is difficult within highly heterogeneous groups and may best form part of a 'personalised' approach.

The overall aim of this study was to seek different signals in the biologic resistant study group; as expected in terms of their available cytokine profile this differs from healthy control patients but were closer in many ways to the DMARD good responder group than the DMARD resistant group. Indeed only a small number of cytokines tested demonstrated significant differences between healthy controls and DMARD good responders. The DMARD resistant group did however display higher composite DAS scores driven by subjective assessments (similar CRP and ESR). **In many ways these observations are in keeping with the microRNA findings of Chapter 4.**

There are two considerations from this part of the analysis;

- Why are the biologic resistant and DMARD good responder profile not more dissimilar?
- That the relatively higher DAS28 values seen in the DMARD resistant group do reflect in the serum profile of these common analytes.

Firstly, the biologic resistant and good responder groups may diverge less as a reflection of disease duration. Alternatively, the biologic resistant group are *not* as inflamed yet the DAS28 ESR and its inherent weaknesses fail to reflect this.
This is explored further in Chapter 6.

In contrast with this hypothesis are the improvements noted in subjective disease activity, ESR/CRP after treatment regimen changes and reduction seen in many cytokines. The observations from examination of the longitudinal analysis suggest that in the biologic resistant group a number of analytes fall appropriately and statistically significantly. These are not however the more 'traditional' pro inflammatory cytokines such as TNFa, Il-17. IL-6 and IL-1. Several explanations could be put forward;

- This may reflect a treatment effect (specific anti-cytokine therapy)
- The biologic resistant group drive inflammation though alternate pathways
- The biologic resistant group are not actually inflamed
- Serum values are not representative of the synovial compartment
- Sampling/methodological/data quality

A similar observation can be made in the DMARD resistant group, albeit different cytokines change in the observation period. At all time-points, differences between the cytokines would appear to fall when the error bars are viewed although closer statistical

examination suggests this difference is small. Again these may represent small serum changes not reflecting the synovial compartment. However, the large reductions in ESR, CRP and clinical improvement are not as impressive when the cytokine profile is viewed in this way.

Correlation between the composite DAS28 score and selected clinical components was sought but not demonstrated. This may reflect the previously discussed weaknesses of the DAS28 or that blood and synovial compartments (reflected as tender or swollen joints) do not relate.

Differences in cytokine profiles according to clinical 'categories' were also sought. It could be hypothesised that differences may be apparent according to treatment category as specific cytokines were targeted. In the small numbers in each treatment category this could not be demonstrated. Both smoking and the presence of autoantibodies confer a poorer disease prognosis but even within this phenotypically severe cohort, it could be hypothesised that greater than one disease subtype may exist. In this way alterative inflammatory pathways may be involved. A further study of mRNA outputs or GWAS studies may explore this finding.

The significance of different profiles observed when autoantibody status is considered is not clear. It is in some ways in keeping with the observations of Hueber et al. This group studied an early arthritis cohort and related the high cytokine group to high CRP, ESR, RF and ACPA titres (Hueber et al. 2007). This may simply be a false positive on the basis of small sample size. Alternatively, it may represent a real phenomenon whereby different RA subtypes persist and exhibit different serum profiles reflecting different pathophysiology and disease subtypes.

The lack of association between common pro-inflammatory cytokines such as TNFa and IL-6 in serum and fatigue suggests other factors may drive on fatigue at this disease stage as hypothesised in Chapter 3. It is presumed that circulating cytokines cross the blood-brain barrier and in this way influence central fatigue and mood. We did not identify any differences in serum cytokine values between those more fatigued, depressed or anxious.

Therefore, in biologic resistant longstanding disease, the circulating cytokine profile may not be reflective of central processes such as mood and fatigue but is reflective of significant observed clinical improvements (however no better than readily available tests such as ESR and CRP).

6.1 Chapter 6- How does coping, mood and illness perception contribute to the 'resistant' phenotype in severe Rheumatoid Arthritis?

6.1.1 Chapter Aims

During the process of patient interview and data collection, the means by which the resistant phenotype could be characterised was refined. It was evident that in the face of disability, loss of employment and pain, individuals displayed a wide range of disease beliefs, traits and outward coping. To capture and quantify this, and study the impact on disease characteristics, two additional questionnaires were administered.

The Brief COPE questionnaire and Illness Perception Questionnaire (IPQ) were chosen as two validated questionnaires that would capture various dimensions of coping and illness perception to allow conclusions about the biologic resistant study group to be drawn.

The hypothesis behind this chapter is that the significant pain levels, fatigue, poor vitality, disability and impaired quality of life experienced by this cohort would have lead to the development of coping strategies that would offset these negative experiences. Additionally, by examining coping strategies and illness perception, it was hypothesised that the range of disability and function observed may be explained. Finally, the influence of the high prevalence of anxiety and depression identified in the main biologic resistant cohort (Chapter 3) upon coping and illness perception was sought.

I will first address the means by which mood and coping may influence disease activity measures before reviewing previously publications addressing this area.

To conclude, the implications of addressing coping, illness perception and mood, are discussed in order to apply these findings both in order to target treatment in those areas most effectively and how they may apply in biologic resistance.

6.2 Introduction

There are factors other than inflammatory disease activity that may influence the subjective disease process in RA. For example, both pain and prevailing mood may influence a number of disease dimensions, in particular the subjective components of he DSA28 scoring system. Both function and severity are a composite of many different contributory factors from the physical to the psychological. Perception of the severity and implications for the individual of RA symptoms vary widely and also subject to external influence. Coping is thus both shaped and tested by these factors. However not all coping is considered positive and it is on this basis that both coping and illness perception merit evaluation.

6.2.1 The influence of pain in Rheumatoid arthritis

Studies in RA suggest that the experience of pain is a regular one with over three quarters experiencing moderate to severe pain in the last two months. As one would expect this is associated with more severe disease (Taylor et al. 2010) yet despite 'severity', pain remains responsive to biologic therapies. Pain may be experienced as a result of articular damage, biomechanical pain (feet particularly), inflammatory disease or neuropathic pain (compression neuropathies, cervical myelopathy or peripheral neuropathy). The experience of pain is perceived centrally and thus is modulated by a number of factors including coping (personal strategies or support networks), disease or drug beliefs/understanding and even spiritual factors.

6.2.2 The influence of mood in Rheumatoid arthritis

Mood may not only influence subjective pain perception but also a patient's ability to manage daily RA-related symptoms. 42% of the cohort displayed 'possible' depression and 47% 'possible' anxiety disorder as described in Chapter 3. 28% had 'probable' depression and 23% 'probable' anxiety disorder. A number of studies in RA have identified disturbance of mood in RA to a similar degree. (Chandarana et al. 1987) identified a prevalence of 21.4% anxiety and 19% depression also using a score of 8 in the HADS questionnaire. Similarly, (Pincus et al. 1996) found a depression prevalence of 15% also using 11 as the HADS-D cut-off for case identification.

Mood disturbance can also be found at all stages of disease duration. In an early arthritis cohort, (Covic et al. 2012) identified 18.9% 'possible depression', 9.4% 'probable' and 16.7% with 'possible anxiety' and 18.6% 'probable'. (el-Miedany & el-Rasheed 2002) studied an Egyptian, predominately female cohort with average disease duration of 8.4 years and mean age of 41.9 yrs old. They found the prevalence of depression to be 66.2% and depression 70% using the WHO ICD-10 classification. Isik et al described a cohort of intermediate disease duration of seven years and mean age of 52.3yrs in whom they identified a prevalence of depression of 41.5% and anxiety 13.4% (Isik et al. 2006). Their use of the Hamilton depression and anxiety scale introduces different cut-off values and definition of both diagnoses, which may account for their notably high (70.8%) prevalence of either or both disorder. Finally Hider et al examined RA patients with a disease duration of 13.6 years and identified 47.5% prevalence of

depression in a cohort with active disease commencing TNFi therapy (Hider, Tanveer, Brownfield, Mattey & Packham 2009a).

There are a number of proposed reasons from the bio-psychosocial to the pathophysiological accounting for this finding of increased prevalence of mood disorder in RA. Alteration in mood can be explained simply by the situation those with chronic illness find themselves in- for example the loss of employment and functional capacity could be considered a major influence upon prevalent mood. Additional reasons include social isolation owing to poor function, as a relationship stressors, adverse adjustment to long-term illness and to chronic pain (Isik et al. 2006).
The biological explanation proposes depressed individuals have elevated levels of pro-inflammatory cytokines such as IL-6, IL-17 and TNFa, in addition to persistent HPA axis activation causing relative glucocorticoid insensitivity (Yang Liu et al. 2012; E Leonard 2010; Jehn et al. 2010). As is demonstrated in Chapter 3, the prevalence of depression is increased in RA but not directly related to burden of inflammatory disease. In this way, patients with many chronic illnesses such as diabetes and stroke, and not just inflammatory conditions, have increased incidence of mood disturbance. Biologically this may be plausible as chronic stressors, including low mood, drive elevated cortisol and it's suppressive effect in the immune system.

Identifying mood disturbance is important, as there are significant implications from drug compliance to perception of benefits and side effects of therapy. Low mood is also related to job productivity and absence in a situation where this may also be affected by reduced function and disability. Pain intensity perceived is also elevated with subsequent additional avoidance behaviour and reduction in physical activity (R. R. Edwards et al. 2011; van Lankveld et al. 2000). The implication of this burden of morbidity is also important in healthcare cost planning.

Effective RA treatment to remission is associated with reduction in anxiety and depression (Kekow et al. 2011). However, being depressed or anxious at baseline predicts fewer patients achieving remission and response to TNFi (Hider, Tanveer, Brownfield, Mattey & Packham 2009b). The effect of treatment with biologic therapies may improve measures of mood. Picchianti-Diamanti et al examined the effect of two years Etanercept therapy in RA and psoriatic arthritis patients (Picchianti-Diamanti et al. 2010). Mean mental health scores (SF36) improved from 39.4 to 56 and MCS 36.1 to 42.3 in a cohort with mean disease duration of 8.8years. Similar improvements were seen in earlier disease course and in the PsA group. Mathias et al found similar but less pronounced improvements could be seen after six months treatment with Etanercept (Mathias et al. 2000). Morris et al found higher correlation between those that were depressed and pain scores, swollen joint scores and higher disability (A. Morris et al. 2011). Mattey et al found higher TNFa therapy discontinuation over a 36-month period in those depressed, anxious, smokers and those with a higher baseline DAS28 (Mattey et al. 2010). The strongest association however was with the total HADS score. Furthermore, higher depression and anxiety scores directly influence quality of life

scores (Nas et al. 2011). Thus disease course, response to treatment and disability may be directly influenced by mood.

Only by seeking depression and/or anxiety can the correct treatment be suggested. Therefore annual assessment may be justified at all disease stages for early intervention to improve outcomes.

6.2.3 Weaknesses of using composite disease activity scores

The DAS28 score is currently recommended to evaluate disease activity and response to therapy rather than severity. It is a dynamic score but is subject to influences other than the purely inflammatory, a number of which will be addressed.

6.2.3.1 Patient driven scores

Two component scores in the DAS score are patient driven; the tender joint count and patient global assessment are subjective. They are thus subject to external influence such as psychosocial factors, patient expectation and major life events.

6.2.3.2 Joint swelling

Not only is joint swelling very subjective, old synovial thickening often persists despite being 'non-inflamed' particularly in longer standing disease. This contributes to the SJC making low disease activity more difficult to achieve in established disease.

6.2.3.4 Tender joints

Established joint damage may be tender and thus not reflective of active inflammation yet nonetheless elevating the DAS score. This may have some bearing in composite scores failing to exclude an additional fibromyalgic component of RA.

6.2.3.5 Pain

There is some evidence that pain pathways and perception become increasingly ingrained and unresponsive with time.

6.2.3.6 ESR

The ESR if often elevated in long standing disease as addressed in Chapter 1.

It is necessary to put these limitations in context. As illustration, In the case of a primary 'lack of' response to TNFi, the initial DAS28 score is important. It is far more difficult to achieve low disease activity from a DAS28 baseline score of between 7 and 8 than from 5.2. The factors that have accounted for such high parameters require consideration. These may be low mood, accounting for higher pain perceived and thus tender joint count. Other types of pain such as neuropathic pain or degenerative pain may exist in longer standing disease. The ESR is invariably elevated in longer standing disease and resorting to the DAS28-CRP score may be more appropriate.

In summary, mood disturbance is common and important and there are a number of means by which it may influence response to treatment. This has lead to the central hypothesis in this Chapter,

> ***Does mood actively contribute to perceived lack of response to treatment in this cohort?***

6.4 Coping and Illness Perception

Leventhal and colleagues devised the 'self regulatory' model to define illness perception (Leventhal et al. 1984). In order to form a cognitive representation of a disease, and thus adapt, five domains of the illness must be addressed. These are

- **Identity**; the symptoms and features that the individual associates with their disease
- **Consequences** of the illness
- **Cause** of the illness
- **Timeline**; how long does the individual expect this illness to last
- **Control** (cure); the degree or not to which the treatments and measure the individual takes can influence the experience of the illness.

Coping is the means by which a subjective perceived threat or stressor is diminished. Behaviours to reduce perceived threat are based upon these representations and then re-evaluated for their perceived effect. Coping consists of the sum result of these behaviours.

Being diagnosed with RA places huge physical, emotional and social stressors upon the individual. The challenge is life-long and coping behaviours must start early. Additionally, RA as a disease fulfils those criteria associated with less favourable illness representation such as lack of personal control, perceived long-term condition and often poor outcome. Yet functioning and psychological outcomes vary widely. Prior experience, perhaps in another major illness, may shape this early as does personality, but as the challenges of RA vary often week-to-week and over a lifetime, specific coping behaviours may require to be modified and evaluated in order to affect illness perception and those elements of Leventhal's model.

Illness perception and illness outcome are also related. Orbell et al found the outcome after knee arthroplasty was directly related to patient's beliefs in addition to preoperative function and mood (Orbell et al. 1998). Rozema et al examined illness representations in breast cancer patients and both physical and mental health were affected by health beliefs around their illness (Rozema et al. 2009). Outcome in RA has also been shown to be affected by illness representation (Carlisle et al. 2005). Sharpe et al studied RA patients with disease of less than two years duration. The increasing depression identified in their cohort was related to both coping and illness representations (pain and disability were also important) (Sharpe et al. 2001).

There is therefore a need to assess both coping and illness perception.

6.4.1 Previous studies in RA involving mood, coping and illness perception

(Groarke 2004) examined 75 patients with RA with disease duration of 12 years. Disease activity was based upon subjective physician assessment of disease activity whereby 58% were 'inactive' and a quarter had 'severe' disease. They used the COPE and IPQ questionnaires in addition to the Arthritis Impact Measurement Scale to assess function, pain and depression. They found that depression did lead to less use of more positive coping strategies and also more denial. In their cohort, disease activity did not influence pain or mood but the opposite question was not addressed. They also found that illness perception had a larger influence than disease activity status on mood, pain or disability.

Graves et al examined the illness beliefs of 125 RA patients with a median of ten years of disease (Graves et al. 2009). They applied the IPQ, DAS-28 to measure disease activity and the SF-36 and HAQ to measure quality of life and disability. Illness beliefs were not associated with disease activity at the time of assessment but degree of personal and treatment control related to disability. In this way, assessment of inflammation and illness perception is important.

Scharloo et al measured functioning, coping and illness perception in patients with chronic lung disease, psoriasis and RA. In RA patients with a mean of 12yrs disease they found that illness perception accounted for the greatest variance in functioning above coping and disease activity variables (Scharloo et al. 1998).

Covic et al examined 157 RA patients with mean disease duration of 13years but less disability than the main resistant RA group in this cohort (mean HAQ only 0.54). They set out to determine the main predictors of pain and depression; these were disability (the biggest), helplessness and passive coping (Covic 2003).

Morris et al examined a large cohort of over a thousand RA patients prospectively over an average of eight years and related depression to health and functional outcomes. They found the presence of persistent and intermittent depression significantly worsened these outcomes. After adjustment, no influence of mortality was found however (A. Morris et al. 2011).

Tuncay et al suggest that coping and the role of mood are not restricted to RA. Their Turkish diabetic population displayed similar influences of anxiety and socio-demographics and very little use of negative coping strategies such as denial and substance use (Tuncay et al. 2008).

6.5 Aims of this Chapter

- To determine the influence of mood and clinical assessments
- To establish the role of perceived disease status (illness perception) and relationship to disease activity, mood, pain and function
- To examine the hypothesis that the coping strategies used would relate to the concurrent severe functioning, fatigue and clinical factors identified in Chapter 3.
- Examine the hypothesis that the high prevalence of anxiety/depression would result in more use of negative coping strategies

6.6 Method

In addition to those clinical methods and questionnaires previously described in Chapter 2 (HADS (mood), FACIT-F (fatigue), quality of life (SF-36v2) and HAQ-DI (disability)), two additional questionnaires were sent by post and mailed return to all 50 biologic resistant patients. Ethical approval was given by the West of Scotland Local Research Ethics Committee (REC Ref Number 10/S0703/4, Amendment 2 March 2011). Sample versions of those questionnaires sent are attached in Appendix 7.

6.6.1 Additional Questionnaires

Brief COPE questionnaire (Carver 1997)	Examines 14 dimensions with 2 items (questions). Each item answered on a 1-4 point Likert scale whereby 1= 'not at all' and 4 – 'I've been using this a lot". The dimensions can be further grouped as per Table 6-94 into adaptive and maladaptive strategies, problem based and emotion based strategies.
Brief Illness Perception Questionnaire (Moss-Morris et al. 2002)	Illness perception is based upon cognitive and emotional representations of disease (Broadbent et al. 2006). The B-IPQ has 8 items shown in Table that address Leventhal's model in addition to emotional representations, and a causal question, scored on a 0-10 Likert scale (where zero=no effect and 10=extremely

Table 6-93 Coping and illness perception questionnaires administered, data captured and scoring method

Problem Based	Emotion Based	Dysfunctional Coping	Adaptive	Maladaptive
Active coping	Positive reframing	Self distraction	Active coping	Self distraction
Planning	Acceptance	Denial	Planning	Denial
Instrumental support	Humour	Venting	Positive reframing	Venting
	Religion	Substance use	Acceptance	Substance use
	Use of emotional support	Behavioural disengagement	Humour	Behavioural disengagement
		Self- blaming	Religion	Self- blaming
			Use of emotional support	
			Instrumental support	

Table 6-94 Theoretical dimension grouping to describe coping strategies

Question	Cognitive Illness Representation
1	Consequences
2	Timeline
3	Personal control
4	Treatment control
5	Identity
6	Emotional concern
7	Illness comprehension
8	Emotional effect
9	Causal

Table 6-95 Dimensions examined by the Brief IPQ questionnaire (based on B-IPQ questionnaire, Appendix 7)

6.7 Results

6.7.1 Whole group characteristics

30 patients returned both paired questionnaires with correctly completed responses that could be scored. Descriptive statistics are shown in Table 6-96.

Variable	Median and range (max-min)
Gender	25/30 female
Age	60yrs (43-74)
Disease duration	204 months (72-537)
Previous DMARDs	5.5 (2-9)
Previous Biologic therapies	3 (2-5)
Previous joint surgeries	1 (0-5)
Previous joint replacements	2 (0-7)
HADS A >/= 8	14/30
HADS D >/= 8	13/30
ACPA positive	73%
RF positive	60%

Table 6-96 Study group (n=30) demographics, previous treatments, HADS score and immunology

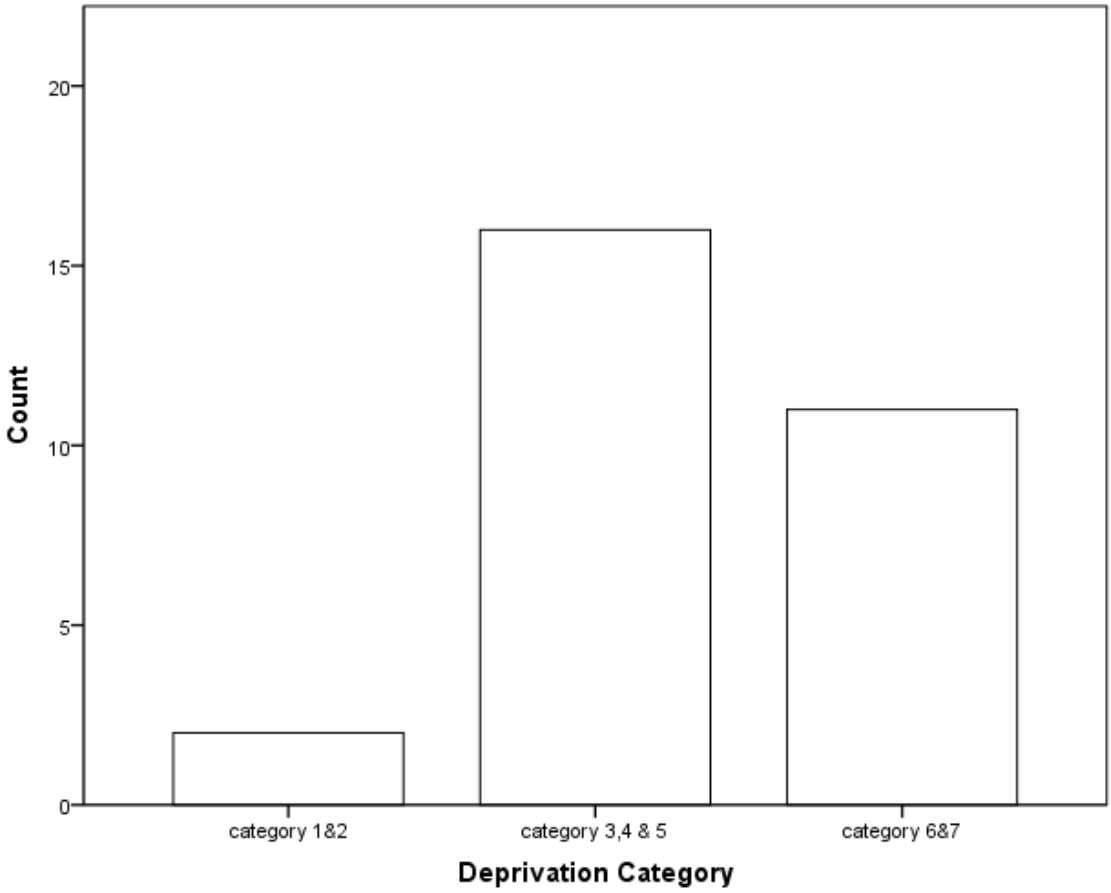

Figure 6-122 Deprivation categories of study group

The group typically displayed significant disease duration in excess of 17 years reflected in multiple prior DMARD and biologic treatments. Of note, the prevalence of depression or anxiety was 50%, of anxiety 43% and those with features of both disorder 40%. At the time of questionnaire administration, three respondents were known to have a diagnosis of mood disturbance and were treated with antidepressant therapy (HADS-A scores were 13, 16 and 10 and HADS-D 17, 10 and 10 respectively). The majority of this group were from more deprived postcodes.

Severe disease reflects in high disability scores yet also the presence of ongoing moderate disease activity in keeping with the main biologic resistant cohort (Table 6-97).

Whole group variable, n=30	**Median**	Range (max-min)	IQR
TJC	**4**	0-19	11
SJC	**4**	0-18	5
Pain	**3.9**	0.2-9.8	48
Patient Global	**39**	2-96	58
ESR	**14**	2-106	23
CRP	**4.25**	0.3-79	8.3
DAS-28 ESR	**4.22**	2.04-7.14	2.62
DAS-28 CRP (n=26)	**4.37**	1.94-6.86	2.83
HAQ	**2.000**	1.000-3.000	0.531
FACIT-F	**25**	3-48	19

Table 6-97 Disease activity component scores, composite disease activity, disability and fatigue of resistant RA group

6.7.2 Influence of mood and clinical outcomes

In order to test the hypothesis that mood influences subjective clinical variables, the components of the DAS28, disability and fatigue levels were evaluated by the presence or absence of mood disturbance as determined by their HADS questionnaire scores.

Clinical Variable (median score)	**Whole group**	Depressed (n=14)	Not depressed (n=16)	Anxious (n=13)	Not anxious (n=17)
DAS28	*4.23*	**4.80**	**3.71***	**5.06**	**3.41***
TJC	*4*	9.5	3	8	3
SJC	*4*	4.5	3.5	5	3
CRP	*4.3*	3.6	6.6	4.1	5.3
ESR	*14*	21.5	12	23	12
Pain	*39.5*	51.5	21	**62**	**19****
Patient Global	*39.0*	**59.5**	**31.5***	**73.0**	**25.0****
HAQ	*2.00*	2.13	1.88	**2.31**	**1.88***
Fatigue (FACIT)	*26*	**17.5**	**33****	**16**	**33****

Table 6-98 Influence of mood and clinical variables
(Mann-Whitney test where * p<0.05, ** p<0.01 and shown in bold font) (a lower FACIT score represents higher fatigue)

Significant differences can be seen in many of the clinical variables (numerically more although not achieving significance owing to the small sample size). The effect on the

DAS28 score is large nonetheless; the ESR contributes to this observation with no supportive CRP differences. There were also large differences in fatigue levels and some numerical differences in disability. To further test this, HAQ categorical grouping (disabled and very disabled where the latter HAQ greater than 2.000) was examined. No differences in in those clinical variables shown in Table 6-98 above were seen to reach statistical significance but higher anxiety was associated with poorer function (not significant in the depressed group but similar trend observed).

6.7.3 Coping (Brief-COPE results)

Cronbach's internal consistency scores (ICC) were calculated for each of the 14 domains and ranged from 0.189 in the 'denial' domain to 0.957 in 'planning'. In general, acceptable ranges were found.

Domain	Items	Cronbach's alpha coefficient
Active coping	1+2	0.819
Planning	3+4	0.957
Positive reframing	5+6	0.727
Acceptance	7+8	0.413
Humour	9+10	0.970
Religion	11+12	0.935
Emotional support	13+14	0.831
Instrumental support	15+16	0.711
Self distraction	17+18	0.647
Denial	19+20	0.189
Venting	21+22	0.792
Substance misuse	23+24	0.869
Behavioural disengagement	25+26	0.874
Self blame	27+28	0.898

Table 6-99 Cronbach's internal consistency score by coping domain (Brief COPE questionnaire)

Most frequently used strategy (domain) and mean score	Least commonly used strategy (domain) and mean score
1- Acceptance-5.10	1- Substance use-0.39
2- Active coping-4.55	2- Denial-0.79
3- Planning-3.72	3- Behavioral disengagement-1.04

Table 6-100 Most frequently and least commonly used coping strategies in study group

Of those strategies shown in Table 6-99 above, those most often used (highest mean scores) were acceptance, active coping and planning as shown in Table 6-100.

Additionally the domains were grouped to form broad coping strategies and Cronbach's score again calculated. Higher scores in the problem based and adaptive based coping strategies were seen to suggest favoured use in this cohort.

Strategy	Cronbach's test	Mean domain score (+SD) (max value 6)
Problem based	0.86	4.04 (1.54)
Emotion based	0.68	3.32 (1.00)
Dysfunctional coping	0.64	1.45 (0.76)
Adaptive coping	0.78	3.59 (0.95)
Maladaptive coping	0.64	1.45 (0.76)

Table 6-101 Cronbach's ICC results and mean coping domain score (n=30)

The effect of mood and preferential use of favourable or less favourable coping strategies was examined and results shown in Table 6-102. Adaptive strategies were favoured more often than maladaptive strategies; there was no influence if depressed, anxious or very disabled. The same, although less pronounced, observation was made between problem based and emotional based strategies. Least use of maladaptive strategies was seen in the very disabled group.

	Adaptive based	MWU test	Maladaptive based	MWU Test	Problem based	MWU Test	Emotion based	MWU test
Depressed	3.33	0.189	1.05	0.677	3.87	0.324	3.01	0.143
Non depressed	3.75		1.12		4.10		3.54	
Anxious	3.63	0.965	1.12	0.763	4.24	0.775	3.26	0.553
Not anxious	3.45		1.05		3.73		3.28	
Very disabled	3.29	0.293	0.98	0.268	3.62	0.264	3.10	0.347
Less disabled	3.72		1.20		4.21		3.43	

Table 6-102 Brief-COPE questionnaire results by coping strategy and effect of mood
(Mean answer response, Mann Whitney tests between mood and disability category)

6.7.4 Illness Perception (B-IPQ results)

Cognitive Illness Representation	Median score (range min-max) Whole group n=30
Consequences	8 (2-10)
Timeline	10 (5-10)
Personal control	3 (0-10)
Treatment control	7 (3-10)
Identity	8 (2-10)
Emotional concern	8 (0-10)
Illness comprehension	9 (1-10)
Emotional effect	8 (0-10)
Causal	n/a

Table 6-103 Illness perception item scoring (Brief IPQ scoring results)

Assessment of illness perception reflected high levels of understanding and of 'timeline' (consistent with long disease duration) but lowest scoring in the domain of 'personal control'. Scoring in the 'treatment control' group could be considered higher than expected when the number of prior treatments is considered. The range of responses observed within the group confirms wide variability within the individual's illness perception however. 'Causal' responses varied widely with the two responses most common responses "genetics/family history" and "stress".

	Not anxious N=16	Anxious N=14	Not depressed N=15	Depressed N=15	Very disabled N=14	Less disabled N=16
Consequences	8 (2)	8.5 (2)	8 (2)	8 (2)	8 (2)	8 (2)
Timeline	8.5 (2)	10 (0)	3 (6)	10 (0)	10 (0)	10 (0)
Personal control	3 (5)	3 (6)	3 (6)	3 (5)	2 (7)	4 (3)
Treatment control	8.5 (4)	5 (3)	8 (4)	5 (2)	5 (4)	8 (4)
Identity	8 (4)	8 (3)	8 (2)	8 (3)	8 (2)	8 (3)
Emotional concern	5 (4)	10 (2)	5 (4)	9 (3)	8 (4)	7.5 (6)
Illness comprehension	8.5 (3)	9 (2)	8 (3)	9 (2)	9 (2)	9 (3)
Emotional effect	3.5 (7)	8.5 (2)	3 (7)	8 (1)	8 (6)	7.5 (6)

Table 6-104 Illness perception and the effect of depression, anxiety and disability
(Category median item response (IQR))

Table 6-104 shows the effect of mood and IPQ response scoring. As expected, both emotional concern and effect were affected by mood but timeline and treatment control median scores were influenced by mood state and functioning. In order to further examine the influence of prevalent mood on illness perception, correlation with clinical factors and the B-IPQ items were sought (Table 6-105).
There was no correlation between the five Brief COPE strategies of Table 6-94 and mood, clinical nor biochemical variables. Disability (HAQ) only correlated with anxiety (0.413, p=0.026) yet with no other variables.

	Variable	Depression (HADS-D)	Anxiety (HADS-A)	Fatigue (FACIT)
Clinical	TJC	0.313	0.276	**-0.476***
	SJC	**0.419***	**0.386***	**-0.465***
	Pain	**0.506****	**0.579****	**-0.579****
	Patient Global	**0.621****	**0.675****	**-0.647****
	Age	0.064	-0.193	0.028
	Disease duration	-0.245	**-0.389***	0.259
Disease Activity	DAS-28 ESR	**0.456***	**0.498****	**-0.656****
Biochemical	ESR	0.071	0.181	-0.124
	CRP	-0.050	-0.007	-0.287
Questionnaires	HAQ	0.347	**0.413***	-0.283
	FACIT-F	**-0.648****	**-0.603****	n/a
	HADS-A	n/a	n/a	**-0.603****
	HADS-D	n/a	n/a	**-0.648****
B-IPQ domains	Consequences	**0.436***	**0.467****	-0.196
	Timeline	-0.313	-0.237	0.226
	Personal control	-0.231	-0.269	**0.408***
	Treatment control	**-0.601****	**-0.600****	**0.587****
	Identity	0.197	0.149	-0.131
	Concern	**0.527****	**0.626****	**-0.160**
	Understanding	-0.023	-0.003	-0.016
	Emotional response	**0.652****	**0.733****	**-0.539****

Table 6-105 Bivariate associations of clinical, disease activity, patient outcome measures and illness perception with anxiety, depression and fatigue
(Spearman's correlation coefficient (where *p<0.05, **p<0.01)).

This exploratory bivariate analysis identified that a number of clinical variables were significantly associated with depression and anxiety and fatigue in this smaller cohort (as demonstrated in the main resistant RA group in Chapter 3). Several of the items of illness perception correlated with mood, namely consequences ("how much does it affect your life", treatment control (a negative influence), concern and emotional response.
Regression analysis was then performed, using those statistically significant bivariate associations, to examine which illness perception variables both independently and overall explained the most variance in mood and fatigue. As a composite of the other clinical variables, the DAS28 ESR was entered only and this variable was entered at Step one to allow the influence of illness perception and other significant variables to be examined.

6.7.4.1 Fatigue

The fatigue 'enter method' model had a high R^2 overall. Overall effect of mood was 19.2% and illness perception variables 14.5% of the variance of fatigue. Of these depression and DAS28 was the most significant when determined in a forward stepwise model.

Fatigue Model	Variable	Overall R^2	Change R^2	Beta
1	DAS28 ESR	0.379	0.379	**-0.507***
2	HADS-A	0.438	0.059	0.134
3	HADS-D	0.571	0.133	-0.203
4	Personal control	0.572	0.001	0.052
5	Treatment control	0.618	0.045	0.114
6	Concern	0.633	0.015	**0.496***
7	Emotional response	0.717	0.084	**-0.629***

Table 6-106 'Enter' method multiple regression model of fatigue
Beta=standardised beta coefficient, (*p<0.05, **p<0.01)

Fatigue model	Variables	Overall R^2	Beta (p-value)
1	-Depression	0.407	-0.655 (<0.0001)
2	-Depression -DAS28 ESR	0.567	-0.480 (0.003) -0.411 (0.009)

Table 6-107 Forward stepwise regression model for fatigue
(Beta= standardised beta coefficient)

6.7.4.2 Anxiety

For anxiety, the overall model R^2 was 0.638 and the contribution of illness perception variables to anxiety was 26.1%. In the forward stepwise model, DAS28 and disability were significant independent variables relating to anxiety outcome.

Anxiety Model	Variable	Overall R^2	Change R^2	Beta
1	DAS28 ESR	0.230	0.230	**0.480***
2	HAQ	0.312	0.082	**0.302***
3	FACIT	0.370	0.058	0.208
4	Treatment Control	0.396	0.026	-0.005
5	Concern	0.484	0.081	-0.281
6	Emotional response	0.638	0.154	**0.821***

Table 6-108 'Enter' method multiple regression model of anxiety
(Beta=standardised beta coefficient, (*p<0.05, **p<0.01))

Anxiety model	Variables	Overall R^2	Beta (p-value)
1	-Emotional response	0.416	0.645 (<0.0001)
2	-Emotional response -DAS28 ESR	0.592	0.575 (<0.0001) 0.424 (0.003)
3	-Emotional response -DAS28 ESR -HAQ	0.659	0.547 (<0.0001) 0.357 (0.007) 0.270 (0.036)

Table 6-109 Forward stepwise regression model for anxiety
(Beta= standardised beta coefficient)

6.7.4.3 Depression

In the final depression model, fatigue accounted for nearly 25% of the variance of depression and was the single strongest variable. The "emotional response' question from the IPQ accounted for 12.6% as expected. This would suggest prevalent mood dose affect illness perception variables.

Depression Model	Variable	Overall R^2	Change R^2	Beta
1	DAS28 ESR	0.182	0.182	0.119
2	FACIT	0.430	0.248	**-0.381***
3	Treatment control	0.437	0.007	-0.005
4	Emotional response	0.563	0.126	**0.419***

Table 6-110 'Enter' method multiple regression model of depression
Beta=standardised beta coefficient, (*p<0.05, **p<0.01)

Depression model	Variables	Overall R^2	Beta (p-value)
1	-Fatigue	0.429	-0.655 (<0.0001)
2	-Emotional response	0.554	0.402 (0.014)
	-Fatigue		-0.465 (0.005)

Table 6-111 Forward stepwise regression model for depression
(Beta= standardised beta coefficient)

6.7.6 Role of Deprivation

To test the hypothesis that social deprivation may influence choice of coping strategy and illness perception, responses were examined and significance calculated. There were no differences observed when examined by deprivation.

B-COPE coping strategy	KW test of significance	B-IPQ item	KW test of significance
Problem based strategy	0.206	Consequences	0.930
Emotional based strategy	0.774	Timeline	0.441
Dysfunctional Strategy	0.871	Personal control	0.486
Adaptive Strategy	0.538	Treatment control	0.435
Maladaptive Strategy	0.871	Identity	0.957
		Concern	0.798
		Understanding	0.690
		Emotional response	0.452

Table 6-112 Influence of deprivation and coping strategy and with illness perception responses (KW-Kruskal-Wallis test)

6.8 Chapter Conclusions and Discussion

Inclusion to the main study cohort was the presence of both DMARD and biologic resistant disease and active inflammatory disease as determined by the DAS28 ESR. Significant disease duration and substantial co-morbidity was noted. It is evident from a small representative cohort of biologic resistant patients that mood has a strong influence not only on disease assessments but also illness perception. Strategies to cope with RA however are less influenced by prevalent mood.

6.8.1 Prevalence and Significance of mood disturbance

The prevalence of depression and/or anxiety in this cohort is over 40% and therefore should be actively sought in similar groups. The HADS questionnaire takes only a few minutes to complete and is thus ideal in the out patient setting. Anxious and/or depressed patients exhibit a higher DAS28 as a result of higher pain scores, patient global assessment and tender joint counts though no correlation with biochemical variables. This should be factored into disease assessment at all stages of the treatment process. Those identified as being anxious or depressed require expert evaluation and education of the impact these co-morbid conditions may have upon RA disease activity and assessment.

There is correlation between both anxiety and disability and between pain and depression in this cohort. This is relevant when considering those reporting worsening function to evaluate concurrent mood and assess pain in detail when mood is reported as low.

6.8.2 Coping

Longstanding RA is associated with huge personal and emotional impact necessitating the use of varied coping strategies, which persist despite disturbance of mood. There is a reported preference within the whole group, as one would expect, toward the use of strategies that may be considered positive such as acceptance and planning. Relatively little use of behaviours such as avoidance and denial was seen.

In view of the high prevalence of depression it was postulated that low mood or anxiety would affect self reported coping strategies. This was not observed and the favoured use of ‘positive’ strategies such as adaptive or problem based approaches was seen irrespective of mood. This would suggest a disconnect between mood (often variable in time and severity with multiple contributing factors) and coping (a set of strategies chosen and tested over many years of disease to overcome the unrelenting challenges of pain and disability)

6.8.3 Illness perception

The IPQ questionnaire quantifies the patient’s experience and understanding of their disease. RA patients with long disease duration experience little control over their disease although have high levels of understanding, perhaps reflecting good education. This reinforces the need for multidisciplinary education at all stages of disease.

Adjusting expectations toward disease control in longstanding RA rather than remission is also important to avoid perceived lack of response to treatment.

Prevalent mood and higher levels of fatigue influence reported domains of illness perception and correlation was observed between clinical, PROMs and illness perception responses. The presence of mood disorder correlates with perceived concern about their condition, perceived treatment control and consequences of RA (how much they see their condition as affecting them), an observation reflected in higher patient global assessments. There were some differences between the depression and anxiety groups highlighting the importance of establishing the correct diagnosis and treatment in view of their common co-occurrence. As noted in Chapter 3, both disease activity and fatigue correlate with the DAS28.

Examining variables individually is important to seek elements that are treatable.

- Reported fatigue should also prompt evaluation of depression scoring and careful examination and consideration of the DAS28.
- A report of anxiety would have similar implications for the DAS28 but consideration of disability and any aid that could be provided.
- Finally, those reporting depression are likely to report high fatigue levels. Consideration of the central and peripheral causes of fatigue should be performed in addition to treatment of mood.

6.4 Limitations

Examination of this small cohort raises a number of discussion points and acknowledgement of limitations. Replication with a larger cohort would of course add power to any findings. The influence of any acute social stressors such as bereavement at the time of completion of questionnaire cannot be excluded. In a similar way, the presence of social assistance such as family support was not assessed. Social support is important in predicting pain and disability and thus outcome (Evers et al. 2001). The influence of age and personality cannot be discerned in this cohort; coping may be most plastic in early life/disease becoming more fixed with time.
I have not set out to assess personality and the influences this has upon coping (Carver & Connor-Smith 2010) although this remains an important consideration.

6.8.5 Implications

6.8.5.1 General Implications

The identification of these findings is not only an opportunity to carefully consider disease assessment but to consider best treatment options. For example, mood disturbance may be the sole factor blunting response to biologic therapy as illustrated above. It is also of interest that although the clinical features of depression and anxiety differ, the effect on clinical variables is similar. This would suggest that screening for either or both is sufficient. Therefore, an assessment *prior* to treatment may help in

managing expectation of treatment response and allow an assessment of mood to direct additional appropriate treatment. In the same way, prospectively studying coping would be of interest to identify those traits demonstrated at baseline that influence disease outcome. In late disease such as this cohort display, and despite favoured use of more positive coping strategies, coping seems less important showing little or no correlation with clinical factors, mood or illness perception.
Finally, the use of the DAS28-CRP may help to minimise one of the variables weakening the reliability of the DAS28 assessment. The choice of ESR or CRP does not bear any importance in assessments of mood, coping or illness perception.

6.8.5.2 Implications for 'biologic resistance'

This sub-study defines a group of patients with concurrent mood disturbance who display elevated components of the DAS28 score. The effect is to blunt reported response to treatment and perception of their illness. However, the biologic resistant group are also defined by 'positive' coping strategies shaped by significant duration of disease but not by prevalent mood.

> *In this way, mood shapes response to treatment measured in the clinic with the DAS28 assessment, and illness perception thereof, but does not determine coping.*

The implication herein is that a proportion of biologic resistant patients have 'severe disease' driven by mood disturbance and high fatigue levels.

7.1 Chapter 7; General Discussion and Conclusions

7.1.1 Conclusions of this study

Chapter 3 presents the heterogeneous 'phenotype' of the main study cohort. I have demonstrated the substantial burden of disease in terms of prior treatments, joint surgery and employment status. The related comorbidity this cohort displays is significant. Within the findings of significant numbers that merit screening for depression, anxiety or merit more aggressive CV risk factor modification there is the opportunity to target these areas. The information gained from PROMs enriches the assessment process. Fatigue, disability and impairment of quality of life are all recognised features in longstanding RA but rarely quantified in daily practice. Unfortunately, these domains are relatively unresponsive to treatments in published literature and within this cohort. The relationship between clinical variables, social factors and PROMs is complex but close inter-correlation in this cohort suggests that a holistic approach is needed.
The DAS28 and weaknesses in assessing the biologic resistant group are presented. The group are inflamed by this measure:- they display response to changes in therapy, a fall in conventional markers of inflammation (not statistically significant), and appropriate cytokines change with therapy. The control groups display key differences as hoped, which is important in relation to understanding the implications of Chapter 4.

Chapter 4 examined the CD14+ microRNA profile of three 'candidate' microRNA and an exploratory microarray of the defined study groups. The candidate microRNA showed some correlation with swollen joint counts and distinct profiles were seen between study groups suggesting integrity of the groups determined by clinical differences. Three novel microRNA, microRNA-423, -3178 and -1275 were validated as being relatively upregulated in the biologic resistant group compared with the other control groups. MicroRNA-423 and -1275 were studied at three and six months and showed comparable reductions as was observed with the DAS28 score. Strong correlation between microRNA suggests co-transcription. Correlation with RANKL, MMP-12 and MCP-1 was observed. These microRNA may represent biomarkers of disease activity in biologic resistant RA.

Chapter 5 examined the serum cytokine profile in cross-section and longitudinally. Examination of cytokines at baseline confirmed significant differences between study groups:- findings of the inflamed clinical phenotype were confirmed. The DMARD good responder and DMARD resistant group profiles appeared distinct but differences from the biologic resistant group were less clear (a function of degree of inflammation?). A number of cytokines followed an observed pattern of reduction that was reflected in the DAS28 however. Correlation with the DAS28 was not seen with clinical, biochemical or selected PROM variables (fatigue or mood disturbance) at baseline.

Chapter 6 examined a subset of the main biologic resistant study group to examine coping and illness perception. This sub-group displayed comparably high rates of mood disturbance with resultant increases in clinical variables such as pain, tender joint counts and global assessments that drove the DAS28. Coping strategies were generally favourable but not influenced by prevalent mood unlike illness perception in which the opposite was observed. Both mood and fatigue influenced current perception of disease again influencing subjective disease assessment (and potentially self-evaluation of treatment past and present).

7.1.2 Limitations and weaknesses

There are of course limitations within this study. The effect of underpowering cannot be excluded. The clinical data is observational and in trying to draw conclusions about mood, fatigue and associations, firm conclusions would be undoubtedly affected by smaller numbers. The Bonferroni correction method is used to reduce the chance of type 1 statistical errors but may increase the likelihood of a type II error (false negative). In the regression calculations, particularly in Chapter 6, larger numbers are always desirable so results should be interpreted with caution. For this reason both types of regression were performed. Furthermore, in the clinical group, selection bias and reporter bias (patients more likely to respond?) may be present. The number of PROM questionnaires (evaluated in Chapter 3) returned at subsequent visits fell slightly. In particular, 30/50 patients returned the coping questionnaires and, although this is in keeping with return rates seen in postal questionnaires in general, applying findings of this group should not necessarily be applied to the whole biologic resistant cohort.

Many of the early microRNA studies were in small series of heterogeneous patients. The observation of different microRNA profiles made between research groups may reflect patient profiles or different methodologies employed. We aimed to minimise this effect by creating study groups that are phenotypically similar and in this way clinically applicable. However, the effect of individual treatments and their changes is not possible to calculate. There were a number of treatment variations between study assessments from intra-articular and intramuscular steroid, DMARD changes and biologic therapy changes. In particular the effect of intermittent B-cell depletion with Rituximab is particularly difficult to estimate. Statins are prescribed primarily to reduce the incidence of cardiovascular morbidity and mortality but have also been shown to reduce disease activity in RA (McCarey et al. 2004; Okamoto et al. 2007). Statin cardio-protective benefits are primarily through the effect of stabilising atheromatous plaque and similar microRNA to those seen in RA are readily identified within. MicroRNA have also been studied in a wide number of medical conditions including vascular disease and diabetes. In view of the significant number of comorbidities recorded in the biologic resistant cohort, an effect on the peripheral microRNA profile cannot be excluded. The effect of concurrent OA should also be considered in any profiling of longstanding RA. There is evidence for circulating microparticles or exosomes containing microRNA as

being potential messengers in view of their potential for uptake by macrophages (Lässer et al. 2011). It is hypothesised the joint and circulating profile may reflect one another in this way. Potentially however the macrophage profile reflect other co-morbidities (cardiovascular, intercurrent infection and obesity are examples).

The choice of tissue and cell type in which to measure microRNA is important. The blood and synovial compartments are assumed separate. Lymph nodes and lymphatics act as important regions of antigen presentation and initiation of the immune response. This has not been systematically studied. Studies to date are conflicting. We chose blood as this is practical and non invasive. However, the findings of Chapter 4 and the joint profile are not mutually exclusive.

Despite this, the resistant microRNA profile identified in this study cohort can be said to represent a cell- and disease-specific profile. It may represent a 'response-to-treatment' profile. The resistant microRNA profile cannot be said to represent a drug response profile or disease stage profile.

7.1.3 Clinical Applications

There are several clinical applications from results of the biologic resistant cohort that have already been highlighted including the weaknesses of the DAS28 score, the challenge of achieving low disease activity in longstanding disease (yet apparent responsiveness of inflammation) and importance of considering mood disorder in the case of a lack of response to treatment. Several specific examples stand out such as the need to consider mood, fatigue and disease activity together. The unmet burden of need with respect to mood and cardiovascular risk has also been highlighted and should prompt more attention to risk modification.

The challenge remains that we are unable to reliably predict outcome. Table 1-2 outlines the range and combination of factors that have been shown to influence outcome. Added to this are epigenetic factors. The results of the biologic resistant group confirm the wide heterogeneity even within a phenotypically similar group and suggest resistance to treatment is a function of many variables. In disease of significant duration, effective treatment should include a comprehensive holistic approach to symptom management, assessment of relevant co-morbidity and assessment of vascular risk.

7.1.4 What is the link between inflammation, mood, epigenetics and cardiovascular disease?

As demonstrated in this cohort of patients, there is substantial co-morbidity and I have focussed on CV disease and mood disturbance. This raises the question of any pathophysiological processes that may be linking these processes in RA?

Inflammation, atherosclerosis and microRNA

The link between atherosclerosis and RA has been observed epidemiologically and referred to in Chapter 1. Pathophysiologically, atherosclerosis is a chronic inflammatory process. It is therefore of no surprise to find publications referring to microRNA mediating this process. Nazari-Jahantigh et al examined microRNA-155 within atherosclerotic plaques (Nazari-Jahantigh et al. 2012). They found microRNA-155 highly expressed in both atherosclerotic plaque and the macrophages within. MicroRNA-155 deficiency leads to plaque volume reduction. MicroRNA-155, -145 and -126 are three important microRNA characterised in atherosclerosis (Wei et al. 2013). Van Empel et al reviewed those circulating microRNA demonstrated in myocardial infarction, stable IHD, stroke, diabetes and heart failure (van Empel et al. 2012). Such microRNA may have a role in diagnosis and prognosis with promise as biomarkers. The challenge lies in establishing the role of the circulating profile representing the target tissue and 'pure' patient/disease cohorts.

Depression and raised inflammatory markers

There is some evidence that depression and inflammatory markers such as CRP, IL-6 and IL-1 (via a link with obesity are linked (Howren et al. 2009). However the direction of causality is not known. Almeida studied an elderly population and despite identifying an elevated CRP in depressed individuals, social factors such as poor physical health were strong confounders. Uddin et al studied depressed and non-depressed subjects and also found raised inflammatory markers (IL-6 and CRP) higher in the depressed individuals (Uddin et al. 2011). They identified unique DNA methylation patterns in those with depression but no correlation with CRP. The same authors demonstrated epigenetic changes in peripheral blood after psychological stressors (post traumatic stress disorder (Uddin et al. 2010). The life changing effect of RA could be hypothesised as having similar effects. Finally, Treharne et al have shown that RA patients who have CV disease are also more depressed and less optimistic (Treharne et al. 2005).

High CRP is linked to increased cardiovascular risk

Bjerkeset et al examined CRP in over 9000 subjects. They identified an association between increased CRP with increased risk of myocardial infarction (Bjerkeset et al. 2011). Empana et al also found those that were depressed had raised inflammatory markers in a prospective study of otherwise healthy middle aged males. They went on to identify an association with ischaemic heart disease (Empana et al. 2005). Miller et al review the links between these observations in their review. They propose that depression induces a mild inflammatory response and encourages CV risk behaviour such as smoking and inactivity. The hypothalamic-pituitary axis activation may increase

susceptibility to infection and elevate IL-6. Depression may lead to obesity through inactivity and the group with the highest inflammatory markers were obese (Miller et al. 2002).
Yudkin et al propose a model whereby elevated IL-6 is the central pathogenic cytokine. As noted in Chapter 1, elevated IL-6 is characteristic in RA and anti-IL-6 therapy effective. Acting on the liver it is the main stimulus of CRP (Yudkin et al. 2000). IL-6 may be in part related to obesity in keeping with the observations of Mohamed-Ali et al who found IL-6 was released systemically, but not TNFa, from adipose tissue (Mohamed-Ali et al. 1997). Additionally IL-6 may be released in response to psychological distress such as mood disturbance. Subsequent activation of the HPA axis leads to hypertension and central obesity and dyslipidaemia.

Rheumatoid Arthritis; a combination of risk

In the case of RA, not only are additive conventional cardiovascular risk factors such as smoking present but also systemic inflammation. Many of the findings above can be seen in the biologic resistant cohort.
The prevalence of depression in this cohort is 42% in keeping with other studies in RA and replicated at other disease stages. 100% of the patients measured have an elevated waist:hip ratios greater than desirable range in keeping with central obesity. 59% had a BMI greater than 30. The effect of depression and CRP, BMI and IL-6 was numerically evident but did not achieve significance as seen in Table 7-112. An elevated BMI and waist:hip ration may also be in keeping with reduced physical activity.

	Not Depressed (HADS<8) median (IQR)	**Depressed** (HADS >8) median (IQR)
CRP mg/dl	9.9 (25.9)	14 (27.2)
BMI	24.5 (9)	28 (10)
IL-6 pg/ml	48.3 (83.3)	58.3 (201.9)

Table 7-113 Effect of depression and BMI/selected inflammatory markers

In summary, RA patients display a combination of many of these features perhaps explaining the clustering of adverse risk and increased morbidity. Molecular links tie epigenetic modifications, inflammatory markers, depression and coronary artery disease. The findings of this study are in keeping with these observations.

The raises the question of which dimensions to treat and when? In reality a multifaceted approach is necessary from prompt suppression of inflammation, to screening for mood disturbance to the identification of biomarkers that predict subclinical atherosclerosis. Aggressive treatment of modifiable CV risk factors is needed. Prospective studies can then answer the hypothesis that better treatment of inflammation leads to less depression and less CV disease (as has been shown in the short term).

7.2 Summary

Figures 7-123 and 7-124 graphically represent the factors that 'define' this biologic resistant cohort. Resistance to treatment is multifactorial. Subjective response is affected by illness perception, mood and fatigue. Deprivation may interact with these variables. Epigenetic factors have been identified in this cohort suggesting biological mechanisms may characterise the inflammatory response and/or mediate the resistance to treatments.

On the other hand severity could be defined by number of joint surgeries and radiological damage quantitatively and directly. Disability and quality of life are both unaffected by clinical variables, inflammatory markers and deprivation suggesting these outcomes are more complex to model in disease of longstanding duration.

Resistance Model

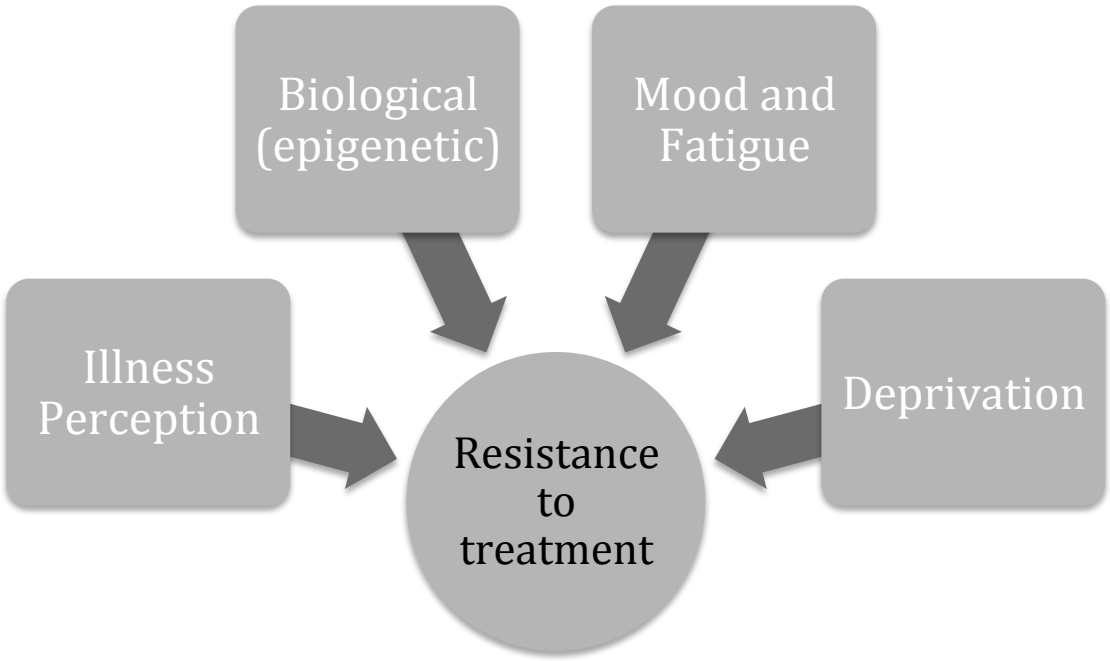

Figure 7-123 Representation of the factors that contribute to resistance to treatment in this study

Severity Model

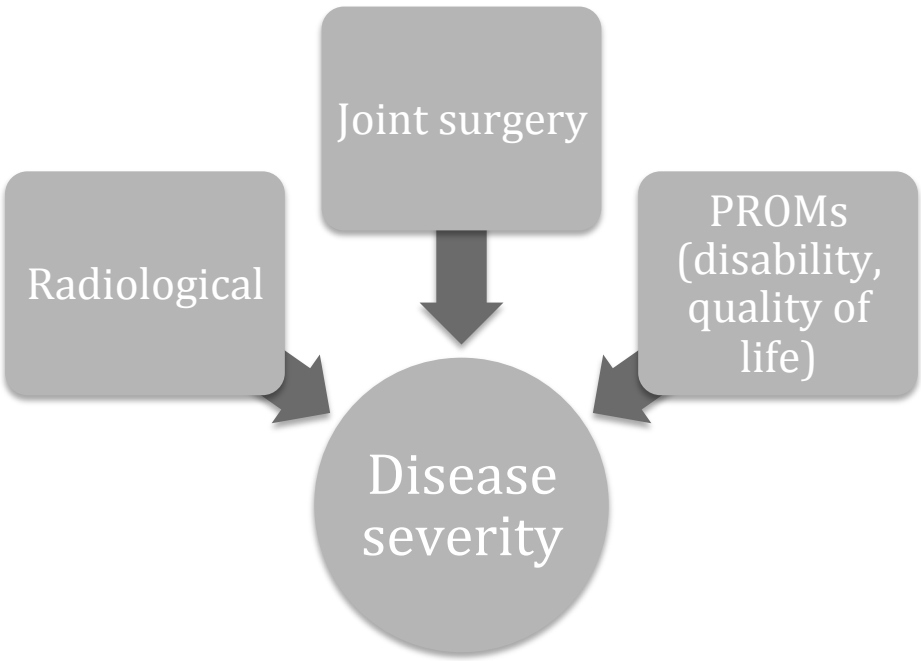

Figure 7-124 Representation of the variables that could be used to determine disease severity

7.3 Future work

There is a wealth of clinical and biological information that can be gathered at each patient encounter in RA. The PROMs and the time to complete and score them raises the question of how best to incorporate their use in daily practice. In disease of longstanding duration, study of this cohort puts forward a case for the assessment of such dimensions but only to understand which domains require focussed interventions. Determining which dimensions are of most value to patients and which dimensions could be used to best determine disease status will be of value.

The clinical application of the identification of the 'resistant microRNA' is dependent upon similar observations being made in cohorts with earlier disease. As illustration, the question of RA becoming milder has been raised by previous authors and as patients are referred and treated earlier, overall outlook for the disease has improved (Wolfe & Pincus 2001; Welsing et al. 2005; Alcorn et al. 2009). Without doubt however there remain individuals with severe and aggressive disease. Such aggressive disease *will* often become clear in the first few years but should a unique microRNA profile of resistant disease be present in early disease, there is the potential to seek this resistant microRNA signature with clinical assessments and genotyping to target most aggressive therapy. Profiling the resistant microRNA in a cohort of early RA patients would be of value.

Of more value and as discussed in Chapter 4, elucidation of the biologic pathways targeted by the resistant microRNA will be key to answering the question of a distinct resistant inflammatory network. Within this, novel treatment targets may lie.

The effect of DNA methylation and other epigenetic modifications have not been examined within this thesis. In view of the regulatory effect of DNA methylation status and microRNA profile referred to in Chapter 1, the relation to observed microRNA profile would be of enormous interest in this population. A systematic examination of the epigenetic profile of treatment resistant RA patients is likely to reveal additional findings that may serve to advance the understanding and treatment of this condition.

8.1 Abbreviations used

ACPA- anti citrullinated protein/peptide antibodies
ACR- American College of Rheumatology
ANA- anti nuclear antibody
BMI- body mass index
BP- blood pressure
BSR- British Society for Rheumatology
COX- cyclo-oxygenase
CRP- C-reactive protein
CTLA 4- cytotoxic T-lymphocyte antigen 4
CV- cardiovascular
DAS- disease activity score
DCs- dendritic cells
DMARD- disease modifying anti-rheumatic drug
DNA- deoxyribonucleic acid
ELISA- enzyme linked immunosorbent assay
EMS- early morning stiffness
ESR- erythrocyte sedimentation rate
EULAR- European League Against Rheumatism
FACIT-F- Functional Assessment of Chronic Illness Therapy Fatigue Scale
FLS- fibroblast-like synoviocytes
FOXP3- forkhead box P3
GM-CSF- granulocyte/macrophage colony stimulating factor
GWAS- Genome wide association studies
HADS- Hospital Anxiety and Depression Scale
HATs- histone acetyltransferases
HDAC- histone deacetylase
HDACs histone deacetylase inhibitors
HAQ-DI- Health Assessment questionnaire (DI- disability index)
IFX- Infliximab
Ig- immunoglobulins
IHD- Ischaemic heart disease
Interleukin-2 receptor alpha- IL2-RA
ISH- Isolated systolic hypertension
IM- Intramuscular
LVSD- left ventricular systolic dysfunction
MAP kinases- mitogen activated protein kinases
MDR-1- Multidrug resistance protein
MetS- Metabolic syndrome
MHC- Major histocompatibility complex
MMPs- matrix metalloproteinases
mRNA- messenger ribonucleic acid

MRI- Magnetic Resonance Imaging
MTX- methotrexate
MyD88- myeloid differentiation primary response gene (88)
NSAIDs- non steroidal anti inflammatory drugs
PADI4- peptidyl arginine deiminase, type 4)
PI3 Kinase- phosphatidylinositide 3-kinase
PROMs- patient related outcome measures
PTPN22- protein tyrosine phosphatase, non receptor type 22
PVD- peripheral vascular disease
QALYs- quality adjusted life years
QoL- quality of life
RANKL- receptor activator of nuclear factor kappa-B ligand
RASF- Rheumatoid arthritis synovial fibroblasts
RF- Rheumatoid factor
RISC- RNA-induced silencing complex
RNA- ribonucleic acid
SCORE- Systemic Coronary Risk Evaluation
SE- shared epitope
SIMD- Scottish Index of Multiple Deprivation
SLE- systemic lupus erythematosus
SJC- swollen joint count
SNP- single nucleotide polymorphism
SOCS- suppressor of cytokine signalling
STAT 4- signal transducer and activator of transcription 4
TB- tuberculosis
TC:HDL Chol ratio- total cholesterol:HDL (high density lipoprotein) cholesterol ratio
TCZ- Tocilizumab
Th cells- T-helper cells
TFs- transcription factors
TGF-beta 1- transforming growth factor beta 1
TLR- Toll-like receptor
TJC- tender joint count
TNFi- Tumour necrosis factor inhibitor
TNFa- Tumour necrosis factor alpha
TRAF 1- TNF receptor associated factor 1
Treg cell- regulatory T-cell
US- Ultrasound
VAS- visual analogue score
VEGF- vascular endothelial growth factor
WHR- waist to hip ratio

Appendix 1- Questionnaires employed in Biologic resistant study group

Questionnaire	Aims and Scoring	Administrations
HADS (Hospital Anxiety and Depression Score)	To examine the prevalence of psychological morbidity in the group. Total score 0-21 (>8 suggesting pathology) having added anxiety and depressive scores together. This can be examined separately	**0&6 months**
HAQ (Health Assessment Questionnaire)	Examines how the individual manages day-to-day activities. Responses tally a score translated to a value between 0 and 3 in increments of .125	**0&6 months**
SF-36v2 (Short form questionnaire)	Examines 8 domains (4 physical, 4 mental). Uses a norm based scoring system. Assumes population mean=50, <45 then health status would be less than the population average.	**0&6 months**
FACIT fatigue scale	13 items; weighted and used to calculate total score. Applied versus population average	**0&6 months**
EQ-5D (EuroQol questionnaire)	Applies 5 short responses and a visual analogue score 0-100. Outcome variables are the change in VAS and score across 5 dimensions	**0&6 months**
Rose angina questionnaire	Direct questioning regarding presence of chest pain and subsequent detailed questions seek features suggestive of angina	**Once at baseline**
Brief COPE questionnaire	See Chapter 6.6.1	**Once during study period**
Brief IPQ (Illness Perception Questionnaire)	See Chapter 6.6.1	**Once during study period**

Appendix 2- Venepuncture and Sample Transport

Standard Operating Procedure; Venepuncture

Patient identification checked and confirmed

Local Infection Control policies are to be adhered to (thorough hand washing, use of gloves, trays and appropriate needle disposal)
Vein identification
Application of tourniquet
Mediswab applied to area to be used
Venepuncture; using of BD Vacutainer® Push Button Blood Collection system where possible, 21g or 23g if vein access poor. Tubes used are BD Vacutainer® and are drawn in the following order;

- Yellow top SST (serum separator tubes); invert up to 5 times
- Green top (lithium heparin tubes) invert 8 times after drawing
- Purple (EDTA) tubes; invert 8 times
- PaxGene RNA® (PreAnalytiX) tube (tourniquet released prior to PaxGene tube) followed by 8-10 inversions

Samples transported to the GBRC laboratory are labelled using their unique study reference number.
Samples are stored and transported at room temperature.

Transport to be arranged within 1 hour to allow prompt analysis and appropriate storage at GBRC

Those samples being analysed by local laboratories (Haematology and Biochemistry) are labelled using their standard NHS patient identification including date of birth, hospital reference number and/or CHI number as standard practice. Samples delivered to laboratory within one hour.

Appendix 3- Laboratory Specimen Standard Operating Procedure

Blood Sampling; each patient and study visit		
Bone and cytokine	See 'GBRC handling'	2x clotted serum tubes
Epigenetics	See 'GBRC handling'	4x Lithium heparin tubes
Haematology	Local Laboratory	1x EDTA
Biochemistry	Local Laboratory	1x Clotted serum
RNA	See 'GBRC handling'	1x PaxGene RNA tube
Baseline visit only		
Immunology	Regional Laboratory, Gartnavel General Hospital	2x clotted serum tubes

GBRC Handling

- Epigenetic samples; refer to 'Epigenetic clinical specimen collection and processing'
- Clotted serum tubes; following centrifugation; aliquot to Eppendorf serum tubes; -80deg freezer for later analysis
- PaxGene tube; prompt freezing at -80deg freezer

1. EPIGENETIC CLINICAL SPECIMEN COLLECTION AND PROCESSING

1.1. Sample collection (Glasgow Royal Infirmary, Rheumatology Clinic)

20 ml of patient/control blood should be collected into lithium heparin containing tubes (4 green cap tubes). Blood samples should be kept in room temperature (RT). Cell isolations must be conducted on the day of blood collection.

1.2. Sample processing (GBRC)

1.2.2. Isolation of PBMC

PBMC's will be extracted using RPMI wash and Histopaque creating a layer on the diluted blood. This is centrifuged at 2100 rpm for 15 minutes and the opaque interface containing mononuclear cells, is transferred with a Pasteur pipette, into a clean tube. This is further washed, centrifuged and cells re-suspended in cold MACS buffer. Cells are then counted and after a further centrifuge, cell type isolation can proceed

1.2.2a CD14+ cells isolation

PBMC are re-suspended in MACS buffer and CD14+ beads (Miltenyi Biotec, 130-050-201) added and incubated cells for 15 minutes in refrigerator. By using an autoMACS Separator CD14+ cells and a negative (CD14-) fractions can be obtained.
Flow cytometry is then required to check cell fraction purity.

1.2.2b Archiving CD14+ cells for microRNA, transcriptomics, DNA methylation and SNP studies

microRNA study (priority)

incubate the samples in RNA*later* solution overnight at 4^0C to allow thorough penetration of the cells then transfer to –20°C. Samples can be stored at–20°C indefinitely.

transcriptomics

-pallet at least 0.5 x 10^6 cells in RNAse/DNAse free eppendorf tubes by spinning down at 2500 rpm, 5 min, 4^0C and proceed as above.

DNAmethylation/SNP study

-pallet 0.2x10^6 cells in RNase/DNase free eppendorf tube by spinning down at 2500 rpm, 5 min, 4^0C;
This will be done using a QIAcard FTA Spot matrix, allowed to dry and stored ambiently

1.2.2d CD4+ cells isolation

The process is similar to that described in 1.2.2a

1.2.2e Archiving CD4+ cells for microRNA, transcriptomics, DNA methylation and SNP studies

(The same as for CD14+ cells)

1.2.2f Archiving CD4- CD14- cells for microRNA, transcriptomics, DNA methylation and SNP studies

(same as for CD14+ and CD4+ cells)

Appendix 4- Study Protocol (final version June 2011 as submitted for ethics review) and ORBIT inclusion/exclusion criteria

THE ROLE OF EPIGENETICS IN RESISTANT RHEUMATOID ARTHRITIS
PROTOCOL FINAL VERSION JUNE 2011

FULL TITLE	An Observational, Multiple-Centre Study to investigate the Clinical, Pathological, Immunological and Epigenetic Characteristics of patients with biologic therapy-resistant Rheumatoid Arthritis
SPONSOR	Greater Glasgow and Clyde Health Board
INDICATION	Rheumatoid Arthritis
HYPOTHESIS	Patients with therapy-resistant RA show greater plasticity in gene expression regulation in response to inflammation and treatment with current standard-of-care therapies as evidenced by differences in the pattern and extent of DNA methylation, post-translational modifications of histone structure and micro RNA expression. Such epigenetic changes will be evident in accessible circulating peripheral blood leukocyte subsets.
OBJECTIVES	**Primary:** • To describe the clinical and epigenetic characteristics of rheumatoid arthritis (RA) patients who have 'biologic therapy-resistant' disease • To explore how any epigenetic profile identified is related to the presence of RA, the disease duration, or disease severity (or a combination thereof), by comparing them to control groups detailed below: **Secondary:** • To characterize the peripheral blood plasma / serum biomarker signature and examine its' stability over time within this RA patient population
STUDY DESIGN	This is a multi-centre, observational study in RA patients (n=50) previously treated with conventional DMARDs and at least two previous biologic therapies. Patients will be admitted to the study regardless of reason for prior drug continuation and will therefore include toxicity and efficacy failures. This will ensure that a broad patient population is captured for subsequent analysis. Enrolled patients will undergo three (3) assessments on Day 1

	(baseline), and at approximately Weeks 12 and 24 (see table 1), including: • Clinical and laboratory disease activity assessment • Biomarker blood sampling • Assessment of co-morbidity and presence thereof
CONTROL PATIENTS	There will be three comparator groups recruited: 1. Normal subjects – all patients recruited to the 'therapy-resistant' group will be asked to bring a close friend of the same gender and similar age. 2. DMARD-resistant RA patients – up to 50 patients with moderate/severe RA who meet the BSR eligibility criteria for starting anti-TNF therapy 3. DMARD-sensitive RA patients – 25 patients with longstanding RA (meeting the ACR 1987 definition) who have responded to conventional therapy (see below)
NUMBER OF SUBJECTS	Up to 200 RA patients (50 biologic therapy resistant RA patients, 25 DMARD sensitive RA patients and up to 50 DMARD resistant RA patients) 25 normal controls will be enrolled in total.
LENGTH OF STUDY	Up to 28 weeks from screening through to the end of the observational period for biologic therapy resistant group and group 2 above: • Screening: Up to 4 weeks prior to baseline visit • Observational period : 24 weeks
INVESTIGATIONAL MEDICAL PRODUCT(S)	Not applicable
NON-INVESTIGATIONAL MEDICAL PRODUCT(S)	Patients will continue to receive standard of care altered according to the judgement of the treating physician.
INCLUSION CRITERIA	Biologic therapy-resistant RA 1. Diagnosed with RA by the 1987 American Rheumatism Association (ARA) criteria for the classification of RA 2. Active RA at screening as defined by: • Disease activity score (DAS28) ≥ 3.2 3. Multiple treatment failure as indicated by: • Prior receipt and failure of conventional DMARD treatment. • Previously received at least two (2) biologic therapies for rheumatoid arthritis, such as, but not necessarily limited to, etanercept, adalimumab, infliximab, rituximab, abatacept, anakinra and /or tocilizumab. Normal controls • participant should not display any features of active arthritis, inflammatory or degenerative, or any current symptomatic joint injury DMARD Therapy-resistant RA 1. Diagnosed with RA by the 1987 American Rheumatism

	Association (ARA) criteria for the classification of RA 2. Active RA at screening as defined by: • Disease activity score (DAS28) ≥ 3.2 3. No previous biologic therapy, but with active disease despite the use of at least two conventional DMARDs DMARD Sensitive RA Group 1. Diagnosed with RA by the 1987 American Rheumatism Association (ARA) criteria for the classification of RA 2. Disease duration of >10 years 3. Demonstrate a good response to 2 or fewer conventional DMARD 4. DAS <3.2 reflecting low disease activity Inclusion criteria related to all participants 1. Able and willing to give written informed consent and comply with the requirements of the study protocol 2. Age 18 and older to 75 yrs
EXCLUSION CRITERIA	Active cancer or acute/chronic infection Surgery within one month prior to screening.
ASSESSMENTS PERFORMED: **Resistant RA Group Only**	
- DEMOGRAPHICS	Data will be collected on age, gender, ethnicity, employment, years of formal education, marital status, smoking status (never, ex-smoker, <15cpd, >15cpd) and social deprivation (Carstairs score).
- CLINICAL PHENOTYPE & COMORBIDITY	All patients will have a full detailed clinical history and clinical examination that will collect data on: 1) Rheumatoid arthritis (articular): • age of symptom onset; disease duration (diagnosis made) • ESR at disease onset • ACR core set (Swollen joint count [SJ], Tender joint count [TJ], Health Assessment Questionnaire [HAQ], Patient's pain score [PS], Patient's Global Assessment [P Global], Physicians Global Assessment (Inv Global), ESR and hsCRP • Disease activity scores (DAS, DAS28, CDAI and SDAI) • Total Sharp Score on plain hand & feet radiographs 2) Rheumatoid arthritis (extra-articular) • history, signs or diagnosis of: - nodulosis - pulmonary fibrosis

- sicca syndrome
- cutaneous vasculitis including nail-fold vasculitis
- pleuro-pericarditis
- scleritis or corneal melt

Additionally, all patients will have data collected on:

3) Quality of life questionnaires
- SF-36v2
- FACIT-Fatigue
- EQ-5D

4) Cardiovascular profile
- clinical history and examination and blood pressure
- BMI and waist:hip ratio
- lipid profile
- Rose questionnaire and ECG

5) Psychological profile
- Hospital Anxiety & Depression (HAD) questionnaire

6) Bone profile
- history of fracture
- bone densitometry (if DEXA performed within previous 2 years)
- vitamin D, (PTH assays if appropriate)

7) Drug therapy
- current therapy
- history of previous DMARD/biologic therapy; start/stop dates with reason for stopping
- exposure to oral/IA/IM steroid
- postmenopausal HRT use and duration

8) Co-morbidity
- History of other auto-immune disease (including diabetes and thyroid disease), cardiovascular, cerebrovascular, renal, pulmonary, malignant, psychiatric or infectious disease.

BIOMARKERS and PHARMACODYNAMICS

On the days and times specified in 'Table 1', the following tests will be included, but not limited to, all patients in the **Biologic-therapy Resistant group, DMARD sensitive group, DMARD resistant group and healthy control groups.**

- **Epigenetic analysis**

- **Acute Phase Panel**: hsCRP

- **Auto-antibodies**: Rheumatoid Factor (RF), anti-cyclic citrullinated peptide (CCP) antibodies, anti-nuclear antibodies (ANA), immunoglobulins and their isotypes, reflex ENA testing as appropriate. Processing will be undertaken at the Immunology Department at Gartnavel General Hospital, 1053 Great Western Road, Glasgow, G12 0YN

- **Bone Metabolism Panel**: may include CTX-I, CTX-II, MMP-3, TIMP-1, DKK-1, OC, COMP, PDP, DPD, NTX-I, BAP, PIINP, Helix-2, Vitamin D, PTH. Analysis of bone markers will be undertaken by Roche. Samples will not be stored for longer than five years and remain within the Rheumatoid Arthritis Inflammation Discovery group.

- **Core Inflammatory Cytokines**: may include IL-1, TNF, sTNFR1, sTNFR2, IL-6, sIL-6R, sgp130, BAFF, APRIL, sBCMA, sBAFF-R, sTACI, IL-12p40, IFNg , TGFb (to be extended pending multiplex methodology to include other novel disease relevant markers)

Samples to assess exploratory biomarkers will be collected on the days and times specified in 'Table 1'. These samples will be used only for research purposes to identify dynamic biomarkers that help characterize the molecular and patho-physiological mechanisms of the disease process - such as, but not limited to, TNFα, IL-1β and IL-6. These samples will be stored for up to 5 years after database closure, and include:

- DNA for pharmaco-genomic analysis / drug resistance-toxicity / epigenetic modifications
- Serum/plasma and mRNA for the analysis of disease-related analytes (whole genome profiling)
- Whole blood for cell subset purification and subsequent protein/mRNA processing

Further exploratory analysis during or after 5 years will only be undertaken after consent has been obtained.

Normal Control Group

Processing and analysis of samples will be undertaken at the Glasgow Biomedical Research Facility unless stated above.

Healthy controls will undergo demographic assessment as outlined above and limited clinical examination.
Blood sampling will be collected at single baseline visit only and reflects those samples listed above.

DMARD Resistant Group	Blood Sampling at following visits (weeks); separate consent obtained through linked ethics application • Acute phase panel; 0, 12, 26 • Bone Metabolism panel; 0, 12, 26 • Core Inflammatory cytokines; 0, 12, 26 • Autoantibody and Immunology profiling; 0 • Epigenetic sampling; 0, 12, 26
DMARD Sensitive Group	DMARD-sensitive controls will undergo demographic assessment as outlined above and limited clinical examination. Blood sampling will be collected at single baseline visit only and reflects those samples listed above.

PROCEDURES:A detailed schedule of assessments and procedures is tabulated below (Table 1).

Informed Consent:
It is the responsibility of the investigator, or a person designated by the investigator (if acceptable by local regulations), to obtain written informed consent from each subject participating in this study, after adequate explanation of the aims, methods, anticipated benefits, and potential hazards of the study. The informed consent must be obtained prior to initiating screening procedures. Participants will be given written and verbal information about the study, and will have a minimum of 48 hours to consider the information before deciding whether or not to participate

Study Procedures and Assessments

Subjects will be enrolled based on the inclusion/exclusion criteria detailed above in accordance with prevailing standard operating procedures. Only those subjects who fulfil all entry criteria and none of the exclusion criteria will be enrolled into the study.

At Visit 1 (day 1)
- Full clinical history, drug history and examination (as above)
- ECG
- RA disease activity assessment (DAS, DAS28, CDAI, SDAI, ACR core set)
- Questionnaires – Rose, HAQ, HAD, SF36v2, EQ5-D, FACIT-fatigue
- Blood for Antibodies (RF, anti-CCP, Immunoglobulins and isotypes), Acute Phase Panel, Bone Metabolism Panel and Core Inflammatory Cytokines.
- Epigenetic blood collection.

At visit 2 (12 weeks)
- Clinical and drug history
- RA disease activity assessment (DAS28)
- Sampling for Acute Phase Panel, Bone Metabolism Panel & Core Inflammatory Cytokines
- Epigenetic blood Collection

At visit 3 (24 weeks, end of study)
The study will end at Visit 3.
- Full clinical history, drug history and examination
- RA disease activity assessment (DAS, DAS28, CDAI, SDAI, ACR core set)
- Questionnaires – HAQ, HAD, SF36v2, EQ5-D, FACIT-fatigue

- Blood for Acute Phase Panel, Bone Metabolism Panel & Core Inflammatory Cytokines.
- Epigenetic blood collections.

Patients may visit the outpatient clinic on additional occasions during the course of this study or before Visit 2 for other treatments or assessments, as dictated by standard of care. No specific procedures are mandated by this study protocol for those visits.

STATISTICAL ANALYSES	
Disease Activity/Safety	Composite disease activity scores (e.g. EULAR Disease Activity Score [DAS]) will be calculated. Summaries will also be prepared. As appropriate, listings, summary tables and graphs (subject plot and/or mean plots) will be provided for clinical assessments
Other	Descriptive statistics will be performed including calculation of mean, median, standard deviation for assessment of population gene expression levels. Analysis of variance techniques may be used in an exploratory context to assess relationships between selected variables. These data may additionally be used as a reference control dataset for future analyses of clinical, synovial, and biomarker parameters in this RA population.

SAMPLE SIZE JUSTIFICATION:

The number of subjects to be enrolled was based on pragmatic consideration.

STUDY SYNOPSIS

Title of Study:	Optimal management of RA patients who require Blologic Therapy (ORBIT study)
Study Centre:	Multi-centre
Duration of Study:	3 years
Objectives:	An open label randomised controlled trial comparing rituximab with anti-TNF therapy in biologic naïve patients over 12 months
Primary Objective:	To compare the efficacy and cost effectiveness of anti-TNF therapy and rituximab therapy in the treatment of 'biologic-naïve' patients with active rheumatoid arthritis.
Secondary Objectives:	➢ To prospectively evaluate the influence of mood on response to, and side effect profile from, anti-TNF and rituximab. ➢ To identify whether synovial immuno-histology at baseline predicts differential response to rituximab and anti-TNF therapy.
Study Endpoints	The primary outcome measure will be the mean change in DAS28 between 0 and 12 months
Methodology:	Randomised controlled trial
Sample Size:	302
Registration/Randomisation:	via IVRS
Inclusion Criteria	Patients with active RA who are eligible for biologic therapy according to BSR guidelines and are sero-positive for RF and/or anti-CCP antibodies
Exclusion Criteria	Patients will be excluded if they have any contraindication to anti-TNF therapy or rituximab therapy: ➢ women who are pregnant or breast-feeding ➢ unwillingness to use effective contraception ➢ history of or current inflammatory joint disease or autoimmune disease other than RA ➢ treatment with any investigational agent ≤ 4 weeks prior to baseline or < 5 half-lives of the investigational drug ➢ intra-articular or parenteral corticosteroids ≤ 2 weeks prior to baseline. ➢ active infection ➢ septic arthritis within a native joint within the last 12 months ➢ sepsis of a prosthetic joint within 12 months or indefinitely if the joint remains in situ ➢ known HIV or hepatitis B/C infection ➢ latent TB infection unless they have completed adequate antibiotic prophylaxis ➢ malignancy (other than basal cell carcinoma) within the last 10 years ➢ New York Heart Association (NYHA) grade 3 or 4 congestive cardiac failure ➢ demyelinating disease ➢ latex allergy or allergy to excipients in any of the study medications ➢ any other contra-indication to the study medications as detailed in their summaries of product characteristics

Appendix 5; Laboratory sample storage and quality control form

Roche RA Study – Lab Form

Date: Patient ID: Time:
PB Volume: Processed by: Serum: **R** **G**

MNC Count: Dilution: Volume:

Buffer 80ul/1 x10^7 cells

CD14+ Isolation (from MNC)

CD14- cell count: CD14+ cell count:
Dilution: Dilution:
Volume: Volume:

Buffer 80ul/1 x10^7 cells

CD4+ Isolation (from CD14- fraction)

CD4+ cell count: CD14-, CD4- cell count:
Dilution: Dilution:
Volume: Volume:

Aliquots

Min. cell conc. for Q = 1.5x10^6, T = 0.5x10^6, P = 0.2x10^6, F = rest (200,000). Q and T max. 3x10^6 in 700ul volume, so may need to have multiple aliquots

1/X	**CD14+** Volume (ul)	**CD14+** Cell No.	**CD4+** Volume (ul)	**CD4+** Cell No.	**CD14-CD4-** Volume (ul)	**CD14-CD4-** Cell No.
(Q) QIAzol						
(T) TRIzol						
(P) PBS						

FACS Staining

	Purity (%)	**Comments**
Isotype	Sample	
CD14+	CD14+	
CD4+	CD4+	
Neg	Neg	

Appendix 6- Study visits 'biologic resistant' group

101	19/08/2010	23/11/2010	05/04/2011
102	20/08/2010	16/11/2010	25/03/2011
103	25/08/2010	30/11/2010	15/03/2011
104	26/08/2010	26/11/2010	14/04/2011
105	14/09/2010	11/01/2011	26/04/2011
106	16/09/2010	06/01/2011	02/06/2011
107	23/09/2010	22/12/2010	16/03/2011
108	28/09/2010	12/01/2011	11/05/2011
109	30/09/2010	11/01/2011	12/04/2011
110	01/10/2010	21/12/2010	18/03/2011
111	01/10/2010	10/02/2011	16/06/2011
112	05/10/2010	26/01/2011	20/05/2011
113	07/10/2010	02/02/2011	04/05/2011
114	08/10/2010	20/01/2011	14/04/2011
115	12/10/2010	31/03/2011	09/06/2011
116	15/10/2010	06/01/2011	27/04/2011
117	18/10/2010	07/01/2011	01/04/2011
118	21/10/2010	11/02/2011	01/07/2011
119	27/10/2010	16/02/2011	15/04/2011
120	04/11/2010	19/01/2011	13/04/2011
121	09/11/2010	10/02/2011	15/06/2011
122	11/11/2010	[redacted]	28/07/2011
123	15/11/2010	06/04/2011	18/07/2011
124	16/11/2010	24/05/2011	26/07/2011
125	23/11/2010	24/02/2011	22/07/2011
126	03/12/2010	16/02/2011	13/06/2011
127	09/12/2010	23/02/2011	08/07/2011
128	13/12/2010	01/06/2011	28/07/2011
129	14/12/2010	10/03/2011	29/06/2011
130	17/12/2010	24/02/2011	31/05/2011
131	21/12/2010	24/03/2011	15/07/2011
132	21/12/2010	10/03/2011	11/07/2011
133	22/12/2010	15/03/2011	07/06/2011
134	10/01/2011	23/03/2011	14/07/2011
135	12/01/2011	28/04/2011	[redacted]
136	14/01/2011	28/04/2011	28/06/2011
137	18/01/2011	08/04/2011	[redacted]
138	18/01/2011	04/04/2011	14/07/2011
139	19/01/2011	13/04/2011	05/07/2011
140	19/01/2011	24/03/2011	[redacted]
141	20/01/2011	27/04/2011	18/07/2011
142	20/01/2011	01/06/2011	19/07/2011
143	21/01/2011	10/04/2011	07/06/2011
144	21/01/2011	03/05/2011	08/07/2011
145	21/01/2011	30/03/2011	18/07/2011
146	24/01/2011	29/03/2011	05/07/2011
147	25/01/2011	06/05/2011	07/07/2011
148	26/01/2011	04/05/2011	29/06/2011
149	27/01/2011	03/06/2011	21/07/2011
150	04/02/2011	03/06/2011	25/07/2011

[redacted] declined visit

venepuncture not possible

Appendix 7 Sample questionnaires

Hospital Anxiety and Depression Scale

FOLD HERE

Hospital Anxiety and Depression Scale (HADS)

GL assessment
the measure of potential

Name: _____ Date: _____

Clinicians are aware that emotions play an important part in most illnesses. If your clinician knows about these feelings he or she will be able to help you more.

This questionnaire is designed to help your clinician to know how you feel. Read each item below and **underline the reply** which comes closest to how you have been feeling in the past week. Ignore the numbers printed at the edge of the questionnaire.

Don't take too long over your replies, your immediate reaction to each item will probably be more accurate than a long, thought-out response.

FOLD HERE

A	D		A	D
		I feel tense or 'wound up'		
3		Most of the time		3
2		A lot of the time		2
1		From time to time, occasionally		1
0		Not at all		0
		I still enjoy the things I used to enjoy		
	0	Definitely as much		
	1	Not quite so much		
	2	Only a little		
	3	Hardly at all		
		I get a sort of frightened feeling as if something awful is about to happen		
3		Very definitely and quite badly		
2		Yes, but not too badly		
1		A little, but it doesn't worry me		
0		Not at all		
		I can laugh and see the funny side of things		
	0	As much as I always could		
	1	Not quite so much now		
	2	Definitely not so much now		
	3	Not at all		
		Worrying thoughts go through my mind		
3		A great deal of the time		
2		A lot of the time		
1		Not too often		
0		Very little		
		I feel cheerful		
	3	Never		
	2	Not often		
	1	Sometimes		
	0	Most of the time		
		I can sit at ease and feel relaxed		
0		Definitely		
1		Usually		
2		Not often		
3		Not at all		
		I feel as if I am slowed down		
		Nearly all the time		3
		Very often		2
		Sometimes		1
		Not at all		0
		I get a sort of frightened feeling like 'butterflies' in the stomach		
		Not at all	0	
		Occasionally	1	
		Quite often	2	
		Very often	3	
		I have lost interest in my appearance		
		Definitely		3
		I don't take as much care as I should		2
		I may not take quite as much care		1
		I take just as much care as ever		0
		I feel restless as if I have to be on the move		
		Very much indeed	3	
		Quite a lot	2	
		Not very much	1	
		Not at all	0	
		I look forward with enjoyment to things		
		As much as I ever did		0
		Rather less than I used to		1
		Definitely less than I used to		2
		Hardly at all		3
		I get sudden feelings of panic		
		Very often indeed	3	
		Quite often	2	
		Not very often	1	
		Not at all	0	
		I can enjoy a good book or radio or television programme		
		Often		0
		Sometimes		1
		Not often		2
		Very seldom		3

Now check that you have answered all the questions

A	D

TOTAL

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FACIT-F questionnaire

Below is a list of statements that other people with your illness have said are important**. Please circle or mark one number per line to indicate your response as it applies to the past 7 days.**

		Not at all	A little bit	Some-what	Quite a bit	Very much
HI7	I feel fatigued	0	1	2	3	4
HI12	I feel weak all over	0	1	2	3	4
An1	I feel listless ("washed out")	0	1	2	3	4
An2	I feel tired	0	1	2	3	4
An3	I have trouble starting things because I am tired	0	1	2	3	4
An4	I have trouble finishing things because I am tired	0	1	2	3	4
An5	I have energy	0	1	2	3	4
An7	I am able to do my usual activities	0	1	2	3	4
An8	I need to sleep during the day	0	1	2	3	4
An12	I am too tired to eat	0	1	2	3	4
An14	I need help doing my usual activities	0	1	2	3	4
An15	I am frustrated by being too tired to do the things I want to do	0	1	2	3	4
An1	I have to limit my social activity because I am tired	0	1	2	3	4

FACIT-F Scoring

FACIT-Fatigue Subscale Scoring Guidelines (Version 4) – Page 1

Instructions:* 1. Record answers in "item response" column. If missing, mark with an X
2. Perform reversals as indicated, and sum individual items to obtain a score.
3. Multiply the sum of the item scores by the number of items in the subscale, then divide by the number of items answered. This produces the subscale score.
4. **The higher the score, the better the QOL.**

Subscale	Item Code	Reverse item?		Item response	Item Score
FATIGUE SUBSCALE	HI7	4	-	_____	=_____
	HI12	4	-	_____	=_____
	An1	4	-	_____	=_____
	An2	4	-	_____	=_____
Score range: 0-52	An3	4	-	_____	=_____
	An4	4	-	_____	=_____
	An5	0	+	_____	=_____
	An7	0	+	_____	=_____
	An8	4	-	_____	=_____
	An12	4	-	_____	=_____
	An14	4	-	_____	=_____
	An15	4	-	_____	=_____
	An16	4	-	_____	=_____

Sum individual item scores: _____
Multiply by 13: _____
Divide by number of items answered:
=Fatigue Subscale score

HAQ

HEALTH ASSESSMENT QUESTIONNAIRE (HAQ-DI)©

Name: _____ Date: _____

Please place an "x" in the box which best describes your abilities OVER THE PAST WEEK:

	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
DRESSING & GROOMING				
Are you able to:				
Dress yourself, including shoelaces and buttons?	☐	☐	☐	☐
Shampoo your hair?	☐	☐	☐	☐
ARISING				
Are you able to:				
Stand up from a straight chair?	☐	☐	☐	☐
Get in and out of bed?	☐	☐	☐	☐
EATING				
Are you able to:				
Cut your own meat?	☐	☐	☐	☐
Lift a full cup or glass to your mouth?	☐	☐	☐	☐
Open a new milk carton?	☐	☐	☐	☐
WALKING				
Are you able to:				
Walk outdoors on flat ground?	☐	☐	☐	☐
Climb up five steps?	☐	☐	☐	☐

Please check any AIDS OR DEVICES that you usually use for any of the above activities:

☐ Devices used for Dressing (button hook, zipper pull, etc.)
☐ Built up or special utensils
☐ Crutches
☐ Cane
☐ Wheelchair
☐ Special or built up chair
☐ Walker

Please check any categories for which you usually need HELP FROM ANOTHER PERSON:

☐ Dressing and grooming ☐ Arising ☐ Eating ☐ Walking

Please place an "x" in the box which best describes your abilities OVER THE PAST WEEK:

	WITHOUT ANY DIFFICULTY	WITH SOME DIFFICULTY	WITH MUCH DIFFICULTY	UNABLE TO DO
HYGIENE				
Are you able to:				
Wash and dry your body?	☐	☐	☐	☐
Take a tub bath?	☐	☐	☐	☐
Get on and off the toilet?	☐	☐	☐	☐
REACH				
Are you able to:				
Reach and get down a 5 pound object (such as a bag of sugar) from above your head?	☐	☐	☐	☐
Bend down to pick up clothing from the floor?	☐	☐	☐	☐
GRIP				
Are you able to:				
Open car doors?	☐	☐	☐	☐
Open previously opened jars?	☐	☐	☐	☐
Turn faucets on and off?	☐	☐	☐	☐
ACTIVITIES				
Are you able to:				
Run errands and shop?	☐	☐	☐	☐
Get in and out of a car?	☐	☐	☐	☐
Do chores such as vacuuming or yard work?	☐	☐	☐	☐

Please check any AIDS OR DEVICES that you usually use for any of the above activities:

☐ Raised toilet seat ☐ Bathtub bar ☐ Long-handled appliances for reach

☐ Bathtub seat ☐ Long-handled appliances in bathroom ☐ Jar opener (for jars previously opened)

Please check any categories for which you usually need HELP FROM ANOTHER PERSON:

☐ Hygiene ☐ Reach ☐ Gripping and opening things ☐ Errands and chores

Your ACTIVITIES: To what extent are you able to carry out your everyday physical activities such as walkin climbing stairs, carrying groceries, or moving a chair?

COMPLETELY	MOSTLY	MODERATELY	A LITTLE	NOT AT ALL
☐	☐	☐	☐	☐

Your PAIN: How much pain have you had IN THE PAST WEEK?
On a scale of 0 to 100 (where zero represents "no pain" and 100 represents "severe pain"), please record the number below.

Your HEALTH: Please rate how well you are doing on a scale of 0 to 100 (0 represents "very well" and 100 represents "very poor" health), please record the number below.

EQ5D

By placing a tick in one box in each group below, please indicate which statements best describe your own health state today.

Mobility

I have no problems in walking about ❑

I have some problems in walking about ❑

I am confined to bed ❑

Self-Care

I have no problems with self-care ❑

I have some problems washing or dressing myself ❑

I am unable to wash or dress myself ❑

Usual Activities (e.g. work, study, housework, family or leisure activities)

I have no problems with performing my usual activities ❑

I have some problems with performing my usual activities ❑

I am unable to perform my usual activities ❑

Pain/Discomfort

I have no pain or discomfort ❑

I have moderate pain or discomfort ❑

I have extreme pain or discomfort ❑

Anxiety/Depression

I am not anxious or depressed ❑

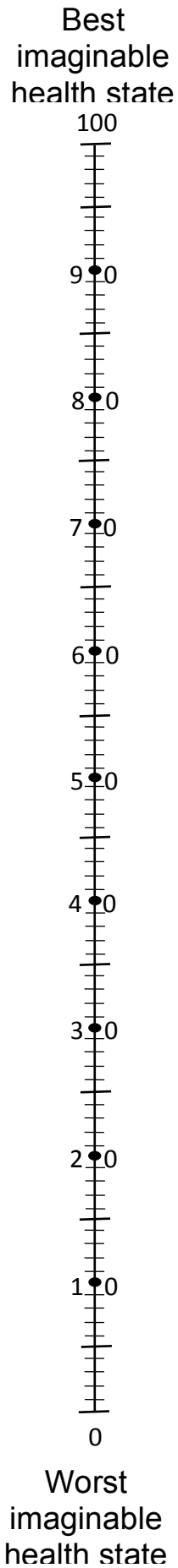
I am moderately anxious or depressed ❑

I am extremely anxious or depressed ❑

We would like you to indicate on this scale how good or bad your own health is today, in your opinion. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your health state is today.

To help people say how good or bad a health state is, we have drawn a scale (rather like a thermometer) on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

Your own health state today



SF-36v2

SF-36 v2™ Health Survey

(SF-36 v2 Standard, US Version 2.0)

To be completed by the PATIENT

Name (Last, First, Middle Initial)
Identification Number
Event

Directions: *Answer every question by filling in the correct circle or writing in the information. If you need to change an answer, completely erase the incorrect mark and fill in the correct circle. If you are unsure about how to answer a question, please give the best answer you can.* **Mark only one answer for each question unless instructed otherwise.**

Today's Date (MM/DD/YY)
☐☐ / ☐☐ / ☐☐

Shade circles like this: ● **Not like this:** ⊗ ✓

Mark only one answer for each question. Please do not mark outside the circles or make stray marks on the questionnaire.

01. In general, would you say your health is:

○ Excellent ○ Very Good ○ Good ○ Fair ○ Poor

02. Compared to one year ago, how would you rate your health in general now?

○ Much better ○ Somewhat better ○ About the same ○ Somewhat worse ○ Much worse

The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?	Yes, limited a lot	Yes, limited a little	No, not limited at all
03. Vigorous activities, such as running, lifting heavy objects, participating in strenuous sports	○	○	○
04. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	○	○	○
05. Lifting or carrying groceries	○	○	○
06. Climbing several flights of stairs	○	○	○
07. Climbing one flight of stairs	○	○	○
08. Bending, kneeling, or stooping	○	○	○
09. Walking more than a mile	○	○	○
10. Walking several hundred yards	○	○	○
11. Walking one hundred yards	○	○	○
12. Bathing or dressing yourself	○	○	○

During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of your physical health?	All of the time	Most of the time	Some of the time	A little of the time	None of the time
13. Cut down on the amount of time you spent on work or other activities	○	○	○	○	○
14. Accomplished less than you would like	○	○	○	○	○
15. Were limited in the kind of work or other activities	○	○	○	○	○
16. Had difficulty performing the work or other activities (for example, it took extra effort)	○	○	○	○	○

Please continue on next page

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Patient Last Name: _____

During the past 4 weeks, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or anxious)?	All of the time	Most of the time	Some of the time	A little of the time	None of the time
17. Cut down the amount of time you spent on work or other activities	○	○	○	○	○
18. Accomplished less than you would like	○	○	○	○	○
19. Did work or activities less carefully than usual	○	○	○	○	○

20. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

○ Not at all ○ Slightly ○ Moderately ○ Quite a bit ○ Extremely

21. How much bodily pain have you had during the past 4 weeks?

○ None ○ Very mild ○ Mild ○ Moderate ○ Severe ○ Very severe

22. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

○ Not at all ○ A little bit ○ Moderately ○ Quite a bit ○ Extremely

These questions are about how you feel and how things have been with you during the past 4 weeks. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the past 4 weeks...	All of the time	Most of the time	Some of the time	A little of the time	None of the time
23. Did you feel full of life?	○	○	○	○	○
24. Have you been very nervous?	○	○	○	○	○
25. Have you felt so down in the dumps that nothing could cheer you up?	○	○	○	○	○
26. Have you felt calm and peaceful?	○	○	○	○	○
27. Did you have a lot of energy?	○	○	○	○	○
28. Have you felt downhearted and depressed?	○	○	○	○	○
29. Did you feel worn out?	○	○	○	○	○
30. Have you been happy?	○	○	○	○	○
31. Did you feel tired?	○	○	○	○	○
32. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting friends, relatives, etc.)?	○	○	○	○	○

How TRUE or FALSE is each of the following statements for you?	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
33. I seem to get sick a little easier than other people	○	○	○	○	○
34. I am as healthy as anybody I know	○	○	○	○	○
35. I expect my health to get worse	○	○	○	○	○
36. My health is excellent	○	○	○	○	○

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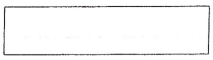
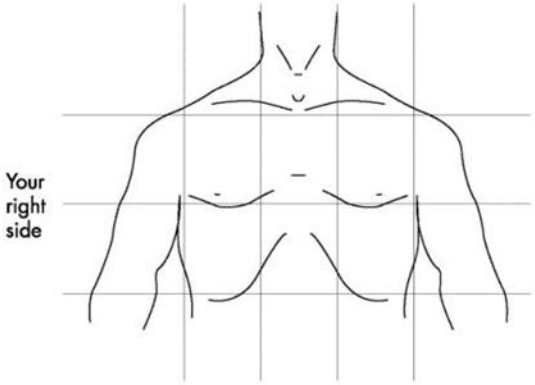
14 - 

ROSE questionnaire

1 Do you ever have any pain or discomfort in your chest?

Yes/No

2 Where do you get this pain or discomfort?
Please mark **X** on the appropriate places



3 When you walk at an ordinary pace on the level does this produce the pain?

Yes/No/Unable

4 When you walk uphill or hurry does this produce the pain?

Yes/No/Unable

5 When you get any pain or discomfort in your chest on walking, what do you do?

Stop Slow down Continue at same pace Not applicable

6 Does the pain or discomfort in your chest go away if you stand still?

Yes/No

7 How long does it take to go away?

10 minutes or less
more than 10 minutes

Brief Illness Perception Questionnaire

Appendix A. The Brief Illness Perception Questionnaire

For the following questions, please circle the number that best corresponds to your views:

How much does your illness affect your life?

0	1	2	3	4	5	6	7	8	9	10
no affect at all										severely affects my life

How long do you think your illness will continue?

0	1	2	3	4	5	6	7	8	9	10
a very short time										forever

How much control do you feel you have over your illness?

0	1	2	3	4	5	6	7	8	9	10
absolutely no control										extreme amount of control

How much do you think your treatment can help your illness?

0	1	2	3	4	5	6	7	8	9	10
not at all										extremely helpful

How much do you experience symptoms from your illness?

0	1	2	3	4	5	6	7	8	9	10
no symptoms at all										many severe symptoms

How concerned are you about your illness?

0	1	2	3	4	5	6	7	8	9	10
not at all concerned										extremely concerned

How well do you feel you understand your illness?

0	1	2	3	4	5	6	7	8	9	10
don't understand at all										understand very clearly

How much does your illness affect you emotionally? (e.g. does it make you angry, scared, upset or depressed?)

0	1	2	3	4	5	6	7	8	9	10
not at all affected emotionally										extremely affected emotionally

Please list in rank-order the three most important factors that you believe caused your illness.

The most important causes for me:-

1. _____
2. _____
3. _____

Brief COPE

We are interested in how people respond when they confront difficult or stressful events in their lives. There are lots of ways to try to deal with stress. This questionnaire asks you to indicate what you generally do and feel, when you experience stressful events. Obviously, different events bring out somewhat different responses, but think about what you *usually* do when you are under a lot of stress. Plese circle the response that best describes your answer.

	I haven't been doing this at all	I have been doing this sometimes	I have been doing this often	I've been doing this a lot
I've been concentrating my efforts on doing something about the situation I am in.	0	1	2	3
I've been taking action to try to make the situation better.	0	1	2	3
I've been trying to come up with a strategy about what to do.	0	1	2	3
I've been thinking hard about what steps to take.	0	1	2	3
I've been trying to see it in a different light, to make it seem more positive.	0	1	2	3
I've been looking for something good in what is happening.	0	1	2	3
I've been accepting the reality of the fact that it has happened.	0	1	2	3
I've been learning to live with it.	0	1	2	3
I've been making jokes about it.	0	1	2	3
I've been making fun of the situation.	0	1	2	3
I've been trying to find comfort in my religion or spiritual beliefs.	0	1	2	3
I've been praying or meditating.	0	1	2	3
I've been getting emotional support from others.	0	1	2	3
I've been getting comfort and understanding from someone.	0	1	2	3
I've been trying to get advice or help from other people about what to do.	0	1	2	3
I've been getting help and advice from other people.	0	1	2	3
I've been turning to work or other activities to take my mind off things.	0	1	2	3
I've been doing something to think about it less, such as going to the movies, watching TV, reading, daydreaming, sleeping or shopping.	0	1	2	3
I've been saying to myself "this isn't real".	0	1	2	3
I've been refusing to believe that it has happened.	0	1	2	3
I've been saying things to let my unpleasant feelings escape.	0	1	2	3
I've been expressing my negative feelings.	0	1	2	3
I've been using alcohol or other drugs to make myself feel better.	0	1	2	3
I've been using alcohol or other drugs to help me get through it.	0	1	2	3
I've been giving up trying to deal with it.	0	1	2	3
I've been giving up the attempt to cope.	0	1	2	3
I've been criticizing myself.	0	1	2	3
I've been blaming myself for things that happened.	0	1	2	3

Appendix 8- PEAC biobank application for matched ORBIT samples

Application to obtained samples from the PEAC Biobank
Iain McInnes & Duncan Porter
December 2011
Request
As part of a study investigating the role of epigenetic changes in conferring resistance to therapeutic intervention in patients with rheumatoid arthritis (RA), we request access **to baseline PAXgene Blood RNA tubes from 26 patients** recruited to the ORBIT study from Glasgow.
Details of request
Formal study title: An Observational, Multiple-Centre Study to investigate the Clinical, Pathological, Immunological and Epigenetic Characteristics of patients with biologic therapy-resistant Rheumatoid Arthritis (Sponsor: Greater Glasgow & Clyde Health Board).
This study is testing the following hypothesis, namely that patients with therapy-resistant RA show greater plasticity in gene expression regulation in response to inflammation and treatment with current standard-of-care therapies as evidenced by differences in the pattern and extent of DNA methylation, post-translational modifications of histone structure and micro RNA expression. Such epigenetic changes will be evident in accessible circulating peripheral blood leukocyte subsets.
The endpoints for this study are as follows:
Primary:
- To describe the clinical and epigenetic characteristics of RA patients who have 'biologic therapy-resistant' disease
- To explore how any epigenetic profile identified is related to the presence of RA, the disease duration, or disease severity (or a combination thereof), by comparing them to control groups detailed below

Secondary:
- To characterize the peripheral blood plasma / serum biomarker signature and examine its' stability over time within this RA patient population

This is a multi-centre, observational study in RA patients (n=50) previously treated with conventional DMARDs and at least two previous biologic therapies. Patients will be admitted to the study regardless of reason for prior drug continuation and will therefore include toxicity and efficacy failures. This will ensure that a broad patient population is captured for subsequent analysis.

Enrolled patients will undergo three (3) assessments on Day 1 (baseline), and at approximately Weeks 12 and 24 (see table 1), including:
- Clinical and laboratory disease activity assessment
- Biomarker blood sampling
- Assessment of co-morbidity and presence thereof

There will be three comparator groups recruited:
4. Normal subjects – all patients recruited to the 'therapy-resistant' group will be asked to bring a close friend of the same gender and similar age.

5. DMARD-resistant RA patients – up to 50 patients with moderate/severe RA who meet the BSR eligibility criteria for starting anti-TNF therapy
6. DMARD-sensitive RA patients – 25 patients with longstanding RA (meeting the ACR 1987 definition) who have responded to conventional therapy (see below)

We have now successfully recruited the study group together with control groups 1 and 3. The RA population entering the ORBIT study in Glasgow comprise control group 2 above and as such we request access to the PAXgene Blood RNA tubes retrieved from 26 patients thus far recruited from Glasgow into the ORBIT study. From these tubes we will extract total RNA, including small RNAs. We wish to use one of the three PAXgene Blood RNA samples (2.5mls) stored from each patient at baseline to examine the microRNA and mRNA expression profile in matched samples. By this means we aim to minimise the use of the irreplaceable component of the sample resource.

In parallel, we are conducting miR/mRNA and DNA methylation analysis of separated blood cell populations from all 4 groups. Thus, our study will reveal cell type unique epigenetic changes related to arthritis pathogenesis and treatment. Moreover, matched analysis of epigenetic modifications of whole blood will allow exploring the potential of these modifications as potential diagnostic/prognostic biomarkers.

Statistical considerations
This is a descriptive study – no prior data are available to inform the sample size and on this basis our numbers can only be based on pragmatic considerations. Studies of similar size have been successful in unravelling biomarker signatures in cancer cohorts e.g. in lung cancer.

Supporting statement
We currently have little notion as to why some RA patients do well on initial DMARD therapy and remain essentially stable over a prolonged period of time, whereas others exhibit an aggressive disease course marked by intolerance or poor response to a variety of therapeutics. Specific drug related factors are likely to operate e.g. by biologics inducing neutralizing antibodies, as may genetically defined pathway specific elements e.g. concerning the biology of the target or of the metabolism or other pharmacology of a given agent. It is also possible that acquired epigenetic changes *within the patient* will influence the capacity of RA patients to respond to any agent per se. This study examines this latter possibility using a variety of clinical phenotypes and in particular will test the idea that epigenetic changes underlie at least in part the biology of the clinically observed variance of therapeutic response. As such the study addresses a question of critical clinical importance, with substantial individual patient benefit and health economic impact if promising data are obtained. As such we further contend that it meets the core criteria set for access to samples within the PEAC biobank.

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