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Translational research in rheumatoid arthritis:

Exploiting melanocortin receptors

By

Tazeen Jahan Ahmed

A thesis submitted for the degree of Doctor of Philosophy in the University of London

Queen Mary University, Barts and the London School of Medicine and Dentistry, William Harvey Research Institute

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DECLARATION

I declare that the materials contained in this thesis have not been used in submission for any other academic award, that it embodies the results of my own work and it has been composed by myself. All sources of investigation have been duly acknowledged and the thesis does not exceed 100000 words.

This thesis has been written by me and the work presented in this thesis is the result of my own investigation with the exception of some of the RNA extractions and immunophenotyping which were carried out routinely by staff members of Experimental Medicine and Rheumatology.

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ABBREVIATIONS

Abbreviation	Extended version
µl	Microlitre
ACPA	Anti-citrullinated peptide antibody
ACR	American College of Rheumatology
ACTH	Adrenocorticotropic hormone
AID	Activation induced cytidine deaminase
ALX	Lipoxin receptor
AP1	Activator protein 1
APRIL	A proliferating inducing ligand
Arg	Arginine
ASPIRE	Active-Controlled Study of Patients Receiving Infliximab for the Treatment of Rheumatoid Arthritis of Early Onset
ATL	Aspirin triggered lipoxin
ATTRACT	anti-tumor necrosis factor trial in rheumatoid arthritis with concomitant therapy
BAFF	B cell activating factor (same as BLyS)
Bcl 6	B cell lymphoma 6
BLK	B lymphoid tyrosine kinase
BLyS	B lymphocyte stimulator (same as BAFF)
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CCL	Chemokine ligand CC motif
CCP	Cyclic citrullinated peptide
CCR	CC chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
cGCR	Cytosolic glucocorticoid receptor
CIA	Collagen-induced arthritis
COBRA	Combinatietherapie Bij Reumatoide Artritis
COX	Cyclooxygenase
CRH	Corticotropin-releasing hormone
CRP	C reactive protein
C_T	Cycle threshold
CTLA4	Cytotoxic T lymphocyte antigen 4
CTX	C terminal cross linking
CXCL	Chemokine ligand CXC motif
CXCR	CXC chemokine receptor
DAB	Diaminobenzidine
DANCER	Dose-Ranging Assessment: International Clinical Evaluation of Rituximab in Rheumatoid Arthritis
DAS28	Disease activity score 28
DC	Dendritic cell
DMARDS	Disease modifying antirheumatic drugs
DMEM	Dulbeccos Modified Eagles Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E.Coli	Escherischia coli

ECL	Enhanced chemoluminescence solution
ELISA	Enzyme linked immunosorbent assay
ERA	Early rheumatoid arthritis
ERK	Extracellular signal related kinase
ESR	Erythrocyte sedimentation rate
FC	Flow cytometry
FITC	Fluorescein isothiocyanate
FLS	Fibroblast like synoviocytes
FOX P3	Forkhead box p3
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GC	Glucocorticoid
GCRβ	Glucocorticoid receptor β
Gln	Glycine
GPI	Glucose 6 Phosphate isomerase
HA	Haemagglutinin
HAQ	Health assessment questionnaire
HBS	Hepes buffered Saline
HEK	Human embryonic kidney
HEPES	hydroxyethyl piperazineethanesulfonic acid
HETE	Hydroxyeicosotetranoic acid
His	histidine
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IκBα	I kappa B alpha also known as nuclear factor of *kappa* light polypeptide gene enhancer in *B*-cells inhibitor, *alpha*
IL	Interleukin
IUPHAR	International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification.
JAK	Janus Kinase
KC	Keratinocyte derived chemokines
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
LX	Lipoxin
M	Mole/litre
MAPK	Mitogen activated protein kinase
MC	Mast cell
MCCT	Connective tissue mast cells
MCM	Mucosal mast cell
MCP1	Monocyte chemotactic protein 1
MCR	Melanocortin receptor (human)
Mcr	Melanocortin receptor (mouse)

MHC	Major histocompatibility complex
Min	Minute
Ml	Millilitre
MMP	Matrix metalloproteinase
MRAP	Melanocortin receptor accessory protein
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSH	Melanocortin stimulating hormone
MT	Melanotan
NCT	National clinical trial
NFAT	Nuclear factor of activated T-cells
NFKB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NICE	National Institute for Health and Clinical Excellence
NK	Natural killer
Nle	Norleucine
NOD	Nucleotide oligomerisation domain
Nos2	Mouse nitric oxide synthase
OA	osteoarthritis
Oligo dT	Oligo Deoxythymine
PACE4	Paired basic amino acid cleaving enzyme 4
PADI	Peptidyl deiminase
PAM	Peptidylglycine alphaamidating monooxygenase
PBS	Phosphate buffered saline
PCA	Primary component analysis
PCR	Polymerase chain reaction
POMC	Proopiomelanocortin
PTPN22	Protein tyrosine phosphate non receptor 22
QMUL	Queen Mary's University of London
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RANTES	Regulated upon activation normal t cell expressed and secreted
RAPID1	Rheumatoid Arthritis Prevention of Structural Damage
REFLEX	Randomized Evaluation of Long-Term Efficacy of Rituximab in RA
RF	Rheumatoid factor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RTPCR	Real time polymerase chain reaction
SAP	Saponin
SCID	Severe combined immunodeficiency
SD	Sprague Dawley
SIRS	Systemic inflammatory response syndrome
SLE	Systemic lupus erythematosus

SNP	Single nucleotide polymorphisms
SRC	Sarcoma
STAT	Signal transducer and activator of transcription
STIVEA	Steroids in very early arthritis
TBS	Tris buffered saline
TCR	T cell receptor
TEMPO	Trial of Etanercept and Methotrexate with Radiographic Patient Outcomes
TGFβ	Transforming growth factor β
Th1	T helper 1
TIMPS	Tissue inhibitors of metalloproteinases
TLR	Toll like receptor
TNF	Tumour necrosis factor
TNFAIP3	Tumour necrosis factor alpha induced protein 3
TNFRSF	Tumour necrosis factor receptor superfamily
TRAF 1	Tumour necrosis factor receptor associated factor 1
TRAP	Tartrate resistant acid phosphatase
UK	United Kingdom
UNG	Uracil DNA glycosylase
US	Ultrasound
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
WB	Western Blot
WHRI	William Harvey Research Institute

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting 1% of the population. The aetiology of rheumatoid arthritis is unknown, although there are multiple postulated theories. In 1950, Philip Hench won the Nobel prize for treating patients with rheumatoid arthritis with cortisone. He also treated 6 patients with adrenocorticotropic hormone (ACTH) with good results. ACTH is a melanocortin. The melanocortin system describes the five melanocortin receptors, their ligands, agonists and antagonists and the accessory proteins. The aim of this study was to explore the melanocortin receptors in rheumatoid arthritis synovium.

Methods

HA-tagged stable cell lines were created for MC1R, MC3R and MC5R. Multiple antibodies were tested for their utility using Western Blot, immunohistochemistry and flow cytometry. Samples of synovium from 28 patients with RA were tested using RTPCR for the presence of MC1R and MC3R. Gene expression was correlated with clinical characteristics, cytokine (RTPCR) expression and immunohistochemical score.

Results

The stable cell lines expressed MC1R, MC3R and MC5R respectively. Of the antibodies tested none were found to be of utility in detecting MC1R or MC3R .The MC1R RQ values in rheumatoid synovium appear to split into two groups, high and low. The medians of the two groups are significantly different (p=0.0005). There is almost a 5 cycle, or 64 fold, difference in gene expression between the medians of the two groups (1.59 v 6.23). Of note no MC3R positive samples were CD138 high (i.e. no MC3R positive samples had a significant plasma cell infiltrate) (p=0.006). Categorical analysis using Fishers Exact test revealed an association between MC1R high samples and CD68 lining high scores, (i.e. MC1R high samples also had a high macrophage score in the lining of the sample) (p=0.02). MC1R low samples were associated with not being on combination therapy,

this did not quite reach significance (p=0.07). Linear regression analysis confirmed these associations for MC1R. PCA analysis did not show any grouping of samples according to any of the variables tested, likely due to sample size.

Conclusion

MC1R and MC3R are found in human synovium. Current commercial antibodies are not of utility in detecting MC1R or MC3R. Synovial samples can be split into high and low MC1R gene expression groups. MC3R was either present or absent. High expression of MC1R was associated with a high macrophage score and MC3R expression was associated with a low plasma cell score. MC1R and MC3R expression in RA synovium could be used as biomarkers of disease state or severity as well as a target for therapy.

Chapter 1. Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory condition affecting approximately 1% of the population with the prevalence being stable or declining over ten years before and after the turn of the century[3-4]. The incidence of RA peaks at 50 -80 years old although RA can appear at any time throughout a lifetime[5]. There is a female predominance as in many other autoimmune conditions with a female to male ratio of approximately 3:1[6]. RA is found worldwide with a predominance in European and North American populations with some populations of North American Indians having a particularly high prevalence and some Asian and African areas having particularly low prevalences with an associated difference in pattern of age incidence[7]. Non-white populations may have a greater female to male ratio and have an earlier age of onset in the incidence of RA. The aetiology of rheumatoid arthritis is unknown, although there are multiple postulated theories.

RA is a disease that manifests primarily in the synovial joints as well as having extra-articular features including eye, skin, neurological, psychological and respiratory involvement. It produces a symmetrical arthritis especially affecting the small joints of the hands. Patients with RA present with swollen joints associated with systemic features such as fatigue and stiffness (particularly early morning stiffness improving over the day, but stiffness can last all day). Other features are raised inflammatory markers, associated positive immunology (i.e. a positive rheumatoid factor (RF) and/ or anti cyclic citrullinated peptide (CCP/ACPA) antibodies). The older classification criteria[8] (table 1.1) include erosive changes seen on X-ray although this is not part of the newer criteria[9] (table

1.2). The new diagnostic criteria allow earlier diagnosis of rheumatoid arthritis allowing the earlier initiation of disease modifying therapy.

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

Table 1.1 Classification criteria (1987) for Rheumatoid Arthritis [8]. 4 criteria of 7 required.

Classification criteria for RA (score-based algorithm: add score of categories A–D; a score of ≥6/10 is needed for classification of a patient as having definite RA)[‡]	Points
A. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)[#]	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF ***and*** negative ACPA	0
Low-positive RF ***or*** low-positive ACPA	2
High-positive RF ***or*** high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP ***and*** normal ESR	0
Abnormal CRP ***or*** abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

Table 1.2 Diagnostic Criteria (2010) for Rheumatoid Arthritis [9]

Pathologically, RA is characterised by synovial inflammation leading to hyperplasia of the synovial lining and the classical pannus formation of infiltrating leukocytes and transformed fibroblast-like synoviocytes. Inflammatory cytokine production and growth factors have effects on chondrocytes and osteoclasts leading to cartilage and bone destruction and the formation of pits which appear as erosions on xrays. The autoantibodies, RF and anti CCP have not been proven to be directly pathogenic in human models although there are strong associations with erosive disease and antibody positivity[2, 10].

Current treatments including disease modifying therapies, biologics (monoclonal antibodies) and joint replacement are expensive and contribute to the economic burden of the disease. The overall prognosis in RA is unclear. ACPA positive and ACPA negative diseases have different prognoses. Seropositive RA is associated with erosive disease and disability [11]. Overall life expectancy is shortened in RA. RA is associated with an increased morbidity and mortality particularly related to immunoproliferative and cardiovascular disease[12]. In fact, uncontrolled or improperly managed RA couples with a higher incidence of lymphoma and is a cardiovascular risk factor for accelerated atherosclerosis. RA is also an important cause of disability which can lead to personal, social and physical limitations. There is an association of RA with depression which can further compound functional limitations[13]. RA has socioeconomic complications both in the loss of work by patients and compromise of working conditions[14].

1.1.1 Aetiology

The aetiology of RA is unknown and is thought to be a complex interplay between genetic and environmental factors such as stress (involving the hypothalamic–pituitary–adrenal axis) and infection.

Genetics

Twin studies have shown that the incidence in monozygotic twins is 15% compared to a dizygotic incidence of 3.6% suggesting the importance of genetic factors in the onset of RA[15]. Heritability of RA is thought to be between 53 and 65%[16] with an increased odds ratio of disease in siblings of 1.7[17].

There are five main genomic regions of interest in seropositive RA. The two main areas are HLADRB1 and PTPN22 (protein tyrosine phosphatase non receptor 22). Genome wide association studies confirmed by independent cohort studies have also found an association with chromosome 6q23- an area containing the genes oligodendrocyte lineage transcription factor 3 (OLIG3) and tumour necrosis factor α induced protein 3 (TNFAIP3), as well as TRAF1/C5 and STAT4[18]. Seronegative RA has been shown to be associated with HLA DRB 103, interferon response factors and lectin binding proteins[2].

Since the 1970's, HLA DRB1 has been identified as a significant factor in all patient populations. This gene encodes for the 3rd hypervariable region of the class II MHC DRβ1 chain, with the areas of interest being part of the antigen binding site. Polymorphisms HLA DRB 0101 and 0104 give the greatest risk of RA and act synergistically, with heterozygotes having the greatest risk of RA. The shared epitope QKRAA confers particular susceptibility to RA[19]. Other alleles associated with RA are 0102, 0401, 0404, 0405, and 0408, all of which have the shared epitope sequence[20]. See figure 1.1.

PTPN22 is a significant association in multiple European populations [21-23] although not in a Japanese population[24]. It is also important in other autoimmune diseases, including systemic lupus erythematosus (SLE), but not in psoriasis or psoriatic arthritis. HLA DRB1 and PTPN22 polymorphisms act synergistically to increase disease risk[18]. See figure 1.1.

Four single nucleotide polymorphisms (SNPs) in the STAT4 gene associated with RA have been reported, two in distinct North American Cohorts, and the other two in a Swedish and a Korean

cohort [25-26]. TNF receptor associated factor 1, (TRAF1) / complement component 5 (C5) SNPs are shown to have an important association with RA in two North American cohorts [27]. See figure 1.1.

There are multiple other genes that have been implicated in the pathogenesis of RA. The majority are genes which are involved in T cell activation (e.g. similarly to PTPN22) or involved in the NFκB pathway (like TRAF1). Other associated genes involved in T cell activation include CTLA4, IL2, IL2RA, CD28 and CD40, whereas another example of a gene involved in the NFκB pathway is REL. Other pathways advocated to be relevant to RA pathogenesis include B lymphoid tyrosine kinase (BLK), CCL21 and TNFRSF14 a TNFα receptor superfamily member. In any case, it is worth remembering that the strongest associations remain with HLA DRB1 and PTPN22[2]. See figure 1.1.

Environmental Factors

The strongest environmental association with RA is smoking but there are also associations with other forms of respiratory stress such as silicosis. Periodontal disease may also precede RA. Both these mechanisms seem to be related to post-translational modifications of proteins particularly to citrullinated forms, an action carried out by peptidyl arginine deiminase 4 (PADI4), an enzyme found in some forms of bacteria (*Porphyromonas gingivalis*) causing periodontal disease[28]. There is a link between citrullinated enzyme α-enolase, HLA DRB1 *04, PTPN22 and smoking, thus tying genetic and environmental factors together [29-30]. There is also an association with some forms of bacteria found in the gut and CCP positive disease[31]. Other environmental factors are infections and there is a large amount of research into viruses such as Epstein Barr virus (EBV)[32] cytomegalovirus (CMV)[33-34] and bacteria such as *E. Coli* or *Proteus* species[35]. Parvovirus B19 gives a symmetrical polyarthritis in adults indistinguishable from RA. However, although these associations suggest molecular mimicry as the cause of disease it is not clear why systemic infection and inflammation might lead to joint specific disease. There may also be a link between immunisation and onset of RA[36]. The involvement of hormonal factors cannot be underestimated in view of the disease preponderance in women. In any case onset of RA may or may not be associated with adverse life

events. There are some accounts of dietary influences on RA pathogenesis but this may be confounded by the association between obesity and severe disease[37]. Intriguingly, moderate alcohol intake may protect against onset of RA[38]. See figure 1.1.

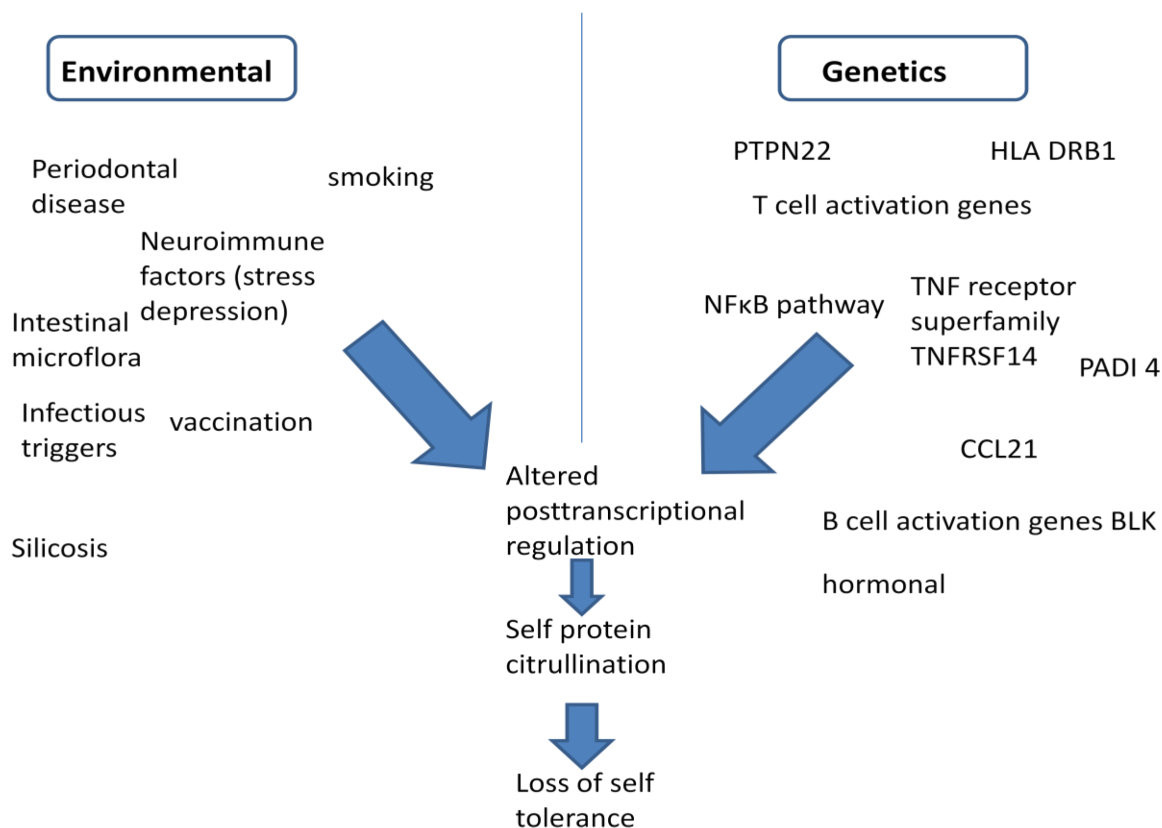


Figure 1.1. Triggers for RA.

Periodontal disease, smoking and the members of the intestinal microbiome are known predisposing factors for rheumatoid arthritis. The enzyme PADI peptidyl arginine deiminase 4 is both an environmental and genetic factor for the loss of self tolerance with the production of citrullinated peptides. HLA DRB1 and PTPN22 (protein tyrosine phosphatase non receptor 22) are the strongest genetic links to rheumatoid arthritis. HLA DRB1 is involved in the MHC molecule antigen presentation, responsible for self peptide selection. CCL21 is a chemokine implicated in germinal centre formation and there are multiple single nucleotide polymorphisms related to T cell activation. NFκB related genes include Rel (a protooncogene), TNFAIP3 (a negative regulator of NFκB activation) and TRAF 1 (a regulator of TNFα superfamily signalling). TNFRSF14 is a TNFα receptor superfamily member with proinflammatory activity. BLK is a tyrosine kinase involved in B cell receptor signalling and B cell development. Infectious triggers include parvovirus B19, cytomegalovirus and Epstein Barr virus and bacteria such as *E.coli* or *Proteus* species. Hormonal triggers are thought to relate to the higher incidence of RA in women over men (3:1).

1.1.2 The Rheumatoid joint

Clinically, the rheumatoid joint is painful, swollen and tender. The swelling can be due to synovial hypertrophy (pannus formation), or because of an excess of synovial fluid. There may be soft tissue oedema and radiologically there may be bone marrow oedema. The joint is made up of bone, synovial fluid, cartilage-containing components, and synovium, containing a synovial lining layer of type A and type B synoviocytes or macrophages, and resident synovial fibroblasts, and a stromal layer of connective tissue and cells. Each part of the joint can be affected in RA with cartilage destruction, bone destruction and synovial changes. These changes are thought to be secondary to pro-inflammatory cytokines and chemokines along with the production of damaging enzymes such as matrix metalloproteinases from the overgrown synovial tissue.

Cells in RA

Macrophages, T cells, B cells and dendritic cells all comprise part of the infiltrate of leukocytes found in RA. There are also fibroblast-like synoviocytes which have changed from their usual behaviour to a more aggressive phenotype.

T cells

T cells are found as part of lymphocyte aggregates with B cells, follicular dendritic cells and plasma cells, which sometimes form functional ectopic lymphoid structures with the presence of germinal centres and class switching of B cells. They comprise 40% of the cellular infiltrate. It was thought that the major player in RA was the T helper type 1 (Th1) cell but this has now been overtaken by the Th17 cell, which are present in the RA joint and peripheral blood. Th17 cells are induced by TGFβ, IL1, IL23 and IL6 and themselves produce TNFα, IL21, IL22 and IL17a and IL17f - all major pathogenic cytokines. TNFα suppresses the function of regulatory T cell subsets and indeed regulatory T cells from the RA joint are less capable of suppressing inflammation. This suppressor activity is recovered after treatment with anti-TNFα therapy. See figure 1.2. T cell depleting therapies have not proven to

be beneficial [39] although T cell co-stimulation blockade has been shown to be beneficial in clinical trials[40].

The majority of data regarding the pathogenicity of T cells is gained from mouse models - particularly collagen-induced arthritis models. These models have clarified that adoptive transfer of naive or primed T cells is disease causative with the first signs of arthritis as early as 3-5 days after transfer. Below I comment on a few examples of adoptive transfer studies in arthritis mouse models.

The SKG model (a strain which spontaneously develops a chronic arthritis) has an arthritis transferable to nude mice and severe combined immunodeficiency mice *via* lymph nodes, or splenic T cells, particularly CD4+ T cells, producing an arthritis that is symmetrical with pannus formation. Almost 90% developed a pneumonitis and 10-20% displayed subcutaneous nodules, features consistent with human RA. These SKG mice bear a loss of function mutation in a tyrosine kinase essential for T cell receptor signaling which leads to selection of autoreactive T cells that would usually be deleted during development [41]. The mutation is in the SH2 domain of ZAP 70 signal transduction molecule. T cell related cytokines are pivotal for this model with IL6, TNFα and IL1 as well as IFNγ and IL4 being important in the initiation of arthritis [42]. In the mouse collagen-induced arthritis model, adoptive transfer of CD4 T cells and γδ T cells showed homing to the joint and interaction with osteoclasts present in arthritic recipients. The CD4 T cells comprised more Th17 cells and were apposed to osteoclasts with a specific antigenic distribution if ovalbumin primed, whereas non-specific T cells gave a diffuse distribution of T cells [43]. In a modified K/BxN arthritis mouse model, naive T cells induced arthritis 10 days after transfer into T cell deficient recipients and Th17 polarised T cells induced arthritis in all mice with production of auto-antibodies [44].

Adoptive transfer studies have also been used to reveal the role of regulatory T cells in the suppression or absence of suppression of disease. Adoptive transfer of FoxP3-expressing T regulatory cells and FoxP3 and Bcl6-expressing T regulatory cells both caused suppression of collagen-induced arthritis with reduced paw inflammation [45]. Mouse models have also given an

idea of how current biologic treatments may work on regulatory T cells in vivo. In the collagen-induced arthritis model, treatment of mice with cytotoxic T lymphocyte antigen 4 immunoglobulin (CTLA 4 Ig) not only reduced the incidence of disease but increased the regulatory T cell population in the joints of affected animals [46].

Although there is a large body of work implicating T cells in mouse models of RA, there is less characterisation of T cells in humans with RA. The Th17 cells are now being characterised in humans and there is data that indicate a functional relation between T cells and the synovium and specifically effector cells such as osteoclasts [43]. There is longstanding data regarding the presence of T cells in the synovium, appearing in distributions varying from diffuse to organised germinal centres. The majority of these T cells were CD4+ although there were CD8+ T cells present[47]. Also the efficacy of abatacept, a CTLA4 decoy molecule, affecting T cell co-stimulation, is strongly indicative of an aetiological role of T cells in RA. See figure 1.2.

B cells

The importance of B cells in RA has been brought to the forefront with the efficacy of rituximab, an anti-CD20 biologic, in the treatment of seropositive RA patients. The majority of the data regarding the pathogenicity of B cells in RA is again from mouse models, mainly collagen antibody-induced arthritis and serum transfer-induced arthritis. See figure 1.2.

Auto-antibodies rheumatoid factor (RF) and antibodies to citrullinated protein antigens (ACPA/CCP) are found in RA. ACPA are predictive of disease severity and erosions, RF are predictive of erosions. B cells are also found in the synovium, again from a low number in the diffuse type of infiltrate to concentrated areas in ectopic lymphoid structures which can become germinal centres in some cases[47]. Although B cell function is to produce antibodies, in the rheumatoid joint B cells can also play roles in cytokine production and antigen presentation that can elicit T cell help to promulgate disease. B cell stimulation factors such as B lymphocyte stimulator (BlyS/ BAFF) and a proliferating inducing ligand (APRIL) are found in human synovial fluid at a concentration greater than that seen

in peripheral blood, BlyS also correlates with autoantibody levels[48]. APRIL is expressed in germinal centres in RA synovium [49]. The presence of these two factors is suggestive of a prominent role for B cells in RA[50].

If serum from the CIA mouse is transferred to a severe combined immunodeficiency (SCID) mouse, the recipient mouse develops an arthritis[51]. Transfer of only anti collagen II antibodies leads to arthritis in the SCID recipient mice [52]. Serum and anti glucose 6 phosphate isomerise (GPI) antibodies from the KRN TCR transgenic mouse model to an immunodeficient recipient leads to arthritis. Transfer of monoclonal anti GPI antibodies to healthy mice resulted in a limited arthritis with a severe arthritis resulting from the injection of multiple monoclonal antibodies [53]. These are just a few examples of adoptive transfer antibody models that illuminate the role of antibodies and therefore B cells in auto-antibody associated arthritis (see figure 1.2).

Ectopic lymphoneogenesis

RA synovitis is associated with a lymphocytic infiltrate. This can vary from a diffuse infiltrate (53.6%) to aggregates of T cells and B cells (20.3%) to lymphoid follicles to active germinal centres (23.4%)[54]. The infiltrates become more and more complex in their microstructures as they become more histologically complex. The microstructures are similar to secondary lymphoid organs such as the lymph node and so can be thought of as ectopic lymphoneogenesis. These ectopic lymphoid structures are comprised of B cells, T cells, macrophages and follicular dendritic cells.

Cytokines are important for the formation of these ectopic lymphoid structures. This has been illuminated by investigation of mice with various defects in the TNF superfamily of cytokines, some of which fail to form germinal centres others which have other lymphoid structural abnormalities[55]. The lymphotoxin α and β pathways seem particularly involved in secondary lymphoid structure generation. Chemokines are also implicated in the genesis of these structures sustaining the trafficking of T cells and B cells to the correct areas. For example, mice lacking CCL21 have few lymph node T cells and have defects in the movement and organisation of T cells[56]. B

cells seem to be particularly controlled by CXCL13. Mice with a disrupted receptor for CXCL13 have disrupted lymph nodes and splenic follicles, thus strongly implying a critical role for CXCL13 in their formation [57]. Human synovial tissue graded with immunohistochemistry techniques for diffuse infiltrates, aggregates and follicles showed higher CXCL13 and CCL21 in germinal centre positive samples with the presence of CD21 being consistent with follicular dendritic cells, a cell type only found in germinal centre positive samples. Furthermore, germinal centre positive samples expressed lymphotoxin β. This supports the importance of the duo CXCL13/CCL21 and of the lymphotoxin pathway in the formation of ectopic lymphoid structures in humans [54].

There is uncertainty about the significance of the presence of these structures in the RA synovium. Possibly the synovium here is not initiating this autoantibody response but is acting as a reservoir of antibody producing cells. It is possible that these structures are present but not functionally active.

There is evidence for clonal activation of B cells in these germinal centres[58] and ACPA is found in synovial fluid in RA patients [59] suggesting local production of autoantibodies. However, this could still mean the germinal centres are acting as reservoirs of antibody producing cells. There is however, evidence that these germinal centres are functional. RA tissue continues to produce AID (activation induced cytidine deaminase required for class switching) and to maintain class switch. This can occur after transplantation of human RA tissue into SCID mice, therefore in the absence of contact with the human blood stream, with continued production of human ACPA. This strongly supports the notion that these structures are indeed functional [60].

There is direct and indirect evidence for the role of B cells in the pathogenesis of RA, with direct evidence for the functionality of the follicular structures found in about 25% of RA synovial samples. There is also evidence for the role of T cells giving B cell help and being involved in the initiation of RA synovitis. It may be that there are T cell predominant subtypes of RA and B cell antibody rich subtypes of RA and that RA itself is a heterogeneous disease at the cellular level with similarities at the phenotypic level. Further research is ongoing in this area.

Macrophages

Macrophages are members of the innate immune system that interact with members of the adaptive immune system, specifically, T cells to produce the adaptive immune response. They are essential in the initial response to external pathogens and are normal residents of the synovium comprising type A synoviocytes (type B are fibroblast-like synoviocytes.) Macrophages consume pathogens by phagocytosis and display peptide antigens on MHC class II molecules prior to interaction with the T cell receptor. They can also scavenge the immune complexes and other antigens *via* Fc receptors and scavenger receptors. These cells express Toll-like receptors (TLR) and NOD (nucleotide oligomerisation domain)-like receptors which identify and interact with pathogen-associated molecular patterns and disease-associated molecular patterns including apoptotic debris. Macrophages can also be activated by lipoproteins and proteases *via* specific receptors[2]. They are found in the intimal lining layer of synovium and become more prominent in inflamed tissues. Macrophages can also act as important effector cells in RA with the production of multiple enzymes such as matrix metalloproteinases (MMPs) that cause direct tissue damage. See figure 1.2. They also produce cytokines and chemokines, thus attracting other cellular components of both the innate and adaptive immune systems. Macrophages can also produce damage by the production of reactive oxygen intermediates as well as nitrogenous intermediates, contributing to the pro-inflammatory milieu of the rheumatoid joint.

The importance of macrophages in RA can be illustrated by their accumulation in the synovium with active disease. This may be due to a number of reasons such as increased recruitment of circulating monocytes, decreased emigration once recruited or decreased apoptosis (implying prolonged life span at the tissue site). Synovial macrophage infiltration correlates with radiographic progression[61] and synovial lining macrophage numbers are reduced following treatment with conventional disease modifying anti-rheumatic drugs, DMARDS, and biologics such as anti-TNFα therapies [62]. This is indirect evidence that macrophages are implicated in the pathogenesis of RA.

There are subsets of macrophages that predominate in the RA joint. Normally activated macrophages are known as M1 macrophages and are pro-inflammatory in nature. See figure 1.2. These predominate in RA. There are however, also M2 macrophages which have anti-inflammatory and repair roles. It is likely that these are also present in the synovial joint but are overwhelmed by the pro-inflammatory M1 phenotype [2].

Treatment with anti-macrophage compounds such as liposomal clodronate, which selectively deplete macrophage numbers, has been shown to suppress disease development in a streptococcal wall antigen induced arthritis rat model [63]. Furthermore, pre-treatment with clodronate makes mice resistant to disease in the K/BxN serum transfer arthritis models whereas disease re-emerges if the mice are replenished with naive macrophages[64]. Rabbits with antigen-induced arthritis have reduced joint swelling, reduced synovial lining macrophages and reduced radiological progression when treated with low dose injected liposomal clodronate. In this specific case, however there was no evident difference seen in pannus formation[65]. Clodronate has been shown to reduce the synovial macrophage lining layer infiltrate when injected into humans joints prior to joint replacement therapy[66]. These experiments indicate that macrophage numbers are modifiable and modulation of macrophage numbers leads to resistance or amelioration of disease, supporting a role for this cell type in the pathogenesis of RA.

Dendritic cells

Dendritic cells (DC) are the bridge between the innate and adaptive immune systems. These cells uptake antigen and process it, displaying both MHC class I and MHC class II molecules thereby interacting with both CD4+ and CD8+ naive T cells to elicit the adaptive response. They are also important in the development of tolerance both at a central level, i.e. in the thymus, and in the periphery where T cells that react too strongly to the self-antigen presented by DCs are removed by induction of apoptosis. DCs can skew the immune response to Th1 or Th2 by the production of the

appropriate cytokine. They are also involved in B cell selection with the trapping of antigen-antibody complexes and produce CXCL13 a B cell attracting chemokine. See figure 1.2.

There are two main types of DC: the classical or conventional DC and the plasmacytoid DC. Classical DCs are resident in lymphoid tissues but can migrate. The plasmacytoid DC skews the immune response to an anti-viral response by the production of interferons directing a Th1 response[67]. Both types of DC can present antigen and initiate an immune response.

The significance of DCs in humans is suggested mainly through immunohistochemistry work associating the presence of plasmacytoid DCs in perivascular areas with ACPA antibodies, although overall numbers were not increased [68-69]. Also patients with RA have few DCs in their blood compared to healthy controls but treatment increases the number of classical DCs in circulation [70]. It seems that therapeutic treatment with methotrexate or infliximab (an anti-TNF agent) leads the classical DCs to produce a more regulatory phenotype which would then inhibit T cell proliferation [71]. Increased numbers of DCs are also measured in synovial fluid. The role of DCs in RA seems to be priming of the adaptive immune response in pro-inflammatory ways. This can be adapted by treatment to priming the adaptive immune response in a more tolerogenic way. Further studies could elicit ways of modifying the DCs and therefore the ensuing T cell and B cell reactivity.

Mast Cells

Mast cells (MC) are numerous in the rheumatoid joint. They constitute a few cells in the normal joint and expand in number in a diseased joint particularly in the sub-lining areas and around blood vessels. They can then constitute up to 5% of cells in the diseased synovial joint [72]. There are two main subsets of mast cell: connective tissue mast cells (MCCT) and mucosal mast cells (MCM). MCM cells express tryptase, MCCT cells express tryptase and chymase. The main subset found in the rheumatoid joint is MCCT although MCM cells are found later in the inflammatory reaction. MCM cells increase in density in the superficial synovium and remain in the superficial areas of the

synovium after resolution whereas MCCT cells decrease[73]. Mast cell derived mediators are abundantly found in synovial fluid and synovial tissue [74-75]. See figure 1.2.

Fibroblast-like synoviocytes

Fibroblast-like synoviocytes (FLS) are normal residents of the human synovium. They are type B synoviocytes (type A being macrophages). Their normal function is to produce synovial fluid, remodel matrix and wound repair and healing. The normal synovial lining layer is 1-3 cell layers thick, but in RA this can expand to 15 cell layers thick. This expansion is due to both types of synoviocyte. Fibroblasts are thought to proliferate within the joint but have been shown to be present in the blood of RA patients suggesting that they may be able to migrate to sites of inflammation. Although not an inflammatory cell itself, the FLS starts to produce molecules consistent with the ability to interact with immune cells such as MHC class II molecules. FLS can be found at sites of cartilage erosion directly producing MMP 1, 3 and 13 which cause direct damage to cartilage [76]. FLS also produce the tissue inhibitors of metalloproteinases (TIMPs) but it seems that the ratio of MMP to TIMP favours the MMP and therefore is damage-inducing rather than remodelling. See figure 1.2. FLS are part of the invasive pannus seen in RA and are modified from FLS found in synovial tissue. They lose cell-to-cell contact inhibition. They also become resistant to apoptosis through a variety of mechanisms but many of which involve mutations in tumour-related genes such as p53. FLS also express RANKL meaning they can interact with osteoclasts [77] and affect bone erosions indirectly. FLS implanted with cartilage into SCID mice showed cartilage destruction without the need for other cellular or antibody responses [78] and are stimulated by exposed extracellular matrix.

FLS express TLR 2[79], 3[80] and 4[81].The effects of these receptors is enhanced by the presence of TNFα and IL1. Activating TLR2 leads to the expression of multiple pro-inflammatory mediators including MMP1, 3 and 13, adhesion molecules such as ICAM1, and chemokines such as CCL8. TNFα itself with IL1 can activate FLS. Prostaglandins activate FLS [82]and FLS are the main producer of

cyclo-oxygenase 2 (COX-2)[83]. FLS contribute to neoangiogenesis and are themselves activated by angiogenic factors RA FLS can migrate to sites of matrix damage and also through the blood stream contributing to the spread of RA in the body [84].

FLS interact with T cells via direct cell-cell contact and activate them. This interaction also occurs with the production of cytokines[85]. FLS also produce B cell activating factors such as BAFF (BLyS) [86] and APRIL and interact with the proliferation of B cells[87].

Mediators

There are multiple mediators of disease in RA. These include cytokines and chemokines which activate the cells of the immune response. Other mediators include prostaglandins and effector mediators such as MMPs and collagenases, growth factors and hormones. More recently discovered mediators include micro-particles. Micro-particles are small membrane-bound vesicles with potent biological activity. Cells that produce cytokines are T cells, B cells, FLS and macrophages and DCs, all of which have a positive feedback loop for cytokine production. The major players are TNFα, IL6, IL1, VEGF (vascular endothelial growth factor) and IL17. They work through their specific receptors to activate an inflammatory response and tissue degradation.

TNFα plays a crucial role in RA as evidenced by the effectiveness of the anti-TNFα therapies in humans. It acts via two different TNF receptors, types I and II. There is a direct association between TNFα levels in the blood and activity of RA[88]. It regulates the RANKL/RANK system with the induction of osteoclast differentiation and proliferation[89]. It increases monocyte activation[90], cytokine, prostaglandin and MMP release, affects T cell apoptosis and clonal regulation[91]. There is also increased endothelial cell adhesion molecule expression[92] and synovial fibroblast proliferation[93]. TNFα also has systemic effects on acute phase protein production and the hypothalamic-pituitary-adrenal axis. It also has effects on angiogenesis *via* the production of VEGF [94] and has effects on the suppression of regulatory T cells. It is involved in the induction of pain [95].

IL1 is present in the rheumatoid joint and is a pleiotropic cytokine with functions overlapping that of TNFα and IL6. Incongruently, anti-IL1 therapies have not been as successful as anti-TNF therapies- this is thought to be due to redundancy in the IL1 system[96]. IL1 is involved with the activation of leukocytes, endothelial cells, chondrocytes and osteoclasts [97] in a manner similar to TNFα. Other cytokines of the IL1 family are IL1α, IL1β, IL18 and IL33. IL1 has local effects on production of cytokines, chemokines, MMPs and prostaglandins. These mediators are released from FLS and monocytes. IL1 has been implicated in cardiovascular disease perpetuation [98] and produces systemic effects on the acute phase response. The cognate cytokine IL18 promotes Th1 cells [99] but also activates neutrophils, osteoclasts and NK cells, whilst IL33 activates mast cells and neutrophils[2].

IL6 also has local and systemic effects. In the joint, IL6 activates osteoclasts, and recruits neutrophils. IL6 is implicated in pannus formation due to its effects on VEGF (vascular endothelial growth factor) production. It also has effects on B cell antibody production and proliferation *via* pre-B cell colony enhancing factor, as well as T cell proliferation via chemokine expression. IL6 acts *via* a non signalling α-receptor unit and two signal transducing subunits called glycoprotein 130 (gp130). IL6 acts via its own receptor on a small range of cells but acts on a larger range of cells by binding to soluble IL6 receptor which then binds to membrane bound gp130[100]. These mechanisms have been described for synoviocytes and endothelial cells. IL6 acts in combination with other cytokines in the induction of Th17 cells [101] whereas it can act on neutrophils with direct activation and indirectly by acting on synovial fibroblasts and chemokine expression [102].

IL17 recruits monocytes and neutrophils to sites of inflammation by enhancing local chemokine production. It facilitates T cell activation and attraction. It acts on FLS to increase their cytokine and MMP production and also acts on osteoclasts enhancing erosion formation and cartilage damage. IL17 is a marker for Th17 cells, a cell type now thought to be predominant in the pathogenesis of RA.

CIA is ameliorated in a IL17-deficient mouse model [103]. IL23 promotes IL17 production by Th17 cells and IL21 activates Th17 cell subsets[104].

TNFα, IL1, IL6 and IL17 act in concert to increase the expression of RANKL and therefore osteoclastogenesis. Macrophage colony stimulating factor, IL11 and IL6 act in concert to cause osteoclast formation in a RANKL-independent manner. IL1, IL6 and TNF α all promote the production of VEGF and therefore angiogenesis. TNF α, IL1 and IL6 activate FLS and chondrocytes with the release of damaging MMPs. TNFα, IL1 and IL6 all act on non-synovial tissue as well and this can lead to changes in adipose tissue, skeletal muscle (rheumatoid cachexia) and vascular endothelium (cardiovascular disease)[105]. See figure 1.2.

Chemokines

Chemokines are small molecules that act as chemotactic mediators for the attraction of cells and are also involved in lymphoid organisation (chemotactic cytokine). They can also be involved in angiogenesis and act locally on chemokine receptors. Chemokine receptors are present on synovial macrophages, FLS and lymphocytes with some chemokines acting on more than one chemokine receptor. They are classified by genes and structure into families depending on the number and position of cysteines in the molecules. The identification of chemokines is complex with many having traditional names as well as a cysteine derived name. They can also be classified into inflammatory and homeostatic mediators. CXC chemokines have two cysteines separated by an unconserved amino acid. CC chemokines have two cysteines together and CX_3C chemokines have two cysteines separated by three amino acids. The receptors for the chemokines are named in a similar way i.e. CXCR, CCR, CR and CX_3CR[106].

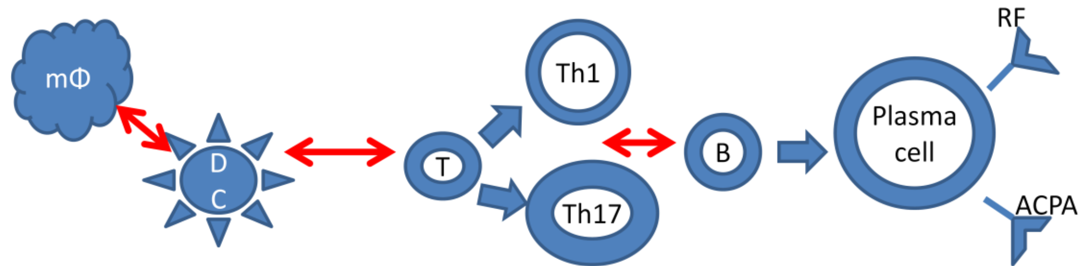
CXCL8 or IL8 is an important inflammatory chemokine in RA. It is found abundantly in serum, synovial fluid and synovial tissue[107]. It is produced by macrophages predominantly but is also produced by FLS and endothelial cells. CXCL8 can induce articular inflammation on its own[108]. CXCL8 attracts neutrophils and may act on endothelial cells during leukocyte trafficking.

CXCL13 is a B cell attractant and activator as well as playing an important role in lymphoid tissue organisation. It acts on CXCR5. It is thought of as a homeostatic chemokine.

Multiple CC chemokines are expressed in RA sera and synovium [109-110] and they act mainly to attract T cells, monocytes and natural killer cells. Examples include CCL2 or monocyte chemoattractant protein (MCP) and CCL5 or Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES). They are produced by macrophages and can induce arthritis when directly injected [111]. Their production can be increased by pro-inflammatory cytokine release. CCR1 is abundantly expressed in RA synovium and is thought to be involved in monocyte recruitment. CCR5 is found on FLS suggesting it is important for monocyte retention. CCR2 and CCR3 are found on chondrocytes [112].

Other CCL chemokines include CCL8, CCL7, CCL13, CCL14 and CCL16, CCL18, CCL19 and CCL20, all found in the rheumatoid joint [113]. XCL1 and XCL2 are the two members of the C chemokine family and are involved in T cell movement. XCR1 is found on lymphocytes, macrophages and fibroblasts.

Fractalkine, CXC_3CL1, is the only member of the CXC_3C family. It acts on monocytes and lymphocytes and particularly T cells and FLS [114]. It is produced by macrophages, FLS, endothelial cells and dendritic cells. Its receptor CX_3CR1 is found on macrophages and dendritic cells[106].



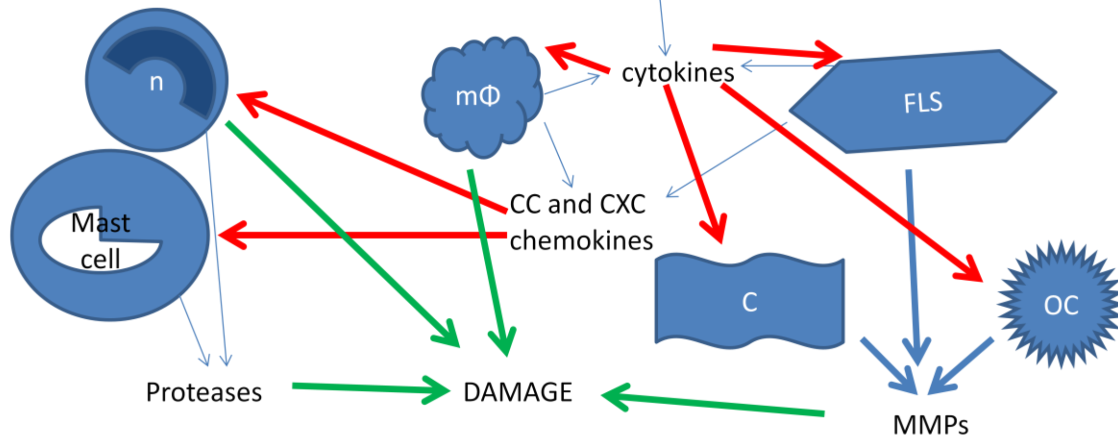


Figure 1.2 The rheumatoid joint.

Macrophages (mΦ) present antigen via MHC class I to Dendritic cells which process the antigen and present to T cells via MHC class II and a costimulation signal of CD80/86. The antigen is thought to be citrullinated self peptides. Naive T cells then become polarised into Th1 or Th17 cells which express cytokines and interact with B cells. B cells are then stimulated to produce antibody-producing plasma cells. These produce RF (rheumatoid factor) and ACPA (anti-citrullinated protein antibodies).

Both innate and adaptive immune system cells are involved with the pathogenesis of RA, with the presence of neutrophils and mast cells. Fibroblast-like synoviocytes (FLS) develop a new profile secreting cytokines, matrix metalloproteases, (MMPs) and expressing NOD-like receptors and Toll-like receptors. Th17 (T helper 17) cells produce IL17, FLS produce IL6, IL1, TNFα and TGFβ. Macrophages produce IL1, IL6 and TNFα, IL15, IL18 and IL32. The action of cytokines on chondrocytes (C), FLS and osteoclasts, (OC) promotes the production of damaging MMPs. Neutrophils (n) and mast cells produce proteases which also cause damage. Neutrophils also produce prostaglandins and reactive oxygen intermediates. Mast cells also produce cytokines such as TNFα, vasoactive amines and arachidonic acid metabolites. The interactions amongst dendritic cells, T cells and B cells are thought to occur in local lymphoid tissue but may occur in ectopic lymphoid tissue in the joint. A positive feedback loop between leukocytes, FLS, chondrocytes and osteoclasts drives a chronic phase[2].

1.1.3 Current Disease Biomarkers

Treatment of rheumatoid arthritis has been much advanced by the advent of biologics. Biologics are mainly monoclonal antibodies used in the treatment of RA and other diseases. However, these drugs are expensive, can have serious side effects, and only a proportion of patients treated with each drug respond adequately, although there are some who respond to the point of remission, whether on drugs or drug free. There are multiple areas where biomarkers would be useful. Initially, it would be helpful to be able to predict those who had a high risk of developing rheumatoid arthritis. A biomarker would be useful in the normal population as well as a higher risk population such as those with undifferentiated arthritis. Once inflammatory arthritis had been established it would be useful to be able to predict those who would progress to the chronic disease and the small percentage of patients who have a flare of arthritis and burn out without on-going disease. Another area would be response to treatment, it would be useful to have a test that would be able to predict whether the patient would respond to monotherapy, combination therapy or would be better off initiating biologic therapy early on. This would enable the patient to avoid unwanted side effects or comorbidity due to the drugs themselves and allow adequate treatment, without loss of function, quickly and effectively. Biomarkers for prognosis would also be helpful. There is a large heterogeneity in the outcome of RA, from those who have non-erosive mild disease that does not affect function, to those that have rapidly progressive erosive disease with rapid-onset disability and comorbidity. It would be helpful for the physician to know from early on how aggressive treatment should be and it may be helpful for the patient to know what to expect from their disease.

Although there is a large amount of research in the field of biomarkers, there is not yet a biomarker that meets all the criteria required. A biomarker should be easy to acquire, inexpensive, with a high sensitivity and specificity for the outcome expected, acceptable to the patient and with a low complication rate from any procedure required. Biomarkers may be clinical, or biological. Clinical markers could include specific points in the history or examination. Other clinical markers could be

radiological such as X-ray, magnetic resonance imaging (MRI) or ultrasound (US) changes. Biological markers could be genetic markers taken from swabs for gene polymorphisms or blood tests for antibodies or molecular markers. Blood tests could look at serum-based soluble markers or peripheral blood mononuclear cell markers, these could be at gene expression or protein expression level. Biomarkers could also be assayed from the synovium itself as part of a synovial biopsy testing system. From the synovium it is possible to test for gene polymorphisms, molecular markers including cytokine profiles and specific molecules such as MMPs as well as patterns of disease markers such as a panel of cytokines or a panel of enzymes. These tests could be of gene expression or messenger ribonucleic acid expression (mRNA) or protein expression through proteomics. A test could be as simple as the presence or absence of a specific molecule or pattern of markers or could account for a molecule plus specific translational modifications. The other power of synovial biopsy is that of the evaluation of the cellular infiltrate. It is possible to label various cell populations such as T cells, B cells, macrophages and plasma cells and look for specific patterns of synovial infiltrate. It may be possible to predict response to rituximab from B cell patterns of expression, for example follicular patterns as opposed to a diffuse infiltrate, or the presence of oligoclonality in B cell populations. This may be a change in infiltrate from before to after treatment or may be a pattern seen in the tissue before treatment [115]. There is a huge interest in patient stratification both at a clinical level for the benefit of the patient with the avoidance of using toxic medications unnecessarily, and at a financial level, to avoid the use of expensive drugs on patients that will not respond to them. See figure 1.3.

Clinical Biomarkers

Biomarkers currently in common usage are rheumatoid factor (RF) and ACPA antibodies. These are simple blood tests done on presentation of the patient to a rheumatologist. The test for rheumatoid factor has been around since the mid 1970's. Rheumatoid factor is used to predict the likelihood of having rheumatoid disease and of being erosive on X-ray. 70% of patients have a positive IgM rheumatoid factor which is tested for by the latex agglutination test or enzyme-linked

immunosorbent assay (ELISA). There are conflicting reports of the sensitivity and specificity of the test. A cohort of 8000 patients revealed a 97.9% specificity for an inflammatory rheumatic disease at a titre of 1 in 80 and a positive predictive value of 80%[116]. Sensitivity was lower at 78.0%. The positive predictive value for RA over other inflammatory disorders was 96.8%. However, a study of 1988 patients showed a sensitivity and specificity of IgM rheumatoid factor of 69% and 86% by ELISA and 66% and 91% with the latex agglutination test. The positive predictive value (PPV) of RA was 49% for the latex agglutination test and for erosive versus non-erosive disease 58% [117]. For IgM ELISA the PPVs were 40% and 47% respectively. An ACPA ELISA was found to be 98% specific with a sensitivity of 68% and seemed to have a higher specificity than an IgM RF ELISA (96% versus 91%). The sensitivities of the tests were similar (48% and 54%) The combination of both tests gave a positive predictive value for RA of 91% and a negative predictive value of 78%. The tests had a positive predictive value for predicting erosions of 91% [118].

The biologics registers have exposed certain clinical features as being predictors of response to TNFα inhibitors. For example the British Society for Rheumatology Biologics Register has shown that baseline DAS28, HAQ and concurrent use of disease-modifying anti-rheumatic therapy and smoking had associations with response [119]. The Swedish registry gives similar information with the use of methotrexate or concurrent DMARDS being consistent with a good response as well as a low HAQ. DAS28 was negatively associated with a good response [120]. However, these features represent associations and need to be clarified as to whether they are prognostic markers or progression factors by further focussed studies. See figure 1.3.

Genetic biomarkers

Genetic biomarkers have been discussed earlier with the shared HLA epitope, PTPN22, STAT4 and TRAF1/C5 polymorphisms being investigated as susceptibility factors and also possibly progression factors in RA. Other polymorphisms already discussed include CTLA4, IL2, IL2RA, CD28, CD40 and TNFRSF14. The shared epitope has been investigated by the British Society of Rheumatology

Biologics Register and showed no association between the shared epitope and PTPN22 and response to anti- TNF therapy [121].

TNF gene polymorphisms at SNP position 308 have been associated with response to anti-TNF agents but a meta-analysis of the 12 studies showed no association between polymorphisms at position 308 and response to TNF therapies[122]. However a more recent meta-analysis of 15 studies suggested that patients with the G allele had a better response to TNF inhibitor treatment[123] and a smaller meta-analysis suggested that those with the A allele did worse on anti -TNF blockers[124]. It is important that further studies are done to dissect out the importance of these polymorphisms for response to therapy. It may be that these patients will do worse regardless of treatment rather than specifically do worse with anti-TNF treatment. There is more evidence for the same polymorphisms as prognostic markers (markers of severe disease). Two studies have shown an association of SNP position 308 polymorphisms and severe disease so screening for this polymorphism may be of use to screen for those with a worse prognosis [125-126].

As discussed TNFα is an important cytokine in the pathogenesis of RA and therefore has been of great interest with regards to polymorphisms within the TNF gene itself but also in terms of the receptor and the TNF receptor superfamily. Other polymorphisms investigated include the TNF receptor II 676 TG genotype [127] and treatment with anti-TNF therapy and TNF superfamily 1B genotype and response to TNF therapy [128].

Gene expression biomarkers

A further way of stratifying patients into prognostic or progression categories is by messenger ribonucleic acid (mRNA) analysis of peripheral blood mononuclear cells or of the synovium itself. Again this could be used as a marker of likelihood of response to treatment or as a marker of prognosis or progression. This can be used to monitor single gene expression [129] or it could be used in microarrays to look at panels of gene expression numbering hundreds to thousands of genes [130]. In studies such as these it is important that the patient samples are classified stringently

allowing the population studied to be homogenous. It is also important that the results of these studies are replicable. As messenger RNA analysis is a snapshot of gene expression at a certain point in time, it is important to establish whether these associations are bystander markers of inflammation or actual prognostic markers.

In a study of the synovium of 50 patients with early DMARD-naïve arthritis, synovial samples were profiled into self-limiting and persistent arthritis. There were differences in mRNA expression of CD44, collagen (1A1, 4A2, 5A1, 6A1 and 16A1) and laminin between the two groups. This suggested that markers of turnover were higher in the persistent arthritis group than the self-limiting arthritis group.[131]

Other studies have used synovial gene expression to look at inflammatory cytokines before and after treatment [132]. This showed a trend towards reduction in a small panel of cytokines that was not statistically significant after treatment with prednisolone. There have also been studies into messenger RNA profiling before and after anti-TNF therapy which have shown differences in groups of responders and non-responders [133], in multiple genes however, a larger sample from the same group showed no difference between responders and non-responders [134]. There was however a difference if the immunohistochemical grade of the sample was taken into account. A study looking at peripheral blood gene expression did find a difference between responders and non-responders to infliximab over a 22 week period identifying a set of genes classified as “inflammatory” that decreased and remained low for responders but that decreased and returned to baseline for non-responders [135].

There has also been a study comparing peripheral blood biomarkers to synovial gene expression profiling to examine whether peripheral blood could be used to predict the inflammatory state of the tissue. This study found no associations with any single gene studied [130] but did find associations at the level of groups of genes or pathways. The patients could be split into a high inflammation and low inflammation group on the basis of immunohistochemistry and this coincided

with the split into two groups of T and B cell gene expression and developmental marker gene expression. These two groups based on immunohistochemistry and gene expression analysis of 256 genes was not reflected in gene expression analysis of the peripheral blood mononuclear cells. The high inflammation phenotype did correlate with CRP (C reactive protein) and ESR (erythrocyte sedimentation rate) and platelet count. Low inflammation subtypes were associated with longer disease duration.

One issue is that gene expression results can be expressed quantitatively or qualitatively. It is important to know what standards are used for comparison. When comparing before and after treatment it is easy to use the pre-treatment sample as the baseline for the relative change of expression. When labelling samples at one set time point it is important to have a set standard for comparison so that measurements can be compared between samples run in different experiments on different days.

It may be that RA patients have a gene expression signature rather than a specific molecule signature [136-137]. Given the heterogeneity of RA, large sample groups are required to power studies sufficiently. As yet there is no consensus biomarker available.

Protein Biomarkers

Similar analysis can be carried out at protein level by proteomic analysis, again looking for a protein signature for RA patients as compared to other inflammatory arthropathies [138] and healthy controls [139]. There have been proteomic studies into response to treatment with etanercept in patient sera and a 24 biomarker signature [140] and a 6 protein profile for response to infliximab including apolipoprotein a1 and platelet factor 4, again from patient sera[141].

Cellular infiltrate as a biomarker

Another group of biomarkers emerging in the prognosis and assessment of response to therapy is the pattern of cellular infiltrate found by immunohistochemistry. Samples can be classified into aggregate-containing and non-aggregate-containing groups or into high and low inflammation

groups. It is known that sampling from one joint can be informative of the processes going on in another joint [142], leading to the idea that sampling from a large joint such as a knee may be indicative of the whole disease process. There is also evidence that sampling from the pannus of synovium and a site distant from this junction gives similar results (e.g. suprapatellar pouch of the knee versus cartilage pannus junction) [76, 143].

Multiple scoring systems have been in use for the categorization of synovial tissue. They take into account the synovial lining layer, the cellular density of the stromal layer and the infiltrate [144]. The infiltrate can be immunophenotyped into B cells, T cells, macrophages , and plasma cells by specific markers for these cells and semi-quantitative scoring of each of these cell populations[145]. There is the Rooney score[146] which has been used in a study of 60 patients with established RA although there was no correlation with joint damage at 2 years[147]. There is also the Tak score[145, 148] which has been used extensively by the Tak group itself[149] and other groups as well[60, 150-151]. There is also the Koizumi score [152] which has been used in cross-sectional studies to assess the difference between OA and RA samples and also compared the results to the Rooney score. There was a correlation between erosive damage and CRP with no associations being found for the Rooney score [153]. The study of the immunohistochemical diagnosis and grading of RA synovium requires standardization across studies and clinical trials [154].

Macrophages as a biomarker

Macrophage numbers in the synovium have been related to response to treatment in a number of studies. They have also been associated with pain sensation in RA [145]. Two studies have associated macrophage numbers with erosive damage although they were small studies [61, 155]. Another study has associated synovial macrophage scores to clinical course in RA[62] and factors in the synovium such as IL6[155] or MMP1[156] and erosions. A study of 36 patients has shown no relationship between macrophage numbers and radiological outcome but did produce a model suggesting number of granzyme B positive cells, T cells and FLS discriminated between minimal

progression and severe progression groups. A study of 88 patients from different trial groups suggested an association between changes in sub-lining macrophage score but not intimal macrophage score and being a good responder, as well as DAS28 changes. It also suggested that changes in the sub-lining score in a subset of control patients was less likely to be affected by the placebo effect than the clinical score [62]. CD68 (a specific macrophage marker) scoring has been shown to be consistent over two centres with the correlation between the DAS28 change and sub-lining CD68 count confirmed [157]. These examinations were carried out before and after rituximab therapy. A small study has shown decrease in macrophage numbers correlating with fall in DAS28 score after prednisolone treatment [158]. A clear distinction can be made between effective and ineffective treatment with regards to the CD68 sub-lining marker and seems less susceptible to placebo effects than clinical evaluation with DAS28 [159]. As many of these studies come from a single group, a proof of concept study was carried out by a group in Dublin who could not conclusively show the relationship between the change in disease activity and change in sub-lining macrophages but did support a role for CD68 staining as a biomarker[157].

Ectopic lymphoneogenesis as a biomarker

Another potential biomarker is the presence or absence of lymphoid follicles, whether active or not as discussed earlier, and disease activity. It has been known for some time that lymphoid follicles are found in the synovium in a proportion of patients with RA [54, 160-161] and this has been correlated to clinical activity and MMP activity in a study of 37 patients [162]. Diffuse synovitis was seen in patients with milder seronegative RA and extra-articular RA was associated with granuloma formation. Follicular RA had elevated values of TNF and more severe activity than diffuse infiltrate RA[163]. However, it is probably too simplistic to grade patient samples as aggregate or non-aggregate and it is quite likely that the functionality of the aggregates and the presence of germinal centres are important for antibody production and severity of disease.

Lymphoid follicles and aggregates have been investigated as a marker of response to treatment with anti-TNF therapy and two studies have reported synovial lymphoneogenesis is reversible with anti-TNF treatment. However although both studies agreed on this, they disagreed with regards to whether lymphoneogenesis predicted a higher [164] or lower [165] response to therapy. However, the two studies labelled aggregate-positive and aggregate-negative samples differently with the study that detected a lower response to therapy only acknowledging those samples with large aggregates. Also there was heterogeneity in the anti TNF treatments used, so these studies may not be directly comparable. It is likely that larger scale studies are required with homogeneity of treatment between groups and standardisation of immunohistochemical grading to clarify the ability of lymphoneogenesis to predict response to a particular anti-TNF therapy rather than anti-TNF therapy as a group of treatments.

At present, although there is data suggesting the usefulness of synovial ectopic lymphoneogenesis, it has not been accepted across the board as a key component of the pathological process and there are those who suggest that the formation of ectopic lymphoid follicles is in fact a bystander effect from chronic inflammatory processes[166]. Ectopic lymphoneogenesis is found in other diseases, including those that are not antibody-mediated or antibody-associated diseases [167]. Larger, well described prospective studies with well-defined immunohistochemical outcomes in DMARD-naive patients are required to settle this issue.

Response to rituximab, an antibody against human CD20, which depletes B cells, has also been investigated for predictors of response to treatment. Studies before and after rituximab treatment have looked at clinical response and depletion of synovial B cells and immunoglobulin synthesis[115] as well as numbers of plasma cells[168] and numbers of circulating pre-plasma cells as predictors of response. Biopsies from 24 patients were assessed at 4 and 16 weeks post treatment with rituximab, there was a reduction in RF and ACPA after treatment and B cells were depleted in the blood of all the patients. There was a trend towards reduced lymphoid aggregates in synovium and follicular

dendritic cells (taken as markers of ectopic lymphoneogenesis). The change in plasma cells differed between responders and non-responders and could predict the decrease in DAS28 at 24 weeks of treatment. A reduction in intimal macrophages also showed a trend for predicting decrease in DAS28 in a univariate but not a multivariate model [168]. This study confirmed the presence of a wide variety of responses of the synovial tissue not seen in the peripheral blood. Another study of 24 patients treated with rituximab showed that high levels of inflammation with regards to inflammation score, high CD3, CD68, CD138 and CD79a scores were associated with high activity post-treatment. This study also showed that although the cell scores (Tak) generally decreased there was a trend towards a higher inflammatory score and CD3 score post treatment in those with high disease activity pre-treatment. Of significant interest though is that CD79+ cells were significantly different between high and low activity groups post treatment with higher CD79 scores being associated with high disease activity. The CD79+ CD20- cells appeared to be plasma cell-like cells. Another finding from this paper was that B cell repopulation was reduced in those that had low activity post-treatment compared to those with high activity post-treatment [169]. Treatment with rituximab is consistently associated with peripheral B cell depletion, however, it is not clear whether this can be used as an indicator of effectiveness of therapy or whether synovial depletion of B cells is a more valuable measure of efficacy.

Clinical Prediction Models

A comparison of the ACR (American College of Rheumatology) 2010 classification criteria, the van Der Helm and Visser criteria for RA, showed that all three algorithms gave good diagnostic properties with the Van der Helm criteria being the most specific but least sensitive. The outcomes were use of methotrexate and persistent synovitis at one year. All criteria were robust, with good levels of correlation with each other [170]. They recommended the use of the ACR criteria for uniformity among future data sets. The ACR criteria displayed a sensitivity of 0.74 and a specificity of 0.66.

Clinical prediction models have also been developed for the clinical course of RA once the disease has been diagnosed. As discussed above, RF and ACPA are associated with erosions. Baseline erosions can predict future erosions [171]. High inflammatory markers are also associated with a higher erosive load [117, 172]. They are not however independent predictors of outcome. Combinations of clinical parameters entered into statistical models have been formulated in order to overcome the lack of discrimination by single parameters. However, no single model has become foremost in the prediction of the clinical course of disease

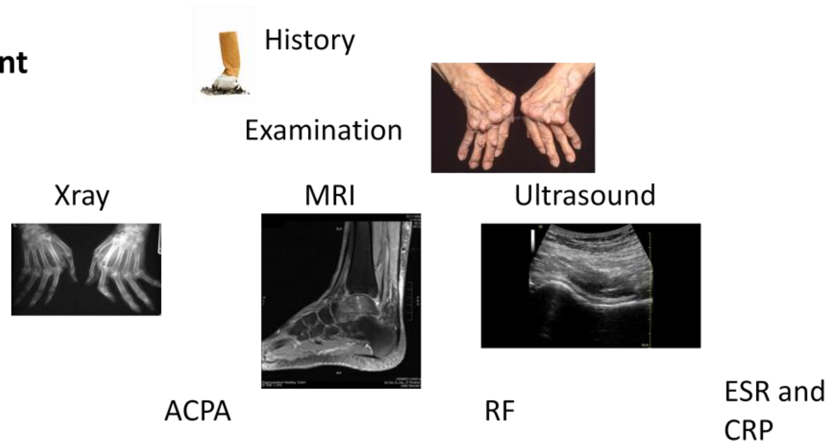
Some models have tried to include genetic polymorphisms for the shared epitope or SNP analysis to increase the predictive value of the models or to find new variables for prediction [173].

There have been some attempts to use single biological markers as predictive agents for the progression of disease. For example, baseline CXCL13 serum protein was used to predict radiological progression in an initial cohort of 74 patients and then a validation cohort of 155 patients with early rheumatoid arthritis. Three other markers were also tested and were not found to have a relationship with radiological outcome [174]. Baseline MMP3 levels have also been shown to be associated with radiological progression and have been used in association with CCP and baseline radiographic damage to produce a model with a predictive accuracy of 0.81. The same group has used this model over a two year period and an eight year period. They also looked at cartilage oligomeric protein 1 and tissue inhibitor of matrix metalloproteinase 1, but these markers were not independent predictors of radiological progression when used in a stepwise logistic regression model. It is of note that a combination of variables gave greater predictive power than either ACPA or MMP3 alone [175]. Urine markers have also been used as predictors of radiological outcome such as C terminal cross linking of type I and type II collagen (CTXI and CTX II) in a cohort of 155 patients that was followed for 11 years. These were used in combination with the RANKL and osteoprotegerin ratio in the serum to produce a model that predicted 36-39% of the observed variance in radiological progression [176].

No single biomarker has been found that predicts either the likelihood of disease or the progression of disease. Given the heterogeneity of disease in RA cohorts it may be that biomarkers will be available for specific subgroups of patients rather than for the whole spectrum of rheumatoid arthritis. At the moment, clinical, radiological and autoantibody characteristics are used to guide treatment with the ACR 2010 criteria being at the forefront of treatment algorithms. Matrix models have been published and validated but are not in routine use given their low predictive values. There is a need for more biomarkers to enable patient stratification, not only for prognosis but for progression and for treatment. Biomarkers could delineate populations that need more aggressive treatment and those that will do well following more conservative management. They could enable patients to avoid the use of toxic medication that would be predicted not to work for them. They could also enable the physician to avoid using expensive drugs where they are unnecessary. Biomarkers may enable better patient stratification and targeting of therapy.

Histological grading and analysis of tissue samples is common in other areas such as cancer or renal disease allowing patient specific decision making. A consensus opinion on biomarkers in rheumatoid arthritis would allow a similar treatment protocol with decisions regarding diagnosis, treatment and follow-up being based on combinations of clinical, serological, radiological and pathological markers, or single markers should they be found in the future. Newer less invasive methods of obtaining synovial tissue, needle biopsies *versus* arthroscopy, have opened up this area as a source of potential biomarkers whether this be gene expression, protein expression, patterns of protein expression, patterns of cell infiltrates or expression of specific signatures of genes or proteins. There is much interest in this field and great potential for new discoveries.

Current patient stratification



Experimental patient stratification

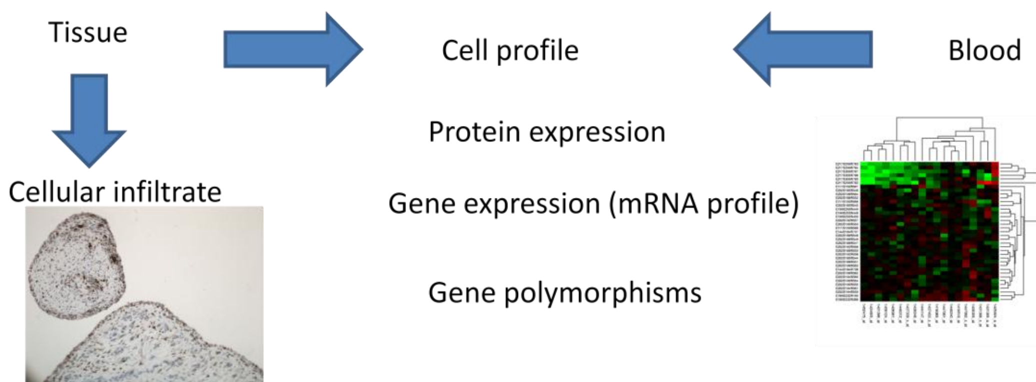


Figure 1.3: Patient stratification and potential biomarkers.

A patient can be stratified into multiple categories by using various factors. Techniques available to all are features in the history (e.g. smoking) and examination (e.g. Metatarsal-phalangeal squeeze) and imaging (e.g. erosive disease or synovial hypertrophy with increased Doppler signal) with the testing of rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA) in the blood. Less well-established and less widely available techniques are the examination of blood or tissue for cell expression, protein expression, gene expression (i.e. mRNA) and gene polymorphisms (e.g SNP 308 of the TNF gene). Synovial tissue can also be tested for a presence of the cellular infiltrate. (images courtesy of en.wikipedia.org)

1.1.4 Current Treatments for Rheumatoid Arthritis

Treatments for rheumatoid arthritis have increased exponentially in the last 20 years. With the development of the biologic era of therapy, this has made a significant difference to the expectations of both rheumatologists and patients. Current UK National Institute for Health and Clinical Excellence (NICE) guidelines state that patients with a diagnosis of rheumatoid arthritis should be offered combination therapy with disease-modifying anti-rheumatic drugs (one to be methotrexate) with or without a short course of glucocorticoids (whether this be oral, intramuscular or intra articular). Other disease-modifying anti-rheumatic drugs are sulphasalazine, hydroxychloroquine and leflunomide. See figure 1.4. Glucocorticoids can also be thought of as disease-modifying in early disease [177]. Regular review of medication, disease status and serum inflammatory markers such as ESR and CRP can lead to escalation of therapy to anti-TNF therapy within six months if disease is not adequately controlled to a DAS28 score below 5.1 on combination therapy. The DAS28 is a combination of tender joint count, swollen joint count, patient global disease activity score and ESR or CRP. An adequate trial of DMARD combination therapy is 6 months with 2 months at a standard dose unless side effects have limited escalation. Current anti-TNF medications recommended by NICE are etanercept, infliximab, adalimumab, certolizumab pegol and golimumab. Tocilizumab, an anti-IL6 preparation, can be used after failure of combination DMARDs, after failure of one anti-TNF or after failure with rituximab. Rituximab can be used after anti-TNF failure but not earlier. Abatacept, a CTLA4 decoy, preparation is recommended for use by NICE and can be used as a first line biologic. Abatacept is currently under review for use after anti-TNF failure. Anakinra, an anti-IL1 preparation, is not recommended by NICE and can only be used as part of clinical trials. Tofacitinib, an oral Janus kinase inhibitor is currently under review by NICE for use in RA. The British Society of Rheumatology Guidelines broadly agree with NICE guidance although they recommend anti-TNF therapy could be used for patients with a DAS28 over 3.2 rather than 5.1 [178]. See figure 1.4.

The common measures in trials of medications for RA are DAS28, ACR20, ACR50 and ACR70, HAQ and erosion scores. The HAQ is the health assessment questionnaire which is a measure of disability. The ACR 20, 50, and 70, are improvements of 20%, 50% and 70% respectively, in the patients tender joint count, swollen joint count and in three of the following: patient pain assessment, patient global assessment, physician global assessment, patient self-assessed disability and an acute phase reactant such as CRP or ESR[179]. The erosion scores commonly used are the Sharp score or the Larsen score. These terms will be used in the following discussion of treatments for RA.

Non-Steroidal Anti-Inflammatory Drugs

Non-steroidal anti-inflammatory drug (NSAID) medications are the first treatment that many patients receive for their RA. They are non-disease-modifying and in the context of RA are used as effective analgesics or adjunctive therapy. They act on the cyclo-oxygenase (COX) pathways (COX 1 and COX 2) to reduce the production of prostaglandins, thus reducing pain and swelling [180]. They are antipyretic, analgesic and anti-inflammatory. By blocking COX enzymes, NSAIDs inhibit the synthesis of prostaglandins, prostacyclin and thromboxane A2. Thromboxane A2 causes platelet aggregation and prostacyclin causes vasodilatation. Prostaglandins are gastroprotective as well as being involved in inflammation, pain and fever. Aspirin is a non-selective NSAID which is primarily used for its anti-platelet effect in diseases such as myocardial infarction and stroke. However, although these drugs are effective analgesics it should be noted that the NSAIDs have a class effect on increasing gastrointestinal haemmorhage and long term treatment with these drugs is not recommended. There are also concerns regarding liver toxicity and long term cardiovascular risks with continuous treatment with NSAIDs. An improvement of pharmacology was sought by the development of selective COX-2 inhibitors, since this isoform was associated with inflammatory and tissue stress states [181]. The COX2 inhibitors are said to be associated with fewer gastrointestinal events with similar symptomatic relief, although a higher cardiovascular risk profile [182]. Both

selective and non-selective NSAIDS increase the risk of renal impairment and worsening cardiac failure. All NSAIDS cause an increase in blood pressure dependent on the dose given[183]. Examples of therapeutics are the non-selective NSAIDs such as ibuprofen, diclofenac and naproxen, and the newer COX-2 inhibitors such as celecoxib and etoricoxib.

Glucocorticoids

Glucocorticoids (GC) are commonly used in the treatment of RA usually in the form of high dose treatment for flares, an initial intramuscular dose for rapid alleviation of symptoms or, as being used more often, low dose adjunctive therapy to disease-modifying anti-rheumatic therapy (DMARDs). Glucocorticoids are also used as local therapy in the form of intra-articular injection. Large bolus doses of methylprednisolone are used in the treatment of severe flares or serious complications of RA such as interstitial lung disease or Still's disease. The use of glucocorticoids varies from Country to Country and there is no consensus guidance on the use of glucocorticoids in the treatment of RA. The recent American guidelines for treatment do not mention glucocorticoids as they are not considered to be DMARDs[184], however, there are European guidelines for the use of systemic glucocorticoid therapy in the rheumatic diseases which discuss the use of glucocorticoids in giant cell arteritis, polymyalgia rheumatica and RA[185].

Glucocorticoids act in several ways, cytosolic glucocorticoid receptor (cGCR) mediated classical genomic effects, cGCR non-genomic effects, membrane-bound GCR non-genomic effects and non-specific non-genomic effects[186]. These effects down-regulate the synthesis of proinflammatory cytokines such as TNFα, IL1 and IL6 via the NFκB pathway. Glucocorticoids also act on AP1 and NFAT pathways or transrepression actions. It was thought that the anti-inflammatory actions and the actions that lead to complications of therapy were via different routes of action, however, this is still

undetermined. There are some patients who are glucocorticoid-resistant, with a recent study showing that there is a difference in the expression of the GCRβ isoform in these patients [187].

There is also debate about whether glucocorticoids are indeed DMARD therapy in themselves. Although the American guidelines state that they are anti-inflammatory and not DMARD, they have been criticized for this and recent data suggests that glucocorticoids may actually have DMARD activity. A Cochrane review published in 2007 reviewed 15 studies and concluded that although glucocorticoids were mostly added to other disease modifying therapy the standardised mean difference in radiological progression was 0.4 in favour of glucocorticoids and that all the studies except one showed a numerical effect of treatment in favour of glucocorticoids. They did however state that there was concern about long-term adverse reactions to glucocorticoid therapy[186]. The STIVEA (steroids in very early arthritis) trial suggested that glucocorticoids could prevent early arthritis patients from progressing to RA and delay the prescription of DMARD therapy [177]. The COBRA (Combinatietherapie Bij Reumatoide Artritis) group used high dose prednisolone in one arm of their treatment groups and showed a sustained difference in radiographic damage score at 11 years with similar mortality and prevalence of osteoporosis in groups using prednisolone or an anti-TNF agent. They did however note a higher prevalence of hypertension, diabetes mellitus and cataracts in the prednisolone group compared to a higher prevalence of hypercholesterolaemia, cancer and infection in the single treatment group. It is of note that prednisolone was not used alone in this group but as part of an aggressive combination therapy regime [188-189]. It has been suggested that glucocorticoids are useful in early RA and there is a window of opportunity where they have disease modifying effects[190], though it remains to be determined if low dose retarded release of glucocorticoids can indeed modify the progression of RA[191] This study published in 2013 showed that giving modified release prednisolone at night to patients with significant early morning stiffness reduced morning stiffness and morning and evening pain. Over 12 weeks there was a greater ACR20 response (48% in the active arm compared to 29% in the placebo arm) and ACR50 response. More patients achieved low disease activity as defined by a DAS28 less than 2.6. This

confirmed the findings of a similar study comparing modified release prednisolone to immediate release prednisolone which showed a significant reduction in morning stiffness in the modified release arm. There were similar advserse events in both arms in both studies [192].

DMARDS

Methotrexate is the drug of choice in the treatment of RA. It inhibits the action of tetrahydrofolate reductase which impairs the ability of cells to divide and multiply. Its effectiveness has been investigated in a French early arthritis cohort of 777 patients. The authors concluded that in this "real-life" use of methotrexate despite suboptimal dosing, those treated with methotrexate had a lower radiological erosion score at 1 year than those not treated with methotrexate but with other DMARDs. Physicians chose to treat patients with more active and severe disease with positive CCP antibodies and at least one erosion with methotrexate [193].

Sulphasalazine is an aminosalicylate and acts to reduce the production of prostaglandins. A comparison of sulphasalazine use to methotrexate use in 1102 Norwegian patients with DMARD-naive arthritis showed that physicians used methotrexate in patients that scored worse in most disease measures. Superior responses were seen to methotrexate at 6 months in the ACR20 criteria with a higher percentage of patients reaching an ACR50 response and remission, but this did not reach significance. Drug survival was higher with methotrexate than sulphasalazine [194]. Two randomised controlled trials have however shown no difference in efficacy between sulphasalazine, methotrexate or a combination of both [195-196]. However, a larger observational cohort of inflammatory polyarthritis showed a significant difference in the erosive status between sulphasalazine and methotrexate of 31%[197]. Overall methotrexate is thought to be superior to sulphasalazine in preventing erosions but they have similar symptomatic efficacy.

A large study of 1000 patients compared methotrexate and leflunomide (10-15mg per week versus 20mg per day) over a two year period, this showed significantly greater clinical benefit at 1 year of methotrexate over leflunomide with greater retardation of radiological progression with

methotrexate over a 2 year period [198]. 64.1% of patients on leflunomide reached an ACR20 response and 71.7% of patients on methotrexate reached an ACR20 response. There were two treatment-related deaths in the methotrexate group: one from pneumonitis and another from pancytopenia, there were no deaths in the leflunomide group. Leflunomide was particularly associated with diarrhoea, methotrexate with mouth ulcers and studies commented on an increase in liver function tests in a percentage of patients. Alopecia was seen with methotrexate and leflunomide and was a reason for discontinuation of medication. Agranulocytosis is a known complication of sulphasalazine and occurred in several studies requiring discontinuation of the drug.

There is plenty of evidence on the efficacy of current DMARD therapy in the treatment of RA. There is also evidence that these treatments come with a known range of side effects that must be monitored for and that patients must be made aware of before starting any treatment. Monthly blood tests for methotrexate and leflunomide can be time-consuming and costly for patients and is a reason that some do not initiate treatment. Complications of medication can lead to death. Another issue is that although these drugs alone and in combination have an effect on disease progression, study outcome is often measured by the ACR20 response which is a 20% improvement in symptoms. Although clearly this is a marker of response fewer patients reach an ACR50 or ACR70 response or indeed remission. Even fewer reach drug-free remission. Although there is a consensus opinion that early treatment with tight control can make a large difference to outcome, it is yet to be seen whether this outcome is in terms of drug-induced or indeed drug-free remission status or rather a control of disease to a level of better function.

Biologics

Biologic drugs, which are immunomodulators specifically targeting cytokines or signalling molecules, or their receptors, have been a breakthrough in treatment of the rheumatic diseases. There is substantial evidence in favour of their use with many high quality studies, especially in RA.

Infliximab was the first monoclonal antibody against TNFα. It is a murine-human chimera given by infusion every 8 weeks. The ATTRACT (anti-tumour necrosis factor trial in rheumatoid arthritis with concomitant therapy) trial showed ACR20 responses of 53%, ACR50 of 26% and ACR70 of 8% at 6 months versus placebo (20%, 5%, 0% respectively). Infliximab is used with methotrexate to help prevent immune reactions to the infliximab itself. There were improved radiographic scores at 12 months. Infliximab does produce a higher incidence of anti-nuclear antibodies and double-stranded DNA antibodies. It is not clear whether these antibodies are by-products or pathogenic [199].

Etanercept is a human TNFα receptor attached to an Fc portion of an antibody. It is given as a subcutaneous injection once or twice a week at doses of 50mg or 25mg. The TEMPO (Trial of etanercept and methotrexate with radiographic patient outcomes) study showed a significant response in ACR20, ACR50 and ACR70 and HAQ and modified Sharp score in the etanercept / methotrexate group versus the etanercept versus the methotrexate group. 85% of patients on combination etanercept and methotrexate therapy achieved an ACR20 compared to 75% and 76% with methotrexate or etanercept alone respectively. At 52 weeks, 43% of those on combination therapy reached an ACR70 compared to 19% and 24% in the methotrexate and etanercept groups. The number of patients reporting adverse events was similar in all three trial groups [200].

Adalimumab or humira is a humanized monoclonal antibody to TNFα. A Cochrane review was published in 2005, reviewing 6 high quality double blind multi-centre randomised controlled trials covering from 12 to 52 weeks. Overall 43% had an ACR50 at 24 weeks compared to 9% placebo and that after 52 weeks there was slowing of radiological progression compared to placebo[201]. The PREMIER study in 2006 was a multi-centre double-blind clinical trial of adalimumab or methotrexate alone versus combination therapy over a 2 year treatment period. At the end of year 2, 69% of the combination group reached an ACR20 compared to 46% and 56% in the adalimumab and methotrexate groups. The ACR50 with combination therapy was 59% and the ACR70 was 47% at two years. Combination therapy had a greater effect on radiological progression that either adalimumab

or methotrexate alone and led to higher rates of remission (49% versus 25%) over 2 years [202]. This study was continued as an open label study for a further three years and results showed that initial combination therapy led to persistently lower HAQ scores, persistently higher rates of remission and less radiographic progression [203].

Golimumab is a newer humanized monoclonal antibody against TNFα. Its unique selling point over and above the previously mentioned is that it can be given as a subcutaneous injection once a month rather than every 2 weeks. A Cochrane review reviewed three high quality studies and found overall that 38% reached ACR50 on golimumab compared to 15% placebo and that there was an 18% absolute improvement in remission rates after 12 to 24 weeks on golimumab. They also found that fewer people dropped out on golimumab than on placebo, for any reason. Golimumab has been studied with regards to efficacy after failure with anti TNF medications [204] and also as a first line therapy instead of methotrexate with promising results [205]. A randomised controlled trial in Japanese patients confirmed the efficacy of combination therapy of golimumab and methotrexate over placebo and methotrexate over 14 weeks [206].

Certolizumab pegol is a newer anti-TNF agent which is a humanized antibody fragment that is attached to polyethylene glycol. Polyethylene glycol does not cross the placenta and increases the half-life of the antibody fragment against TNFα to about 2 weeks. A Cochrane review in 2011 reviewed the current literature for certolizumab and concluded that 35% of people reached an ACR50 response compared to 6% of placebo and 11% reached remission compared to 1% placebo. Serious adverse events were seen in 10% of active treatment arms compared to 5% of placebo [207]. The RAPID1 (rheumatoid arthritis prevention of structural damage) trial was an international 52 week randomized double-blind placebo-controlled phase III trial. The primary endpoint was the ACR20. There were two doses of certolizumab given 200mg and 400mg every two weeks and the ACR20 for these groups were 58.8% and 60.8% compared to 13.6% for a placebo and methotrexate

group. The mean change from baseline in radiological scores was smaller in the treated group than the placebo group. The frequency of infectious events were comparable between groups [208].

Rituximab is a humanised chimeric monoclonal antibody to CD20 used as an infusion at a minimum of 6 month intervals in the treatment of RA. CD20 is a B cell marker found on all B cell lineage cells except for pro B cells and plasma cells. The REFLEX (randomised evaluation of long term efficacy in RA) study is a randomised placebo-controlled trial looking at the long term efficacy of rituximab over 24 weeks with TNFα failures. This study showed a response to rituximab using the ACR criteria with an ACR20 response of 51% versus 18% placebo, ACR50 response of 27% versus 5% placebo and ACR70 response of 12% versus 1% placebo. Rituximab was well tolerated and there was also an improvement in HAQ, SF36 and the DAS28 as well as modified Sharp score [209]. The DANCER (dose ranging assessment international clinical evaluation of rituximab in rheumatoid arthritis) study was designed to assess dose response and reported an AC20 of 55%, ACR50 of 33% and ACR70 of 13% at 500mg dose with similar results at 1000mg dose. Again this study was carried out in DMARD and anti-TNF resistant patients[210].

Tocilizumab is a humanised chimeric monoclonal anti-IL6 receptor antibody which binds to both the membrane-bound and soluble forms of the receptor thereby blocking IL6 activity. A Cochrane review published in 2010 summarised the current randomised clinical trials: 31% of patients reach ACR50 compared to 10% with placebo with 5% having side effects compared to 2% with placebo [211]. Initial trials of monotherapy were carried out in Japanese patients and showed that 78% of the 8mg/kg dosage group reached ACR20 while only 11% of the placebo group did. They also noted normalization of the CRP in 76% of the tocilizumab group. The percentage of patients reaching good to moderate DAS28 response was 91% compared to 19% in the placebo group. They also noted increases in cholesterol, and liver enzymes as well as decreases in white cell counts [212].

Anakinra is an IL1 receptor antagonist, a recombinant form of a naturally occurring molecule. A Cochrane review of five trials was published in 2009 and reported that 38% of patients on anakinra

achieved an ACR20 compared to 23% of those on placebo. They reported that although anakinra was a safe and modestly efficacious drug, the amount of improvement is less than that seen using other biologic therapies[213]. It is not currently approved by NICE for use in the UK for the treatment of RA other than in clinical trials.

Abatacept is not an anti-cytokine therapy. It is a fully human soluble co-stimulation molecule modulator, a CTLA4 protein attached to an IgG1 Fc fragment. It inhibits the co-stimulation of T cells. A Cochrane review of seven trials published in 2009 reported that 37% of patients achieved an ACR20 on abatacept compared to 17% placebo and that abatacept had moderate efficacy in the treatment of RA[40]. It is currently approved by NICE, for use in the UK for the treatment of RA especially in those patients with contraindications to rituximab.

Although there is a range of biologic therapies that can now be used for the treatment of rheumatoid arthritis, it should be noted that all the trials use an ACR20 response as being the primary endpoint, fewer patients reach an ACR50 or ACR70 which may be more consistent with a return to normal function rather than an improved disease state. It is also of note that some patients reach ACR20 on placebo, this reiterates the fact that some patients will be over treated if given biologics or indeed combination DMARD therapy. The biologic drugs also have their own side effects. Risk of infection is an issue for all of the biologics and some have individual risks such as worsening of interstitial lung disease with anti-TNF medication or reactivation of tuberculosis. There have been warnings regarding rituximab and progressive multifocal leukoencephalopathy caused by the JC virus in rare cases. This reiterates the need for careful patient selection and the need for patient stratification or biomarkers to predict those who require aggressive biologic therapy, those who would do well on methotrexate monotherapy or indeed those who would remit without treatment. There is also a clear need for head-to-head trials of biologics both within a group such as the anti-TNFs and between groups of biologics e.g anti-TNF *versus* rituximab.

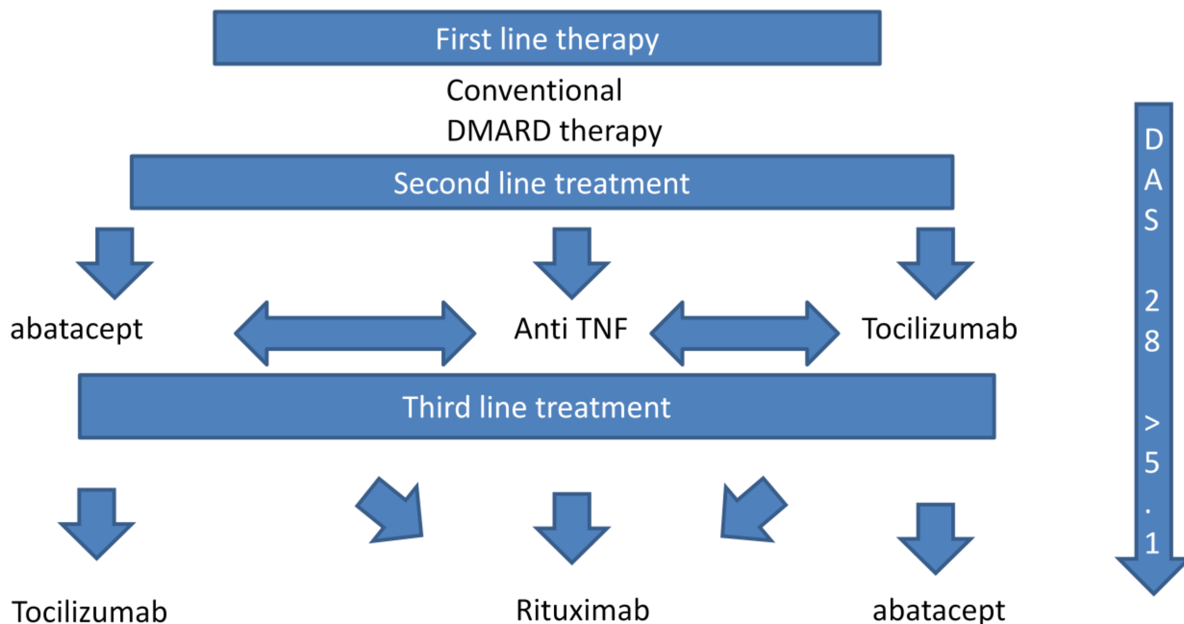


Figure 1.4: NICE guidance for treatment of Rheumatoid arthritis.

Conventional disease-modifying anti-rheumatic therapy includes drugs such as methotrexate, sulphasalazine and leflunomide. This should be started within 3 months of onset of symptoms.

If the DAS28 score remains above 5.1 at two visits one month apart after a trial of conventional DMARD therapy for at least 6 months with two DMARDS at standard dose then the patient may be placed on second line therapy, usually anti TNF such as etanercept, adalimumab, golimumab or certolizumab pegol but this can also be abatacept or tocilizumab. A successful therapy allows a drop in the DAS28 of 1.2. This is measured at three months. Anti-TNF therapy is usually used with methotrexate. Should the DAS28 remain above 5.1 the patient may be moved onto third line therapy, in most cases rituximab with methotrexate but if there are contraindications to rituximab use then tocilizumab, abatacept or an alternative anti-TNF may be used as third line therapy. Tofacitinib is being appraised by NICE for use as second line treatment.

1.1.5 Future Directions of Therapy

There are several different approaches to new therapies in RA. It is a widening field and as more is understood about the pathogenesis of RA, new pathways are opened for exploration.

New approaches include new formulations of old drugs, for example both abatacept and tocilizumab are at present given intravenously, but there have been studies looking at using them subcutaneously to increase patient acceptability and reduce overall costs of the drugs [214].

There has also been interest in new drugs for currently known targets. For example ocrelizumab is an anti-CD20 humanized antibody that targets an overlapping epitope to that of rituximab. Ofatuzumab also targets CD20 but targets a different epitope on the membrane proximal loop of CD20. REGB 88 is a fully human anti-IL6 receptor antibody which is currently in phase II trials which would be a competitor for tocilizumab.

Another approach is to target the same pathways currently in use at different points. Atacicept targets the B cell pathway by binding to Blys and APRIL, activators of B cell proliferation. In phase I trials only a trend to clinical improvement was seen and the phase II trial did not reach its primary endpoint in that there was no significant difference between the placebo and the atacicept arms. A significant decrease in immunoglobulins and rheumatoid factor was seen [215] which is consistent with the drug's presumed mode of action.

ALD 518 is a humanised monoclonal antibody that potently binds IL6 itself. It has been tested in a phase II study to determine its efficacy and safety in active RA with an inadequate response to methotrexate. In this study of 127 patients 82% reached an ACR20 on 320mg of ALD518 compared

to 27% on placebo, at week 12, and 44% reached DAS28 remission. Side effects of a rise in liver enzymes and serum cholesterol were noted [216].

There are also new drugs for new targets identified by pathophysiological processes in RA. One of these is to target the IL17 pathway since the importance of Th17 cells is better understood. Secukinumab is a full human monoclonal anti-interleukin 17A antibody. In a phase II dose finding study, the primary endpoint of ACR20 response was not reached although a secondary endpoint of CRP was found to be reduced in active treatment compared to placebo [217].

Another approach is to target the negative modulation of the inflammatory response by targeting the regulatory T cells known to be suppressed in active disease. A humanized agonistic monoclonal antibody BT061 has been trialled in phase II studies with potential benefits [218].

Some agents are closer to clinical use. These include the JAK 3 (Janus kinase) inhibitor tofacitinib which has recently been in phase III trials for use in conjunction with methotrexate in comparison to adalimumab and placebo. Results for the percentage of people reaching ACR20 on tofacitinib was favourably comparable to adalimumab and statistically significantly greater than placebo [219]. The same group has trialled tofacitinib as monotherapy versus placebo with 65.7% reaching ACR20 versus 26.0% in the placebo arm [220]. Both studies showed that tofacitinib had side effects of lowering the neutrophil count and raising cholesterol levels. The benefit of this medication is that it is oral and should therefore be more acceptable to patients. Also as it is a small molecule rather than a monoclonal antibody it is theoretically cheaper to produce than current biologic therapy. VX509 is also a predominantly JAK3 inhibitor that is currently in phase II trials [221].

Fostamitinib is a spleen tyrosine kinase inhibitor. It has been studied in phase II trials with varying results. Two studies showed a significant benefit to patients with 65% to 67% of patients reaching ACR20 compared to 32-35% of the placebo arm. Both studies reported diarrhoea and neutropenia as unwanted side effects of active treatment [222-223]. A third study of 219 patients did not reach the primary endpoint of a difference in ACR20 but this lack of efficacy was thought to be due to a high

placebo response from patients with a normal CRP but high ESR. There was a potential benefit seen if a subgroup of patients with a high CRP was analysed [224].

Another pathway that has been targeted is the p38 MAPK (mitogen activated protein kinase) pathway, however phase II trials of drugs including pamapimod have not reached the ACR20 primary efficacy end point. Other drugs in this group are SCIO-469, and VX702 [225]. They are MAPK inhibitors, it is thought that although these drugs are successful in animal models, it is possible that adequate dosing is an issue in humans or that the kinase is too downstream and other signalling pathways can take over the role of the MAPK.

Other new targets are the sphingosine 1 phosphate lyase molecule which is the final degradative step in the sphingolipid pathway. This has reached phase II trials. CCX354-C a chemokine receptor 1 antagonist is also under trial for safety and efficacy in humans[221].

1.1.6 Summary

There are multiple new pathways and targets for treatment but as in the case of current DMARD and biologic therapy, side effects, expected or otherwise remain an issue for the treatment of RA. There is no one size fits all medication for the treatment of RA and it may be that this is not an achievable goal. The area is still in need of cheaper, orally administered possibly small molecule drugs that have fewer side effects than those that are currently or soon to be available. As many new medications come out of the exploration of the pathophysiology of RA it is important to continue the search for new molecules and pathways as novel targets for pharmacological intervention.

The treatment of rheumatoid arthritis has evolved over years from the era of being unable to treat inflammation and ensuing deformation and disability adequately to the advent of DMARDs which made a difference to disease progression. This then went on to using the DMARDs in combination with synergistic effects to suppress disease more adequately [188]. The advent of biologic therapy has had a huge impact on the treatment of RA but so has the concept that there is a window of

opportunity for treatment of RA in early disease. Treating patients early (within 3 months to 1 year of onset of symptoms) and treating them to target (e.g a DAS28 of less than 2.6) has also been a major contributor to higher disease remission rates, quality of life and physical function rates [226]. Treating patients aggressively within the first few months of disease has also been shown to have long term effects on radiographic progression of erosions and consequently disease progression [189]. Treating patients during their window of opportunity leads to higher remission rates, drug-free and otherwise, and also better disease control so better responses to therapy [202-203]. There are, however, patients who will do badly regardless of treatment strategy – aggressive, early or otherwise [189]. It is especially for these patients that novel treatment paradigms and therapies are still required.

1.2 Novel Approaches: Endogenous Anti-inflammatory Pathways

One novel approach to the area of inflammation that has appeared over the last twenty years is the concept of resolution of inflammation. As discussed above, current therapies, including those under development and yet to reach the market, are devised to block specific pathways and processes operative in joint inflammation, with anti-TNFα and anti-CD20 therapies being archetypal. However, it is emerging that this is only half the story.

The story of inflammation begins with some sort of tissue insult, whether this be an infection, trauma or damage of some sort. The tissue secretes signals such as chemokines as a response to trauma. There are multiple molecules that determine a distress signal. This leads to an invasion of initially neutrophils (or eosinophils) which mop up any initial infection and call in macrophages which are also inflammatory. This is the role of the innate immune system in general, the activation of the innate immune system is immediate and it is only when the innate immune system is

overwhelmed that the adaptive immune system comes in to play. Once the neutrophils and macrophages have cleared the inflammation, the neutrophils undergo apoptosis, the macrophages leave and the tissue should return to its baseline un-inflamed state [227]. However this return to baseline is not, as was once thought, the absence of inflammatory insult but a positive process with its own armamentarium of mediators that bring the tissue from an inflammatory state back into its normal resting state.

There are several processes of clearance of inflammation that lead to the return to the normal state (catabasis)[228]. Exclusion of the primary insult is foremost as this will stop the synthesis of pro-inflammatory mediators. This would then halt further leukocyte infiltration. There is then the breakdown of the pro-inflammatory stimuli and also the cessation of production of these pro-inflammatory cytokines and chemokines and other inflammatory mediators such as MMPs and proteolytic enzymes. This is the process that is targeted by most current therapy. Then there is the removal of the inflammatory cell infiltrate. There are a couple of ways that cells may leave the site of active inflammation. This can be local death, usually by apoptosis followed by phagocytosis by macrophages (M2, phagocytic anti-inflammatory) that then leave the site by lymphatic drainage[229]. Some of these macrophages themselves may die by apoptosis and be cleared themselves by other macrophages. The important factor is that the ingestion of the apoptotic neutrophils by macrophages is non-phlogistic i.e. it does not induce an inflammatory response[230]. Some cells might re-circulate systemically and leave the site of inflammation [231]. The resolution phase of an acute inflammatory process can be defined in histological terms as the interval from maximum neutrophilic infiltration to the absence of neutrophilic infiltration and the resolution index can be defined as the time it takes for the infiltration of neutrophils to fall by half from maximum [227].

The resolution of inflammation can be targeted at various checkpoints during its course, to reduce the maximum peak of inflammation or to reduce the resolution index and speed up the catabasis of

the tissue. It can be targeted at the catabolism of the pro-inflammatory cytokines and chemokines, a pathway which is currently in use as a drug target. It can be targeted at the pro-inflammatory receptors, the Toll-like receptors and the NOD-like receptors. The apoptosis of the neutrophils can also be targeted ensuring that there is no over-expression of damaging enzymes or oxidative bursts in the healing tissue. A further target is to encourage the phagocytosis of neutrophils by macrophages to aid clearance of the tissue in a non-phlogistic fashion. Further, the clearance of the macrophages or the neutrophils themselves could also be a target to ensure clearance of inflammatory and potentially hazardous cells from the tissue[232]. Once these cells have been cleared and the local cells cease production of pro-inflammatory mediators because of completion of the inflammatory response (with ideally the removal of the primary insult) the tissue can repair and return to its resting state. See figure 1.5.

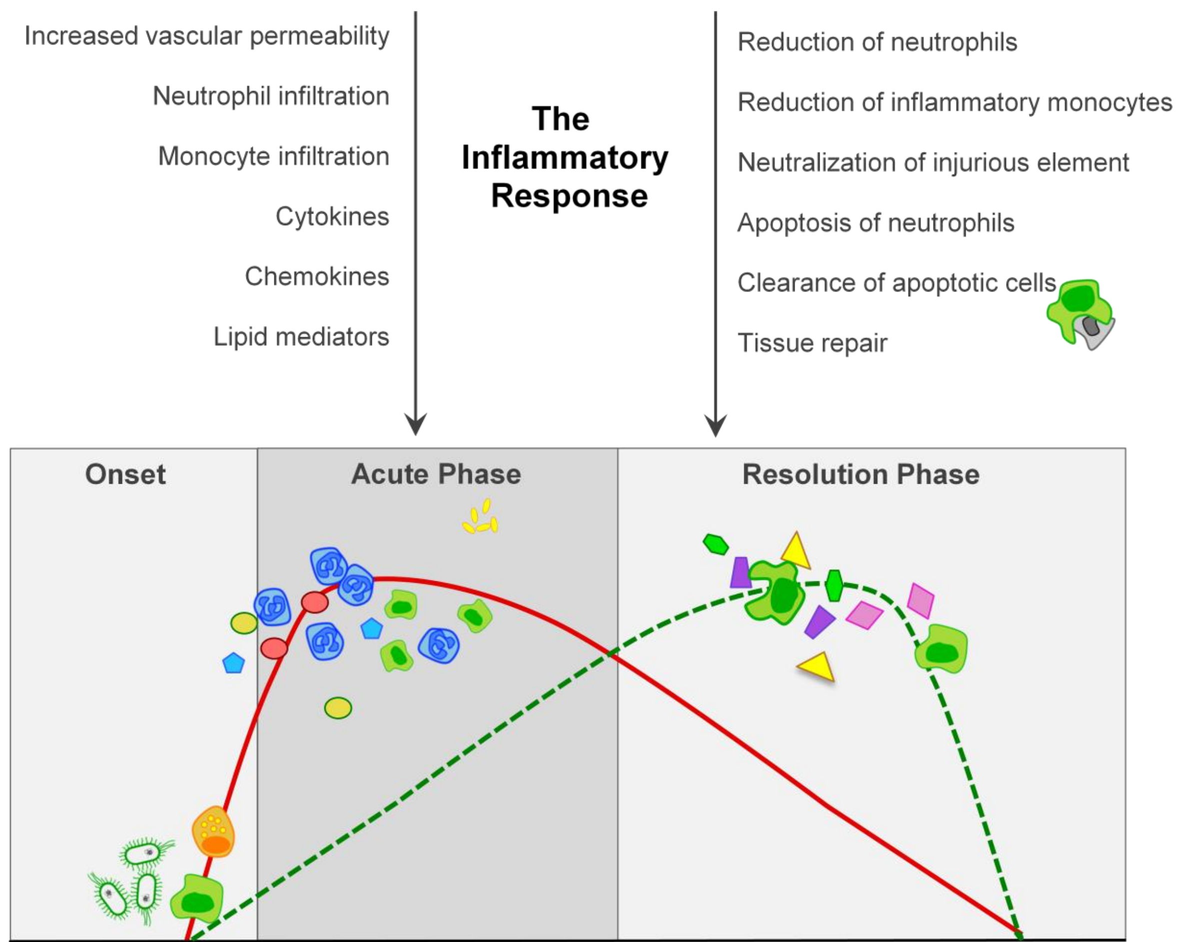


Figure 1.5: The inflammatory response.
Stimuli such as tissue injury or microbial invasion trigger the release of chemical mediators (complement, cytokines, eicosanoids, and other autacoids) that activate the leukocyte recruitment (onset). Neutrophils are the first cell type to be recruited, and then peripheral blood monocytes also accumulate at the inflammatory site (acute phase).These monocytes will eventually differentiate into a more phagocytic phenotype helping to neutralize the injurious element and to clear the tissues of apoptotic neutrophils (resolution phase). This proresolving macrophage (and the involvement of stromal cells cannot be excluded here either) orchestrate resolution, by releasing and/or responding to proresolving mediators. Eventually, fully differentiated cells clear the site of debris, dead cells, and bacteria and leave (via the lymphatics?). The previously inflamed tissue or organ regains its functionality, with return to homeostasis [1].

There are now known to be a host of mediators that are involved in the resolution phase of inflammation. Some of these are the lipoxins, resolvins, protectins and maresins. Others include heme oxygenase 1, annexin A1, galectins and melanocortins. Here, I will concentrate on melanocortins, the main focus of my thesis, though recognising that there are several mediators/pathways operative in resolution with a potential impact on human joint disease.

1.3 Melanocortin System

Introduction

In 1950, Philip Hench was awarded the Nobel prize for treating patients with rheumatoid arthritis with cortisone [233-234]. What is less well known is that he treated 6 patients with adrenocorticotropic hormone (ACTH) with good results. ACTH is a melanocortin. ACTH has subsequently been used in the treatment of inflammatory arthritides, mainly in the treatment of gout where it is still used in America today. A placebo-controlled trial of synacthen, a synthetic version of ACTH, in patients with RA showed an additional benefit in the treatment of RA (ACR50 response) which lasted three months after two injections on alternate days [235]. ACTH was evaluated in the treatment of gout patients with relative contraindications to NSAIDs and was found to have good effect over and above that which would be expected from the release of endogenous cortisol alone[236]. The later discovery of the pro-opiomelanocortin system with the melanocortins and melanocortin receptors (MCR) led this observation to have a mechanistic basis.

The Hypothalamic-Pituitary-Adrenal Axis

The hypothalamic-pituitary-adrenal axis is a complex multi-organ neuroendocrine system involved in a wide range of core body functions, particularly homoeostatic mechanisms. It has been studied in an immunological context because of the well-described anti-inflammatory effects of cortisol, an

endogenous glucocorticoid produced by the cortex of the adrenal gland. The effects of glucocorticoids in RA have been discussed above in Section 1.1.4.

Adreno-corticotropic hormone (ACTH) is short polypeptide released from the anterior pituitary gland. It is a tropic hormone for the release of cortisol from the adrenal gland. ACTH mediates cortisol release by binding to MC2R receptors in the adrenal gland. Release of ACTH from the pituitary is itself controlled by corticotropin-releasing hormone (CRH), produced by the hypothalamus. ACTH and cortisol both function in a negative feedback loop, themselves regulating CRH release [237].

Hench's initial interest in ACTH was as a potential therapeutic agent for RA. However, currently ACTH is also being investigated for its endogenous biological role within the hypothalamic-pituitary-axis in rheumatoid arthritis rather than as a treatment. ACTH has been investigated particularly in regard to its specific pharmacological actions on the melanocortin receptor MC2R, for which it is the only known endogenous ligand. However, it is also an agonist at other melanocortin receptors, with actions overlapping those of melanocortin-stimulating hormones. The biology of this melanocortin system is discussed in further detail below.

Comparisons between healthy subjects and patients with RA have suggested a difference in the diurnal variation in ACTH and cortisol with a temporal relationship with IL1b and TNF α[238]. The adrenocortical response to ACTH also seems to be impaired in RA patients, with the reduced production of cortisol and other steroid hormones [239-241]. There are many studies into the reaction of adrenal function to treatment with exogenous steroids[242].

Melanocortin receptors

The melanocortin system describes the five melanocortin receptors, their ligands, agonists and antagonists and the accessory proteins.

The melanocortin receptors (MCR: gene, MC_n: protein; IUPHAR database nomenclature, http://www.iuphar-db.org/) are a family of five small stimulatory G protein-coupled receptors, termed MC1R to MC5R, initially identified as neuropeptide receptors in mouse and human in the early 1990s [243-249]. They are encoded by intronless genes. Each receptor has seven transmembrane domains, with an extracellular amino-terminus and short cytosolic carboxy-terminus. The melanocortin receptors have been shown to dimerise, MC1R with itself and also with MC3R [250]. The melanocortin 2 receptor (MC2R) is the only one of the five which has been shown to require an accessory protein for translocation to the cell membrane[251]. It is also the exception in that it has introns. There are two accessory proteins identified for the MC2R either of which can help MC2R reach the cell surface. However the presence of the accessory proteins (MRAP1 or MRAP2) may have no effect or indeed inhibit the translocation of the other melanocortin receptors to the cell surface and their ability to be activated by agonists[252].The melanocortin 1 receptor (MC1R) has a C-terminal region required for translocation to the surface membrane [253], while mutations of the trans-membrane domains of the MC3R have been shown to have an effect on signalling and expression at the cell membrane [254].

All of the melanocortin receptors signal via the cyclic AMP (cAMP) pathway, activating adenylate cyclase resulting in increased intracellular cyclic AMP [255-257]. In certain cell types, activation has also been shown to mobilise calcium from intracellular stores (human MC1R, MC4R, mouse Mc1r, Mc3r, Mc4r, Mc5r) [258-260] . There is evidence from transfection studies that melanocortin receptor activation can interact with the MAPK signalling pathway and the phosphorylation of ERK (MC4R) [261]. Activation of the MC3R can result in interaction with more than one pathway depending on the dose of agonist given [255]. This can include protein kinase A mediated phosphorylation leading to a calcium dependent inositol triphosphate signalling (MC3R)[255]. Activation of the JAK/STAT pathway occurs when MC5R is stimulated in B lymphocytes[262] and activation of the MAPK/ ERK pathway has been shown to give a biphasic response when MC5R is activated in a transfected cell line or alternatively, monophasic response in adipocytes[263]. If the

cAMP pathway is blocked then MC1R can signal via intracellular calcium mobilisation or the inositol trisphosphate pathway [264-265]. MC1R has been shown to affect the NFκB pathway by protecting IκBα from degradation leading to a down-regulation of inflammatory cytokines and chemokines [257, 266].

Melanocortin Receptor Distribution

The melanocortin receptors are found in the brain and in peripheral tissues as shown below in Table 1.3. It is notable that MC1R, MC3R and MC5R are expressed on multiple cells of the immune system suggesting a role for them in inflammation. They have been shown on mouse macrophages localised to the peritoneum and rat macrophages in the synovium.[267-268] MC5R is expressed in macrophages, CD4 T cells and NK cells. Of note to the rheumatologist, MC1R and MC5R are present in human articular chondrocytes [269], and rheumatoid synovial fibroblasts. MC1R, MC3R and MC5R are all present at gene expression level in macrophages, dendritic cells, T and B lymphocytes as well as neutrophils. These cells are known to be part of the chronic immune response of rheumatoid arthritis and represent a source of effector cells for endogenous (or indeed exogenous) ligand. MC3R has been shown to be present by Western Blot in both rat neuronal[270] and chondrocyte cell lines[271]. I will concentrate on MC1R, MC3R and MC5R in this thesis.

Table 1.3. Distribution of the five melanocortin receptors. m=mouse h=human

	MC1R	**MC2R**	**MC3R**	**MC4R**	**MC5R**
Brain	Glial cells Astrocytes Pituitary Periaqueductal grey	n/a	Periventricular grey matter, hypothalamus Lateral septal nucleus, ventral tegmental area	Hypothalamus spinal cord, cortex, septal region, brainstem	Cortex cerebellum
Cell	Melanocytes (h) Keratinocytes (h) [272] Endothelial cells (h) Adipocytes (h) [273] Mucosal cells Chondrocytes(h)[269] Osteoblasts (h)[274] Macrophages (m) Monocytes (h) Dendritic cells (h) [275] Mast cells (h) Neutrophils (h) CD8 T cells (h) NK cells(h) B lymphocytes (h)[276]	Adipocytes (m) Osteoblasts (h)[274] Dendritic cells (h) Chondrocytes (h)[269]	Macrophages (m) CD4 Th cells (h) Monocytes B lymphocytes (h) [276] Dendritic cells (h) Neutrophils	Osteoblasts (h) [274] Dendritic cells (h)	Macrophages Sebocytes Chondrocytes (h)[269] CD4 Th cells NK cells [276] Mast cells(h) Dendritic cells (h) B lymphocytes(h)[262] Neutrophils Adipocytes
Organ	Brain, gut, skin, testis[243]	Adrenal cortex	Placenta, gut, brain, heart [245] Testis[243]	Brain	Peripheral tissues[246] Exocrine glands, testis [243]

Melanocortin Receptor Ligands

The ligands for the melanocortin receptors are derived from the pro-opiomelanocortin system. Pro-opiomelanocortin (POMC) is the precursor protein, from which prohormone convertases cleave the melanocortin stimulating hormones (MSH) alpha-, beta- and gamma-MSH and ACTH as well as non-melanocortin peptides, beta-lipotropin, gamma-lipoptropin and beta-endorphin. POMC and its related components were thought initially to be found only in the pituitary but now have been shown to have a wider distribution. The melanocortin peptides share a common amino acid sequence of His-Phe-Arg-Trp. Prohormone convertase 1 (also known as 3) is a serine type protease that cleaves POMC into pro-ACTH and β lipotropin. Prohormone convertase 2, another serine proteinase, cleaves pro-ACTH to ACTH1-17, corticotrophin like intermediate lobe peptide (CLIP) and αMSH. PC2 also acts on the γlipotropin hormone to produce βMSH and the N terminal peptide of POMC to produce γMSH[277]. Other proteases can also act in the POMC system such as the furin convertases and the paired basic amino acid cleaving enzyme 4 (PACE4). αMSH is the first 13 amino acids of ACTH [278] but it is acetylated at the N terminus and amidated at the C terminus. Thus, ACTH 1-17 generated by prohormone convertases is then cleaved by carboxypeptidase E to generate an αMSH precursor. N-acetyltransferase then acetylates ACTH1-13 and PAM (peptidylglycine alpha-amidating monooxygenase) amidates ACTH1-13. It is only functional when amidated and acetylated. Recently a further enzyme (PRCP, prolyl–carboxy-peptidase) has been shown to cleave αMSH and is thought to be a further mechanism of regulation by causing inactivation of the peptide[279]. (See figure 1.6)

An important issue is that of receptor selectivity or lack thereof. MC2R only responds to ACTH while the other melanocortin receptors respond to the melanocortin stimulating hormones to different degrees [280]. MC1R responds to αMSH>ACTH>γMSH as does MC5R. MC3R responds to γMSH=ACTH>αMSH. As well as endogenous agonists, there are endogenous antagonists in both the mouse and human systems [281]. These are known as Agouti and Agouti-related protein in the

mouse and agouti signalling protein (ASP) in the human. In mice, Mahogany and syndecan 3 have been shown to modulate agouti and AGRP. A further regulator is the mahogunin ring finger 1 which negatively modulates the function of αMSH, it is a negative regulator of MC1R and MC4R by competing for the MCR with the Gα subunit of the G protein [282].

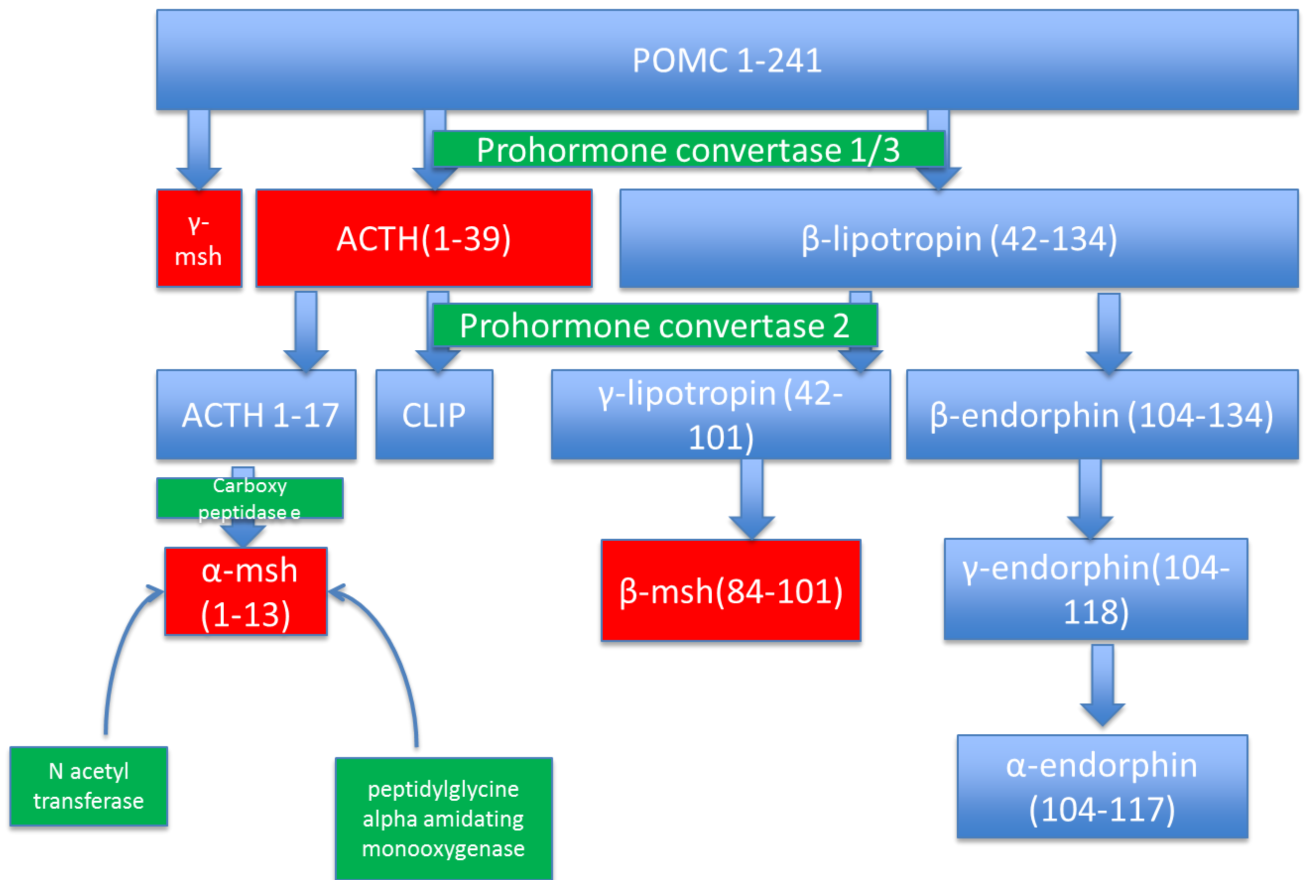


Figure 1.6: POMC cleavage network for the production of the melanocortin peptides.

Enzymes are green, melanocortin peptides are red, other peptides are in blue. POMC is cleaved by prohormone convertases 1 to ACTH and γMSH. ACTH is cleaved to ACTH 1-7 by prohormone convertase 2 to ACTH 1-17. This is then cleaved to αMSH by carboxy peptidase E.

1.3.1 Actions of MCRs

MC1R

MC1R was originally found on melanocytes and found to be the receptor responsible for skin pigmentation. MC1R is found on melanocytes and keratinocytes and has been shown to respond to UV light and damage by triggering the production of anti-inflammatory cytokines[283]. MC1R polymorphisms are related to melanoma with those with the red hair and freckles phenotype being particularly susceptible and this is thought to be due to loss of function of the MC1R. Those with MC1R loss of function mutations are more susceptible to UV damage [284].

MC1R loss-of-function mice have worse experimental colitis than wild type mice [285]. MC1R has been shown to be partially the target of treatment when autoimmune colitis is treated with αMSH derivatives (KPV)[286]. It has also been shown to be involved in ischaemia reperfusion injuries of the heart and blood vessels and the melanocortins can induce a situation similar to ischaemic preconditioning which is cardioprotective [287]. MC1R has been found in the duodenum of human subjects with celiac disease along with αMSH with more intense staining in celiac patients than normal subjects [288].

MC1R polymorphisms have been associated with multiple sclerosis [289] and response to inflammatory pain [290]. MC1R has been shown to be up-regulated in skin models of injury at the site of injury as has αMSH in both mice and humans[291]. There is also work on MC1R agonists and antagonists for treatment of vitiligo and hyperpigmentation respectively. Polymorphisms of MC1R may be important in the pathogenesis of vitiligo [292]. See figure 1.7.

MC3R

The majority of work at the moment into MC3R is to do with obesity and the effect that MC3R polymorphisms have on lean mass and fat mass. Mc3r knockout mice are of normal weight but their body composition is changed with greater fat mass and less lean mass. They have increased feed efficiency but do not have a change in food intake. They do not have fatty liver disease unlike Mc4r

knockout mice [293]. A similar phenotype is seen in humans with MC3R polymorphisms, although it is controversial whether there is a change in body composition. MC3R polymorphisms have not been monogenically linked to obesity unlike MC4R. The role of MC3R in obesity is thought to be complex and does not functionally overlap with MC4R[294].

There is data regarding involvement in ischaemia reperfusion studies in the Mc3r knockout mouse with an increase in myeloperoxidase (MPO) levels, cell adhesion and emigration in Mc3r knockout mice compared to wild type with superior mesenteric artery ligation. An Mc3r agonist attenuated the cell adhesion, emigration and chemokine generation secondary to superior mesenteric ligation but this effect was not seen in Mc3r knockout mice under the same conditions [295].

There is evidence to suggest the involvement of Mc3r in central pain processing pathways with possible nociceptive effects [296].

Polymorphisms of MC3R are associated with susceptibility to tuberculosis,[297] confirmed in a case control study in a South African population, but this study does not describe if this SNP association leads to loss of or gain of function in the MC3R gene.

MC5R

MC5R has been found in sebocytes and sebaceous glands and is instrumental in the secretion of sebaceous fluids. Antagonists to MC5R may have a role in the treatment of acne vulgaris as might MC1R[298] [299]. Mc5r may also have effects on aggressive behaviour in mice and deficiency of Mc5r has been shown to increase defensiveness and decrease aggression [300-301]. Mc5r has been implicated in sneezing due to pollen allergy with αMSH suppressing sneezing and IgA expression[302]. Mc5r expression increases with allergy induction in the trachea of mice. MC5R has also been found in the duodenal biopsies of coeliac patients along with MC1R [288].

Mc5r is thought to be important in the immune-privileged area of the eye, there has been evidence to suggest that Mc5r is necessary for the protection of mice from autoimmune uveitis by αMSH

[303]. Mc5r has been shown to be expressed in retinal pigment epithelial cells and ganglion cells of retinas and Mc5r knockout mice have severe retinal damage after induction of experimental autoimmune uveitis[304]. The protection of the retina was found to be dependent on Mc5r as was the induction of CD4 T reg cells.

1.3.2 Anti-inflammatory actions of melanocortin peptides

αMSH and related drugs

The anti-inflammatory actions of αMSH have been shown in cell lines, human cells, animal models of disease and also in the human population in stroke and dermatological diseases. αMSH was initially found to be an antipyretic, able to counteract the pyrogenic activities of IL6 and TNFα [305]. Manna and Aggarwal initially showed that αMSH suppressed pro-inflammatory cytokine production by monocytes in response to bacterial lipopolysaccharide by inhibiting NFκB translocation to the nucleus [257]. See figure 1.7. Not only does αMSH suppress proinflammatory cytokines, it can activate the production of anti-inflammatory cytokines such as IL10 from monocytes[306] and keratinocytes[307].

αMSH has been shown to be effective in several inflammatory models. It has been shown to be effective in an experimental contact dermatitis and suppresses the sensitisation and elicitation phase of the immune response. αMSH induces hapten-specific tolerance when given intravenously and is dependent on the induction of IL10[308]. Derivatives of αMSH, KPV and K(D)PT, can act in a similar fashion to αMSH. This finding has been taken forward in the nickel-induced contact eczema model in humans where a topical application of αMSH gave reduced disease[309]. αMSH has been used in a model of cutaneous vasculitis induced by LPS and was able to reduce vascular damage and haemorrhage by downregulating cell adhesion molecules crucial for the extravasation of leukocytes to the site of inflammation[310]. αMSH has been topically applied to an airways model of allergy

sensitised to ovalbumin, pro-allergic cytokines were found to be reduced and the anti-inflammatory action of αMSH was dependent on IL10[311].

Melanocortin agonists have been investigated in models of stroke encompassing mouse, rat and gerbil models and also global ischaemic models and local ischaemic models. Gerbils given 10 minutes of global cerebral ischaemia by the occlusion of both carotid arteries had reduced neuronal death, hippocampal damage and improved functional recovery if treated with an αMSH derivative with a longer half-life (NDP-MSH(Nle4, D-Phe7 αMSH)) between 3 and 9 hours after insult. Interestingly Mc4r blockade abrogated the effects of the NDP-MSH suggesting the activity of Mc4r in this process [312]. In human studies, αMSH has been used as a biomarker for predicting functional recovery from stroke [313].

αMSH and its analogues have also been used in preclinical models of renal injury and lung injury secondary to sepsis or other forms of injury. It has been shown in multiple models to ameliorate injury with improvements in histology and plasma creatinine compared to controls. AP214, an analogue of αMSH with a longer half- life, has been used in a sepsis induced kidney injury model. Treatment with AP214 was delayed until 6 hours after the onset of sepsis and still reduced damage to the kidney as assessed by histology, reduced the rise in serum creatinine, reduced tubular damage and liver function tests. It also showed that AP214 caused a rise in mean arterial pressure with reduction of bradycardia in septic mice. There were also effects on cytokines and evidence of reduced NFκB activation. There was also an improvement in survival rate in both lethal sepsis groups (improved from 0% survival to 10% survival) and sublethal sepsis groups (an improvement from 40% survival to 70% survival)[314].This has been reflected in other papers treating kidney injury models with αMSH up to 6 hours after injury with increased recovery and protection against renal injury[315].

αMSH also ameliorates liver inflammation even if given 30 minutes after onset, with decreased neutrophil infiltration and also decreased gene expression of chemotactic cytokines such as MCP1

and IL8 as well as TNFα [316]. Severe tissue injury can lead to acute respiratory distress syndrome as can renal ischaemic reperfusion injury with similar pathways activated in both organs. αMSH can inhibit lung oedema, decrease injury score and leukocyte infiltrate as well as decreasing serum creatinine and improving histology score in the kidney. Gene expression of TNFα and ICAM1 is reduced in the lung after treatment with αMSH. αMSH also prevented the destruction of IκBα, phosphorylation of p38 MAPK and decreased AP1 binding suggesting that αMSH works via various pathways to modulate the inflammatory response, rather than just inhibiting one method of dampening inflammation[317].

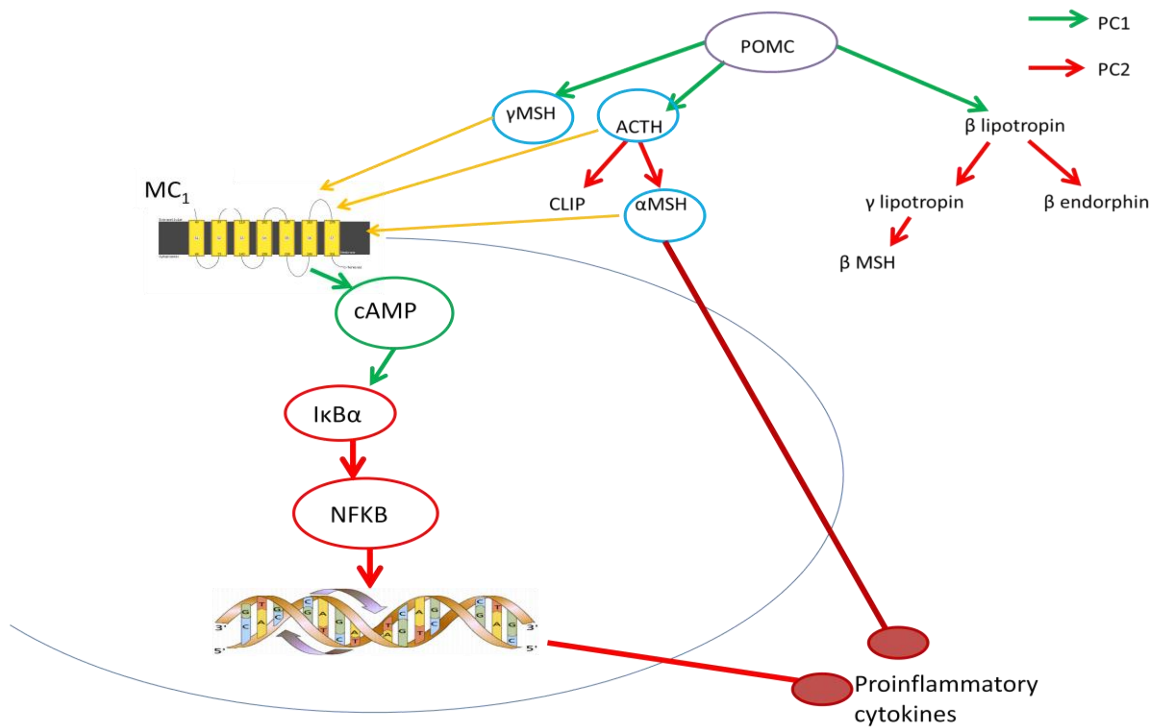


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Figure 1.7: Mechanism of action of MC_1.

Proopiomelanocortin, POMC, is cleaved by prohormone convertases 1 (PC1) to adrenocorticotropic hormone (ACTH) and then cleaved by prohormone convertase 2 to α melanocortin stimulating hormone, (MSH), and corticotrophin-like intermediate peptide (CLIP). γMSH is cleaved directly from POMC. The melanocortins known to act on MC_1 are circled in blue. These three ligands can act on MC_1 to increase the production of cAMP (cyclic adenosine monophosphate) to protect IκBα which has an inhibitory effect on NFκB and has a negative effect on proinflammatory cytokine production.

1.3.3 Melanocortin ligands as therapeutics

Given the data from preclinical models and the success of αMSH and its derivatives as well as de novo agonists, melanocortin ligands have been taken forward into clinical trials for further investigation in humans. The minimum peptide sequence from αMSH that can activate a receptor is a tri-peptide, (KPV). However, although active, it has a very short half-life and much work has been based on modifying αMSH and its derivatives to extend their duration of action. There are many research groups working on the melanocortin peptides to produce a preparation that is easy to deliver, specific to its target tissue and with a longer half-life. Table 1.4 summarises melanocortin agonists under development.

KPV and KPT are tri-peptides that have the anti-inflammatory properties of αMSH without the activation of skin pigmentation. They are small peptides and therefore easy to deliver locally, but have unfavourable pharmacokinetics in terms of short half-life and so other approaches have been used. αMSH has been used as the basis of αMSH-transferrin and NDP αMSH (MT-I) both of which have longer half-lives than αMSH. Other attempts to increase the half-life include novel delivery systems such as polystyrene beads attached to KPV[318] or dimers of peptides linked by a cysteine-cysteine linker[319].

The minimal sequence that binds all the receptors except MC2R is αMSH 6-9, adaptations of this peptide include fatty acylation of the N terminus and the production of peptides such as AP214 (contains six lysine residues at the N terminus) and HP228 (Ac-Nle_4Gln_5[D-Phe][D-Trp_9] αMSH). Cyclic peptides such as MT-II and SHU-9119 have potent anti-inflammatory activity, longer half-life and agonist activity (e.g. SHU-9119 is an antagonist at MC3R and MC4R and agonist at MC_5). γMSH has been used as the basis for MC3R-specific agonists. Examples include [D-Trp_8]γMSH and a novel cyclic peptide with selective MC3R activity[320].

Bristol Myers has produced, BMS 470539, an MC1R agonist for its anti-inflammatory actions. Melacure Therapeutics AB have produced the small molecule ME10501. AP214 is being tested by Action Pharma and Palatin Technologies are looking at bremelanotide in ischaemia reperfusion[321].

Two phase II trials have been completed for treatment with AP214. There is a study of prevention of kidney injury in patients (NCT01256372) and a study of the pharmacokinetics of AP214 acetate (NCT00903604) in patients undergoing cardiac surgery. No results have been published as yet.

Possible side effects of melanocortin receptor stimulating drugs are skin pigmentation for activation of MC1R, hypertension and behavioural disturbances and pan-melanocortin receptor agonists may activate the yawning and stretching reflexes stimulated by MC4R. There are also effects on food intake and energy metabolism, although for MC3R this could be a beneficial effect in the treatment of inflammation-related cachexia. An important aspect of drugs for MC3R would be the inability of the drug to cross the blood-brain barrier, where it would inhibit the action of the agonist on food intake and central processing of blood pressure[322] allowing only the peripheral anti-inflammatory actions to occur. It is important for the drug to be receptor specific e.g. targeting MC3R would avoid the skin pigmentation side effects, or using the active tri-peptides KPV and KPT would also avoid this. It would be important for the drug to be tissue or cell specific in a tissue to avoid unwanted effects in places other than the tissue of interest. The advantage of small molecule drugs over peptides is the increase in half life and the possibility that these drugs may be taken orally.

Melanotan II, a pan-melanocortin receptor agonist, is available on the internet and is used for tanning and as a sexual stimulant. There have been case reports of melanoma associated with its use[323] and also recently a case of rhabdomyolysis, renal dysfunction and agitation with overdose[324]. Bremelanotide has been used for the treatment of female sexual dysfunction, nasal administration caused hypertension leading to termination of the trial (Palatin Technologies).

Table 1.4 Current melanocortin agonists under potential development.

Compound	Classification	Activity	Effects	References
αMSH	Endogenous	Pan agonist	Anti inflammatory Skin pigmentation	[325]
βMSH	Endogenous	Pan agonist		
γMSH	Endogenous	Pan agonist with increased MC3R selectivity	Anti inflammatory	[326]
Agouti-related peptide	Endogenous	Antagonist, MC3R, MC4R	Skin pigmentation	
Agouti-signalling protein	Endogenous	Antagonist, MC1R,MC3R, MC4R	Skin pigmentation	
D Trp^8-γMSH	Synthetic peptide	Agonist mainly MC3R	Anti-inflammatory Arthritis	[327]
NDP-αMSH (MT-I)	Synthetic peptide	Agonist mainly MC3R and MC4R	Anti-inflammatory	[325]
MT-II	Synthetic peptide	Pan-Agonist	Anti inflammatory	[328]
KPV	Synthetic peptide	MC1R agonist	Anti-inflammatory	[325]
KPT	Synthetic peptide	Agonist	Anti-inflammatory	[325]
(CKPV)2	Synthetic peptide	Agonist	Anti-inflammatory on neutrophils	[319]
GKPV	Synthetic peptide on beads	Agonist	Anti-inflammatory on melanoma cells	[318]
AP214	Synthetic peptide	MC1R, MC3R, MC4R, MC5R agonist	Anti-inflammatory , Sepsis and arthritis	[314, 329]
HP228	Synthetic peptide	Pan agonist	Protective in acute models of inflammation and organ damage	[330]
AP1189	Small molecule	Positive allosteric modulator for MC1R	Beneficial effects on SIRS IBD and RA	Synact Pharma
BMS-470539	Small molecule	Agonist MC1R	Inhibits LPS induced cytokine accumulation in mice	[331]
ME10501	Small molecule	High affinity Mc1r, MC4R	Neuroprotective	[332]
Bremelanotide	Small molecule	Agonist MC1R and MC4R	Prevents organ dysfunction	Palatin Technologies
SHU-9119	Synthetic peptide	Antagonist MC3R and MC4R, agonist MC1R and MC5R	Experimental tool	
Scenesse	Synthetic peptide	Pan agonist	Vitiligo	Clinuvel
Czen001 and 002	Synthetic peptide	Agonist	Anti infection and anti-inflammatory	MSH pharma

1.3.4 Anti-inflammatory actions in arthritis models

Melanocortin agonists have been used in the treatment of some models of experimental arthritis. AP214 is a peptide modelled on αMSH and acts as a pan-agonist to all MC receptors. AP214 has been shown to be effective in a mouse model of inflammatory arthritis (KBxN serum), diminishing clinical score and inducing pro-resolving properties (increased phagocytosis) in macrophages [329]. Another preparation is the use of a protective cover releasing αMSH at sites of high inflammation. Carrier technology has been applied to α and γ MSH and used in the CIA (collagen-induced arthritis) and urate peritonitis model showing effective amelioration of disease [326]. This technique was initially used with IFNβ by surrounding it with the latency-associated protein (LAP) of TGFβ conjugated with matrix metalloprotease (MMP) sites. This has been shown to enable targeting of the cytokine to sites of inflammation where the cytokine can be released [333]. αMSH has been used to treat adjuvant arthritis in rats with an increase in body weight, reduction of the arthritis score and erosions [334]. POMC gene therapy has been used to treat adjuvant arthritis in rats with a reduction in paw swelling after adjuvant injection as well as thermal hypersensitivity[335].

Melanocortins have also been studied in models of gouty arthritis (see next section for their application in human gout). αMSH and a small peptide derivative ((CKPV)2) have been shown to inhibit the ability of monocytes to produce neutrophil chemo-attractants and activating compounds in response to urate crystals [336]. An abridged version of αMSH (ACTH4-10) has been shown to reduce neutrophils accumulation and inhibit macrophage activation with reduced chemokine KC release. This study also identified Mc3r as being responsible for the actions with the use of Mc3r/Mc4r antagonist SHU9119 and showing the absence of the MC4r transcript. Also the agonist melanotan II an MC3R/MC4R agonist gave similar results as the αMSH derivative [337-338]. In the same system MC3R specific agonists MTII and γMSH also inhibited neutrophil accumulation and macrophage cytokine and chemokines release. Furthermore Mc3r was found to be expressed in

C57BL6 mouse and SD (Sprague Dawley) rat peritoneal macrophages by Western Blot. Taking the investigation further into the joint itself, ACTH reduced joint size and inhibited neutrophils accumulation in rat knee joints injected with urate crystals. The same paper isolated rat Mc3r on synovial macrophages using gold staining electron microscopy techniques. SHU9119 abrogated the effectiveness of ACTH in this model. γ-MSH gave similar results in the same model [339]. More evidence suggesting that Mc3r is the important MCR in this model came with the efficacy of non-selective and selective Mc3r agonists in the amelioration of urate peritonitis in a mouse with a non-functional Mc1r. This was further supported by showing the presence of Mc3r in mouse peritoneal macrophages [340]. [D-Trp^{8}]γ MSH (an Mc3r specific agonist) afforded protection when used in the treatment of rat gout arthritis and but not when used in Mc3r-negative mice with urate peritonitis, again suggesting a role for Mc3r in this model of gouty arthritis[341]. The same compound has been shown to be efficacious in murine peritonitis despite a non-functional Mc1r, again guiding us to believe that Mc3r is important in this mouse model of gout[267]. Overall these experiments show the efficacy of ACTH and its derivatives, both natural and synthetic, in the treatment of mouse and rat models of gout and suggest that Mc3r is the receptor mediating these effects.

1.3.5 Melanocortins in human arthritis

Little is known about the effects of melanocortins on human arthritis other than the effects of ACTH in rheumatoid arthritis and gout which have been known about for a very long time [233-234, 342-347]. ACTH was re-evaluated in the 1990s for gout [236] and is still used in the USA for the treatment of gout in patients with contraindications to NSAIDs. Catania et al reported elevated levels of αMSH in the synovial fluid of rheumatoid arthritis patients and juvenile chronic arthritis patients compared to those with osteoarthritis (OA). They also showed that the levels of αMSH were elevated in synovial fluid as compared to serum of the same patients. The concentrations of αMSH were proportional to the degree of inflammation [348]. To remain in the joint, Bohm et al have recently described the presence of melanocortin receptors 1 and 5 in human chondrocytes and

have proposed a role for the melanocortins in the osteoarticular system[349]. Yoon et al showed a reduction in MMP13 production and p38 kinase phosphorylation when human chondrosarcoma cells were pretreated with αMSH and then stimulated with TNFα. This was independent of ERK and JNK kinases but dependent on p38 kinases and NFκB [350]. The treatment of TNFα activated human chondrocytes (cell line) with αMSH reduced the production of proinflammatory cytokines and increased the release of anti-inflammatory cytokine IL10 [351].

Given the knowledge from preclinical studies regarding the melanocortin receptors in experimental arthritis models, there is scope for extending this work into the field of human studies. Melanocortin agonists could be a novel therapeutic for the treatment of inflammatory arthritides. The presence of the receptor in the synovium could not only show that there is a target for these inflammation modulators in this tissue but the presence or absence of them or the pattern of expression could be used as a biomarker for disease, response to treatment or they could be a marker of a high or low inflammatory state.

Hypothesis of Thesis

Melanocortin receptors are present in rheumatoid arthritis synovium and may be used as a biomarker or as a target for therapy.

Aims of Thesis

To optimise tools for the characterisation of the melanocortin receptors in the rheumatoid synovium

To show the presence of melanocortin receptors in the rheumatoid synovium

To correlate the presence of the melanocortin receptors with a) clinical characteristics, b) immunophenotype of the synovium and c) cytokine gene expression

Chapter 2. Materials and Methods.

2.1 RNA extraction

The melanocortin receptor genes are intronless, meaning no exons are excised in the formation of the messenger ribonucleic acid (mRNA). This means that the cDNA that is created in vitro prior to the PCR reaction has the same sequence as the genomic DNA sequence. It is therefore very important to have a stringent method of RNA extraction with adequate exclusion of genomic DNA to ensure that no false signals emerge. RNA is also unstable and easily denatured and so the method of extraction had to be optimised for maximum purity, integrity and concentration of the end product. All RNA extractions were carried out on ice to reduce activity of RNases that may be present in the tissues and to reduce the likelihood of denaturation of the RNA.

Different extraction methods have been tested and optimised: RNA has been extracted using the RNeasy mini kit, the RNeasy plus mini kit, and using the Qiazol kit all as per the manufacturers' protocol (Qiagen, Hilden, Germany). Once optimised, the RNeasy mini kit was used for extraction of RNA from cell lines and a combination of the Qiazol kit and the RNeasy mini kit was used for tissue as detailed below. See table 2.3.

2.1.1 Qiazol extraction method

As tonsil tissue was readily available it was used to optimise the RNA extraction technique. Tonsil tissue was placed in tubes from the Precellys Lysing kit (with 1.4mm ceramic beads) containing 400 µl of Qiazol on ice. The tissues were disrupted with one pulse of thirty seconds on the Precellys®24 homogeniser. The homogenate was left for five minutes at room temperature and then 80µl of chloroform was added. The tubes were shaken vigorously for 15 seconds and then left to incubate at room temperature for 2-3 minutes. The tubes were centrifuged at 12000g at 4°C for 15 minutes and the upper clear phase transferred to a new 1ml tube. 200µl of isopropanol was added, mixed and incubated for ten minutes at room temperature. This was then centrifuged at 1200g for 10 minutes at 4°C. The supernatant was discarded. 400µl of 70% ethanol was added and mixed then centrifuged

at 7500g for five minutes at 4°C. The supernatant was removed, the pellet air-dried and then redissolved in 30µl of RNase free water.

Four forms of DNA exclusion treatment have been tested: on-column RNase-free DNase set treatment (Qiagen, Hilden, Germany), TURBO™ DNA-free kit (Ambion, Applied Biosystems, Warrington, UK), both on-column and TURBO™ DNase, and the DNA extraction column with the Qiagen RNeasy plus mini kit (Qiagen, Hilden, Germany). Each was used as per the manufacturers' protocol. See table 2.3.

HEK293 cells, U937 cells and THP1 cells were pelleted by centrifuging at 1200g for 5 minutes at room temperature. They were then washed with PBS twice, and centrifuged at 1200rpm for 5 minutes at room temperature. RNA was then extracted with the Qiagen RNeasy minikit with on column DNase (Qiagen, Hilden, Germany). Genomic DNA was further excluded using 5u of TURBO™ DNase as per the manufacturer's protocol.

2.1.2 Final RNA extraction protocol

Once optimised, the RNA extraction protocol for synovial tissue consisted of weighing approximately 15mg of tissue and placing them in 400µl Qiazol containing tubes from the Precellys Lysing kit on ice. The tissues were disrupted with one pulse of thirty seconds on the Precellys®24 homogeniser. The homogenate was left for five minutes at room temperature and then 80µl of chloroform was added. The tubes were shaken vigorously for 15 seconds and then left to incubate at room temperature for 2-3 minutes. The tubes were centrifuged at 12000g at 4°C for 15 minutes and the upper clear phase transferred to a new 1ml tube. 200µl of isopropanol was added, mixed and incubated for ten minutes at room temperature. This was then centrifuged at 1200g for 10 minutes at 4°C. The supernatant was discarded. 400µl of 70% ethanol was added and mixed then centrifuged at 7500g for five minutes at 4°C. The supernatant was removed, the pellet air-dried and then re-dissolved in RNase free water. The RNA was then passed through the RNeasy mini kit (Qiagen) including the on-

column RNase free Dnase digestion. The samples were then treated with 5U of TURBO™DNase from the TURBO™DNA-free kit prior to use for reverse transcription. See table 2.3.

For experiments with synovium 300ng of RNA was treated with TURBO™ DNase, with 200mg reverse transcribed for the positive sample and 100ng for the negative control.

To test for integrity, 1µl of RNA was run on a 0.8% agarose gel in RNA free conditions at 80V. Integrity was confirmed by the visualisation of two bands at a 2:1 ratio representing 28s and 18s ribosomal RNA. RNA was quantified with the Nanodrop 1000 spectrophotometer (Labtech International) measuring absorbance at 260nm. Purity was assessed by the evaluation of the 260:280 to account for protein contamination and the 260:230 ratio for contamination with reagents such as phenol.

2.1.3 cDNA synthesis

RNA was used to synthesise cDNA using the Invitrogen Thermoscript RT-PCR system (Paisley, UK) as per the manufacturer's protocol. Oligo dT's were used as primers. The RNA was denatured for 5 minutes at 65°C. The cDNA was synthesised at 60°C for 1 hour. The sample was incubated with RNase H for 20 minutes at 37°C to eliminate residual RNA. Samples were diluted with RNase free water to a concentration of 4.4ng/µl in preparation for real time polymerase chain reaction. cDNA was diluted to a concentration of 1.1ng/µl for cytokine analyses. This technique assumes the linearity of Thermoscript reverse transcription in that 1 molecule of RNA produces 1 molecule of cDNA, whatever starting amount is used. See table 2.4.

2.2 Qualitative Polymerase Chain Reaction

Reverse transcriptase polymerase chain reaction (PCR) is a powerful technique that enables the investigator to visualise the expression of a gene in the form of a specific sized band on an agarose gel. It allows the amplification of very small numbers of messenger RNA and is specific for the gene involved. As RNA is very unstable and easily denatured, RNA is converted to complementary DNA (cDNA) which is more stable. 1 molecule of RNA generates 1 molecule of cDNA. However, in the case of the melanocortin receptors cDNA has the same sequence as the genomic DNA sequence so it is important that the signal from genomic DNA is excluded as otherwise there will be a false positive signal from every sample tested. The polymerase chain reaction starts as an exponential reaction and then once the substrates and reagents start depleting has a linear phase and then reaches a plateau phase when no more product is made. Qualitative PCR is analysed (agarose gels) in this later phase (plateau) when the variability is higher. However, as I will explain later, real-time PCR uses the exponential phase for the analysis as the precision is higher and hence can be used for quantification. Each cycle doubles the previous cycles' products leading to an exponential increase in product (2^n). Polymerase chain reaction for the melanocortin receptors must always be run with an adequate set of negative controls. That is a control which contains the same reagents but that has not been reverse transcribed and so contains only genomic DNA and no cDNA. Ideally these samples would display no band, however it is very difficult to completely exclude all genomic DNA and therefore whether or not there is a band is dependent on the number of cycles the reaction is run for.

For the PCR reaction the protocol used was: 1µl of cDNA, 10µl of Thermoscientific 1.1 x Reddy Mix PCR Master Mix (Epsom, UK) and 1µl of Qiagen Quantitect primers (Crawley, UK) for MC1R, MC3R, MC5R or GAPDH (glyceraldehyde phosphate dehydrogenase) were used. Conditions for PCR were

95° for 5 minutes, 40 cycles 95° 30 seconds, 55° 30 seconds, 72° 30 seconds, then 72° 10 minutes. See table 2.7.

Custom primers were also designed using Beacon Designer 7.7 to the gene sequence contained in commercial pcDNA vectors from the University of Missouri. BLAST searches and design strategy was automated as part of the primer design by Beacon Designer 7.7. Sequences of these primers are seen in table 2.6. Multiple conditions were used for the custom primers to optimise the reaction. Eventually conditions were set as above. 1µl of cDNA, 9µl of Thermoscientific 1.1 x Reddy Mix PCR Master Mix, and 1µl each of forward and reverse primer were combined, the Qiagen GAPDH primer (1µl) was used as an endogenous control. Bands were visualised using GelRed™ (a fluorescent nucleic acid dye) run at 60V on a 3% agarose gel. See table 2.6.

2.3 Quantitative Polymerase Chain Reaction

Quantitative PCR has revolutionised the detection of gene expression and allows the relative or absolute quantification of RNA samples depending on the technique used. Whereas qualitative PCR asks "is the gene expressed or not?", quantitative PCR can measure "how much of the gene is expressed?" A TaqMan® gene expression assay consists of a forward and reverse primer but also a probe with a high energy fluorochrome reporter and a low energy quencher tag. This assay uses fluorescent resonant energy transfer technology. As the Taq polymerase moves 5' to 3' along the cDNA it reaches the probe and hydrolyses the probe so that the fluorochrome is no longer quenched by the quencher and therefore fluorescence occurs (See figure 2.1). Each copy of the cDNA product formed by the Taq polymerase produces fluorescence and this fluorescence can be measured. The increase in reporter signal is directly proportional to the number of amplicons generated.

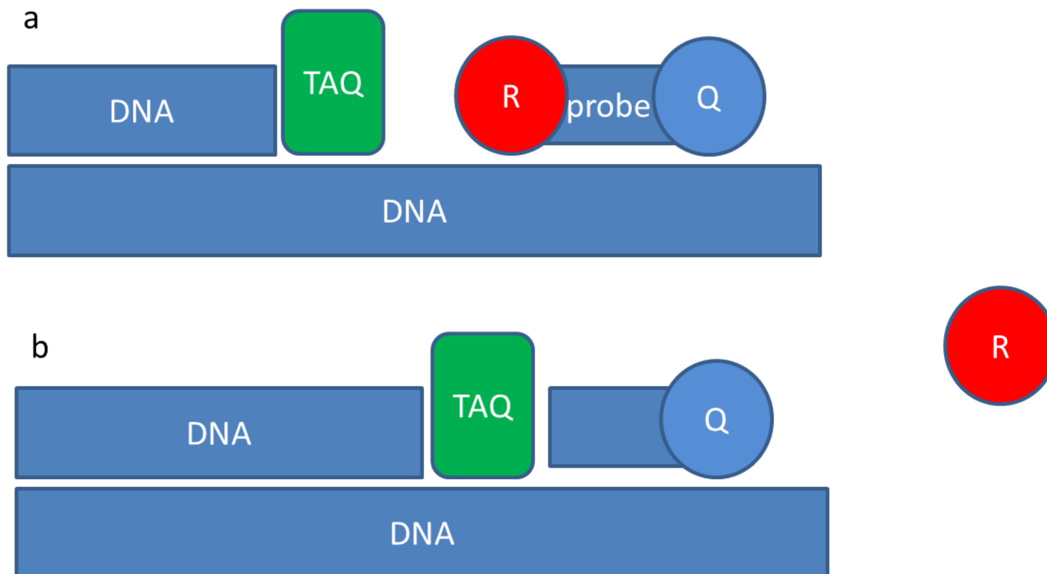


Figure 2.1 The mechanism of fluorescence in TaqMan® gene expression assays.

A) shows the Taq polymerase extending the DNA 5' to 3. R is a reporter probe attached to a probe that sits in the path of the Taq polymerase. Q is the quencher molecule attached to the other end of the probe. No fluorescence occurs as the quencher is in close proximity to the reporter. B) shows the Taq polymerase cleaving the probe releasing the reporter allowing fluorescence to occur as it loses its proximity to the quencher molecule.

Once this fluorescence reaches a certain threshold this can be given a number or the C_T (cycle threshold) value. The threshold is the level of detection or the point at which as reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification. This gives the most accurate reading. The threshold can be set automatically by the SDS 2.0 software or manually by the operator. This value is a reflection of the number of cycles that it takes to reach the threshold of fluorescence.

Absolute copy numbers can be measured if there is a standard curve placed on the plate with a known amount of cDNA copies in the reaction. The other option is relative quantification where an equation is used to calculate the relative expression of a gene compared to a calibrator sample, which may be comparing a sample before and after treatment or to give a comparison between normal and diseased tissue.

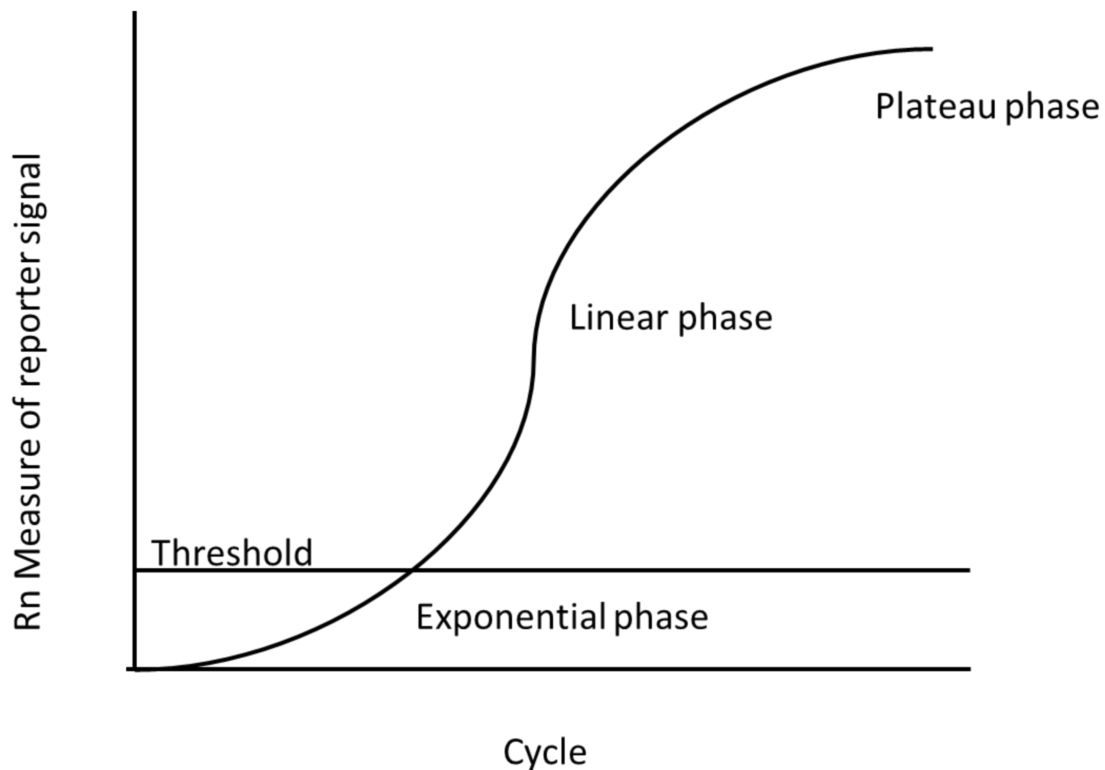


Figure 2.2 Amplification plot for real time PCR.

The threshold is the level of detection at which the reaction reaches a fluorescence intensity above background. The number of cycles can be counted to reach this threshold. This is known as the C_T value.

The ability to quantify the level of gene expression of the melanocortin receptor is very important. As stated previously it is very difficult to ensure complete exclusion of genomic DNA and therefore the presence or absence of the gene expression of interest must always be compared to a negative control of non-reverse transcribed RNA. The difference in cycle threshold number between the positive and negative sample can give an idea of how much of the signal is genomic DNA and how much is actual messenger RNA. For the purposes of this thesis, a sample was only considered to be positive (that is the melanocortin gene was expressed) if there was a three cycle difference between positive and negative samples which is equivalent to an 8 fold difference between numbers of amplicons.

The protocol for real-time PCR was as follows. A 364 well plate (MicroAmp, Applied Biosystems, Cheshire, UK) was used. 0.5µl of 20x TaqMan® Gene expression assay (Applied Biosystems), 4.5µl of cDNA of known quantity and 5.5µl of TaqMan® Universal Master Mix II, no UNG(uracil DNA glycosylase) (Applied Biosystems, Foster City, USA) was used as a finalised protocol. For the melanocortin receptors the final reaction contained 20ng of RNA equivalent. Conditions were 95°C for 10 minutes, then 40 cycles of 95° for 15 seconds and 60° for 1 minute. The PCR reactions were run on the ABi prism 7900HT (Applied Biosystems) thermocycler and data was analysed using SDS 2.0 software. See table 2.5.

2.4 Cell Culture

Cell lines U937, THP1 and HL60 were cultured for use as positive controls for MC receptors by Western Blotting for the tissue of interest. HEK293 cells were cultured for transfection purposes.

Monocytic cell lines U937 cells, THP1 cells and HL60 cells were cultured in RPMI 1640 (Sigma, Pasching, Austria) with 1% non-essential amino acids (Gibco 11140, Invitrogen, Paisley, UK), 1mM L glutamine (Sigma G7513, Poole, UK), 10% fetal bovine serum, 100units/ml units of penicillin and 0.1mg/ml of streptomycin (Sigma P4333, Poole, UK) at 37°C and with 5% CO_2. HEK293 cells (human embryonic kidney) were cultured in DMEM (Sigma, Pasching, Austria) with 10% fetal bovine serum and 100units/ml units of penicillin and 0.1mg/ml of streptomycin at 37°C, with 5% CO_2.

A cell viability assay was carried out to test for the concentration of antibiotic that caused HEK293 cells to die. 1x 10^5 cells were placed in 96 well plates. Each plate had eight replicates of reducing concentration of G418 (Invitrogen, Paisley, UK) a macrolide antibiotic, 1 negative and 1 positive control (without cells or without antibiotic) The plates were read at 1, 2, 5 and 10 days. Cell viability was assessed using cell counting using a Neubauer haemocytometer and trypan blue (Sigma, Poole, UK) as well as the Alamar blue assay. Alamar blue (Invitrogen, Paisley, UK) is a non-toxic dye that is converted by living cells from blue to red (rezasurin to resorufin). 200µl of Alamar blue is added to each well and incubated at 37°C for between 1 and 4 hours. An initial experiment with Alamar blue, showed greatest change in colour at 4 hours and this time-point was used for all further cell viability experiments. After 4 hours of incubation with Alamar blue, the plate was read at 570 and 595 nm wavelengths. This ratio was plotted against antibiotic concentration.

2.5 Bacterial transformation

Bacteria were transformed for the production of a large stock of plasmid prior to the transfection of HEK293 cells. The pcDNA 3.1 vector (Missouri S and T cDNA Resource Center, www.cDNA.org) has an ampicillin resistance site that was useful to select out the bacteria that had been transformed. The vector also had a neomycin resistance site useful for selecting out the cells once transfected. The inserts with the melanocortin receptor sequences were initiated with the sequence for three HA tags. The HA tag is a short peptide from the haemmaglutinin molecule found in the influenza virus and is used as a label for the detection of attached proteins. This sequence is not found in humans and there are many commercial antibodies available for the detection of the HA tag.

TOP-10 cells (Invitrogen, Paisley, UK) are commercially available competent Escherichia coli bacteria. 0.02µg of plasmid (Missouri S and T cDNA Resource Center, www.cDNA.org Figure 2.3 and 2.4) was added to 30µl of TOP10 cells and placed on ice for 10 minutes.

The tubes were then incubated at 42° for 1 minute and placed back on ice for 5 minutes, 300µl of preheated broth was added (Invitrogen, Paisley, UK) (20g in 1 litre)) and agitated for 1 hour at 37°C. 200µl of bacteria was added to 25ml of broth in a Falcon tube and incubated overnight at 37°C. 1ml from the Falcon tube was then added to 200ml of broth with ampicillin (0.05mg/ml) and incubated overnight at 37°C.

The plasmids were purified from the culture using the Qiagen Plasmid Midi Kit (Hilden, Germany) as per the manufacturers' instructions.

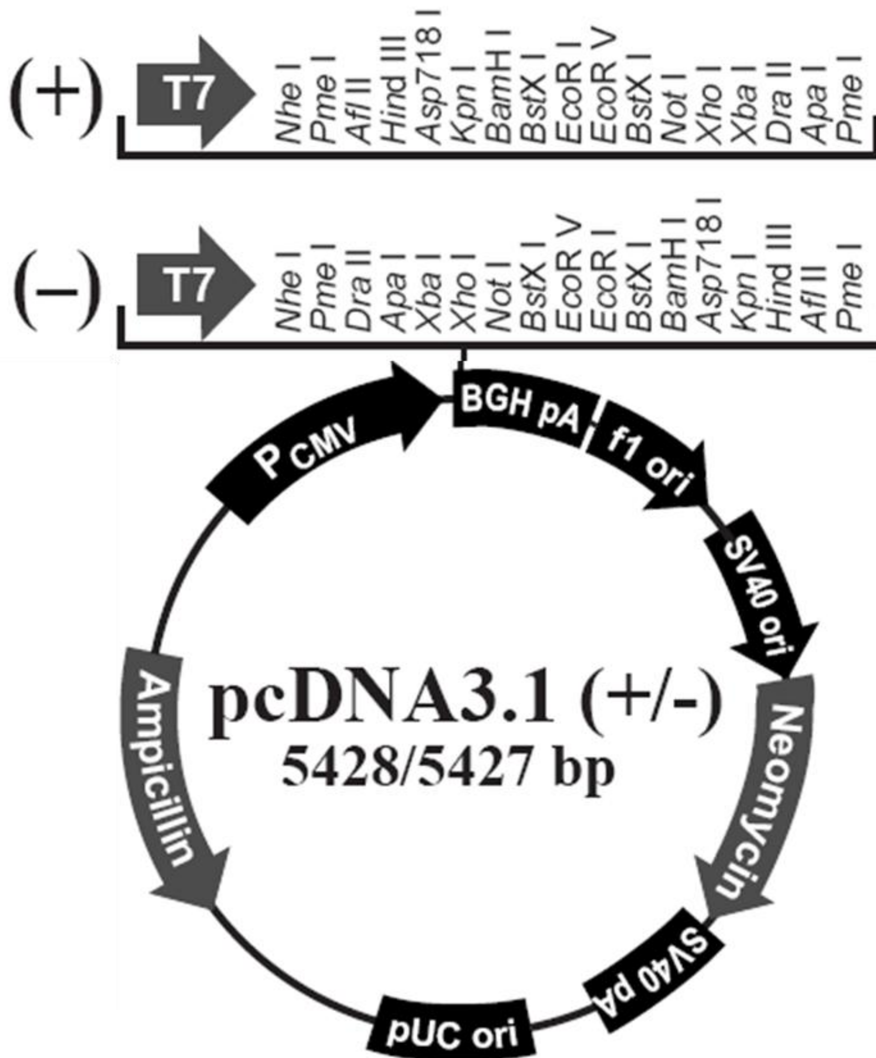
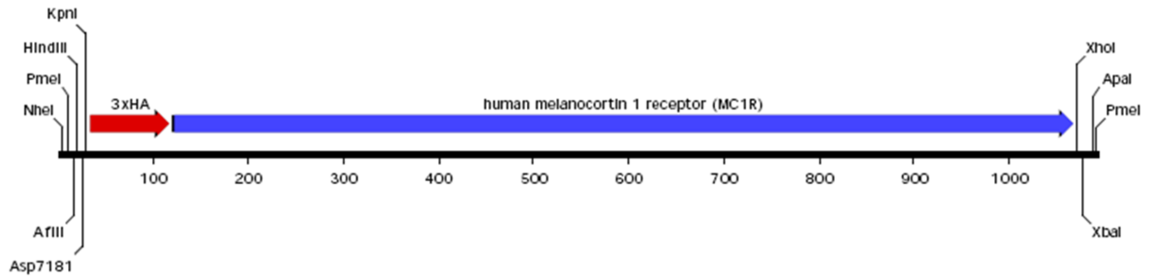
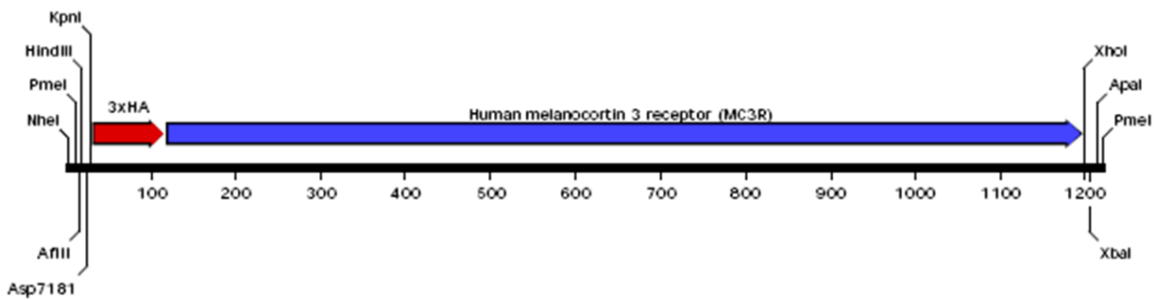


Figure 2.3 Map of pcDNA 3.1 vector including promoter sites (T7, SV40, CMV), origin of replication sites (f1, pUC), resistance inserts (ampicillin and neomycin) and restriction enzyme sites (Pme1 and following).





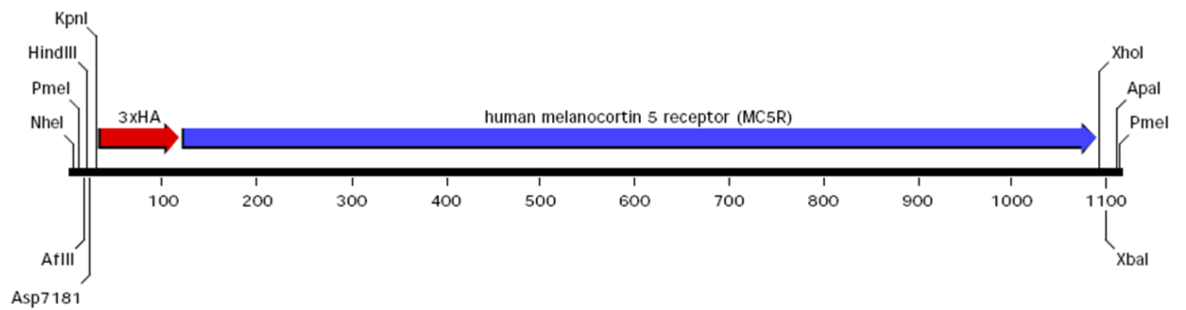


Figure 2.4 Map of inserts placed into vector in previous figure showing position of insertion and position of HA tags at N terminus of protein. Restriction enzyme sites are also shown.

2.6 Restriction Digest of Plasmids

A restriction digestion was carried out to check for the presence of the insert in the plasmid after bacterial transformation. Plasmid DNA was quantified using the Nanodrop 1000 spectrophotometer machine at 260nm and 1µg of DNA was digested with PmeI restriction enzyme (NEB, Ipswich, UK). 0.5µl of PmeI with 0.2µl of bovine serum albumin, 2µl of NEB buffer 4 (NEB, Ipswich, UK) and distilled water, to a final volume of 20 µl, were incubated for an hour at 37°C. 1µl of the digested plasmid was run on a 0.8% agarose gel. Two fold dilutions of the digested plasmid were also run to confirm the concentration of the plasmid using the Quickload NEB 1kb DNA ladder (NEB, Ipswich, UK) as reference. Sequences of the plasmids were also confirmed by Sanger sequencing using the Big Dye 3.1 by the Genome Centre, QMUL.

2.7 Transfection of HEK293 cells

HEK293 cells are a commonly used cell line for transfection experiments and there are reports in the literature of the transfection of HEK293 cells with melanocortin receptor constructs [256, 352]. The transfected HEK293 cells were created to be used as positive controls for experiments with human synovium.

$1x10^6$ cells were cultured in 10 ml of medium overnight on 10 cm plates (Corning, UK). At room temperature, 500µl of sterile 2x Hepes buffered saline (see solutions section) was added to 20µg of plasmid, then made up to 950µl with sterile water. 50µl of sterile 2M calcium chloride was added dropwise to this mix. Medium was aspirated from the cells and 1ml of plasmid mixture was added. The plates were incubated for 30 minutes, tilted every 5 minutes at room temperature. Initial transfection was carried out with a glycerol shock at 24 hours, however, the cells did not survive this procedure, therefore, the plates were incubated for 6 hours at 37°C and then glycerol shocked using the following protocol. The medium was aspirated and the cells were glycerol shocked with filter sterilised 10% glycerol in serum free medium for 4 minutes. The plates were then washed twice in normal medium and then left to incubate in 10mls of medium overnight at 37°C. After 24 hours, the medium was changed to 0.625mg/ml G418 containing medium. Medium was changed twice a week until cells grew to confluency.

2.8 Western Blot

Western Blot technique enables the detection and identification of proteins. It uses gel electrophoresis to separate denatured proteins by the length of the polypeptide chain. It can also separate native proteins by 3D structure. Sodlium dodecyl sulphate, SDS, an anionic detergent, in the polyacrylamide gel keeps polypeptides in a denatured state after they have been reduced by a reducing agent such as dithiothreitol (DTT). The reducing agent removes the secondary and tertiary structure of the protein by removing disulphide bonds. Sampled proteins become covered by negatively charged SDS and move through the polyacrylamide gel according to their molecular weight towards a positively charged electrode. The percentage of the acrylamide gel determines the resolution of the gel. The greater the percentage of acrylamide the better the resolution of lower molecular weight proteins. 12% resolving gels were used throughout this thesis with 4% stacking gels for adequate visualization of the 34-45kDa bands of the melanocortin receptors. 10% and 12% resolving gels are routinely used for SDS polyacrylamide gel electrophoresis, SDS-PAGE.

The melanocortin receptors are G protein-coupled receptors with seven transmembrane domains and therefore should be found in whole cell lysates. Synovial tissue was the tissue of interest. The transfected HEK293 cells were used as a positive control for final experiments with synovial tissue as commercial preparations of human brain or placenta as an alternative positive control were expensive and difficult to source. The HEK293 cells were also an abundant source of protein. See table 2.2.

Transfected HEK293 cells were washed in cold PBS and lysed in 500µl of commercial RIPA buffer (Sigma, Poole, England) with protease inhibitor cocktail (Roche, Mannheim, Germany). The lysates were kept agitated on ice for 5 minutes and then the lysate was centrifuged for 15 minutes at 14000g to pellet cell debris. The supernatant was transferred to a new tube and aliquotted into 100µl samples and frozen at -80°C.

The protein concentration was assayed using the Bradford assay (Biorad, Munchen, Germany) as follows. A standard gradient of bovine serum albumin (Sigma, Poole, England) (0.05µg/ml to 0.5 µg/ml) at reducing concentrations was set up on a 96 well plate. Several dilutions of the sample (1, 1/5, 1/10) lysate were also placed at a volume of 10 µl on the same plate. To each sample 200µl of Bradford solution was added and measured using the plate reader at 595nm. The optical density of the sample was then compared to the standard curve to calculate the unknown protein concentrations.

Known concentrations of proteins were separated with the use of 10% denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (National Diagnostics, Protogel system, Hull, England) under reducing conditions with dithiothreitol (DTT) (conditions for incubation are given with each figure legend) and were then transferred onto polyvinylidene difluoride membranes (Immobilon-P, Bedford, USA). The transfer was a wet transfer in a methanol-containing transfer solution at 4°C (see solutions section) run at 125V for at least one and a half hours. The membranes were then blocked in 5% non-fat milk (Marvel, Dublin, Ireland) PBS -0.1% Tween® 20 (Sigma, Poole, England) for half an hour and then incubated with the primary antibody in 5% non-fat milk PBS 0.1% Tween® 20 at 4°C on an agitator. The conditions for incubation are to be found with each figure legend as the conditions differed depending on the antibody used. The antibodies for the melanocortin receptors were tested at multiple concentrations and incubated overnight with the membrane. (See table 2.2 for all antibody dilutions)

The membranes were then washed in PBS 0.1% Tween® for 5 minutes 6 times and then incubated with the secondary antibody diluted at the manufacturers recommended dilution in 5% non-fat milk PBS 0.1% Tween® 20 for an hour at room temperature. The membranes were washed in PBS 0.1% Tween® 20 for 5 minutes 6 times with a final wash in TTBS (Tween®, tris buffered salt solution) prior to development.

Two substrates were prepared (details are in the solutions section), a hydrogen peroxide containing Tris based solution and a luminol and P coumaric acid Tris based solution. Fluorescence is seen when luminol is oxidised in the presence of hydrogen peroxide and horse radish peroxidase. Under red light conditions these were mixed in a 1:1 ratio to produce an enhanced chemoluminescence solution (ECL). 1 ml was placed on the membrane. A transparent plastic sheet was used to cover the membranes, bubbles were minimised with a roller. The films were then placed on top of the plastic sheet and exposed for 1, 5 and 20 minutes.

Films were developed using a Konica Minolta medical film processor SRX101A.

2.9 Immunohistochemistry

Immunohistochemistry enables the labelling of proteins in cells thus enabling the investigator to both identify the presence of a specific protein and also identify if it is associated with a specific cell type or area of tissue. The technique used throughout this thesis is of precipitation of chromogen giving a dark brown colouring to areas labelled with antibody. Human synovial tissue was the tissue of interest, tonsil was used as a control as it is a source of a large variety of immune cells as well as keratinised epithelium and salivary gland. Human placenta and brain (pituitary), as sources of MC3R were also used as positive controls. Skin was used as a positive control for MC1R. The labelling of immune cells with CD3, CD68, CD138 and CD21 is well-established in human synovium and this laboratory, however there is no immunohistochemical data regarding the MC3R in the literature and methods were set up firstly to optimise the protocol and secondly to optimise the antibodies for the detection of the melanocortin receptors. It is of note that there are no monoclonal antibodies available for purchase for the melanocortin receptors of interest. Although the creation of monoclonal antibodies was discussed, and costed, the time frame and expense precluded this route and therefore only commercially available antibodies specific for human MC3R were tested. See table 2.8.

2.9.1 Method development for melanocortin receptors

The following method was optimised for the melancortin receptors as they were all polyclonal antibodies which required the use of the Vectastain®ABC system to biotinylate the antibodies to enhance the detection of antibody binding. An unlabeled antibody is applied then a biotinylated secondary antibody followed by a preformed Avidin and Biotinylated horse radish peroxidase Complex. The horse radish peroxidase (HRP) is then visualised by the addition of diaminobenzidine.

Paraffin sections of synovial tissue and tonsil were sliced at 3 micrometre intervals and placed on slides and left to dry overnight. The slides were immersed in xylene (Fisher Scientific, Loughborough, UK) twice for ten minutes, then ethanol (Fisher Scientific, Loughborough, UK) twice for five minutes, then rinsed in distilled water. The slides were then placed in pre-heated (95°) target retrieval solution (Dako, Ely, UK) and incubated for 45 minutes at 95° in a waterbath.

The slides were allowed to cool for twenty minutes and then transferred to a slide jar for washing in Tris buffered saline (TBS-see solutions section) for five minutes once.

After washing the slides were dried, the tissue isolated with a hydrophobic pen (Dako, Glostrup, Denmark) and 100µl of 0.3% hydrogen peroxide (Sigma, Poole, England), diluted in phosphate buffered saline (see solutions section), was added to each slide. The slides were incubated for ten minutes in a humidified slide chamber at room temperature.

The slides were washed in TBS twice for five minutes once, dried off and 100µl of protein block (10% horse serum or serum free protein block (Dako) specified in figure legend) was added to each section then incubated for thirty minutes in a slide chamber at room temperature. 100µl of primary antibody diluted in protein block (1µg/ml see table 2.2), was added to each slide and then incubated in the slide chamber for one hour at room temperature. The slides were washed thrice for 5 minutes in TBS.

Excess moisture was removed from the slides, and 100µl of biotinylated rabbit anti-goat antibody (Dako, Ely, UK) or goat anti-rabbit (Dako, Ely, UK) or rabbit anti mouse (Dako, Ely, UK) or donkey anti-goat antibody (Santa Cruz, Middlesex, UK) (1/300) was added and the slides were incubated for 1 hour in a slide chamber at room temperature. See table 2.2. The slides were washed for five minutes three times in TBS, dried off and incubated for 30 minutes with solutions A (avidin solution) and B (biotinylated horse radish peroxidase) from the Vectastain® ABC visualisation system (Peterborough, UK). The slides had three five minute washes in TBS.

100µl of diaminobenzidine (DAB) (Dako, Ely, UK) was added to each tissue section and immediately viewed under the microscope. The reaction was stopped, with the appearance of brown staining, by submersion in tap water.

The slides were placed in haematoxylin (Sigma, Egham, UK) solution for 20 seconds , rinsed in tap water to remove excess stain, then immersed in ethanol twice for two minutes, twice in xylene for two minutes and mounted with DPex (VWR International, Poole, England) solution and left to harden for 24 hours.

The slides were visualised and images were taken with bright field microscopy using the Olympus BX 61 motorised microscope with a ColorView™ II digital camera and Cell P software.

2.9.2 Method for Immunophenotyping

The following method for immunophenotyping is well established in this laboratory. See table 2.2 for antibody dilutions and table 2.8 for reagents.

Paraffin sections of synovial tissue and tonsil were sliced at 3 micrometre intervals and placed on slides and left to dry overnight. The slides were immersed in xylene (Fisher Scientific, Loughborough, UK) twice for ten minutes, ethanol (Fisher Scientific, Loughborough, UK) twice for five minutes, then rinsed in distilled water. The slides were placed in preheated (95°) target retrieval solution (Dako, Ely, UK) and incubated for 45 minutes at 95° in a waterbath.

The slides were allowed to cool for twenty minutes and then transferred to a slide jar for washing in Tris buffered saline (TBS) for five minutes once.

After washing, the slides were dried, the tissue isolated with a hydrophobic pen (Dako, Glostrup, Denmark) and 100µl of 0.3% hydrogen peroxide (Sigma, Poole, England) diluted in phosphate buffered saline (PBS) was added to each slide. The slides were incubated for ten minutes in a slide chamber at room temperature.

The slides were washed in TBS twice for five minutes once, dried off and 100µl of serum-free protein block (Dako, Glostrup, Denmark), was added to each section then incubated for thirty minutes in a slide chamber. 100µl of primary antibody (CD3, CD20, and CD68 1/60 and CD138 1/80 dilution, see table 2.2) diluted in serum free antibody diluent (Dako, Glostrup, Denmark), was added to each slide and then incubated in the slide chamber for one hour at room temperature. The slides were washed thrice for 5 minutes in TBS.

20µl of Envision anti-mouse polymer HRP (Dako, Ely, UK) was added to the slides and the slides were incubated in a dark moist chamber for 30 minutes. The slides were then washed three times with TBS and 100µl of DAB (Dako, Ely, UK) was added to each tissue section and immediately viewed under the microscope. The reaction was stopped with submersion in tap water.

The slides were then placed in haematoxylin (Sigma, Egham, UK) solution for 20 seconds, rinsed in tap water to remove excess stain, then immersed in ethanol twice for two minutes, twice in xylene for two minutes and mounted with DPex (VWR International, Poole, England) solution and left to harden for 24 hours.

The slides were visualised and images were taken with bright field microscopy using an Olympus BX 61 motorised microscope with a ColorView™ II digital camera and Cell-P software.

2.10 Immunofluorescence

Immunofluorescence is a reaction where the antibodies for the protein of interest are labeled with a fluorescent dye and the antigen-antibody complex is visualized using an ultra-violet or fluorescent microscope. The fluorescent dye absorbs ultraviolet rays and emits a visible light which is detected through specific filters by the microscope. See table 2.2 for antibodies.

2.10.1 Immunofluorescence of cells

Indirect immunofluorescence was used to visualise the presence of the HA-tagged receptor on the transfected HEK293 cells. However, the two anti-HA antibodies (clones F7 and 12 CA5) tested could not distinguish between transfected and non-transfected cells and gave a positive signal with untransfected cells. See table 2.2 for antibodies.

$4x10^5$ cells were grown on coverslips overnight in a 12 well plate. After fixing in 4% paraformaldehyde, the cells were washed 3 times with TBS. Protein block (Dako) was applied for 10 minutes then 3 further washes. The primary HA antibody (clones 12 CA5 or F7) was applied at a concentration of 1µg/ml or 5 µg/ml diluted in antibody diluent (Dako) and incubated for 1 hour at room temperature. After three washes with TBS, the coverslips were incubated in the dark at room temperature with the secondary antibody diluted in antibody diluent (goat anti-mouse, Alexa 488 conjugated, (Invitrogen, 1/300 dilution)). After three further washes with TBS, 4',6-diamidino-2-phenylindole (DAPI) was applied for 10 minutes (1ng/ml). The coverslips were washed with TBS and mounted with Mowiol (Calbiochem, Darmstadt, Germany). The slides were imaged using the Olympus BX61 motorised microscope and F View II™ digital camera and Cell-P software.

2.10.2 Immunofluorescence of frozen sections of synovium

To try and reduce the effects of background staining, I investigated the use of immunofluorescence. Of two experiments performed, both had issues with background immunofluorescence and so the technique was not taken further. See table 2.2 for antibodies.

Frozen sections of tissue of 4µm width were sectioned with a cryostat. They were then frozen at -80°C until use. The slides were rehydrated in PBS, isolated with a hydrophobic pen (Dako) and incubated for 30 minutes with Dako serum-free protein block. The slides were incubated for one hour with the primary antibody in Dako serum free antibody diluent (1µg/ml) and then washed three times in PBS for five minutes under agitation at room temperature. Three washes for five minutes in PBS were carried out. A streptavidin conjugated with Alexa® 488 (1/300) in serum free antibody diluent (Dako) was added and the slides incubated for 1 hour in the dark at room temperature. The secondary antibody was incubated for 1 hour in the dark at room temperature. The slides were washed three times with PBS for five minutes isolated from light. The tissue was incubated for ten minutes with DAPI (1ng/ml in PBS), in the dark at room temperature, and washed three times in PBS for five minutes protected from light. Coverslips were mounted with 1 drop of Mowiol (Calbiochem, Darmstadt, Germany) and the sections viewed using an BX61 motorised microscope and F View II™ digital camera and Cell-P software. The excitation wavelength was 488nm and the detection wavelength 530nm for the Alexa® 488 (green) and an excitation wavelength of 350nm and detection wavelength 470nm for DAPI (blue). Fluorescence signals were viewed using the U Plan APO objectives (4x/0.16na, 10x 0.40na, 20x/0.70na) and FITC (for Alexa®488) (HQ480/40-HQ535/50m 510-560nm), and DAPI (EXD360/40-EMD460/50m 435-485nm) filter sets.

2.11 Flow Cytometry

Flow cytometry was used to visualise the presence of the HA-tagged receptor on the transfected HEK293 cells. Cells are labelled with a specific antibody that is either directly conjugated to a fluorochrome or indirectly-labelled with a fluorochrome. The intensity of fluorochrome is detected by detectors enabling antibody labelled cells to be distinguished from unlabelled cells.

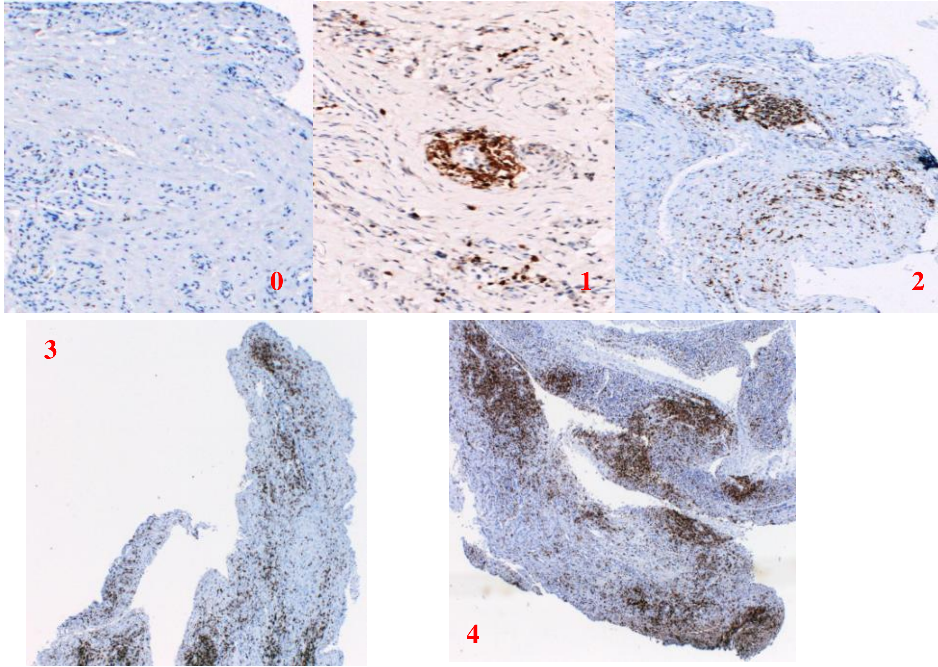
Cells were harvested by cell scraping. $5x10^5$ cells in 20µl of culture medium were added to a 96 well plate. The cells were fixed in 4% paraformaldehyde for 30 minutes and washed in PBS/0.1%BSA (bovine serum albumin) with 0.02% saponin (PBS/BSA/SAP) three times for permeabilisation. For surface staining cells were not fixed and were washed in phosphate buffered saline/ 0.1% bovine serum albumin (PBS/BSA). The cells were then incubated with 20µl of PBS/BSA or PBS/BSA/SAP, 20 µl of blocking human IgG (8mg/ml) and 20µl of monoclonal antibody (4c12) diluted in PBS/BSA or PBS/BSA/SAP. The primary antibodies were diluted to 10µg/ml. The cells were incubated at 4°C for 1 hour in the dark and washed as before 3 times. The cells were then incubated with 40µl of secondary antibody diluted in PBS/BSA (1/200) or PBS/BSA/SAP for 1 hour at 4°C in the dark. The cells were washed twice more as before and then resuspended in 200µl PBS/BSA or PBS/BSA/SAP and analysed within one hour. The cells were analysed using a FACSscalibur (Becton Dickinson, Oxford, UK) flow cytometer and data was analysed using Cell Quest software.

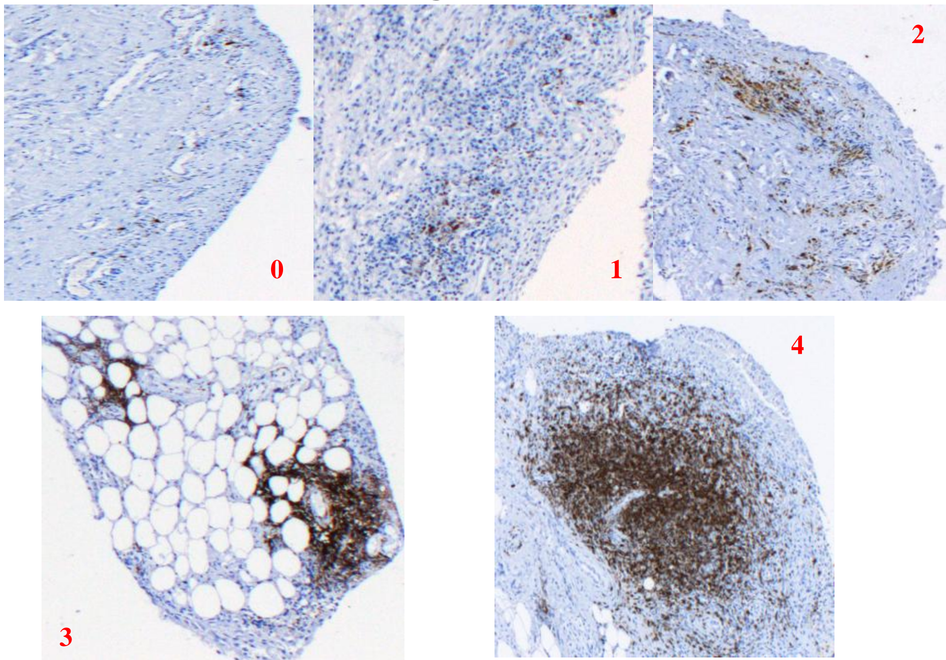
2.12 Synovial Score

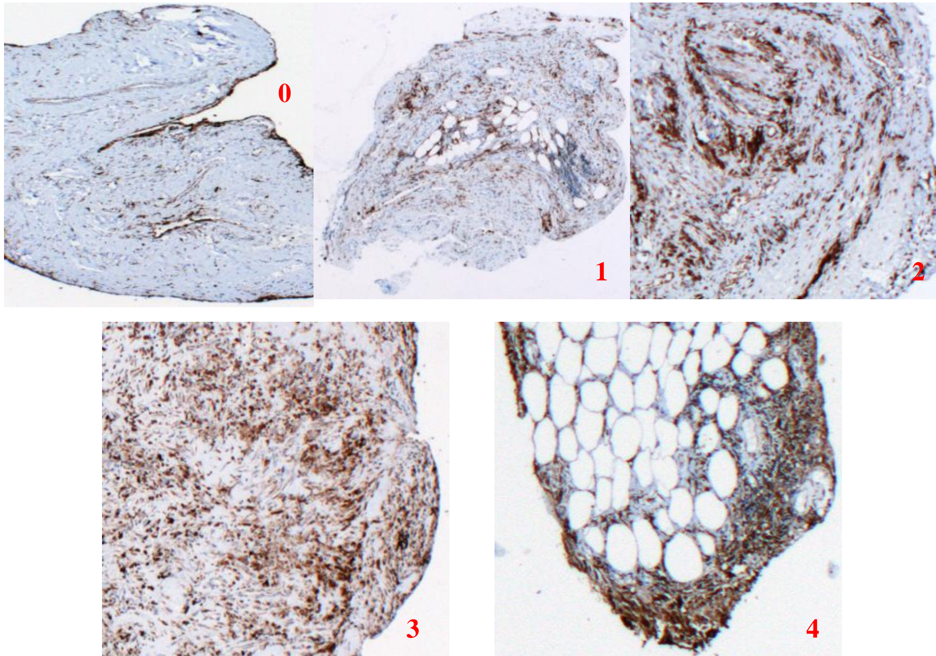
Samples of synovial tissue were scored using a modified Krenn score for grade, inflammatory score and the Tak score was used for the immune cell infiltrate. Each sample was graded by three independent observers, the final score being the median of the three grades. The individual grading scores have not been recorded, the final median score has been used in this thesis. The inflammation score comprises the thickening of the synovial lining layer, the cellularity of the stroma and the inflammatory infiltrate as shown in table 2.1 giving a final score out of 9. The samples were graded according to the degree of organisation of the inflammatory infiltrate. The immune cell infiltrate was graded from 0 for minimal infiltrate to 4 for infiltration by numerous inflammatory cells for T cells (CD3), B cells (CD20), plasma cells (CD138), and macrophages (CD68). These grades were also grouped for analysis into low and high, low being a score of less than 2 and high being 2 and above. CD68 was split into lining (of synovium) and sublining scores.

Table 2.1. Scheme for the histopathological assessment of the three features of chronic synovitis from Krenn et al 2006[144]

Enlargement of the synovial lining cell layer	
0 points	The lining cells form one layer
1 point	The lining cells form 2–3 layers
2 points	The lining cells form 4–5 layers few multinucleated cells might occur
3 points	The lining cells form more than 5 layers, the lining might be ulcerated and multinucleated cells might occur
Density of the resident cells	
0 points	The synovial stroma shows normal cellularity
1 point	The cellularity is slightly increased
2 points	The cellularity is moderately increased multinucleated cells might occur
3 points	The cellularity is greatly increased multinucleated giant cells, pannus formation and rheumatoid granulomas might occur
Inflammatory infiltrate	
0 points	No inflammatory infiltrate
1 point	Few mostly perivascular situated lymphocytes or plasma cells
2 point	Numerous lymphocytes or plasma cells, sometimes forming follicle-like aggregates
3 points	Dense band-like inflammatory infiltrate or numerous large follicle-like aggregates
Sum 0 or 1	No synovitis
Sum 2–4	Low-grade synovitis
Sum 5–9	High-grade synovitis


CD3
0
1
2
3
4


CD20
0
1
2
3
4



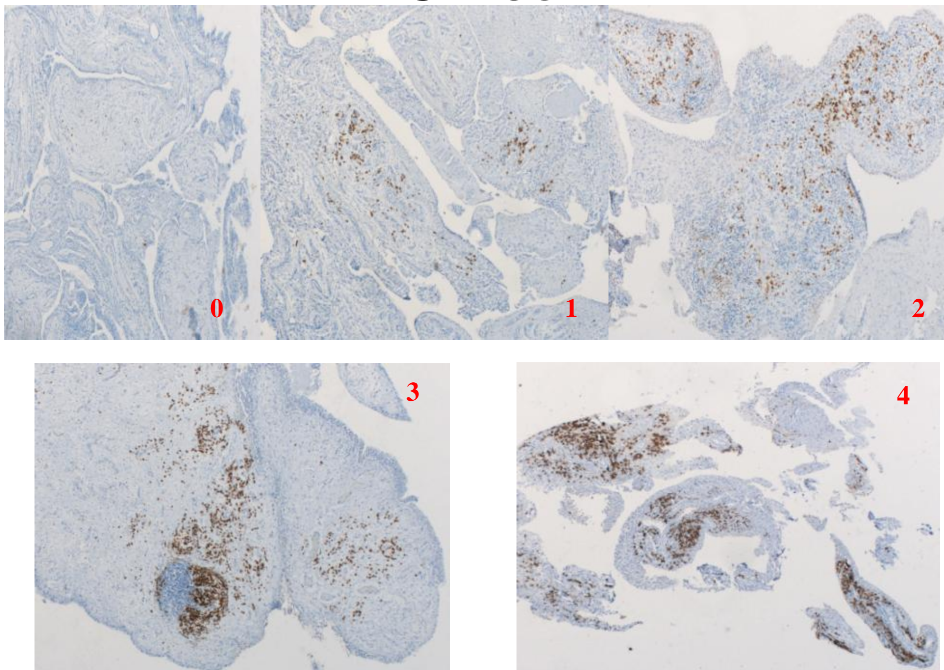


Figure 2.5. Atlas of immunophenotyping scores for synovial tissue. Courtesy of Dr R. E. Hands. (x40)

These photographs were used to immunophenotype and score the synovial tissue samples for CD3, CD20, CD68 and CD138 staining. The samples are graded in red from 0-4. This is a semiquantitative scoring system based on the Tak score as described in the Introduction.

2.13 Collection of Samples

Synovial tissue was collected from 28 patients with rheumatoid arthritis, who fulfilled the criteria for the American College of Rheumatology 1987 revised criteria for RA, and who underwent joint surgery or ultrasound guided tissue biopsy. Patients required 4 out of 7 criteria involving: morning stiffness, arthritis of three or more joint areas, arthritis of hand joints, symmetric arthritis, rheumatoid nodules, erosive radiographic changes.

Samples of skin and tonsil were sourced from the Experimental Medicine and Rheumatology biobank. Tonsil was provided by the Human Tissue Resource Centre, Barts and the London NHS Trust. Placenta and Pituitary paraffin samples were provided by Core Pathology, Barts and the London NHS Trust. Skeletal muscle, Testis, placenta and brain RNA was sourced from Ambion. Brain and skin lysate was sourced from Abcam. See table 2.10.

All patients provided informed written consent. The study was approved by the National Research Ethics Service (07/Q0605/29). Clinical data were collected retrospectively from patient's notes and electronic patient records from a time frame of within two weeks of the patient sample. Samples were stored in RNA later at -80°C for RNA extraction and embedded in paraffin for later immunohistochemical analysis.

2.14 Statistical Analysis

Data has been analysed using Graph Pad Prism 5. Unpaired T-tests were used to compare samples, assuming a normal distribution. Fishers' Exact Test was then applied to assess the difference between categorical variables. Medians have been used for analysis due to small sample size.

Pearson pairwise correlations were calculated using IBM SPSS Statistics 20.0 software. Pearson correlation is a measure of the linear dependence of one variable with another. It has a value

between -1 and 1 where 1 is complete positive correlation and -1 is complete negative correlation. This coefficient can be given a statistical significance or p value. For the purposes of my thesis p values of <0.05 were deemed significant. The p value is a measure of the likelihood of the event occurring by chance alone.

Linear regression was carried out with R software converting the categories into factors to account for any skewing due to categorisation. Linear regression models the relationship between a dependent variable and other explanatory variables. It can be used for prediction and to quantify the strength of association between variables. Important information about the model is the p value of the model or the significance of the model, the r^2 or a measure of the goodness of fit of the model. The r^2 is a measure of the variance in the dependent variable that is explained by the model. It is important to know the number of values missing to more clearly recognise the strength of the association. R enabled the analysis of subsets of each variable and how much each subset contributed to the significance of the model.

Primary component analysis (PCA) was carried out with R software. PCA models the associations and correlations between composite components in a 3 dimensional space. A component reduces the number of variables to a number according to their variation in relationship to each other. Each component is not linearly related to another. The transformation of the data is such that the primary component accounts for the maximum amount of variance in the data, the second the next largest variation and so on. It allows the clustering of data with regards to multiple attributes. It is a summary of data with minimal loss of information. PCA analysis is for linear data but can be applied to categorical data. If it is applied to categorical data the results are reliable but less precise. Only complete data sets are used so this reduces the information available in the plot. MC3R RQ was not analysed in this way as it is dichotomous and therefore there were multiple variables missing. The only data sets that were analysed by PCA were the CD categories, and the cytokine gene expression

as these were numerical values distributed in a linear fashion. However, the CD categories are categorical data and so the analysis was less precise than that with the cytokine data.

2.15 Solutions

Tris buffered saline (10x) pH 7.4-7.5 (TBS),

Trizma Base (T1503, Sigma, Dorset, England) 9.7g/l, Trizma Hydrochloride (T32531, Sigma, Dorset, England) 66.1g/l, Sodium Chloride (S/3160/65, Fisher Scientific, Loughborough, UK) 90g/l

Phosphate Buffered Saline (BR0014G) Basingstoke, England (PBS)

Sodium Chloride 8.0g/l, Potassium Chloride 0.2g/l, Disodium hydrogen phosphate 1.15 g/l, Potassium dihydrogen phosphate 0.2g/l

Hepes Buffered Saline 2x pH 7.2 (HBS)

10 g/l HEPES; 16 g/l NaCl; 0.74 g/l KCl; 0.27 g/l $Na_2HPO_4.2H_2O$; 2.0 g/l dextrose

Methanol transfer buffer

Glycine 54g/l, Tris base 11.5g/l in methanol

Tween® Tris buffered salt solution (TTBS)

50 mM Tris, 500 mM NaCl, 0.05% Tween® 20, pH 7.4.

ECL substrate 1

0.1M Tris, 1%Luminol, 1% p-coumaric acid

ECL substrate 2

0.1M Tris, 0.064% Hydrogen peroxide

Western Blot loading buffer

300mM Tris pH6.8, 36% Glycerol, 10% SDS, 600mM DTT, 0.0012% Bromophenol Blue

Table 2.2 Antibodies used for Western Blot (WB), immunohistochemistry, (IHC) and flow cytometry (FC)

Company	Antibody	Host	Specificity	Dilution/ Concentration
Abcam	HA tag 4C12 Ab1424	Mouse	HA tag	1:1000 (WB)
Abcam	HA tag 4C12-FITC	Mouse	HA tag	10µg/ml (FC)
Abcam	MC3R ab31309	Goat	Human MC3R N terminus	10µg/ml (FC) 5µg/ml (IHC)
Abcam	MC5R ab92287	Goat	Human MC5R N terminus	10µg/ml (FC) 5µg/ml (IHC)
Abcam	AB37373	Goat	Nil	Negative control
Abcam	Ab9110	Rabbit	Anti-HA	1/5000(WB)
AbD Serotec	IgG1 negative control MCA928F	Mouse	Nil	Negative control (FC)
Alomone labs	A023	Rabbit	Anti-human MC3R	1/200(WB),
Alomone Labs	A020	Rabbit	Anti-human MC1R	1/400 (WB), 5µg/ml (IHC)
Dako	P0448 HRP conjugated	Goat	Anti-Rabbit	1:2000 (WB)
Dako	P0447 HRP conjugated	Goat	Anti-Mouse	1:2000 (WB)
Dako	E0432 biotinylated	Goat	Anti-Rabbit	1:300 (IHC)
Dako	E0466 biotinylated	Rabbit	Anti-Goat	1:300 (IHC)
Dako	E0464 biotinylated	Rabbit	Anti-Mouse	1:300 (IHC)
Dako	F0250 FITC conjugated	Rabbit	Anti-Goat	1:40 (FC)
Dako	X0903	Rabbit	Nil	Negative control
Dako	M0814 Clone RP1	Mouse	Human CD68	1/60 (IHC)
Dako	M055 Clone L26	Mouse	Human CD20	1/60 (IHC)
Dako	M7254 Clone F7.2.38	Mouse	Human CD3	1/60 (IHC)
Dako	M7228 Clone MI15	Mouse	Human CD138	1/80 (IHC)
Invitrogen	A21121, Alexa 488 conjugated,	Goat	anti-mouse	1/300 (IF)
Santa Cruz	HA probe F7 sc392	Mouse	HA tag	1:500 (WB)
Santa Cruz	MC1R n-19 sc6875	Goat	Human MC1R N terminus	1:500 (WB)
Santa Cruz	Sc2020 HRP conjugated	Donkey	Anti-goat	1:5000 (WB)

Santa Cruz	sc2042 biotinylated	Donkey	Anti-goat	1:300 (IHC)
Santa Cruz	SC9899	Goat	Anti-human MC1R	1/500 (WB), 5µg/ml (IHC)
Sigma	HPA017431	Rabbit	Human MC3R N terminus	1:20 (IHC)
Sigma-Aldrich	MOPCI 21	Mouse	Nil	Negative control
Sigma-Aldrich	A5441	Mouse	Anti-human β Actin	1/2000(WB)
Unknown	HA probe 12CA5	Mouse	HA tag	1:1000 (WB)

Table 2.3 RNA extraction

Name	Company	Catalogue number	Origin
Chloroform	VWR	100776B	Poole, UK
Ethanol	VWR	10107	Leicester, UK
Isopropanol	VWR	20842.323	Briare, France
Precellys® Lysing Kit Soft tissue homogenizing ceramic beads (1.4mm)	Precellys	03961-1-003	Derbyshire, UK
Qiazol Lysis Reagent	Qiagen	79306	Washington, USA
RNase free DNase set	Qiagen	79254	Hilden, Germany
RNeasy mini kit	Qiagen	74104	Hilden, Germany
RNeasy plus kit	Qiagen	74134	Hilden, Germany
Trizol	Invitrogen	15596-018	Paisley, Scotland
TURBO™ DNA free Kit	Applied Biosystems	AM1907	Warrington, UK

Table 2.4 cDNA synthesis

Name	Company	Catalogue number	Origin
Thermoscript RT PCR system	Invitrogen	11146-024	Carlsbad, USA

Table 2.5 RTPCR gene expression assays

Name	Company	Catalogue number	Origin
CXCL13 TaqMan® gene expression assay	Applied Biosystems	HS00757930_m1	Warrington, UK
GAPDH TaqMan® gene expression assay	Applied Biosystems	Hs99999905_m1	Warrington, UK
IL1β TaqMan® gene expression assay	Applied Biosystems	Hs01555410_m1	Warrington, UK
IL21 TaqMan® gene expression assay	Applied Biosystems	Hs00222327_m1	Warrington, UK
IL6 TaqMan® gene expression assay	Applied biosystems	Hs00174131_m1	Warrington, UK
MC1R TaqMan® gene expression assay	Applied Biosystems	Hs00267168_s1	Warrington, UK
MC3R TaqMan® gene expression assay	Applied Biosystems	Hs00252036_s1	Warrington, UK
MC5R TaqMan® gene expression assay	Applied Biosystems	Hs00271882_s1	Warrington, UK
RANKL TaqMan® gene expression assay	Applied Biosystems	Hs00243522_m1	Warrington, UK
TaqMan® Universal Master Mix II	Applied Biosystems	444038	Warrington, UK
TNFα TaqMan® gene expression assay	Applied Biosystems	Hs00174128_m1	Warrington, UK

Table 2.6 Sequences of custom primers

Sequence of Custom Primer	Name
CCCGTCAGAGATGGACAC	MC1R SG REVERSE
CTTCTGGGCTCCCTCAAC	MC1R SG FORWARD
CTGGTTGCTGAAGAAAGG	MC3R SG REVERSE
TATCTGGAGGGAGATTTTGT	MC3R SG FORWARD
ATCTCAACCTGAATGCCA	MC5R SG FORWARD
CAATGCCCATGTCTTCAC	MC5R SG REVERSE
CTGGGCTCCCTCAAC	MC1R TAQ FORWARD
CCGTCAGAGATGGACA	MC1R TAQ REVERSE
GGATCAGCCCTTCTG	MC3R TAQ FORWARD
CTCCGAGCCATTAGG	MC3R TAQ REVERSE
CCTGTGCCTCATCTC	MC5R TAQ FORWARD
CATGCTGGTCCTCTG	MC5R TAQ REVERSE

Table 2.7 Qiagen primers

Primer Code	Gene	Company
QT01004241	MC1R	Quantitect, Qiagen
QT00209895	MC3R	Quantitect, Qiagen
QT01192646	MC5R	Quantitect, Qiagen
QT01192646	GAPDH	Quantitect, Qiagen

Table 2.8 Immunohistochemistry

Name	Company	Catalogue number	Origin
Antibody Diluent with background reducing components	Dako	S3022	Glostrup, Denmark
Dab Chromogen kit	Dako	K3486	Glostrup, Denmark
Dako pen	Dako	S2002	Gliostrup, Denmark
Donkey serum	Sigma Aldrich	D19663	Irvine, UK
DPex	VWR	369204H	Poole, UK
Envision system + HRP labelled Polymer Anti–mouse	Dako	K4001	Glostrup, Denmark
Ethanol	VWR	10107	Leicester, UK
Goat serum	Sigma Aldrich	G9023-5ml	Irvine, UK
Peroxidase block	Dako	K3954	Glostrup, Denmark
Protein Block Serum free	Dako	X0909	Glostrup, Denmark
Rabbit anti-goat immunoglobulins biotinylated	Dako	E0466	Glostrup, Denmark
Rabbit serum	Sigma Aldrich	R9133-5ml	Irvine, UK
SuperFrost Plus slides	VWR	631-0108	Leicester, UK
Target Retrieval Solution	Dako	S1699	Glostrup, Denmark
Vectastain ABC kit	Elite	PK6100	Peterborough UK
Xylene	VWR		Leicester, UK

Table 2.9 Substances and Materials for Western Blot

Name	Company / Ingredients	Catalogue number	Origin
BCA protein assay	Thermoscientific	23227	Rockford USA
Bradford assay	Biorad	500006	Munchen, Germany
Polyvinylidene difluoride membranes	Immobilon P	IPVH0010	Bedford, USA
Protease inhibitor cocktail	Roche	11836170001	Mannheim, Germany
Protoflowgel stacking buffer	Flowgen bioscience 0.5MTris HCl, 0.4% HCl, pH6.8	H18324	Nottingham, UK
Protogel	National Diagnostics 30% acrylamide,0.8% bisacrylamide	EC890	Hull, UK
Protogel Resolving buffer	National Diagnostics 1.5M TrisHCl, 0.4% SDS pH8.8	EC892	Hull, UK
RIPA buffer	Sigma, 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0.	R0278	Irvine, Uk

Table 2.10 Sources of tissue used in thesis

Tissue/ RNA/ lysate	Source	Ethics number
Tonsil	EMR	07/Q0605/29
Placenta	Core Pathology	07/Q0605/29
Pituitary	Core Pathology	07/Q0605/29
Skin	EMR, QMUL	07/Q0605/29
Testis RNA	Ambion	Applied Biosystems, Warrington, UK
Skeletal muscle RNA	Ambion	Applied Biosystems, Warrington, UK
Placenta RNA	Ambion	Applied Biosystems, Warrington, UK
Brain lysate	Abcam	Cambridge, UK
Skin lysate	Abcam	Cambridge, UK
Synovial tissue	EMR	07/Q0605/29

Chapter 3. Development and validation of novel technical protocols.

3.1 Introduction

The melanocortin receptors are a family of five small stimulatory G protein-coupled receptors. They are encoded by intronless genes, meaning that the messenger RNA sequence is the direct complementary sequence to the genomic DNA sequence with no excisions of introns. MC1R has the untranslated regions (UTR) at either end of the messenger RNA, however MC3R and MC5R are short mRNAs with short UTRs. The melanocortin 1 receptor (MC1R) protein has a C-terminal region required for translocation to the surface membrane, while the trans-membrane domains of the MC3R have been shown to be important for both expression at the cell membrane and downstream signalling. Mutations of MC1R[353] and MC3R[352] have been shown to affect the translocation of the receptor to the cell surface leading to loss of function. This can also have an effect on the abundance of the mRNA itself. The regulation of MC1R and MC3R gene expression is complex and is still being elucidated. Presence of ligand or antagonist can also affect the abundance of the receptors[270].

Each receptor has seven transmembrane domains, with an extracellular amino-terminus and short cytosolic carboxy-terminus. Models have been made using the hydrophobicity of the amino acids in the sequence to predict structure but no structural models have been documented using X-ray crystallography to give an accurate description of the receptor structure. The melanocortin receptors have been shown to dimerise with evidence available for both homodimerisation of MC1R and heterodimerisation with MC1R and MC3R. The melanocortin 2 receptor (MC2R) is the only one of the five which has been shown to require an accessory protein for translocation to the cell membrane. The receptors of particular interest in inflammation are MC1R, MC3R and MC5R and this chapter recounts a search for a positive control for these receptors only.

A positive control as a source of mRNA and protein was required to test the tools to be used. Brain would have been an ideal positive control but specific sections of human brain and human brain RNA and lysate are not easily available. The original paper by Gantz et al showed that placenta, gut and brain expressed MC3R RNA at a high level by Northern blot [245]. MC5R was found expressed in peripheral tissues at a low level, particularly skeletal muscle [246] and more recently has been shown to be an important aspect of the function of exocrine glands and in the sebaceous glands in the skin[354]. MC1R, as discussed in the introduction, is fast becoming the ubiquitous MCR, but is mainly studied in terms of melanoma and its expression in the melanocytes of skin [355]. All of the melanocortin receptors signal via the cyclic AMP pathway, activating a G_s (G stimulatory protein), then adenylate cyclase resulting in increased intracellular cyclic AMP.

HEK293 cells were chosen for the production of a stable cell line. There are multiple instances in the literature of HEK293 cells being transfected with various melanocortin constructs, mostly attached to some form of tag [256]. Confirmation of transfection is investigated using functional assays or Western blot for the tag rather than establishing the presence of the receptor with receptor specific tools. Although it is stated in the literature that the HEK293 cell line is melanocortin free, they do express MC1R at mRNA level. This discrepancy may be due to a functional issue as studies in the literature show a response in cAMP after transfection and not before rather than establishing the presence or absence of MCRs using gene expression or robust protein identification techniques.

A commercial vector containing each of the MCRs was purchased. The vector used for transfection was the plasmid pcDNA 3.1 which contains a penicillin resistance site and a neomycin resistance site. Transfected cells would become resistant to neomycin and this resistance can be used to select a population of cells that are positive for the vector.

My aim here was to identify tools for reliable detection of MC1R, MC3R and MC5R expression at both the messenger RNA and protein level. The initial search for a positive control led to the generation of a stable cell line to provide a standard of reference as well as an easily accessible

source of protein and mRNA. Currently available commercial antibodies were investigated for their utility in detecting the MCRs using various techniques including immunohistochemistry, Western blot and flow cytometry.

3.2 Results

3.2.1 The search for a positive control

A sample of placenta stored at -80°C in RNA later was a kind gift from Giovanna Nalesso (WHRI). As the melanocortin receptors are intronless genes it is important to ensure the RNA is as free of genomic DNA as possible, because the complementary DNA and genomic DNA have the same sequence and are thus indistinguishable by qualitative PCR. Four different methods of DNase treatment were used to eliminate genomic DNA. Firstly, no DNase treatment, on-column DNase as part of the Rneasy mini kit and the Turbo™ DNase used after the RNeasy mini kit, or a combination of on column and Turbo™ DNase. The RNA was measured using a Nanodrop spectrophotometer. Although the same size sample of placenta was used for each treatment there were differing results in RNA concentration with the sample without DNase treatment having double the concentration of that having both DNase treatments suggesting loss of RNA because of the processing of the sample. 1 µg was used in cDNA synthesis and subsequent RTPCR using commercial TaqMan® primers for MC1R, MC3R, MC5R, GAPDH, βactin and 18S. MC1R is present in placenta but MC3R and MC5R do not appear at levels above that seen with genomic DNA. GAPDH and 18s appeared at a C_T appropriate to their function as housekeeping genes whereas βactin appeared at a much higher C_T than expected. Results are not available for the DNase treated samples as MC3R and MC5R were undetectable, again showing that MC3R and MC5R were not present at detectable levels. This experiment was carried out twice, once with a loading amount of cDNA of 10ng and secondly, with 40ng. Similar results were seen with both experiments, with MC1R clearly seen while MC3R and MC5R were not (see figure 3.1).

As placenta could not be used as a positive control for all three receptors with amounts of cDNA that could be obtained from synovium, an alternative approach would be a stable transfected cell line for use as a positive control.

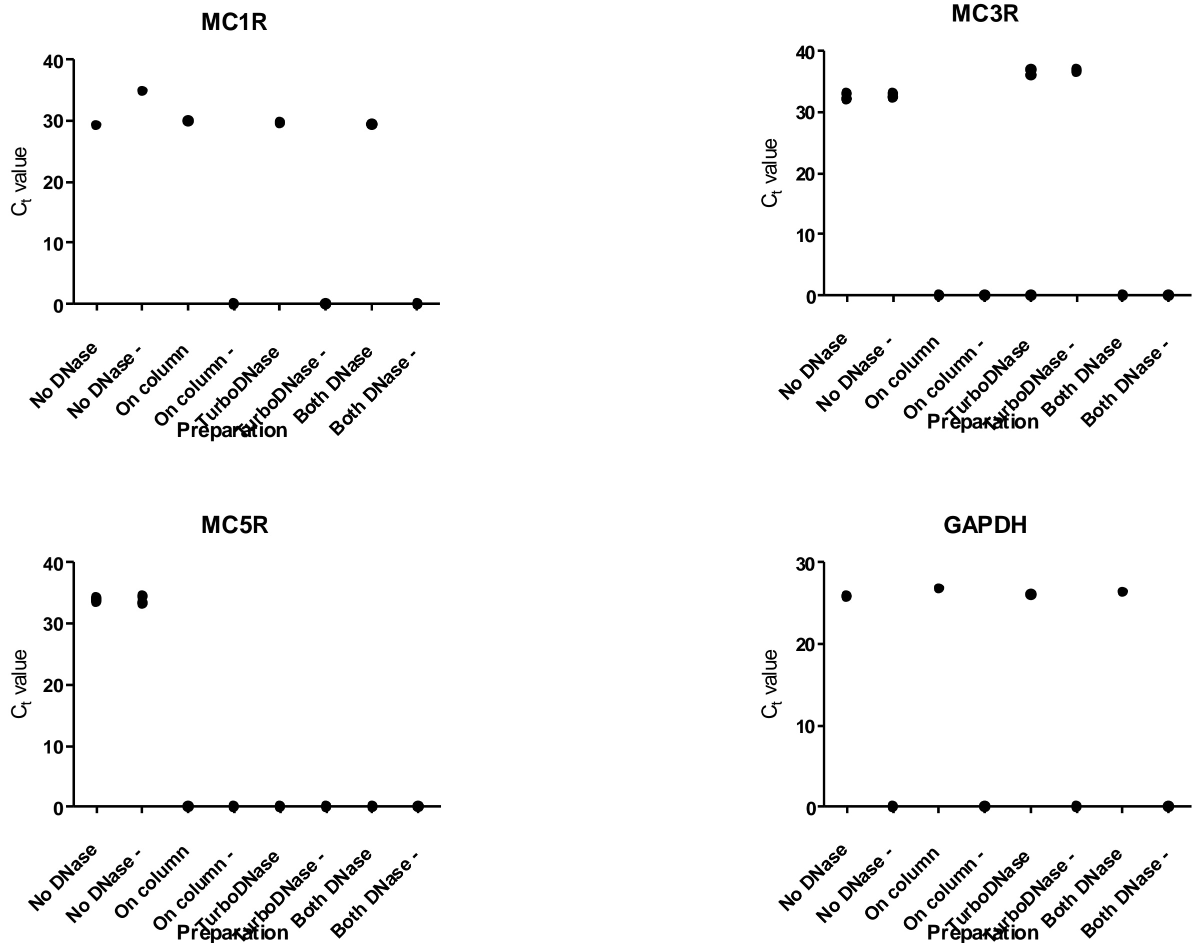


Figure 3.1 Examination of different DNA exclusion techniques on MCR expression.

Samples of placenta RNA were prepared using four different DNA conditions, no treatment, on column DNase treatment, Turbo DNase treatment and both on column Dnase and Turbo Dnase. MC1R and GAPDH are seen consistently regardless of which treatment is used. A genomic DNA signal is seen for MC1R if no treatment is used. No signal is seen for MC3R or MC5R over the genomic DNA signal. For clarity positive and negative controls are next to each other. A signal in the negative control indicates the presence of genomic DNA. This experiment used 40ng of RNA per reaction. This experiment shows that MC1R is present in placenta and that MC3R and MC5R are not detected. Each DNA exclusion treatment gives similar results for MC1R and GAPDH. This is representative of two experiments.

3.2.2 Generation of a stable cell line

A stably transfected cell line was sought for use as a positive control for melanocortin receptor expression in human synovial samples and as a tool to test the primers and antibodies. A first step in creating the cell line was to obtain the vector and check its specificity. The vector would lend neomycin resistance to the transfected cells and so the concentration of G418 (a neomycin-related antibiotic) which caused cell death was established. This concentration of G418 was used to place selection pressure on the transfected cells.

3.2.3 The vectors contain inserts of the appropriate size

The plasmids were digested using the restriction enzyme PME1 to cut out the insert from the vector (see figure 2.3 and 2.4). This substrate was then run on a gel with doubling dilutions to ascertain the concentration of the vector by comparing to a commercial DNA ladder and also to confirm the presence of the insert at an appropriate size. Figure 3.2 shows the dilutions of the vectors and also that each of the lanes shows a band of approximately 1000 bases with the remainder of the vector remaining near the top of the gel. This confirms the vectors contain inserts of a size matching the expected size according to the manufacturer's guidance.

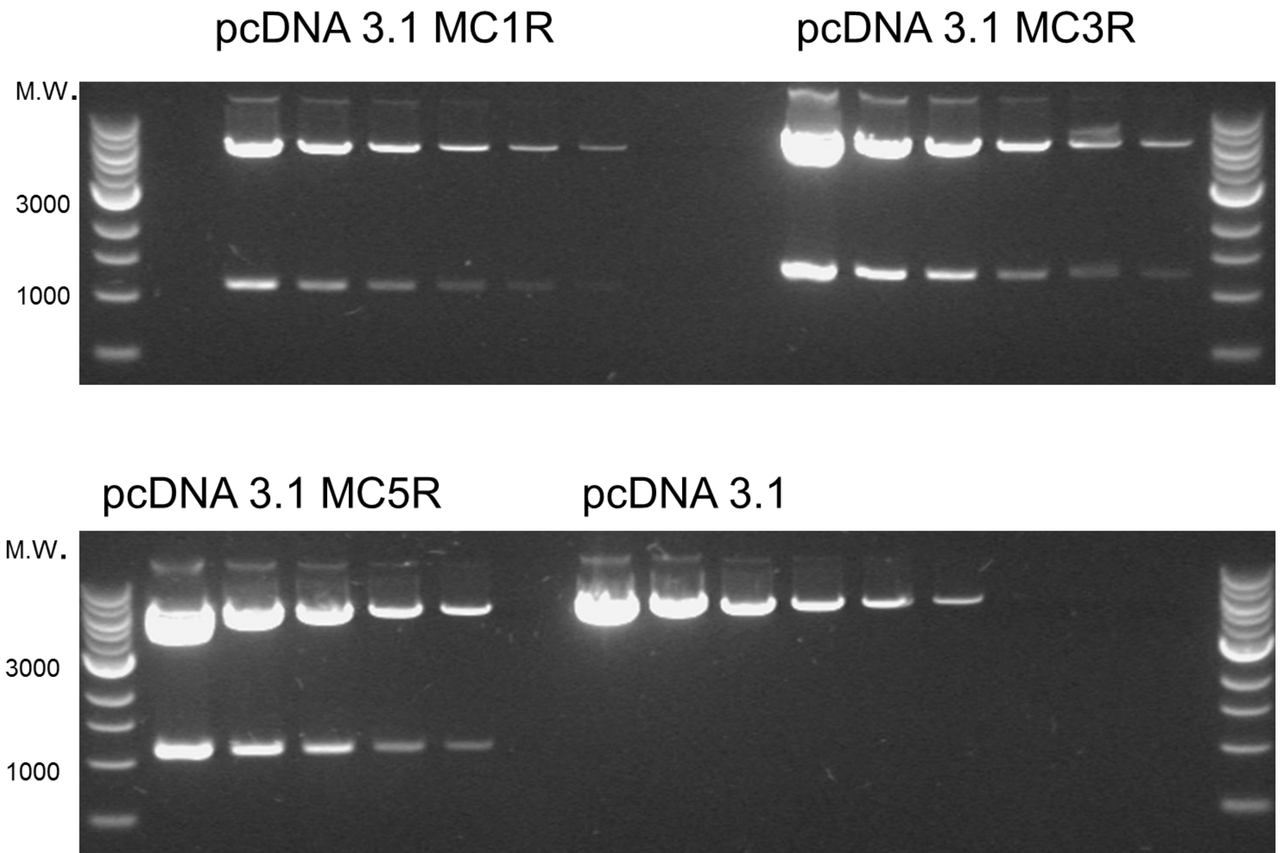


Figure 3.2 Examining the presence of the insert in the vectors.

Agarose gel electrophoresis: Vectors containing each of the MCR sequence inserts were digested with restriction enzyme PME1 and then halving dilutions (represented left to right) were made to ascertain the presence of an insert of the correct size of approximately 1000 base pairs as shown above. This gel is representative of two experiments.

3.2.4 The vectors contain the correct sequences

The purified vectors were amplified using TOP10 E.coli bacteria and purified using the Qiagen plasmid midi kit and then sequenced by the Genome Centre, William Harvey Research Institute, Queen Mary's University of London. In each case the sequence was confirmed as being MC1R, MC3R and MC5R respectively. The samples were sequenced using the T7 promoter, which was present in the vector.

3.2.5 The minimum concentration of genetecin required for cell death is 0.625µg/ml

HEK293 cells were grown in 96 well plates as described in the materials and methods section above. Alamar blue dye was used to ascertain cell death due to genetecin. Two plates were set up on the same day, one was analysed at 5 days the second at 10 days. The cell death was measured using a spectrophotometer as described previously. The ratio of absorbance of 570nm and 590nm was calculated. A value of 0 indicates cell death visualized as a blue well. (A pink colour indicates metabolism of the dye used and therefore living cells). Doubling dilutions of genetecin were used with a maximum of 5mg/ml. Figure 3.3 shows that at 5 days there is some cell death at all concentrations of genetecin, however at 10 days there is complete cell death at concentrations of 0.625mg/ml and above. This concentration of genetecin was therefore taken forward for all further experiments. The experiments had 8 replicates and the error bars represent the standard error of the mean.

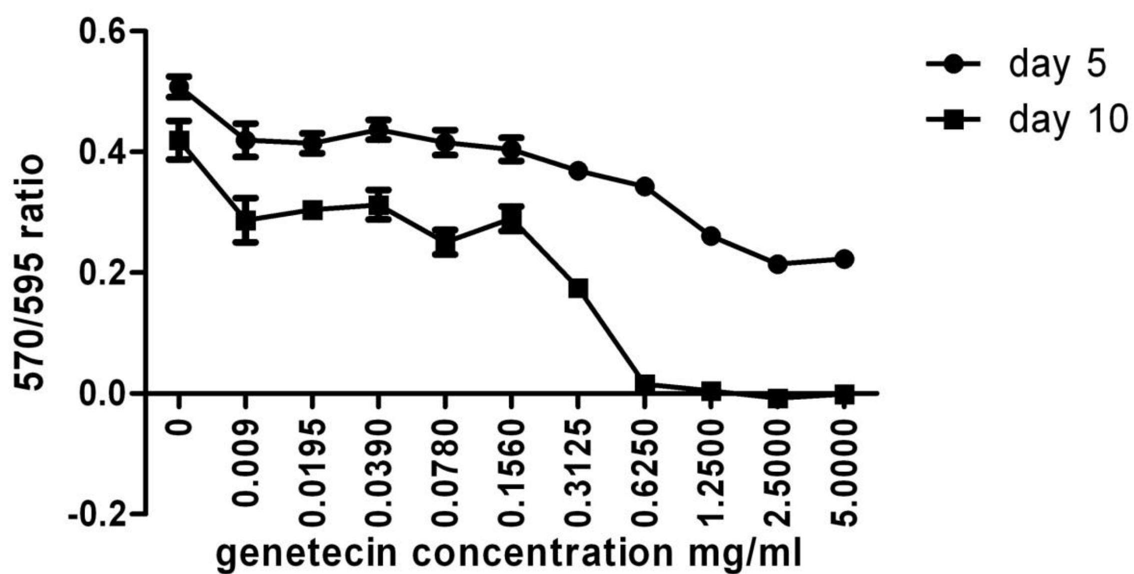


Figure 3.3. Cell viability assay for HEK293 cells using Alamar Blue dye to ascertain the concentration of G418 required to cause cell death at 10 days.

A 570nm/595nm wavelength ratio of 0 indicates a blue well consistent with no live cells. Results are from two plates read at 4 hours after addition of Alamar blue dye. Bars are standard error of the mean from eight replicates. This is representative of one experiment.

3.2.6 HEK293 cells express MC1R but not MC3R or MC5R

Prior to transfection HEK293 cells were initially assumed to be melanocortin-free, as stated in the literature, however it became apparent that HEK293 cells express MC1R at gene expression level before transfection with any construct. This was confirmed by RTPCR using Applied Biosystems TaqMan® primers, and also using custom primers. MC1R appeared at a C_T value of 30.

3.2.7 Transfection of the cells is confirmed by RTPCR

Transfection of the HEK293 cells was confirmed using the Applied Biosystems TaqMan® primers (see figure 3.4) and custom primers (see figure 3.5) for MC1R, MC3R and MC5R. All cell lines expressed MC1R whether transfected with MC1R or not, showing the presence of endogenous MC1R. No MC3R was expressed in MC5R transfected cells and no MC5R was expressed in MC3R transfected cells. No MC3R or MC5R was seen in MC1R transfected cells as expected.

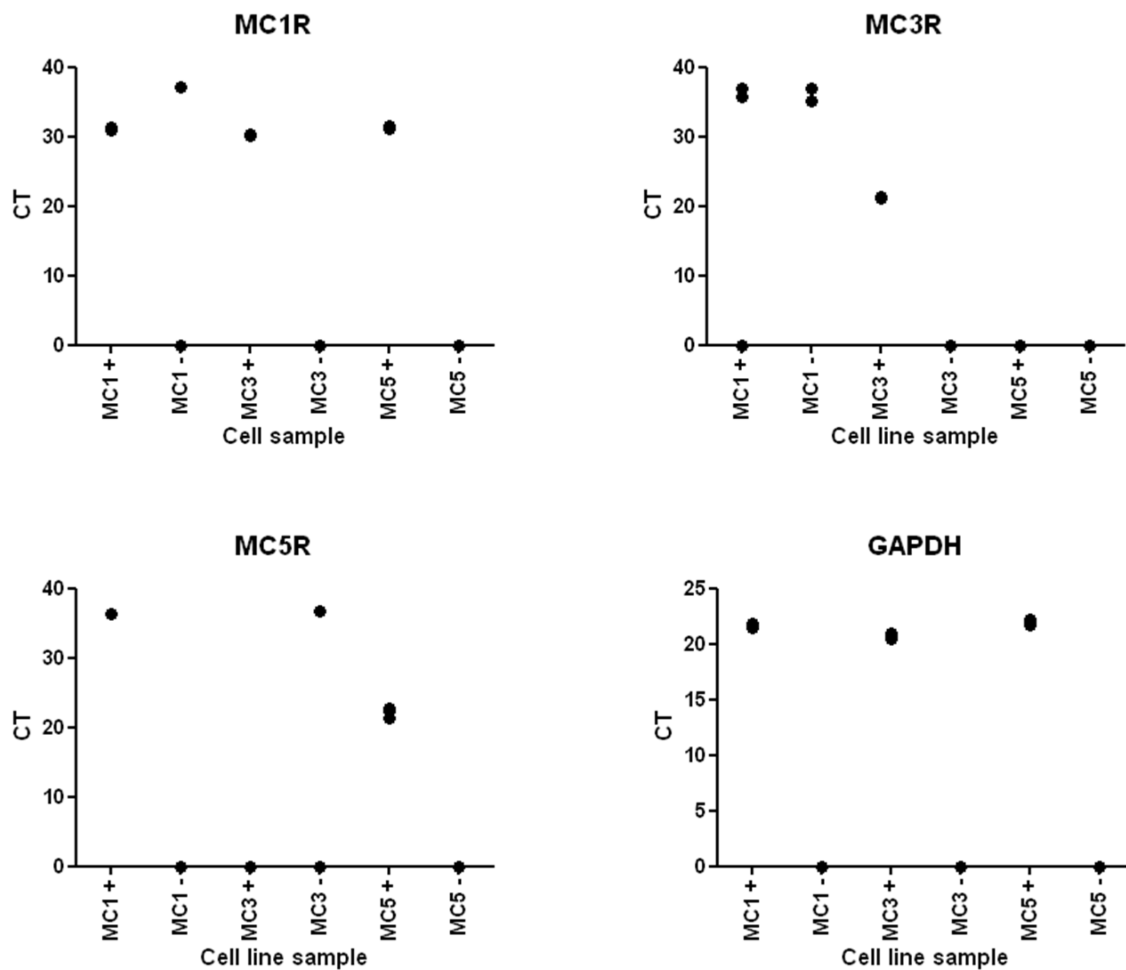


Figure 3.4: RTPCR of HA-MCR cell line cDNA using Invitrogen TAQman® primers. Each plot shows the CT value against the cell line sample. MC1+ is the MC1R transfected cell sample. MC1- is its negative control (No reverse transcriptase) MC3+ is the MC3R transfected cell sample MC3- is its negative control. MC5+ is the MC5R transfected cell sample, MC5- is its negative control. Each sample is plotted next to its negative control for clarity. All cell lines express MC1R, MC3R transfected cells express MC1R and MC3R, MC5R transfected cells express MC1R and MC5R. MC3R transfected cells do not express MC5R and MC5R transfected cells do not express MC3R. All cells express the housekeeping gene GAPDH. This is representative of two experiments.

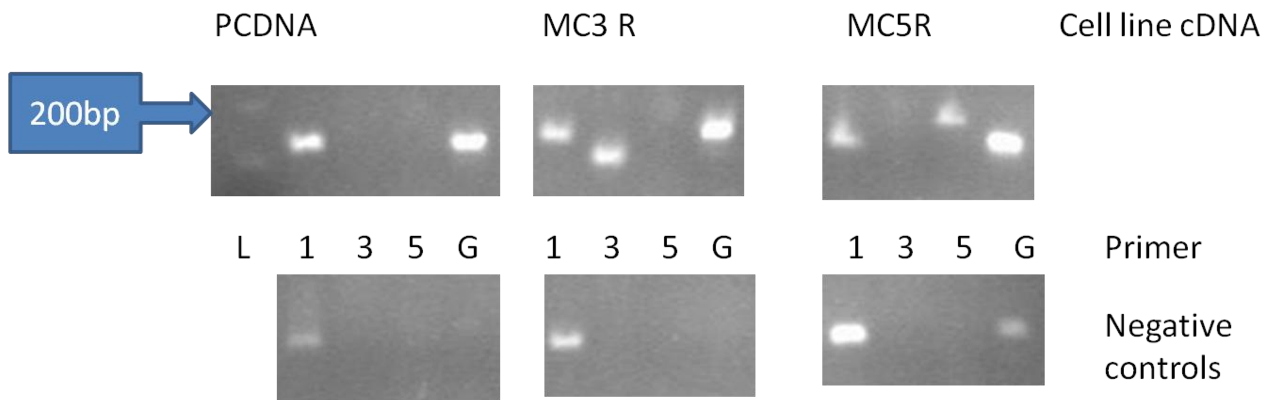


Figure 3.5: PCR of transfected cell line cDNA using custom primers.

L=ladder 1=MC1R primer, 3=MC3R primer, 5=MC5R primer, G=GAPDH primer.

Expected band sizes MC1R= 98bp MC3R=78bp, MC5R=142bp GAPDH=95bp.

PcDNA (empty vector) transfected cells show the presence of MC1R using custom primers. MC3R transfected cells show the presence of MC1R and MC3R, MC5R transfected cells show the presence of MC1R and MC5R. The controls show some genomic DNA contamination for MC1R. This is representative of two experiments.

3.2.8 Transfection of the cells is confirmed by Western Blot for the HA tag

For reference MC1R is expected to report bands at 34-37kDa, MC3R at 40kDa and MC5R at 45 kDa from the manufacturers' literature (Santa Cruz, Sigma).

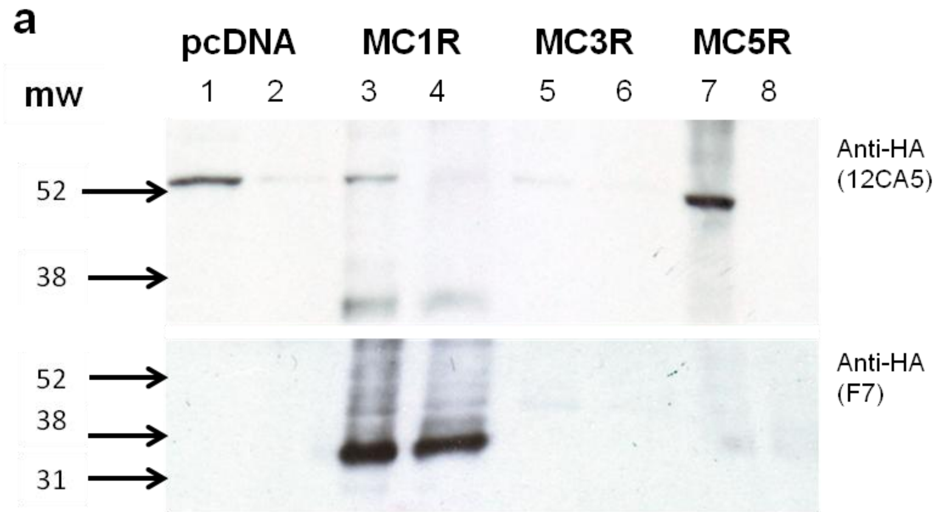
Figure 3.6 displays five representative Western Blots. Panel a shows two Western Blots with samples boiled at 100℃ for five minutes. 100μg and 75μg of each cell lysate was loaded in adjacent wells. A band representing MC1R was demonstrated at just less than 38 kDa, consistent with reports in the literature. No bands are evident for MC3R or MC5R with the two mouse monoclonal anti-HA antibodies F7 and 12CA5. Panel b shows a single Western blot again run with 100μg and 75μg for each cell lysate and boiled at 100°C. With this 4c12 anti-HA antibody clone, two bands emerge; again at 38kDa for MC1R, but also at 31kDa; this would be consistent with a glycosylated and non-glycosylated version of MC1R as previously reported in the literature[250]. A faint band is seen between 38 and 52 kDa consistent with MC3R and no band is seen for MC5R although there is a nonspecific band at 52kDa that may be obscuring a hypothetical MC5R band.

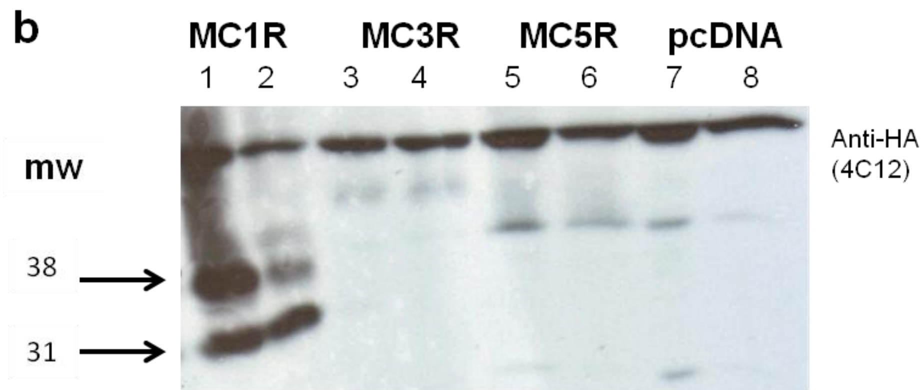
Panel c shows a blot with lysate from 2 million cells incubated with a reducing agent, DTT added to the loading buffer at three different temperatures, normal conditions i.e. at 100°C (reduced and denatured), for 10 minutes at 70°C or for 15 minutes at room temperature (reduced and partially denatured). The blot shows a clear 38kDa band for MC1R for all three conditions, however the second 31kDa band only appears when the lysate has been heated at 70°C or kept at room temperature for 15 minutes. It is also of note that the 38kDa band is far less prominent at the 100°C incubation than the other two temperatures and there is almost a visual gradient from 100°C to 70°C to room temperature. With this antibody (a rabbit polyclonal anti-HA tag antibody) MC3R only

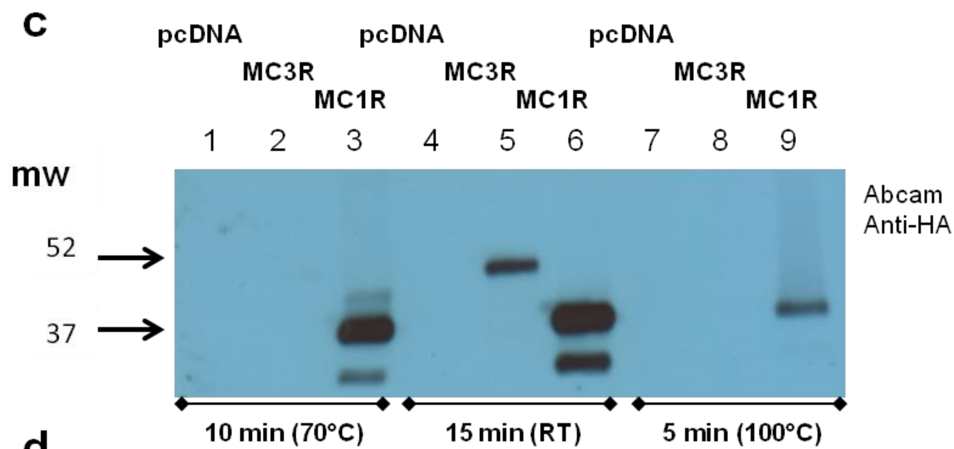
appears when the lysate is incubated at room temperature for 15 minutes and not at all when the lysate is heated either to 70°C or boiled, indicating likely degradation of the protein.

Panel d shows a similar blot run with MC5R lysate and the same rabbit polyclonal anti-HA antibody as panel c. Again 2 million cells were lysed and incubated with DTT-containing loading buffer for 5 min at 100°C, 10 min at 70°C or 15 min at room temperature. A similar pattern to MC3R is seen with no bands visible at 100°C or 70°C incubation but a band less than 52 kDa is seen at room temperature consistent with the expected size of MC5R.

Therefore, I would conclude that MC3R and MC5R seem to be heat sensitive when using the rabbit polyclonal anti-HA antibody and this may explain why no bands or faint bands were seen with the first 3 antibodies. Moreover, these 5 Western blots confirm that there is an HA tagged protein consistent with the expected sizes of MC1R, MC3R and MC5R present in the transfected cells. The proteins are of different sizes consistent with predicted molecular weights. MC1R protein is consistent with that seen in the literature[250].


a
pcDNA
MC1R
MC3R
MC5R
mw
1 2 3 4 5 6 7 8
52
38
Anti-HA
(12CA5)
52
38
31
Anti-HA
(F7)


b
MC1R
MC3R
MC5R
pcDNA
1 2 3 4 5 6 7 8
mw
38
31
Anti-HA
(4C12)


c
pcDNA
MC3R
MC1R
pcDNA
MC3R
MC1R
pcDNA
MC3R
MC1R
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mw
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37
Abcam
Anti-HA
10 min (70°C)
15 min (RT)
5 min (100°C)

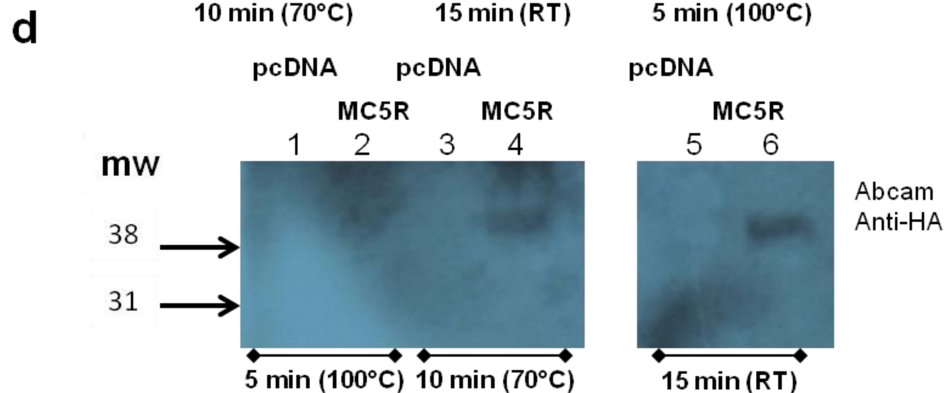

d
pcDNA
MC5R
pcDNA
MC5R
pcDNA
MC5R
1 2 3 4
5 6
mw
38
31
Abcam
Anti-HA
5 min (100°C)
10 min (70°C)
15 min (RT)

Figure 3.6 Western blots immunostained with anti-HA antibodies showing the presence of the HA tagged receptor in the transfected cell lysate.

For reference MC1R is expected to report bands at 34-37kDa, MC3R at 40kDa and MC5R at 45 kDa from the manufacturers literature (Santa Cruz, Sigma).

Panel a shows the presence of HA-MC1R at 37kDa using the anti-HA antibodies F7 and 12CA5 (lanes 3 and 4). Panel b shows presence of two bands for HA-MC1R (lane 1 and 2) and faint bands for HA-MC3R at 52kDA (lanes 5 and 6) using antibody clone anti-HA 4c12. Panel c shows that HA-MC1R is detected at all temperatures for sample incubation (lanes 3,6 and 9), whereas HA-MC3R is seen most clearly when the samples are not boiled but left at room temperature for 15 minutes using a rabbit polyclonal anti-HA antibody (lane 5). Panel d shows the same phenomenon for HA-MC5R (lanes 4 and 6 show bands for HA-MC5R .) For blots a and b, membranes were blocked for an hour prior 1 hour incubation with the primary antibody. For blots c and d membranes were blocked with 5% non fat milk for 3 hours and incubated overnight with the primary antibody. These blots were each independent experiments and were not repeated. No β actin staining was carried out for these blots and so equal loading cannot be verified. MW=molecular weight in kDa.

3.2.9 HA tagged MC1R is detected by two commercial anti human MC1R antibodies

Figure 3.7 shows a Western Blot probed with 3 anti–MC1R antibodies. The rabbit polyclonal anti-HA antibody here is used as a positive control. The lanes with cell lysates contain equal volumes of lysate from 2 million cells and the skin lysate is a commercial preparation containing 40µg of protein. The skin lysate was prepared in a reducing buffer containing β-mercaptoethanol rather than dithiothreitol (DTT). The skin lysate was incubated at room temperature after defrosting from -80°C prior to loading in the gel. The cell lysate was incubated at room temperature for 15 min with a DTT containing buffer prior to loading.

The HA- tag antibody once again shows bands with the cell lysate at 38 and 31 kDa, no skin lysate was included in this blot. The Alomone labs rabbit polyclonal anti-human MC1R antibody displays the same two bands when used as a probe, at 38kDa and 31kDa. There are two bands seen with the skin lysate, one at 52kDa and one of a higher molecular weight but less than 76kDa. The higher molecular weight band is also seen in the control cells and is likely to be non-specific. The goat polyclonal anti -human MC1R antibody sc6875 displays a 38kDa band with the HA-tagged cell lysate and a 52kDa band with the skin lysate. Neither of these bands are seen with the control cell lysate and hence I suggest they are likely to be specific. The goat polyclonal anti-human MC1R antibody sc9899 does not produce any bands with the HA-tagged MC1R cell lysate or in the control cells but does give a 52kDa band with skin lysate.

Out of these three antibodies, the Alomone labs rabbit polyclonal anti-human MC1R antibody most closely resembles the rabbit polyclonal anti-HA antibody when used to probe HA-tagged MC1R cell lysate. All three antibodies give a band of 52kDa with skin lysate which is larger than expected when compared to the HA-tagged protein produced in the transfected cells.

The immunizing peptides were supplied for the Alomone labs antibody and sc6875. The Western Blot in figure 3.8 a shows cell lysate from 2 million control un-transfected cells and from HA–MC1R transfected cells probed with the Alomone labs antibody and with the same concentration of Alomone labs antibody that has been pre-incubated with the immunizing peptide. Pre-incubation with the control peptide should ensure that all the specific binding sites for the peptides are filled. Indeed when the Blot is incubated with the Alomone labs antibody two bands are seen at 38kDa and 31kDa (the anti-HA antibody is used as a control here). These bands disappear when the antibody is pre-incubated and pre-absorbed with the immunizing peptide. No βactin was run and therefore equal loading could not be determined.

A faint band is seen when sc6875 is used to probe the HA-tagged MC1R cell lysate at 31kDa (Figure 3.6b). When the blot is probed with sc6875 that has been pre-incubated and pre-absorbed with the immunizing peptide, this band disappears. These two blots confirm the specificity of the Alomone labs antibody for the immunizing peptide from human MC1R and the specificity of sc6875 for its immunizing peptide from human MC1R. The Alomone labs antibody was chosen to be taken forward for further Western blots for human MC1R.

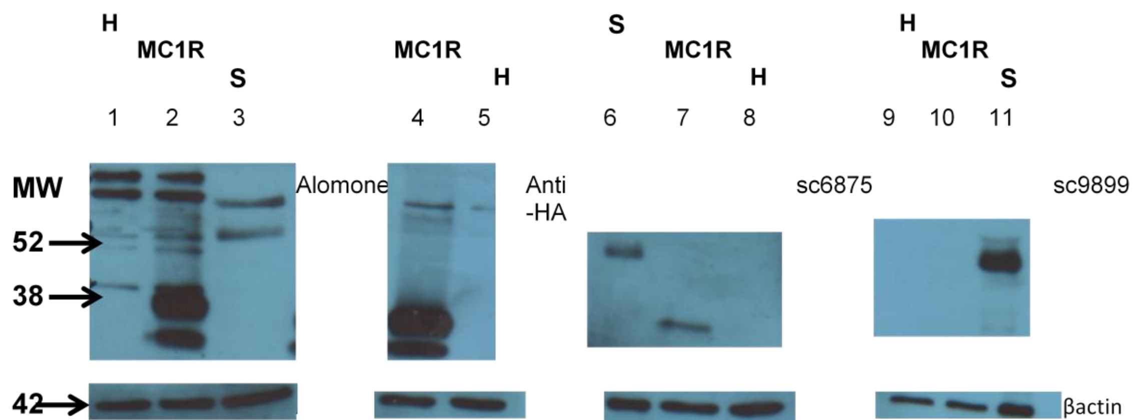


Figure 3.7 Immunoblotting with three anti-human MC1R antibodies showed bands at approximately 52 kDa for skin.

Antibody sc9899 did not show any reactivity for the HA-MC1R lysate (lane 10). Antibody sc6875 detected a 37 kDa band in the HA-MC1R lysate (lane 7) and the Alomone labs detected both the 38kDa and 31kDa bands that were also seen with the HA-antibody (lane 2). Each of the antibodies detetected a band of 52kDa for skin (lanes 3, 6 and 11).Equal loading was confirmed with β actin which had a molecular weight of 42kDa. For this experiment membranes were blocked for three hours with 5% non-fat milk and incubated overnight with the primary antibody. MW= molecular weight in kDa. This experiment was carried out once.

H= untransfected HEK293 cell lysate

MC1R= HA-MC1R transfected HEK293 cell lysate

S= skin

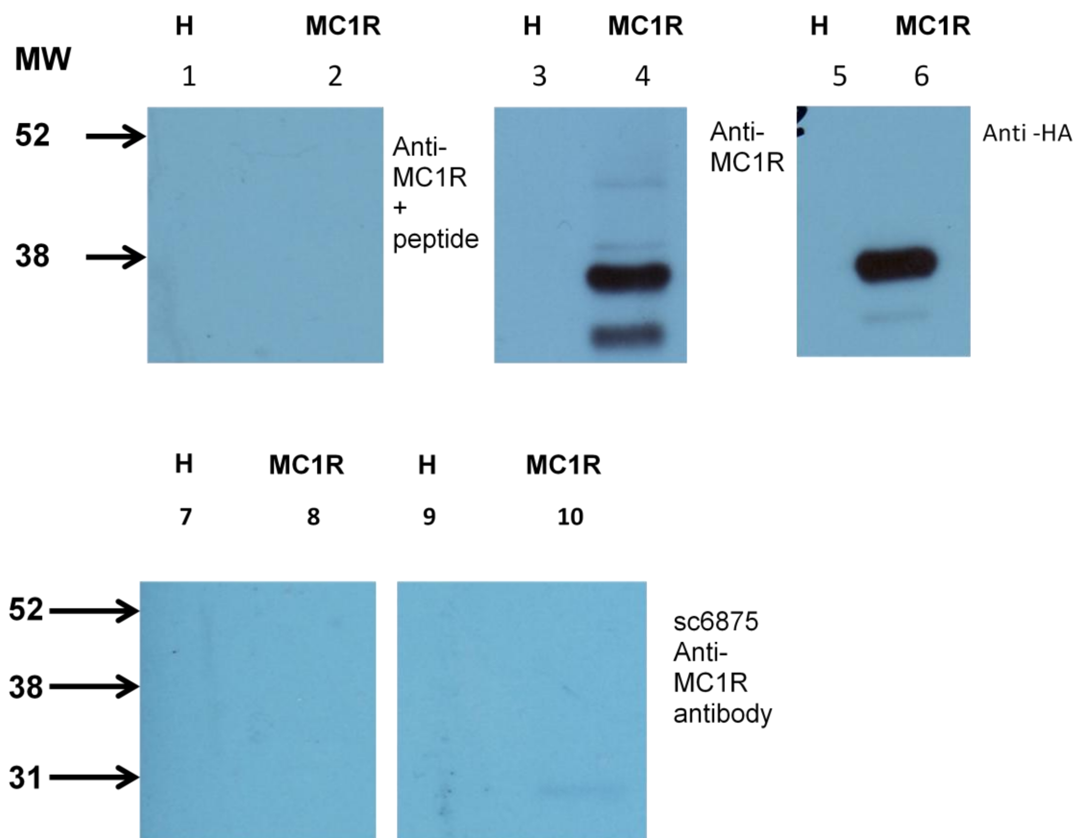


Figure 3.8. Western Blot showing the specificity of the MC1R antibodies Alomone and sc6875.

In the upper blot, Alomone labs MC1R the 37kDa and 31kDa bands (lane 4) are obliterated by incubation of the primary antibody with its control peptide (lane 2). The HA antibody was used as a positive control (lanes 5 and 6).

In the lower blot, Santa Cruz sc6875, a faint band is seen between 31 and 38kDa (lane 10) which is not seen when the primary is incubated with its control peptide (lane 8).

For this experiment membranes were blocked for three hours in 5% non-fat milk and incubated overnight with the primary antibody. No βactin control was run and equal loading cannot be determined. The molecular weights are to the right of the blots. Results of one experiment.

MW=molecular weight in kDa, H= untransfected HEK293 cell lysate, MC1R= HA-MC1R transfected HEK293 cell lysate

3.2.10 HA tagged MC3R is not detected by commercial anti-human MC3R antibodies

Figure 3.9 shows two Western blots using commercial polyclonal anti-human MC3R antibodies. The first experiment uses cell lysate from 2 million cells incubated in DTT-containing loading buffer for 15 minutes. There are two lanes of commercial human brain lysate, both incubated in β-mercaptoethanol-containing loading buffer, one incubated at 100°C for 5 minutes the second incubated at room temperature for 15 min. The rabbit polyclonal anti-HA antibody is here used as a positive control to confirm the presence of an HA-tagged MC3R in the cell lysate. 40µg of brain protein was used in this experiment. With the anti-HA antibody used with the HA-tagged cell lysate, a band corresponding to HA-tagged MC3R is seen at just above 38kDa, approximately 40kDa. When using the goat polyclonal anti-human MC3R antibody to probe a blot containing brain incubated at the specified two temperatures and the HA-tagged cell lysate incubated at room temperature, no bands are seen with the HA tagged cell lysate. This goat polyclonal antihuman MC3R antibody elicits a 52kDa band when the brain lysate has been boiled and a 102kDa band when the brain lysate has been incubated at room temperature.

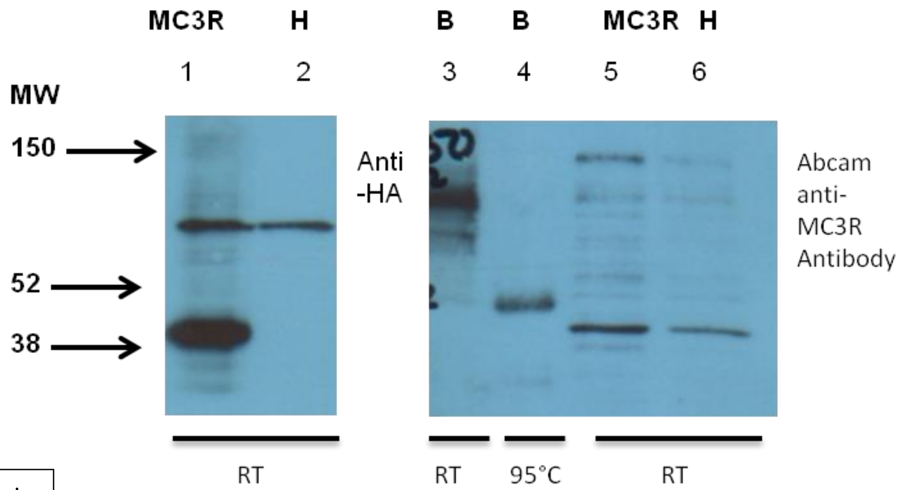
The same blot was re-probed with the rabbit polyclonal anti-HA antibody to confirm that there was an HA-tagged band consistent with HA-tagged MC3R in this sample and indeed this was confirmed. No bands were seen using the HA- tag antibody with brain lysate.

Figure b shows a similar blot using the Alomone lab rabbit polyclonal anti-human MC3R antibody with HA-tagged cell lysate and human brain. Again the cell lysate is from 2 million cells and incubated with DTT-containing buffer at room temperature for 15 minutes. The brain lysate is in β-mercaptoethanol-containing buffer and has either been boiled at 100°C for 5 minutes or incubated for 15 minutes at room temperature. The left side of the blot has been probed with the Alomone labs anti-human MC3R antibody. The right side of the blot has been probed with the Alomone labs anti-human MC3R antibody pre-incubated with its immunising peptide, this should eliminate all binding of the antibody to its specific binding sites, non-specific binding sites remain. The left side of

the blot shows no specific binding with the anti-human MC3R antibody and the HA-tagged MC3R cell lysate. This antibody elicits several prominent bands, for boiled lysate at 38kDa, 40kDa, 52Kda, greater than 52kDa and bands at 76kDa, however some of these bands are non specific as they are also seen in the HEK293 cell lysate. For the brain incubated at room temperature, there are bands at 52kDa, and 76kDa. When incubated with pre-absorbed anti-human MC3R the bands that remain are the 52kDa, 38kDa and 40 kDa although these are all significantly reduced in density, suggesting that the band specific for human MC3R using this antibody are at 76kDa. This would be consistent with the molecular weight of a dimer. This blot was re-probed with the rabbit polyclonal anti-HA antibody to confirm the presence of an HA-tagged MC3R and indeed this was confirmed. No bands were seen with human brain lysate with the HA-antibody. These blots are representative of two experiments with human brain lysate.

Table 3.1 shows all of the antibodies tested for Western Blot using cell lysates and tissue lysates as well as for immunohistochemistry.

a



b

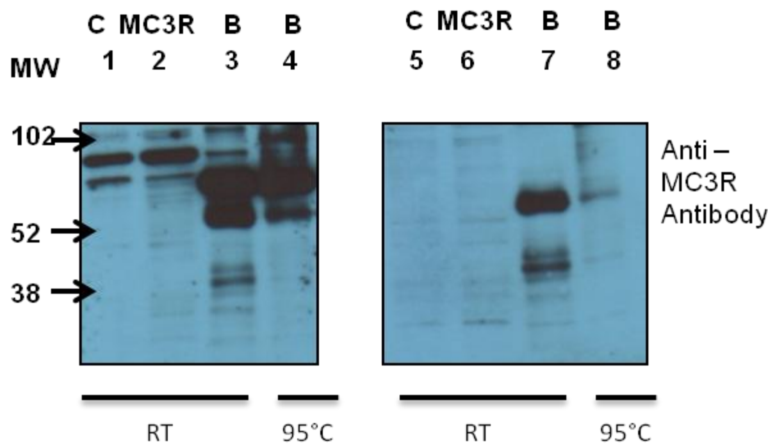


Figure 3.9 Western Blots showing bands with human brain.

Blot a shows on the left the HA-tagged HA-MC_3 band with an HA antibody (lane 1), on the right it shows that when brain is boiled there is a band at approximately 52kDa (lane 4) and when left at room temperature there is a band at 102kDa (lane 3) with the anti-human MC_3 antibody..

Blot b shows on the left a rabbit polyclonal anti-human MC_3 antibody from Alomone labs which gives multiple bands with human brain and no bands with the HA-tagged MC_3 cell lysate(lanes 2 and 6). On the right the antibody has been preadsorbed with its immunizing peptide, however there are still bands visible (lanes 7 and 8). These bands are non-specific and suggest that the actual specific band is at 76kDa (lane 3 and 4). For this experiment membranes were blocked for three hours in 5% non-fat milk and incubated overnight with the primary antibody. No βactin was run for this blot and equal loading cannot be determined.

H=HEK293 cell lysate C=control cell lysate B=brain MW= molecular weight in kDa

MC3R= HA-tagged MC_3 cell lysate

Code	Host	Antigen	Species	Epitope	Western Blot		Immunohistochemistry			
					Cells	Tissue	Placenta	Tonsil	Skin	OA
sc28993	rabbit	MC5R	human	N1-55	X					
sc7644	goat	MC5R	human, mouse, rat	C terminus	X					
ab92287	goat	MC5R	human, mouse,	PNVKNK SSPCED, N terminus	X			√	√	x
sc9899	goat	MC1R	human	N terminus	X	√			√	x
sc6875	goat	MC1R	human, mouse, rat	N terminus	√	√			√	x
M9193	rabbit	MC1R	human, mouse	II cytoplas mic loop	X					
AMR 020	rabbit	MC1R	human, rat	III intracell ular loop	√	√			x	
EPR6530	rabbit	MC1R	human						x	x
ab31309	goat	MC3R	human, chimp	SiQKTYL EGDFV	X	√	√	√		
HPA017431	rabbit	MC3R	human	SiQKTYL EGDFV	X	X	x			
AMR023	rabbit	MC3R	human	NSDSLTL EQFIQH MD	X	√	x			
sc8990	rabbit	MC3R	human	aa 1-88	X	X	x			
MAB3737	rat	MC3R	mouse				x			

Table 3.1 Commercial antibodies tested in Western blot and immunohistochemistry for this thesis. X=did not work , √= did work blank =not tested

3.2.11 Endogenous MC3R is detected in THP1 cells

Unfortunately, the transfected HEK293 cells died after multiple passages and frozen stocks did not re-animate as expected and therefore an alternative positive control was required.

THP1 cells have been noted to be MC3R positive in the literature at gene expression level, however this has not been confirmed at protein expression level. Figure 3.10 shows an experiment carried out with U937, THP1 and HL60 cells, all readily available and prolific in growth rate. The cells were differentiated with phorbol myristate acetate (PMA) at 5µg/ml or left untreated prior to lysing 2 million cells for each sample. The lysate was incubated at room temperature for 15 minutes with DTT-containing loading buffer. The Western blot shows that there is one specific band consistent with MC3R at just above 52kDa molecular weight. There are several non-specific bands also seen just below 76kDa with the THP1 cells but also with the HA–MC1R lysate and HA–MC3R lysate.

These results were confirmed by a second experiment with THP1 and U937 cells. 1 million cells were used this time and incubated with 3 concentrations of PMA. The cells were harvested as per the protocol for HEK293 cells using trypsinisation. RNA was extracted as per the protocol for HEK293 cells. The cells were tested for MCR gene expression using the Applied Biosystems TaqMan® primers. All expressed MC1R, none expressed MC5R. Only THP1 cells expressed MC3R after differentiation with PMA but did not express MC3R before differentiation. MC3R was only expressed by the cells differentiated with 5µg/ml and 10µg/ml of PMA. Cells differentiated with 2.5µg/ml did not express MC3R.

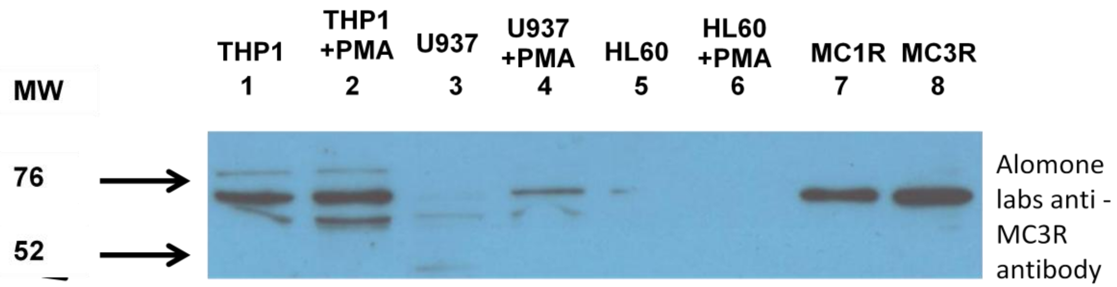


Figure 3.10 Differentiated THP1 cells express MC3R.

This blot shows a band for MC3R between 76kDa and 52kDa in the PMA-treated THP1 cells in lane 2 . It is notable that there are also non specific bands at just below 76kDa which are also seen in the negative controls (the HA-tagged MC1R(MC1R) and MC3R (MC3R) cell lysate (lanes 7 and 8).

For this experiment, membranes were blocked for 3 hours in 5% non-fat milk and incubated overnight with the primary antibody. PMA= phorbol myristate acetate This experiment was performed once. No β actin was used for this blot so equal loading cannot be determined. MW=molecular weight in kDa

3.2.12 Evaluation of RNA extraction technique

Multiple methods of RNA extraction have been tested to ensure the optimal concentration of RNA with the greatest purity and minimal genomic DNA contamination. An experiment with OA synovium was carried out with three forms of RNA extraction and DNA exclusion. One method was Qiazol followed by the RNeasy mini kit with on-column DNase, a second was the Qiazol kit followed by the RNeasy plus mini kit and finally the RNeasy plus kit alone. Qiazol and the RNeasy minikit produced 221.8ng/µl total RNA, Qiazol and the RNeasy plus kit produced 136.0ng/µl and the RNeasy plus kit produced 32.9 ng/µl from 20mg of starting tissue each. The purity in each sample was similar when measured by a Nanodrop Spectrophotometer. A similar experiment comparing Qiazol and the Rneasy mini kit with the RNeasy minikit alone using tonsil as the substrate tissue gave similar results with Qiazol and the minikit producing 281.1ng/µl while the RNeasy kit alone produced 160.4ng/µl. The on-column DNase was used in both methods. Again purity was similar. From these two experiments, the Qiazol kit followed by the RNeasy kit with on-column DNase digestion was chosen as the method of choice for future extractions.

3.2.13 Evaluation of OligodT versus random hexamers

cDNA can be reverse transcribed using either of two types of primers, random hexamers or oligodT's. Random hexamers attach randomly to the RNA whereas oligodTs tend to attach toward the 3'end poly A tail of messenger RNA. An experiment was carried out to ascertain whether the primer made a difference to the cDNA produced. 500ng of tonsil, placenta and testis RNA was reverse transcribed and used at 20ng per reaction for real time PCR. CT values obtained were on average 2 cycles higher for the testis samples for MC1R, MC3R and MC5R and 1 cycle higher for placenta when comparing samples prepared with cDNA with random hexamers compared to that prepared with oligodTs. No MC3R was seen in tonsil and MC5R was present at high C_T values (low expression) in tonsil, testis and placenta. As higher C_T values indicate less product, and random

hexamers produced higher C_T values, it was decided to use oligoDTs as the primer for all reverse transcription reactions with synovium.

3.2.14 Evaluation of Testes as a positive control

Skeletal muscle, placenta, and testis were tested for their suitability as a positive control and calibrator. Testis was seen to express all three receptors. Skeletal muscle did not express MC1R or MC3R but did express MC5R at 100ng per reaction cDNA. Placenta expressed MC1R only. Commercial testis RNA is extracted using a phenol-based technique followed by TURBO™DNase digestion (Ambion). Samples were tested to a concentration of 6.25ng per reaction with results as shown in figure 3.11. Samples were run in triplicate and each fluorescence curve was checked individually for the correct exponential shape. Threshold values were set automatically by the SDS 2.0 software. This experiment was repeated twice with similar results. Further experiments confirm that an RNA equivalent of 20ng per reaction will produce reliable results for all three receptors. Experiments with synovium will have testes RNA used as a positive control and calibrator to allow comparison between experiments.

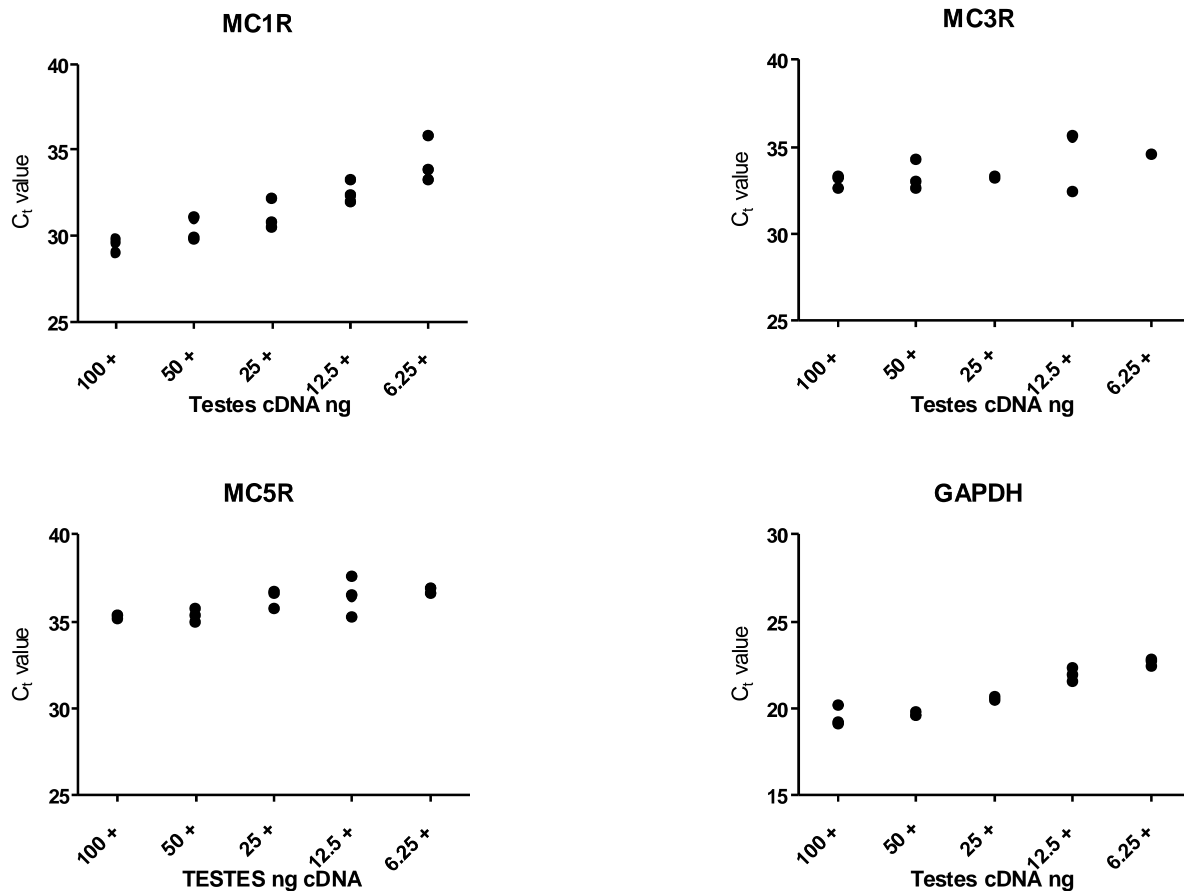


Figure 3.11 Evaluation of a positive control for RTPCR

Doubling dilutions of testes cDNA were used in triplicate to test for the ability to detect MC1R, MC3R and MC5R. GAPDH was detected at cycles below 25 suggesting that it can be reasonably used as an endogenous control at any of these concentrations. MC1R was detected reliably at all concentrations at cycles between 29 and 35. MC3R was detected reliably to a concentration of 25ng/ reaction with a cutoff of 35 cycles used for reliability. MC5R was detected above 35 cycles at all concentrations. Testes were used as a positive control and calibrator for future experiments with synovium. MC5R was not examined in synovium. This experiment was carried out twice.

3.2.15 Evaluation of TaqMan® Primers

Figure 3.12 shows an experiment designed to confirm the relative efficiency of the Applied Biosystems TaqMan® Primers. Commercial testes RNA was reverse transcribed as above. Doubling dilutions were tested with each primer. The graph shows the C_T value plotted against the relative dilution. MC1R and GAPDH primers are equally efficient with 1 cycle increase with each dilution. MC3R and MC5R are not as efficient, suggesting that they are acting at the edge of their detection capability, but can be used to give a qualitative answer. Negative controls are important when looking for the expression of the melanocortin genes as they are intronless. A 5 cycle difference between the positive and negative samples is equivalent to a 32 fold expression level difference if the primer is 100% efficient, a 3 cycle difference would be approximately 8 fold for the MCR primer indicating reduction of genomic DNA. Ideally, no signal would be seen in the negative control if genomic DNA exclusion is stringent.

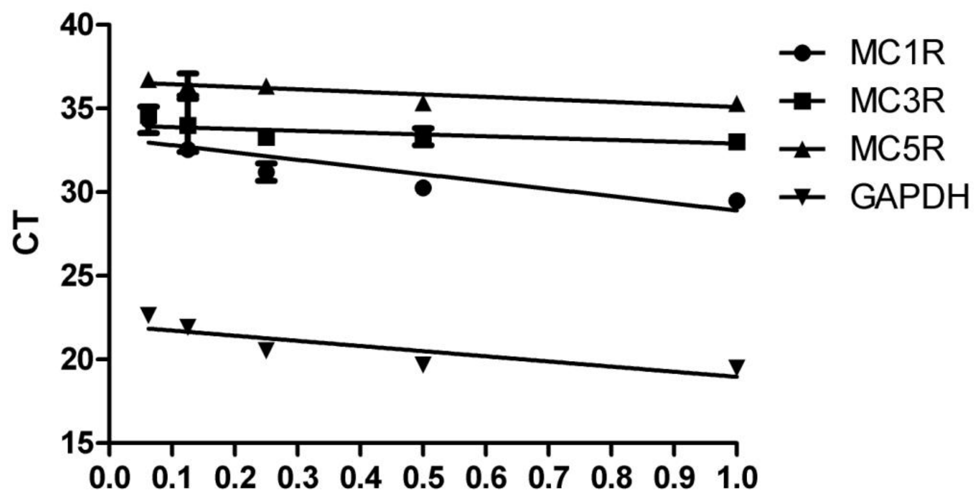
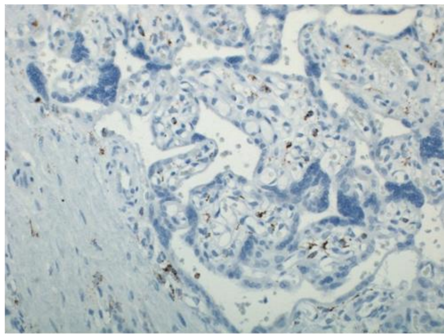


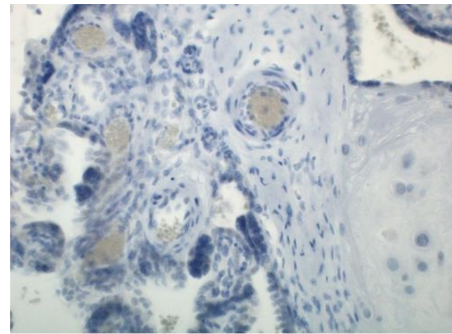
Figure 3.12 The efficiencies of the MCR primers. The Applied Biosystems TaqMan® primers were used with reducing dilutions of testis RNA (Ambion). Testes is shown to express all three melanocortin receptors (negative controls not shown). The MC1R slope is -3.8, MC3R is -1.0 MC5R is -1.5 and GAPDH is -3.1. (A slope of -3.32 indicates an efficiency of 100%). This confirms that MC1R and GAPDH primers may be used for relative quantification. (Data are representative of two experiments.)

3.2.16 Evaluation of ab31309 for paraffin based immunohistochemistry

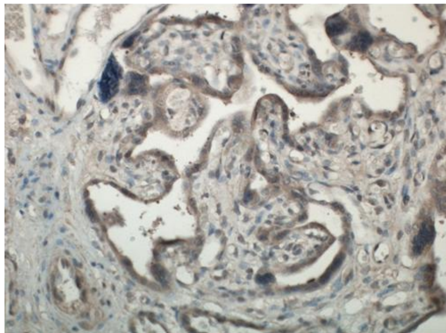
A sample of placenta was stained using the technique for melanocortin receptors as described in materials and methods. Non-specific polyclonal goat IgG was used as a negative control and CD68 as a positive control. CD68 stained macrophages specifically in the placental tissue. Control goat IgG did not stain placenta, ab31309 stained all cell types in placenta but particularly seemed to be prominent in the lining cells. (Figure 3.13)



CD68



Goat IgG control

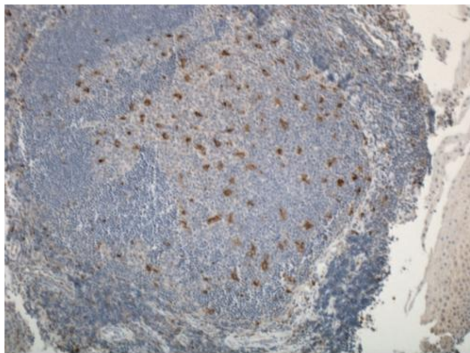


Ab31309

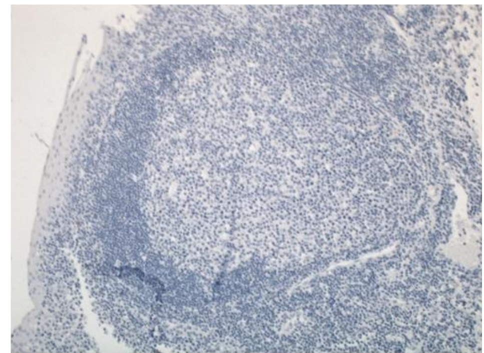
Figure 3.13 Samples of placenta (200x) The left panel is stained with CD68 as a positive control for macrophages, the right panel is the negative control with normal polyclonal goat IgG which did not produce any staining, the bottom panel is placenta stained with ab31309 an antihuman MC_3 antibody. These images are representative of 2 separate experiments.

3.2.17 Ab31309 stains a subset of cells in the tonsil

After establishing that ab31309 gave positive staining with placenta, the antibody was taken forward with an experiment on tonsil (figure 3.14). Here the optimized antibody concentration (5µg/ml) was used to stain a sample of tonsil as a source of all types of immune cells. Although there were no cells stained inside the follicles there were a subset of cells stained that were found outside the follicles in the interfollicular areas. CD68 staining for macrophages was used as a positive control.



CD68



Goat IgG control

Ab31309

Figure 3.14 Samples of tonsil (100x). The left panel shows the positive control CD68 staining tingible macrophages in B cell follicle. The right panel shows the negative control polyclonal goat IgG which shows no staining. The final panel shows ab31309 a goat polyclonal anti human MC_3 antibody stained a subset of cells outside the B cell follicle. These images are representative of 2 experiments.

3.2.18 Rabbit polyclonal antibodies and immunohistochemistry

Sigma produced an anti-human MC3R antibody for use in immunohistochemistry. It was a rabbit polyclonal antibody. It was used in an experiment with human caecum (MC3R is purported to be found in the gut) and was shown to give no staining above that of the normal rabbit polyclonal IgG. It is no longer on the market for purchase.

Professor Bohm (Muenster, Germany) kindly gifted a rabbit polyclonal antibody for human MC1R published with extensively in skin samples[356]. Unfortunately, when tested in synovium normal rabbit polyclonal IgG gives non-specific staining even at the low concentrations used in published work with skin.

There is one rabbit monoclonal antibody for human MC1R. This was tested at the manufacturers recommended dilutions and found not to stain skin, or samples of melanoma or synovium.

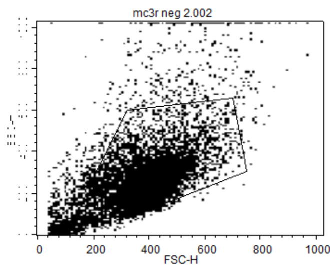
Table 3.1 indicates also the antibodies tested for immunohistochemistry on tissue.

3.2.19 Evaluation of ab31309 for flow cytometry

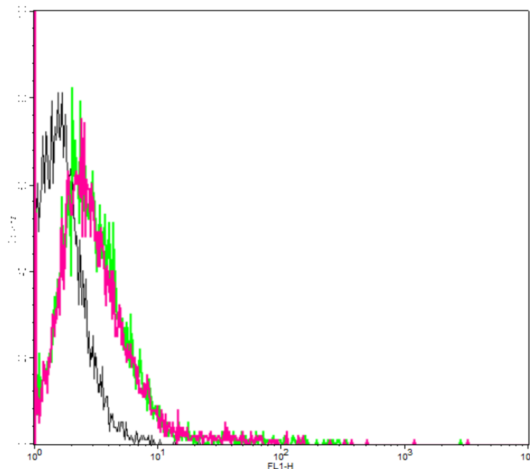
HA-MC3R transfected HEK293 cells were tested for the presence of surface MC3R using ab31309 by flow cytometry. The cells were not trypsinised prior to harvesting to avoid any possible cleavage of the receptor. Cells were not fixed prior to staining.

Figure 3.15 shows that a) there is one population of cells (as expected) and b) ab31309 shows no increased labelling of the cells over and above isotype control. This suggested that either MC3R was not found at the cell surface or that the antibody did not label MC3R. No secondary antibody alone staining was carried out as the isotype control was available.

a



b



Key

Negative control

Isotype control

Polyclonal goat anti human MC3R antibody, ab31309 10ug/ml

Figure 3.15 Evaluation of ab31309 for flow cytometric detection of MC_3

Figure a shows the forward and side scatter plot for this population of HEK293-MC3R cells and the gate used for analysis. There is only one population of cells, as to be expected. Figure b shows that ab31309 gives no increased staining of cells above that seen with an isotype control. This experiment was performed once. There is no secondary antibody alone control as the isotype control is available.

3.2.20 Evaluation of transfected HEK293 cells for surface expression of MC3R

HA-MC3R cells were stained for flow cytometry using an anti-HA antibody directly conjugated to FITC (fluorescein isothiocyanate). Cells were not trypsinised during harvesting to ensure there was no cleavage of the receptor. No secondary antibody was required for this experiment. These cells were not permeabilised and therefore this experiment is looking at the surface expression of the HA tagged receptor in its natural state. Figure 3.16 shows the results of this experiment. A) shows there is one population of cells that was analysed and the gate used for analysis. B) shows that there is a large population of cells that are not labelled with the HA-tag and that there is a smaller population that is. However, there is no difference in labelling between the HA-MC3R transfected cells and the empty vector transfected cells (pcDNA 3.1) suggesting that there is non-specific binding of the antibody to the cells, as these cells do not express the HA-tag by Western blot. This experiment was carried out twice in duplicate. There is no secondary antibody alone control as the isotype control was available and no secondary antibody was used.

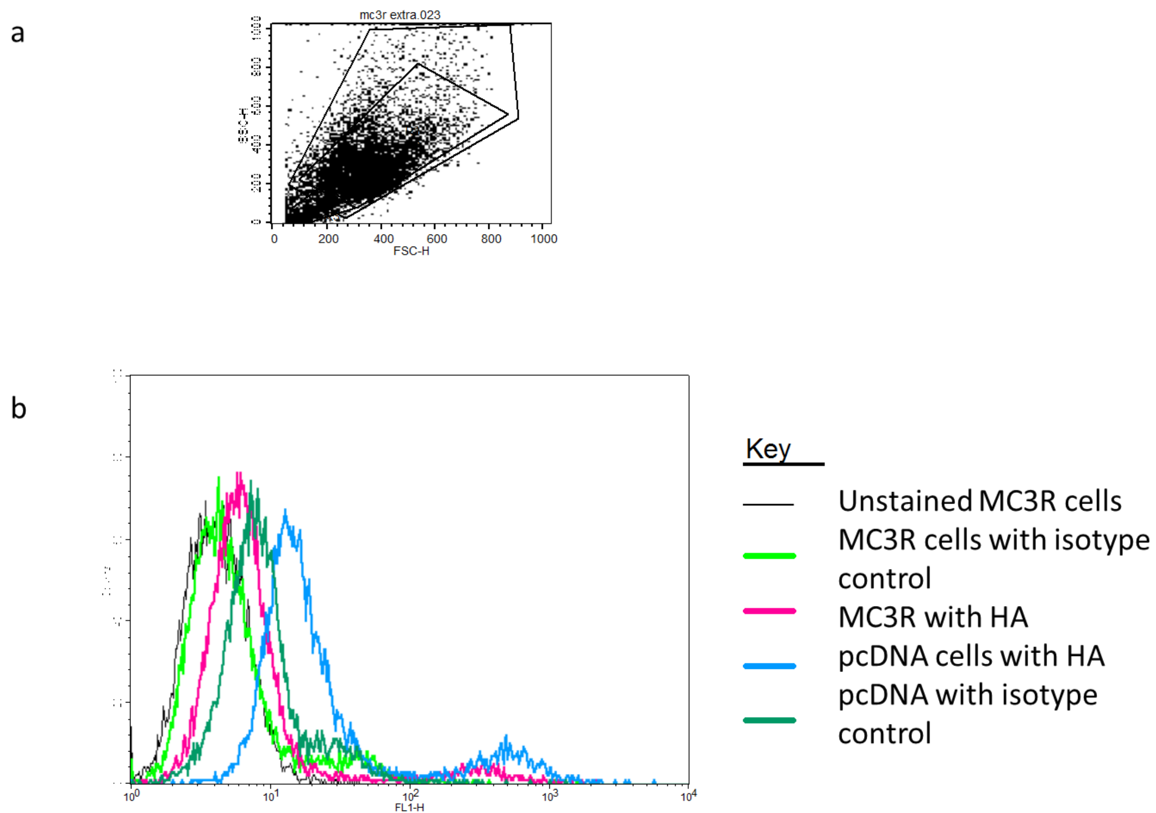


Figure 3.16 Evaluation of surface expression of HA tag using an anti-HA antibody directly conjugated to FITC.

Panel a) shows that there is one population and the smaller gate has been used for analysis. Panel b) shows that there is a large proportion of cells unlabelled by the HA antibody with a small proportion forming the second peak. However both pcDNA and MC3R tagged cells have the same peaks suggesting non-specific binding of the antibody to an antigen in the HEK293 cells. This experiment was carried out twice in duplicate. There is no secondary antibody alone control as the isotype control was available.

3.2.21 Evaluation of the internal expression of HA-MC3R.

To confirm the above findings and to assess the possibility that the receptor was internalised and did not reach the cell surface a further experiment was carried out applying the protocol for cell permeabilisation. Cells were fixed with paraformaldehyde prior to permeabilisation with saponin, thus allowing antibody penetration. This experiment was carried out twice in duplicate.

Figure 3.17 shows similar results to the previous experiment. Panel a shows there is one population of cells and the gate used for analysis. Panel b shows that all of the cells are labelled with the anti-HA antibody suggesting there is intracellular HA-tagged receptor but again the empty vector transfected cells also labelled positive with the HA-antibody suggesting there is non-specific binding to HEK293 cells. There was no secondary antibody alone control used.

These experiments could not confirm the presence of the receptor either on the surface or internally when comparing the MC3R-transfected and the empty vector-transfected cells. These experiments suggested that there was non-specific binding of the HA-antibody to the HEK293 cells. However, the previously described Western Blot experiments using the rabbit polyclonal antibodies to anti-HA confirmed the expression of the protein in each of the cell lines.

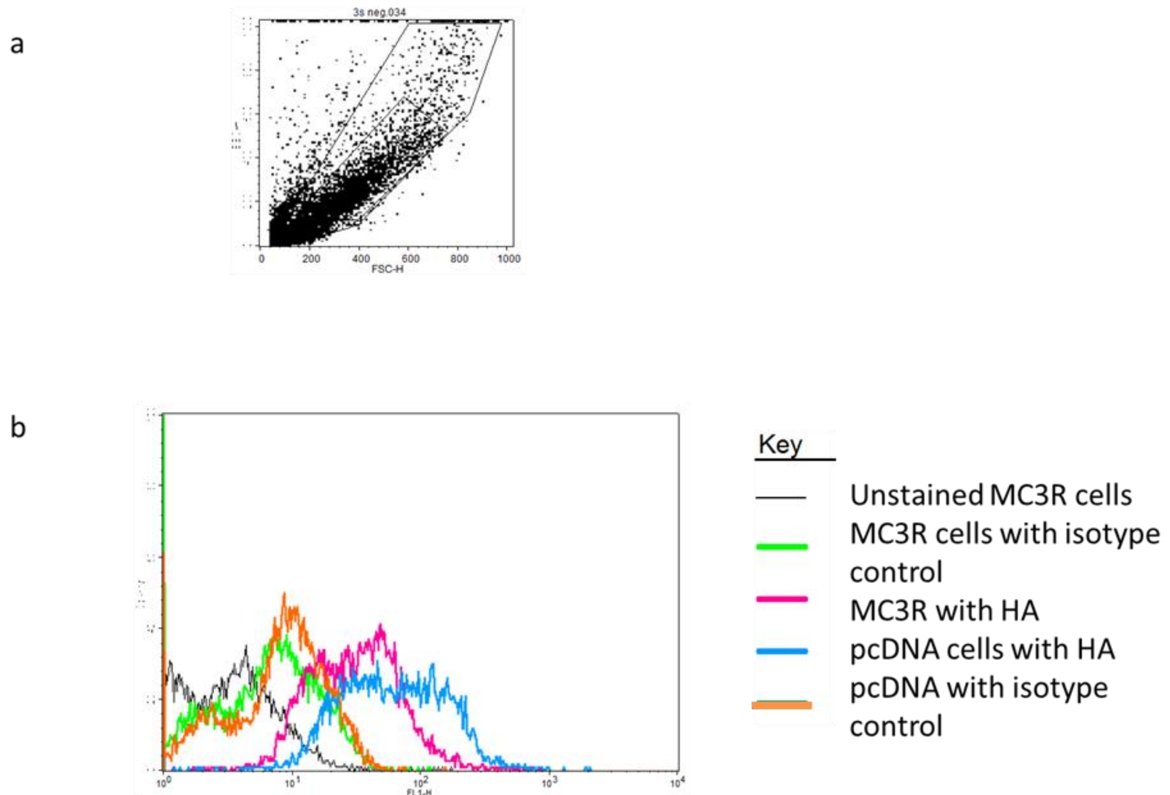


Figure 3.17 Evaluation of intracellular presence of HA-tagged receptor using an anti-HA tagged antibody directly conjugated to FITC.

Panel a) shows there is one population of cells and the gate used for analysis. Panel b) shows that both MC3R and pcDNA 3.1 transfected cells are labelled with the anti-HA antibody above that seen with the isotype and negative control. Unfortunately this suggests non-specificity of the anti-HA antibody There is no secondary antibody alone control.

3.3 Summary

The aim of this chapter was to optimize techniques for the detection of MCRs in the human synovium. This involved creation of a stable cell line for MC1R, MC3R and MC5R which had a triple HA tag at the N terminal end of the receptor. Stable cell lines were successfully created and the HA-tagged receptors were detected with a rabbit polyclonal anti-HA antibody (see figure 3.6). The HA-tagged MC1R was detected with two commercially available anti-MC1R antibodies which also detected a single band with skin (see figure 3.7). The HA-tagged MC3R and MC5R were not detected by any commercially available anti-MC3R or anti-MC5R antibodies.

Two antibodies detected bands with brain tissue (see figure 3.9). One, the Alomone labs antibody detected multiple bands for MC3R. When pre-incubated with its immunizing peptide this antibody revealed there were multiple non-specific bands and a 76kDa band that might have been specific. This might represent a dimer as the expected size of MC3R is 40kDa. The Abcam anti-human MC3R antibody revealed a band at 52kDA with boiled brain lysate and a band at 102kDA when the lysate was left to incubate at room temperature (see figure 3.9a). The Abcam antibody was then taken forward to immunohistochemical experiments (see figures 3.13 and 3.14) and flow cytometry (see figure 3.15). (The Abcam antibody is licensed for use in Western blot but not in immunohistochemistry or flow cytometry). Flow cytometry could not confirm the presence of the HA tag at the surface of the cells due to non-specificity of the anti-HA antibody (See figure 3.16 and 3.17).

The RNA extraction, reverse transcription and polymerase chain reaction techniques were optimized at each step. The MC1R primer was specific for MC1R. The MC3R primer was specific for MC3R and the MC5R primer was specific for MC5R (see figure 3.4 and 3.5). MC1R message was found in untransfected HEK293 cells, placenta and testis, but not found in skeletal muscle. MC3R was found in testis and MC5R was found in testis and skeletal muscle. The primers were examined for their efficiency and range of detection using testis RNA as all 3 MCRs were expressed in this tissue (see

figure 3.12). These experiments confirmed that the primers were suitable for the detection of the MCRs at 20ng of cDNA per reaction (see figure 3.11). Testis was used as a positive control in subsequent quantitative PCR experiments.

In conclusion, the primers were optimized for use on synovial tissue and the antibodies were investigated for their utility in detecting the MCRs. Two antibodies were found that detected MC1R in tissue as well as HA-tagged MC1R lysate. No antibodies were able to detect either HA-tagged MC3R or MC5R. Two antibodies detected MC3R in human brain lysate. Given that the currently available commercial antibodies were unable to detect MC3R or MC5R in the cell lysate, this strongly suggests that the tools available for detecting these proteins are of poor quality.

Chapter 4. Results: The presence of melanocortin receptors in rheumatoid arthritis synovium.

4.1 Introduction

Rheumatoid arthritis is a chronic inflammatory systemic disease mainly affecting the synovial joints but can also affect the eyes, lungs and skin. If uncontrolled it can lead to cardiovascular complications, increased risk of haematological malignancy and severe functional limitation. The advent of anti-TNF therapy, combination DMARD therapy and the recognition of early tight control of disease in combination with the new diagnostic criteria allowing rheumatologists to classify patients and thus possibly instigate therapy earlier has revolutionized current treatment of RA. It is possible now to see patients in remission on medication or even drug-free remission.

However, there is still a significant burden of uncontrolled disease not adequately controlled by current therapies. The biologics and DMARDs also have an associated side effect profile that is not beneficial to all. These two factors enforce the need to find new alternative routes to the treatment of RA, and one viable avenue might be an adaptation of previously tried and tested drugs.

Adrenocorticotropic hormone (ACTH) was first used in the 1950's by Phillip Hench, the Nobel Laureate, for the treatment of RA. ACTH is a melanocortin peptide and part of the melanocortin system. These melanocortin peptides, including α and γ melanocyte stimulating hormone (MSH) have been known to have anti-pyretic and anti-inflammatory effects for many years. They are produced by the action of PC1 and PC2 on the pro-opiomelanocortin peptide. αMSH has been shown to be present at a greater concentration in the synovial fluid than plasma of RA patients with levels associated to anti-inflammatory mediators such as IL1ra[348]. The melanocortin receptors

were discovered in the early 1990's. There are five MCRs, which are stimulatory seven trans-membrane domain G protein-coupled proteins. They activate adenylate cyclase causing an increase in cAMP and protect IκBα, inhibiting the action of NFκB and the production of pro-inflammatory cytokines. MC1R, MC3R and MC5R have been implicated in anti-inflammatory pathways. The mouse homologue, Mc3r, has been localised to mouse macrophages in the synovial knee joint and the Mc3r knockout mice have exacerbated KBxN-induced serum arthritis than wild-type companions, both implicating Mc3r in inflammatory joint disease[327]. Recently, a latency associated peptide (LAP) construct of αMSH has been shown to ameliorate disease in the collagen-induced arthritis model of RA[326]. This is discussed in more detail in the introduction. Although these receptors have been investigated in rodent synovium, there has been no work published in human synovium in RA patients.

Hypothesis

Melanocortin receptors are expressed in synovial tissue. (My focus is on MC1R and MC3R- see Introduction and Chapter 3 for justification)

Aims

To establish and quantify the expression of MC1R and MC3R in synovial tissue from rheumatoid arthritis synovial tissue.

4.2 Results

4.2.1 Demographics of the rheumatoid arthritis patient population

The sample consisted of 28 patients. Table 4.1 shows the descriptive characteristics of the patient sample pool I have used. The median age of the sample was 60.5 years with a range of 32 to 83 years. The majority (23/28) were female with a median duration of disease of 36 months (range 6-336 months). These samples were chosen because data sets were also available for other clinical characteristics with the median ESR being 42.5mm/h (16-132 mm/h) and median CRP being 36 mg/dl (5-274 mg/dl). In addition, 18/22 of patients were RF positive and 11/14 were ACPA positive; 15/21 were erosive on X-ray at the time of the sampling. Of those that had anti-nuclear antibodies (ANA) tested, 7/15 were ANA positive.

Previous and current therapies included glucocorticoids, anti-TNF medication, rituximab and other disease-modifying therapies such as sulphasalazine, hydroxychloroquine and leflunomide. Only 3 patients or 12.5% were DMARD naïve. Just less than half, 11/24 (45.8%) were on methotrexate at the time of sampling. The majority had been exposed to glucocorticoids, 18/21, (85.7%), although fewer were currently taking glucocorticoids, 10/23 (43.5%). Finally, 34.7% (8/15) of patients were currently on an anti-TNF medication and a minority were taking rituximab (2/22, 9%) whilst 25% (6/24) were on a DMARD other than methotrexate (such as leflunomide or sulphasalazine).

Table 4.1 Qualitative Clinical Parameters

Clinical parameter	Yes/no (total)	% positive
Sex: female	23/5 (28)	92.0(female)
Rheumatoid Factor (RF) positive	18/4 (22)	81.8
ACPA positive	11/3 (14)	78.6
ANA positive	7/8 (15)	46.7
Erosions on Xray	15/6 (21)	71.4
Previous Steroid use	18/13 (21)	85.7
Current steroid use	10/13 (23)	43.5
Current anti-TNF treatment	8/15 (23)	34.7
Current rituximab treatment	2/22 (24)	9.0
Current methotrexate treatment	11/13 (24)	45.8
Current other DMARD treatment	6/18 (24)	25.0
DMARD naïve	3/21 (24)	12.5

Table 4.2 Quantitative clinical parameters

Parameter	Median	Minimum	Maximum
Age (years)	60.5	32	83
Disease duration (months)	36	6	336
ESR (mm/h)	42.5	16	132
CRP (mg/dl)	36	5	274

4.2.2 MC1R and MC3R gene expression is present in rheumatoid synovium.

All 28 samples were tested for MCR gene expression using Invitrogen TaqMan® primers, and I ran samples in duplicate. I have used GAPDH as the internal control gene for reasons discussed in Chapters 2 and 3. Regarding the most salient results, all 28 RA synovia were MC1R positive, however, MC3R expression was not evenly distributed: 14 out of 28 samples were MC3R positive leaving 14 MC3R negative samples. Of two patients with early arthritis (symptoms present for less than 1 year) both were MC1R positive and one was MC3R positive indicating expression of these receptors even in early disease. (See table 4.3)

The samples were given a number representing $2^{-\Delta\Delta C_T}$, where ΔC_T is the MC1R C_T value – GAPDH C_T value and $\Delta\Delta C_T$ represents (synovium ΔC_T)- (testis ΔC_T). This number is an RQ (relative quantification) value which represents the fold change of the MC1R value above the calibrator value for each sample. The calibrator has a value of 1. This RQ value is comparable between plates as the same calibrator was used on every plate. It allows the relative quantification of the sample to a calibrator sample. The same calculation was applied for MC3R using the MC3R C_T values.

Sample	Diagnosis	MC1 RQ	MC1R CT	MC3 RQ	MC3R CT
412	RA	5.409893	29.82611	1.71871	35.32527
1511	RA	5.662339	25.80506	15.69728	28.86269
4208	RA	1.263706	24.4403	0.176033	31.94827
4608	RA	10.04252	24.08609	0	
5611	RA	0.151339	32.80439	0.102784	33.92514
6608	RA	0.148732	30.93716	0	
10407	RA	2.381874	23.6974	0.040414	33.66315
10607	RA	6.404096	23.81229	0.170168	34.42516
00210l	RA	1.288602	32.64047	0	
00210r	RA	11.64605	23.87841	0	
511	RA	2.927814	24.33371	0	
2408	RA	0.47721	25.48729	0	
0810	RA	1.388377	25.27858	2.211521	29.93998
3009	RA	2.957446	23.18217	0	
3208	RA	2.396625	25.09623	0	
3508	RA	0.914807	26.56925	19.54839	27.48485
3608	RA	7.156182	23.91451	0	
3708	RA	0.561144	34.99192	128.8032	32.48238
4009	RA	2.524726	25.37855	1.817301	31.18592
4108	RA	2.642567	28.41489	0	
4609	RA	7.128952	23.80526	0.656703	32.13876
4711	RA	4.851642	24.30584	0	
4908	RA	3.640965	23.84011	0	
5508	RA	1.79674	24.63088	31.01927	25.85421
5509	RA	2.455892	30.42302	7.946106	33.57213
5511	RA	8.139555	22.86901	0	
5711	RA	4.212489	30.65455	0	
6111	RA	6.055432	24.59283	0.374069	34.21346

Table 4.3 Expression of MC1R and MC3R in a sample of 28 RA patients. Values are RQ units. RQ represents fold change or $2^{-\Delta\Delta C_T}$ using testis as a calibrator. Raw CT values are next to the calculated RQ values.

4.2.3 MC1R expression can be quantified and separated into high and low expression

Figure 4.1 shows the RQ values of the MC1R positive samples. The MC1R RQ values appear to split into two groups that have been designated high and low. The high samples have higher RQ values representing a larger fold change above the calibrator. Figure 4.1 reports the samples' split by the median of the total sample (2.79). The medians of the two groups are significantly different (p=0.0001). There is almost a 5 cycle in gene expression between the medians of the two groups (1.34 v 5.86) and this corresponds to a remarkable 64 fold difference. By performing this analysis, I could identify 14 samples with high MC1R expression and 14 displaying a low degree of expression for this receptor. There was no relationship between MC1R high and low expression and MC3R positive and negative expression (Fisher's Exact Test, table 4.4).

Figure 4.2 shows the frequency distribution of the RQ values and provides a rationale for splitting the MC1R values into high and low samples.

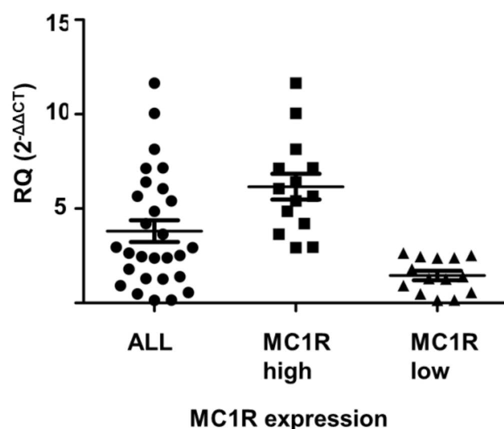


Figure 4.1 Expression of MC1R. All samples were plotted by RQ value. This group was then divided by the median of the group to produce two groups with a 64 fold difference between their medians. (p=0.0005)

	MC1R high	MC1R low
MC3R +	5	9
MC3R -	9	5

Table 4.4. Fisher's Exact Test for MC1R and MC3R shows that there is no relationship between MC1R high and low and MC3R positivity (p=0.257).

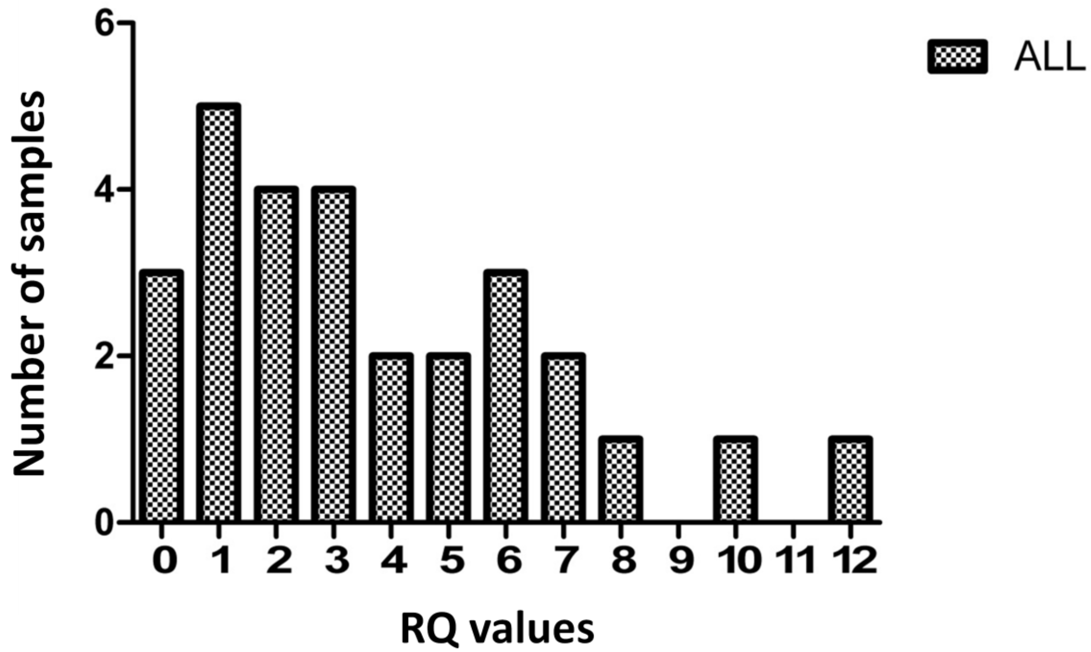


Figure 4.2 Frequency distribution of RQ values. Each RQ value is a category with a number of samples. The median of the distribution is 2.79.

4.2.4 Use of an anti-human MC3R antibody for detection of the MC3R protein in human synovium.

Figure 4.3 shows an experiment with the anti-human MC3R antibody. Three samples of human RA synovium were tested with ab31309 with normal goat IgG used as a negative control, with melanoma used as a negative control and placenta as a positive control for receptor expression. Samples of synovium stained positive for MC3R however, the negative controls also gave the same pattern of staining suggesting that the staining is non-specific. This experiment with synovium has been repeated twice more with polyclonal goat IgG antibodies, which show nonspecific staining with the negative control antibody although this did not stain placenta or pituitary or tonsil (see chapter 3). Of note these three samples of synovium had tested negative for MC3R by RTPCR. Tissue samples were also tested with different lengths of protein block (1 hour to overnight) and also with different protein blocks (2% BSA or 10% horse serum, or 10% rabbit serum), however the background staining with the negative control IgG was not alleviated. This antibody was therefore not taken forward for further experiments.

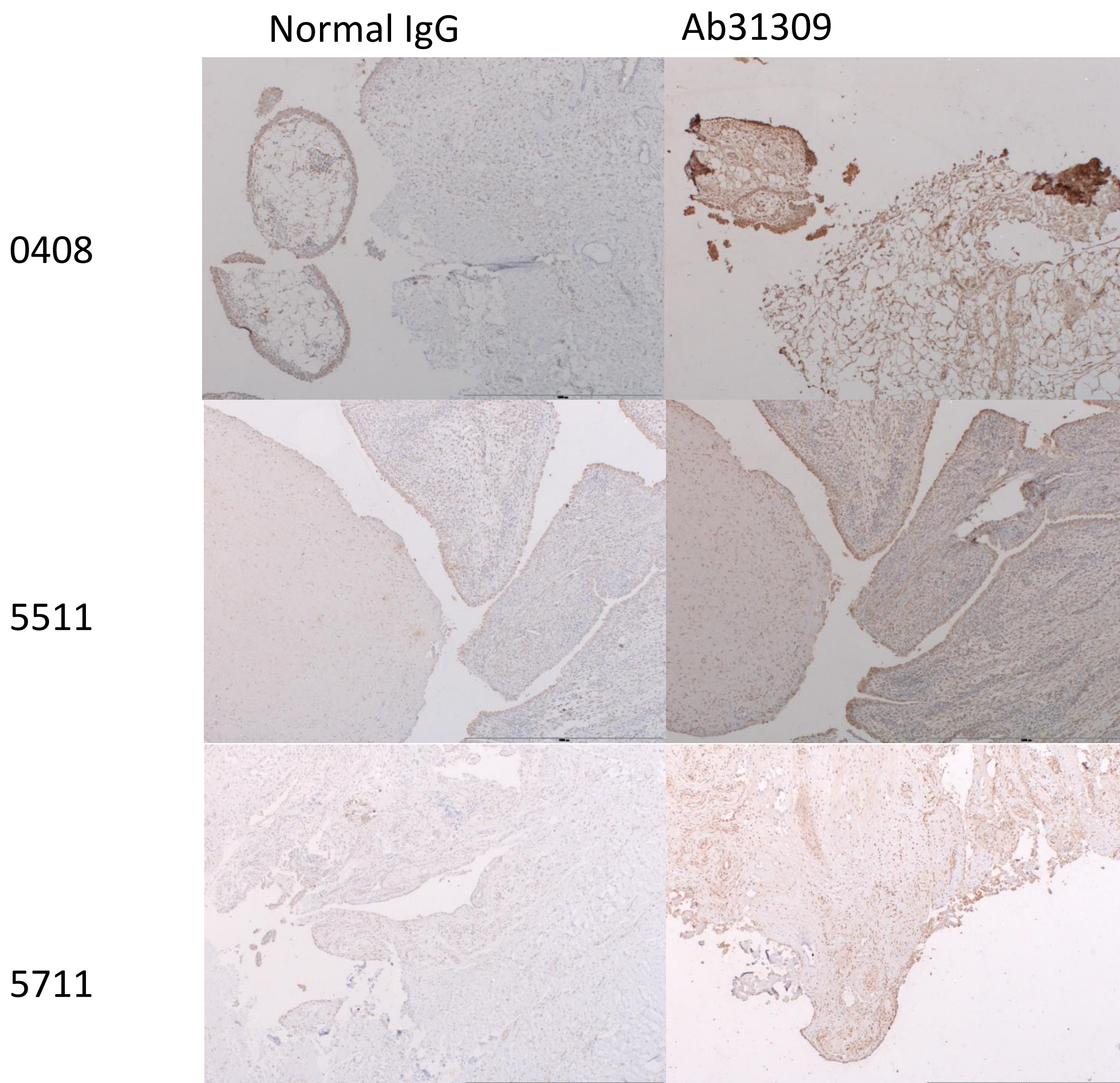


Figure 4.3 Samples of synovium 1.

Patient identification is reported on the left of the figure. Samples were MC3R negative by RTPCR. Samples were blocked for 30 minutes with Dako protein block and incubated with the primary and secondary antibodies for one hour respectively. These were either ab31309 (putatively anti-human MC3R antibody) or normal goat polyclonal IgG. It is notable that with different samples the staining with normal goat IgG is to different extents. This experiment was repeated with different durations of protein block as well as different protein blocks (three experiments).

4.2.5 Use of anti-human MC1R antibody to detect MC1R in human synovium

Three samples of human osteoarthritis (OA) synovium were tested for the presence of MC1R using the antibody sc6875 an anti-human MC1R antibody commercially available from Santa Cruz technology and already tested for Western Blot. (See figure 4.4) OA tissue was used in order to save the scarce resource of RA tissue. These samples had been tested and shown to be MC1R positive by RTPCR. All three samples had MC1R high expression. This experiment used a 10% horse serum protein block for one hour and overnight incubation with the primary antibody as discussed in Chapters 2 and 3. CD68 staining was used here as a positive control. All samples showed some CD68 staining particularly in the lining layer. Although I could detect staining with sc6875, there was also significant background staining with the normal goat IgG as seen with the experiments with the MC3R antibody. It is very difficult to distinguish whether the staining with the MC1R antibody is true or simply background staining. This experiment was repeated with tonsil which gave no background staining with normal goat IgG and with two other samples of osteoarthritis synovium with similar results (using Dako serum-free protein block). A further experiment using proteinase K as the antigen retrieval method produced no staining whatsoever (not shown). Goat polyclonal antibodies were not therefore taken forward for any further use.

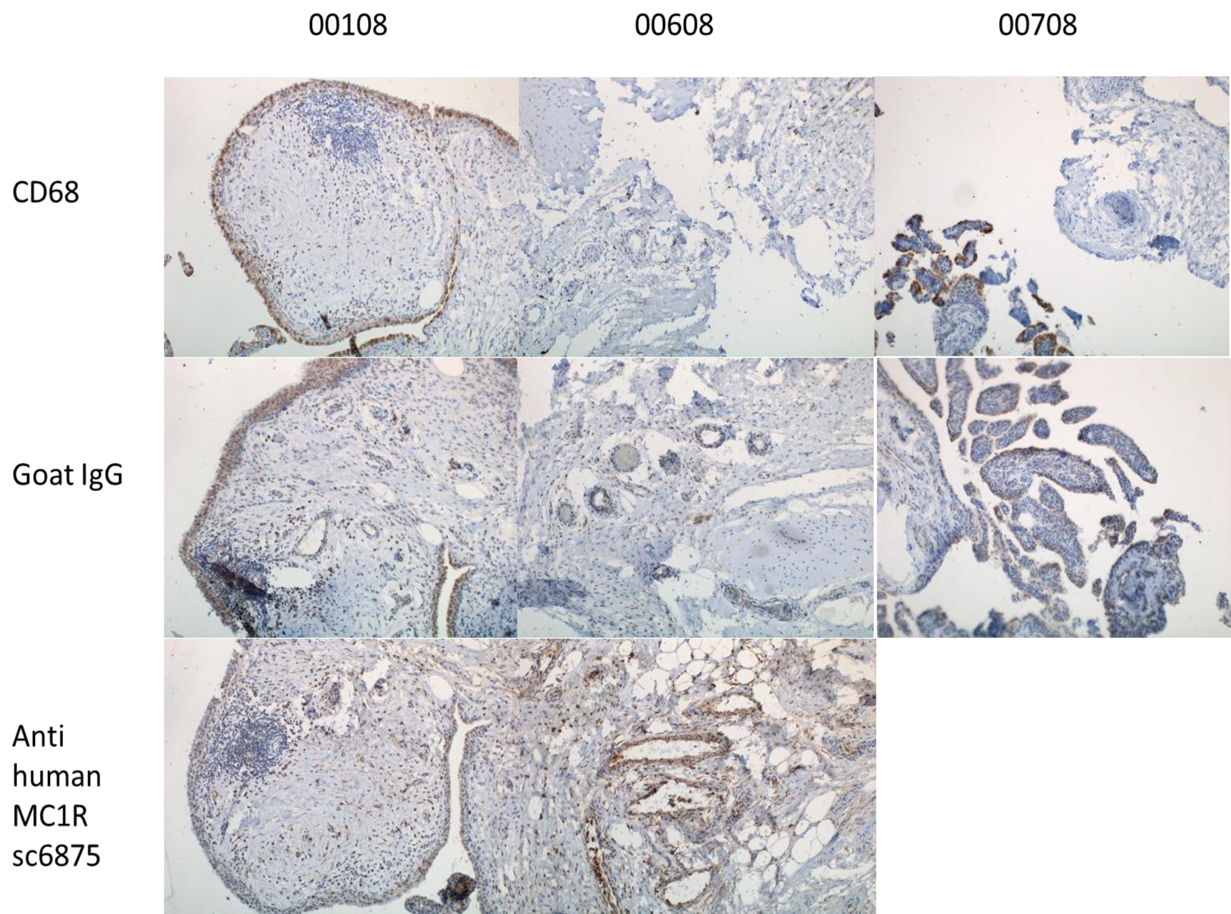


Figure 4.4 Samples of synovium 2.

Patient identification number is on the top of the figure. Samples were MC1R high by RTPCR. Samples stained with both sc6875 the anti-human MC1R antibody as well as with the normal goat polyclonal IgG. It is notable that with different samples the staining with normal goat IgG is to different extents. Samples were blocked for 1 hour with 10% horse serum protein block and incubated with the primary antibody overnight and secondary antibodies for one hour. No staining was done with sc6875for 00708, instead another MC1R antibody sc9899 was used which gave similar results with all three tissues as sc6875.

4.3 Summary

A sample population of 28 RA patients were analysed for quantitative gene expression of MC1R and MC3R using quantitative polymerase chain reaction with Invitrogen TaqMan® primers which I had validated as described in Chapter 3. The majority of these samples were from female patients with a high incidence of RF and ACPA positivity (See table 4.1). All patients expressed MC1R and exactly half the patients expressed MC3R (see table 4.3). MC1R expression was not always to the same level and could be quantified and split into high and low expression groups. There was a significant difference between the median of these groups, with a 64 fold difference between the median values (1.34 *vs* 5.86) (See figure 4.1) .

I have tried to combine these expression data with the degree of protein detection (see figures 4.3 and 4.4), however, as in part discovered in Chapter 3, there is a remarkable lack of good tools to monitor these receptors by immunohistochemistry or Western blotting analyses.

In these settings, antibodies tested for examining the expression of human MC1R and human MC3R in human synovium were found to be non-specific producing a pattern of staining not dissimilar to what I observed with the negative control, normal goat IgG. I conclude that MC receptor protein could not be localized by immunohistochemistry with the currently available commercial antibodies.

Chapter 5. Results: The Relationship of Melanocortin Gene Expression to Clinical and Immunophenotype Characteristics.

5.1 Introduction

There is growing literature that details the validity of immunophenotyping in the analysis of RA synovial samples. This literature has defined immunohistochemical grading as a way of describing the pathological process in the synovia and at the same time as a way of predicting disease or response to treatment.

There are differences between normal and rheumatoid synovium in terms of inflammation and therefore standardised inflammatory score. These scores take into account the synovial lining, the stromal cellularity and the cellular infiltrate. High scores equate to higher degrees of inflammation in the tissue. Macrophage count, both total and sublining, has been purported to be a sensitive marker for response to treatment and is not affected by placebo [62]. They have been related to pain[145], erosions[155-156] and change in DAS28[157-158]. In any case while clearly pathogenic, the use of macrophage count for the tissue pathotyping process has yet to become a consensus biomarker for discrimination between responder and non-responder RA patients.

Plasma cell-like cells (CD79+ CD20-) are associated with high disease activity in a study investigating histological scores before and after treatment with rituximab. Changes in plasma cells were associated with a decrease in DAS28 after 24 weeks of treatment with rituximab and there was a difference between responders and non-responders. Disease responders displayed a significant reduction of plasma cells from their synovial biopsy tissue sample [169]. Again this is not yet a consensus biomarker for the development of RA or for the response to RA treatment. On this basis, I thought that a way forward would have been a detailed analysis of tissue pathotype of the 28 samples I used for MC1R and MC3R expression.

Clinical characteristics for the sample population were identical to those reported in Chapter 4 (table 4.1 and 4.2). The immunophenotype of each sample was determined from matched paraffin samples. These scores included the synovitis score as described in Materials and Methods, grade and CD3, CD68, CD138 and CD20 scores. The CD scores were graded using a semiquantitative Tak score (see Introduction section 1.1.3). Finally, I have used the same cDNA that I verified for MC1R and MC3R mRNA expression to quantify expression patterns for cytokines.

Hypothesis

There is no relationship seen between the clinical and immunophenotype characteristics of rheumatoid arthritis and MCR gene expression in rheumatoid synovium.

Aims

To correlate MC1R and MC3R gene expression with a) Clinical characteristics, b) Immunophenotype and c) Cytokine mRNA expression in rheumatoid synovium.

5.2 Results

5.2.1 MCR expression and clinical parameters

MC1R expression was categorized into MC1R high and MC1R low groups as described in chapter 4 and compared to categorical variables such as samples being obtained from patients on methotrexate, or glucocorticoids. There was an association between being MC1R low and not being on combination therapy that did not quite reach significance (p=0.07). No male patients were MC1R high (p=0.04). Table 5.1 shows that there was no significant pattern emerging when differences between any of the categorical variables were tested for with the Fisher's Exact test. (I selected the Fisher's Exact test as some categories contained less than 10 samples.) Of note I could not observe any significant association with erosive status, rheumatoid factor or ACPA positivity.

Similar results were obtained when the same categorical variables were analysed with regards to MC3R positivity or negativity. However, although there was no significance seen in the relationship between ESR and CRP and MC3R positivity due to the degree of variability in the sample tested, an interesting pattern emerged: MC3R positive samples generally had higher ESR and CRP values (see figure 5.1). However, the variance of values within the groups precluded significance.

Clinical Parameter		MC1R high	MC1R low	P value	MC3R +	MC3R –	P value
RF	**+**	8	10	1.000	11	7	1.000
	-	2	2		3	1	
CCP	**+**	3	6	1.000	6	5	1.000
	-	0	2		2	1	
ANA	**+**	4	3	1.000	3	4	0.619
	-	5	2		5	3	
Erosions	**+**	8	9	1.000	9	6	0.361
	-	3	3		2	4	
Previous	**+**	11	10	1.000	12	9	0.627
steroids	**-**	1	2		3	1	
Current	**+**	6	4	0.680	6	4	1.000
Steroids	**-**	6	8		8	6	
Current	**+**	6	3	0.226	4	4	1.000
anti TNF	**-**	6	10		9	7	
Current	**+**	1	1	1.000	1	1	1.000
rituximab	**-**	11	12		12	10	
Current	**+**	8	4	0.115	5	6	0.682
MTX	**-**	4	9		8	5	
Other	**+**	5	1	**0.073**	4	2	0.649
DMARD	**-**	7	12		9	9	
DMARD	**+**	1	2	1.000	2	1	1.000
Naïve	**-**	11	11		11	10	

Table 5.1 Clinical characteristics and MCR expression.

Twenty-eight synovial tissue samples were analysed for MC1R and MC3R expression (as reported in Chapter 4). This table attempts to define novel correlations between the reported clinical and biochemical parameters and the apparent dichotomy in receptor expression (MC1R high or MC1R low; MC3R+ve and MC3R-ve). The absolute values of reference are in table 4.1 in Chapter 4.

(Of note, totals do not add precisely to 28, as in some cases, information was not available the numbers represent numbers of patients in each category).

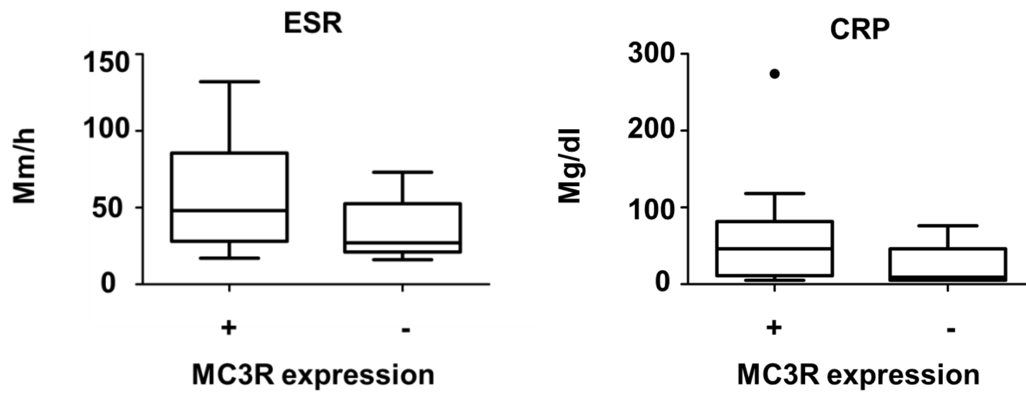


Figure 5.1 Measurements of ESR and CRP in terms of MC3R expression in human RA synovia. Twenty-eight RA synovia were tested for MC3R expression, finding a perfect 50% split. A potential correlation with ESR values and blood levels of CRP was sought. Two sided t-test comparison was non-significant with high variance in values within groups (+n=13, -n=5)

5.2.2 MC1R expression shows associations with synovial macrophage immunohistochemistry scores

Each synovial tissue sample was scored for inflammation score, synovitis score (out of 9), grades of lymphoid organisation (none, diffuse, 1-3), and for the degree of cell infiltrate (from 0 to 4). The latter parameter was obtained by monitoring the presence of CD3 positive cells (T cells), CD20 positive (B cells), CD68 positive (macrophages) and CD138 positive (plasma cell-like cells).

Infiltrate scores were split into two categories of 2 and above or below 2. Categorical analysis using Fisher's Exact test revealed an association between MC1R high samples and CD68 lining high scores, (i.e. MC1R high samples also had a high macrophage score in the lining of the sample) this reached significance (p=0.01). Table 5.2 shows these results.

5.2.3 MC3R gene expression shows associations with synovial plasma cell-like cell scores

The same immunohistochemical scores were used for analysis in relation to MC3R positivity of the RA synovia. Categorical analysis using Fisher's Exact test was used to compare the two categories of infiltrate scores as well as inflammation score and grades with MC3R positive and negative samples. Of striking note no MC3R positive samples were CD138 high (i.e. no MC3R positive samples had a

significant plasma cell infiltrate) and this negative association was highly significant (p=0.006). Table 5.2 shows these results

Immunohistochemistry score		MC1R high	MC1R low	P value	MC3R positive	MC3R negative	P value
CD3	**>2**	7	5	0.695	7	8	1.000
	<1	6	7		5	5	
CD68	**>2**	8	8	1.000	7	7	1.000
	<1	4	5		5	6	
CD68 lining	**>2**	11	5	**0.011**	7	9	0.688
	<1	1	8		5	4	
CD68 sublining	**>2**	9	5	1.000	7	7	1.000
	<1	3	8		5	6	
CD20	**>2**	7	5	0.695	5	8	0.433
	<1	6	7		7	5	
CD138	**>2**	6	7	1.000	0	7	**0.006**
	<1	5	6		11	6	
Inflammation score	**<3**	3	7	0.277	3	3	0.967
	4-6	6	3		4	5	
	7-9	4	4		4	4	
Grade	**>2**	9	6	0.428	6	5	0.692
	<1	4	7		6	9	

Table 5.2 Immunophenotype and MCR expression.

Twenty-eight synovial tissue samples were analysed for MC1R and MC3R expression (as reported in Chapter 4). This table attempts to define novel correlations between the immunohistochemical parameters and the apparent dichotomy in receptor expression (MC1R high or MC1R low; MC3R+ve and MC3R-ve). (of note, totals do not add precisely to 28, as in some cases, information was not available, numbers represent numbers of patients). Highlighted results are significant (p<0.05).

5.2.4 Relationship between cytokine expression and MCR expression

Figures 5.2 and 5.3 shows the ΔCT values for a group of 6 cytokines and chemokines that I have selected for their putative importance in the pathogenesis of RA. Again, MC1R was split into high and low groups and MC3R was split into positive and negative groups.

Figure 5.2 shows box plots comparing the expression of IL1, IL6, TNFα, RANKL, CXCL13 and IL21 in synovial samples with high *versus* low MC1R expression. No significant association was seen for any cytokine studied. Figure 5.3 shows box plots comparing expression of the same cytokines in samples positive or negative for MC3R. Again, no associations were seen. The cytokines were quantified using the ΔC_T method where ΔC_T is equal to the C_T cytokine – C_T GAPDH. As ΔC_T increases, the amount of product decreases. No calibrator sample was used for these calculations.

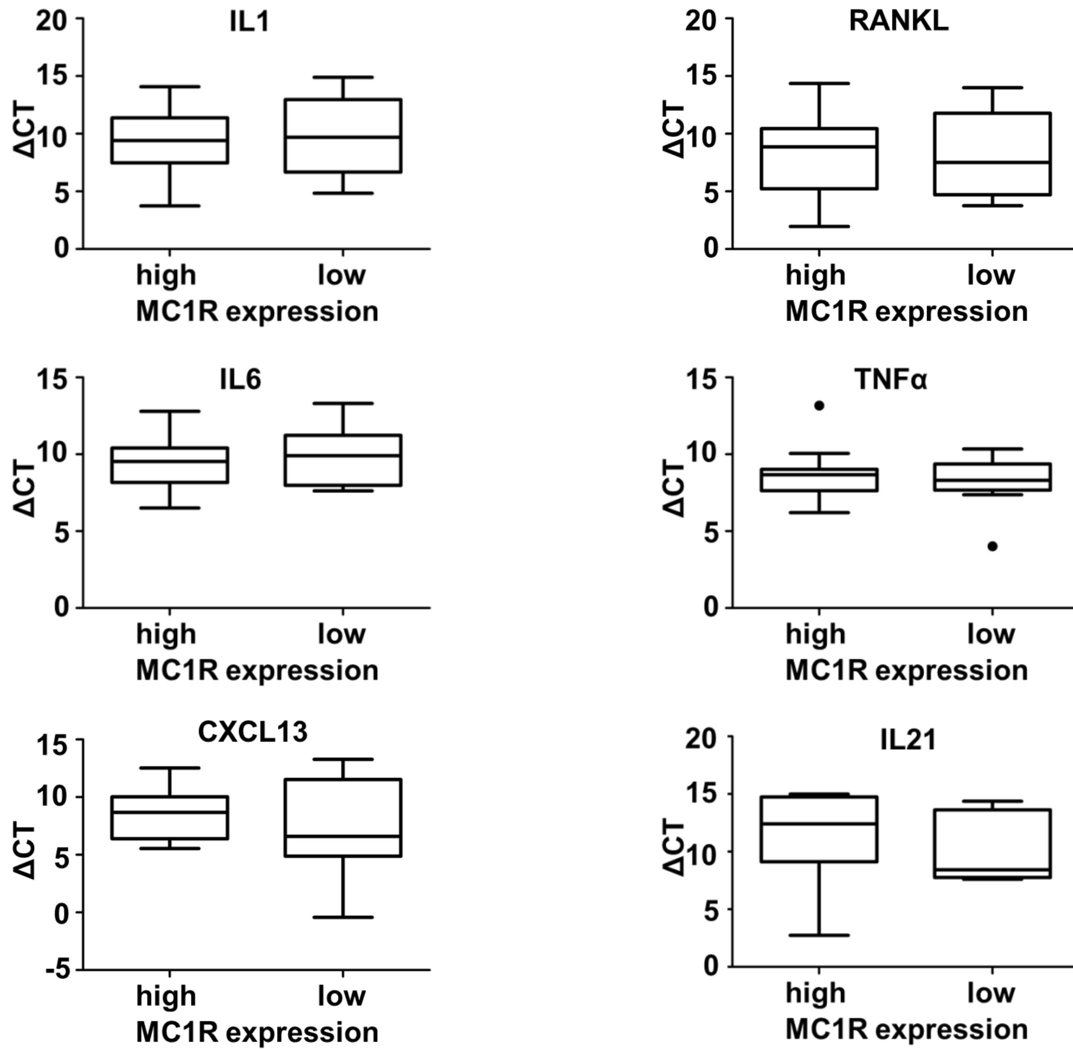


Figure 5.2 Box plots showing profiles of cytokine expression in samples with high or low MC1R expression. Data is represented as Tukey box and whiskers plots with lower and upper quartiles and indicating the exact medians. No relationship emerged between the extent of mRNA expression for any of the six cytokines tested and MC1R high or low positivity in the RA synovia. Only thirteen samples were IL21 positive, these are shown here. Dots represent outliers. n=25

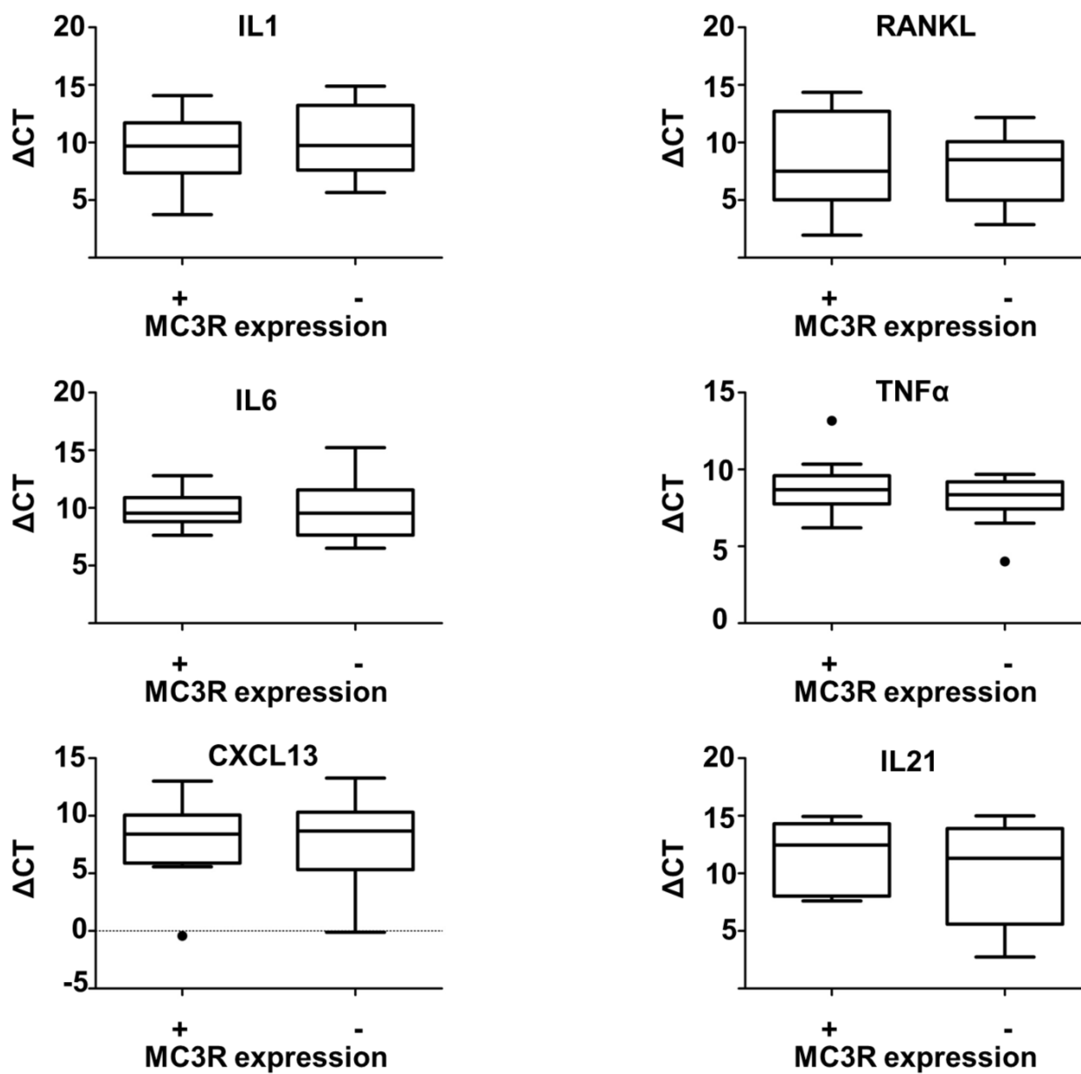


Figure 5.3 Box plots showing profiles of cytokine expression in samples with positive or negative MC3R expression. Data is represented as Tukey box and whiskers plots with lower and upper quartiles and indicating the exact medians. Dots represent outliers. No relationship emerged between the extent of mRNA expression for any of the six cytokines tested and MC3R positivity or negativity in the RA synovia. Only thirteen samples were IL21 positive, these are shown here. n=25

5.2.5 MC1R and its covariates

MC1R has been analysed as a dichotomous variable i.e. either high or low. It can also be analysed as a continuous variable to identify its covariates. Correlation is a way of investigating the relationship between two quantitative continuous variables. There is an assumption that the data samples randomly from a normal distribution. Pearson's correlation plots the two variables on an x-y axis and the closer the scatter of points is to a straight line the higher the correlation. The correlation coefficient can range from -1 to +1. A t-test is used to determine whether this coefficient is significantly different from zero.

Of importance, Pearson's pairwise correlations showed significant associations between MC1R and CD68 lining (Pearson correlation =0.405, p=0.045). There was no association with age and sex or duration of disease. The panel of cytokines with MC1R were tested with an ANOVA (analysis of variance) and still found to be non-significant. Analysis of variance tests the hypothesis that the group means are equal. The dependent variable was MC1R.

Other associations

There is correlation (Pearsons pairwise correlations) within the immunohistochemical groups especially with CD68 which correlates with CD3, CD20, and CD138 (p<0.05).CD138 correlates with CD20 and CD3 and CD68 lining and sublining and total values (p<0.05). This data is shown in table 5.3.

	CD3 (synovial)	CD20 (synovial)	CD138 (synovial)	CD68L (synovial)	CD68SL (synovial)
CD3 **Pearson** **P** **N**	1	0.773 0.000 25	0.689 0.000 24	0.442 0.027 25	0.617 0.001 25
CD20 **Pearson** **P** **N**		1	0.627 0.001 24	0.533 0.006 25	0.441 0.027 25
CD138 **Pearson** **P** **N**			1	0.416 0.043 24	0.274 0.196 24
CD68L **Pearson** **P** **N**				1	0.651 0.000 25
CD68SL **Pearson** **P** **N**					1

Table 5.3 Correlation matrix for immunohistochemistry scores. This table shows the Pearson's pairwise correlations (Pearson) for the immunohistochemistry scores. P represents the significance of the correlation where N is the number of samples tested. Note that not all the samples had immunohistochemistry data available. Correlations in red reach statistical significance ($p<0.05$). L=lining, SL=sublining.

When I analysed the gene expression of the cytokines with the immunohistochemistry scores I found a significant correlation between IL6 mRNA and CD68 sub-lining score (Pearson correlation-0.515, p=0.017). I also observed a negative correlation between IL1 and CD68 sublining score (Pearson correlation -0.461, p=0.047). This data is reported in table 5.4.

		IL1	TNFα	IL6	RANKL	IL21	CD68SL
IL1	Pearson	1	0.583	0.459	-0.517	-0.182	-0.461
	P		0.006	0.042	0.016	0.430	0.047
	N		21	20	21	21	19
TNFα	Pearson		1	0.695	-0.171	-0.214	-0.174
	P			0.000	0.414	0.304	0.427
	N			23	25	25	23
IL6	Pearson			1	-0.344	-0.061	-0.515
	P				0.108	0.780	0.017
	N				23	23	21
RANKL	Pearson				1	-0.129	0.172
	P					0.538	0.431
	N					25	23
IL21	Pearson					1	0.159
	P						0.468
	N						23
CD68SL	Pearson						1
	P						
	N						

Table 5.4 Correlation matrix for cytokines (RQ values) with CD68sl (sublining) score.

This matrix shows the Pearson pairwise correlation values (Pearson) where P is the significance of this value and N is the number of samples tested. Not all samples had matched immunohistochemistry data and therefore not all samples add up to 28. Significant relationships ($p<0.05$) are shown in red. SL=sublining

When I analysed the cytokines I could note a significant correlation between IL6 and TNFα gene expression products (Pearson correlation 0.695, p=0.000). I could also observe a correlation between IL1 and TNFα (Pearson correlation 0.583, p=0.006), IL1 and RANKL (Pearson correlation -0.517, p=0.016) gene expression products as well as IL1 mRNA and ESR (Pearson correlation -0.544, p=0.036). CRP values correlate with ESR which might be expected (Pearson correlation 0.690, p=0.002). ESR correlates with IL21 (Pearson correlation 0.517, p=0.021). This data is reported in table 5.5.

The correlation matrices can be found in the appendices of this thesis.

	IL1	TNFα	RANKL	IL21	ESR	CRP
IL1 **Pearson** **P** **N**	1	0.583 0.006 21	-0.517 0.016 21	-0.182 0.430 21	-0.544 0.036 15	-0.423 0.116 15
TNFα **Pearson** **P** **N**		1	-0.171 0.414 25	-0.214 0.304 25	-0.413 0.111 16	-0.362 0.154 17
RANKL **Pearson** **P** **N**			1	-0.129 0.538 25	0.456 0.076 16	0.446 0.072 17
IL21 **Pearson** **P** **N**				1	0.571 0.021 16	0.322 0.207 17
ESR **Pearson** **P** **N**					1	0.690 0.002 18
CRP **Pearson** **P** **N**						1

Table 5.5 Correlation matrix for cytokines (RQ values) with ESR(mm/h) and CRP(mg/dl).

This table shows the Pearson Pairwise correlations for the named cytokines and clinical markers. P represents the significance of the correlation and N the number of samples used for the correlation (in some cases data was unavailable). Of note the boxes in red show significant correlations (e.g. between IL1 and TNFα) whereas those in green show correlations approaching significance (e.g. between RANKL and ESR and CRP)

Regression analysis.

Linear regression analysis is used to delineate the proportion of variability of a dependent variable (response variable) that can be accounted for by the predictor variables inputted into the model. MC1R expression level is a continuous variable that can be used for linear regression. MC3R expression is a dichotomous variable that could not be analysed in this way.

Several linear regression models were created for MC1R, one for each CD category (CD68lining, CD68 sublining, CD3, CD20 and CD138). Only the variables significant on univariate analysis were included. An initial regression model with CD68lining and sublining showed significant correlation (p=0.007) between these two categories and so only one was used in further models, CD68lining was chosen for further analysis as it was found to be significant on univariate analysis.

All CD categories analysed in linear models as explanatory variables of MC1R (when a single CD category was analysed at a time in each model) showed a significantly high r^2 of over 0.50 (the proportion of variability in MC1R explained by the model). Once the model was corrected for age and sex (included in the model as explanatory variables), only CD138 remained significantly associated with MC1R in all subgroups (factors 0-4). CD68 lining also lost its significant associations. 3 values were missing from these models.

Given the associations on univariate analysis, a model was created using CD68 lining and CD138. This produced a model with a highly significant p value ($p=4.4 \times 10^{-5}$) with an r^2 of 0.79 where 4 observations were missing, CD68 lining factors were all significantly associated with the model ($p<0.05$) as was CD138 grade 2.

This model was corrected for age and sex, the r^2 fell to 0.76, and the significance of the model also reduced to p=0.0004. Factors CD68lining grade 2 remained significantly associated with the variability of MC1R (p=0.03) and grades 3 and 4 approached significance (p=0.06 and p=0.07)

respectively. CD138 grade 2 also approached significance in its part of the variability of MC1R (p=0.09).

A further model was also analysed including all the CD categories, CD68lining, CD138, CD3 and CD20 and age and sex. This model was significant (p=0.006) and had an r^2 of 0.86. In this model CD68lining factors 0.5 and 2 were both significantly associated with the model (p<0.05). CD68lining factor 3 approached significance. CD138 factor 4 and CD3 factor 2 were also significantly associated with this model (p<0.05) CD20 factors 2 and 3 approached significance (p<0.1). Again 4 observations were missing from this model. When analyzing all CD categories as explanatory variables the two most significant variables remain CD68lining and CD138 but now including CD3 and CD20 weakly.

Taken together the models suggest that the association seen with CD68lining on univariate analysis holds true even when corrected for age and sex. There is also a strong association with CD138. Both these models suggest that the correlation is positive in that the higher the CD68 score or CD138 score is the higher MC1R is. A combination of all CD categories gave the best explanation of the variability of MC1R, this is to be expected as the more predictor variables that are inputted the more variance of the dependent variable is explained. CD68 lining and CD138 remained significantly associated with the variability in MC1R. A summary of these models is reported in table 5.6. The models are to be found in the appendix of this thesis.

MC1R adjusted for age and sex	P value of model	R^2	Significant subgroups	Values missing
CD3	0.0014	0.569	no	3
CD20	0.0011	0.575	no	3
CD138	$2.7x10^{-5}$	0.748	yes	4
CD68lining	0.0002	0.666	no	3
CD138 +CD68lining	0.0004	0.756	yes-CD138	4
CD68lining+CD138+CD3+CD20	0.0057	0.860	yes-CD68lining, CD138 and CD3	4

Table 5.6 Summary table of linear regression models where MC1R is the dependent variable.

R^2 represents the goodness of fit of the model and the significance of the subgroups represents whether the factors in each model were also significant. Here, CD138 is shown to be a significant covariate of MC1R regardless of the model used. The first column states which CD categories were included in the model in the order they were included. All models included age and sex.

Primary Components Analysis

Primary components analysis (PCA) was carried out on the linear variables collected as part of the data set. PCA analysis should only be used for linear variables but if applied to categorical data (such as the CD categories) gives reliable but less precise results. The variables included in the analysis were the cytokine gene expression values and CD categories. The rest of the data collected was categorical data. MC3R RQ has not been included in this analysis.

Three analyses were carried out. Firstly, a model was generated using the continuous data. This included the cytokine gene expression data and MC1R RQ. Figure 5.4 shows this model. There is no grouping of the samples by MC1R RQ or by the cytokine data. It is interesting that the variability in IL1, TNF α and IL6 are all in the same direction but this might be expected. IL21 and MC1R RQ vary in the same direction.

The second model generated used the categorical data. Again there was no grouping of samples according to this data, see figure 5.5. It is interesting that the CD categories vary in the same direction reflecting the correlations seen on univariate analysis.

The third model generated used both the linear and categorical data. There was no grouping of samples according to these multiple variables. See figure 5.6.

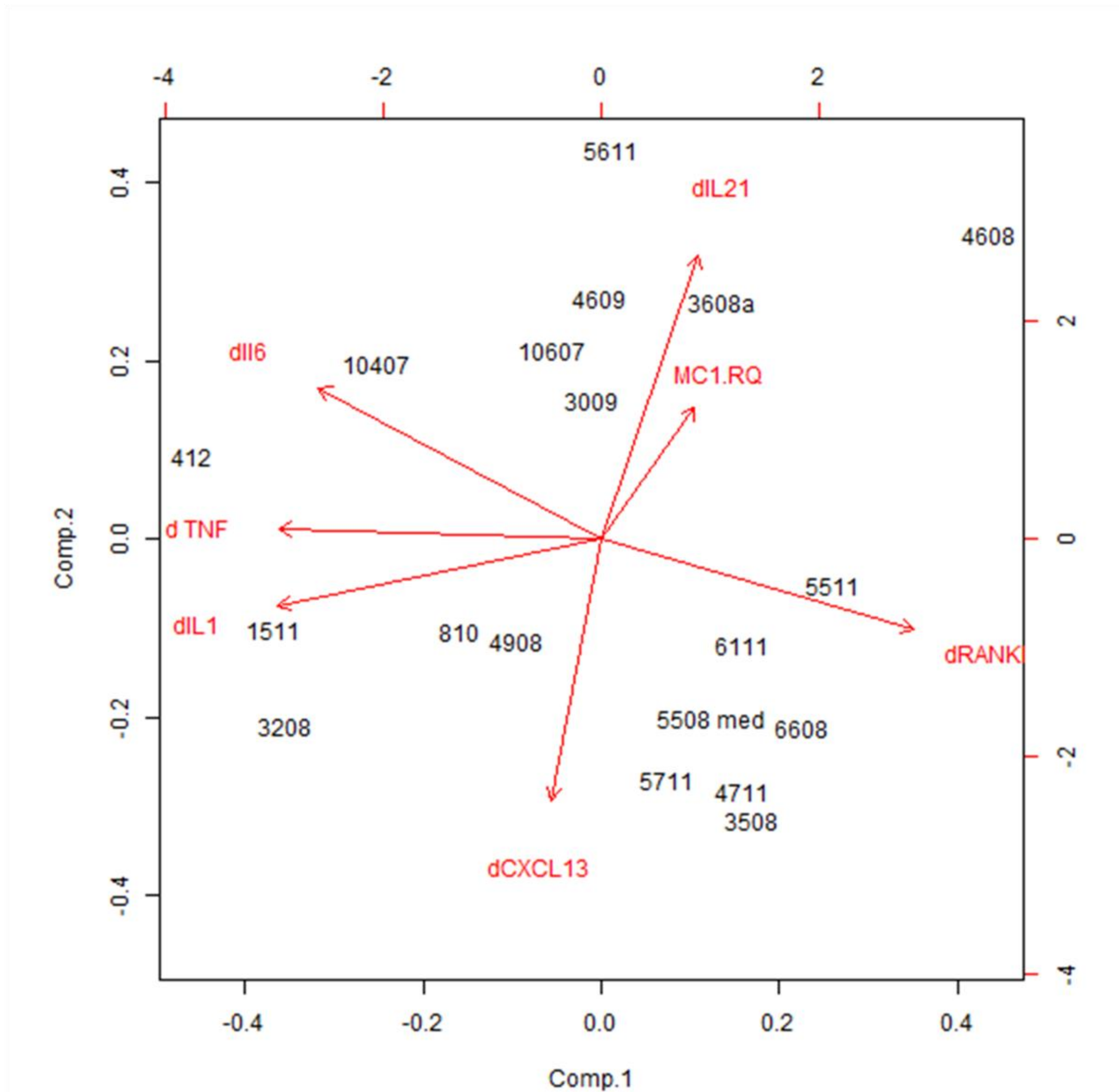


Figure 5.4 PCA analysis of MC1R RQ and gene expression data.

All variables are continuous variables. No grouping of samples is seen according to these variables. Numbers represent sample numbers. dIl6 is the value given to IL6 gene expression. dTNF is TNF gene expression. dRANKL is RANKL gene expression. dIL21 is IL21 gene expression. dIL1 is IL1 gene expression. dCXCL13 is CXCL13 gene expression.

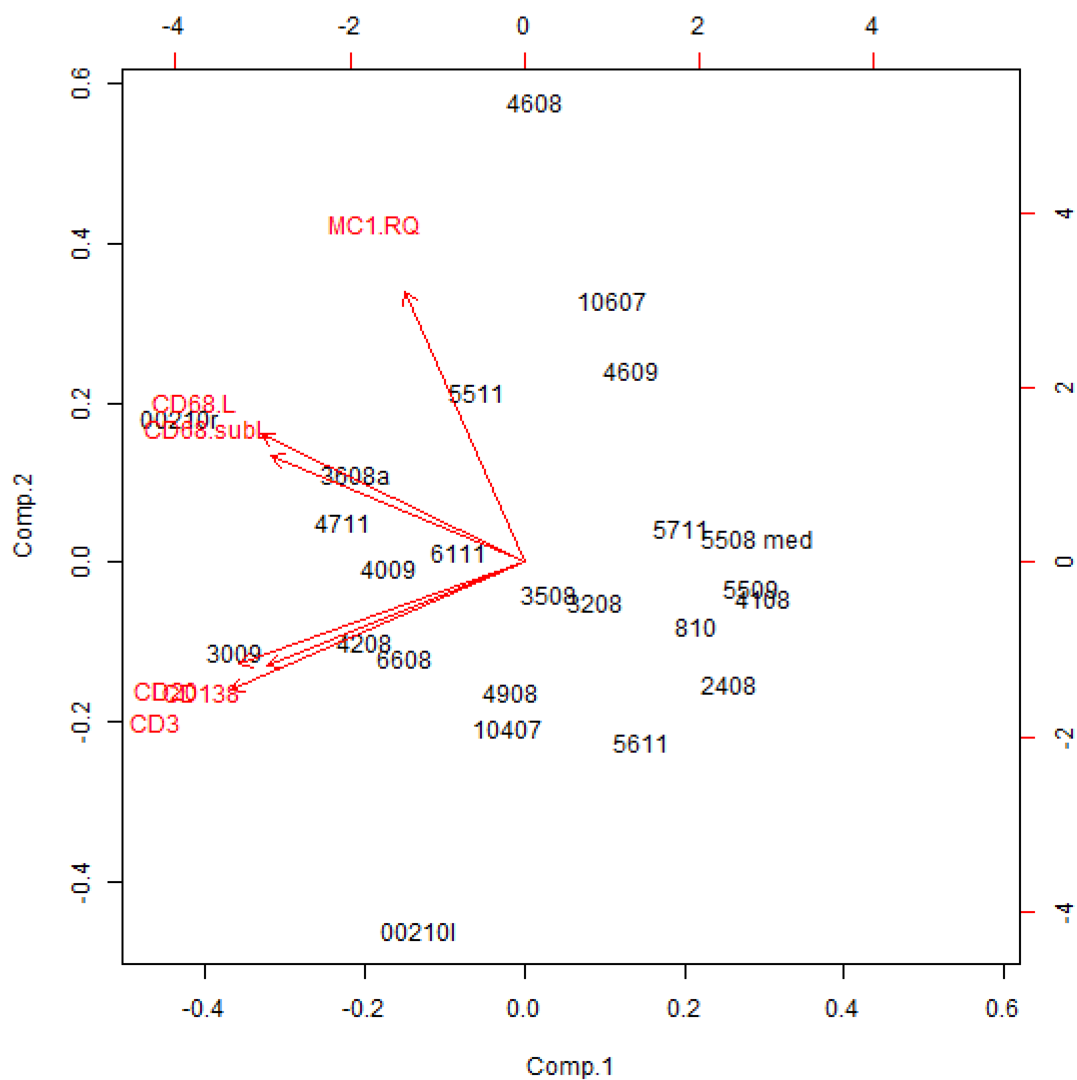


Figure 5.5 PCA analysis of categorical CD variables.

No grouping of the samples is seen according to these variables. Numbers represent sample numbers. CD3 represents the CD3 category, CD68l represents the CD68 lining category, CD68sl represents the CD68 sublining category. CD138 represents the CD138 category, CD20 represents the CD20 immunohistochemical category.

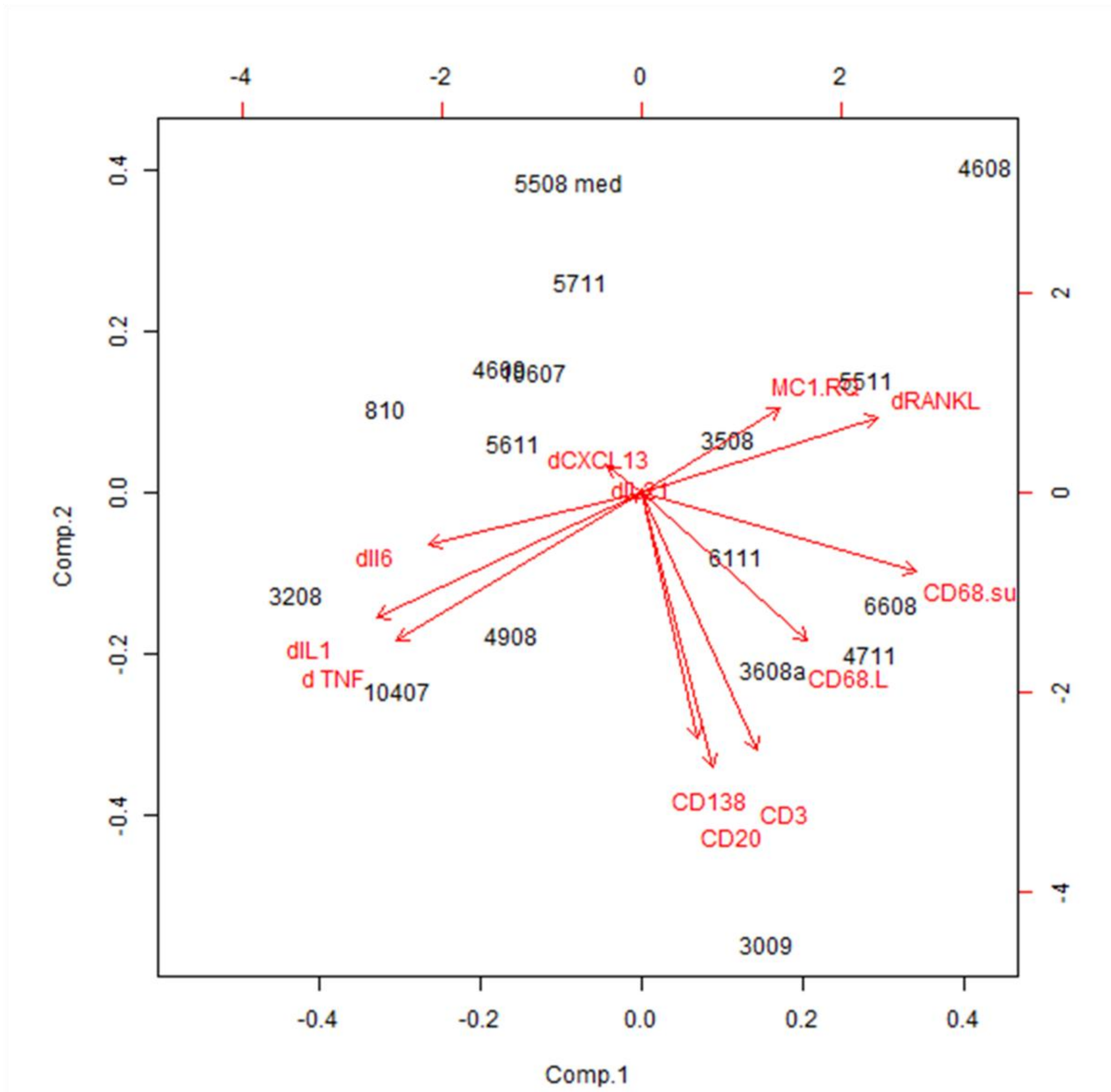


Figure 5.6 PCA analysis of both continuous and categorical values.

Black numbers represent sample numbers. dIl6 is the value given to IL6 gene expression. dTNF is TNF gene expression. dRANKL is RANKL gene expression. dIL21 is IL21 gene expression. dIL1 is IL1 gene expression. dCXCL13 is CXCL13 gene expression. CD3 represents the CD3 category, CD68l represents the CD68 lining category, CD68sl represents the CD68 sublining category. CD138 represents the CD138 category, CD20 represents the CD20 immunohistochemical category.

5.3 Summary

I could not demonstrate correlation between MC1R high and low groups with clinical characteristics or cytokine expression. There was an association between MC1R low groups and not being on combination therapy that did not reach significance. Also there were no men in the MC1R high group. I could not demonstrate any correlation between MC3R positive or negative groups with clinical characteristics or cytokine expression. In this case the null hypothesis could not be excluded.

However, on a positive note, I was able to unveil unprecedented associations between the MC1R high groups and CD68lining scores (see Table 5.2). Linear regression analysis of MC1R revealed that CD68 lining scores and CD138 scores are significant factors in accounting for the variability in MC1R even when taking into account age and sex. See Table 5.6.

A negative association was obtained between MC3R positive status and plasma cell score with not a single MC3R positive samples having a high plasma cell score (See table 5.2).

PCA analysis did not show any grouping of samples according to cytokine gene expression or immunohistochemical categorical data. See figures 5.4 to 5.6. This is likely due to the small numbers in the sample.

I conclude that the null hypothesis is false in that there is an association between MCR expression and immunophenotype.

Chapter 6. Discussion

In this PhD studentship, I have for the first time, investigated expression of the melanocortin receptors in human synovia, reporting on their unequal presence and attempting to define patterns/ profiles of association with established clinical markers and markers of synovial pathotype. I have learnt a wide variety of different techniques, being able to provide new tools that will be of use to colleagues who will continue my work. I discuss below the outcomes of my experimental data and their implications, starting from the work done to validate the tools to be used with precious human samples, the data obtained with the RA samples and their potential significance.

6.1 Creation of a Stable Cell Line

The MCRs are small transmembrane receptors that have short N terminal and C terminal domains which are distributed as indicated in table 1.3 in the Introduction. The ideal tissue for an MC3R positive control would have been brain but this is difficult to source and expensive to purchase. It was important to have a reliable positive control for experiments with specimen tissue and since placenta did not appear to be a viable positive control for RTPCR, I had to create stable transfected cell lines for MC1R, MC3R and MC5R.

There are multiple reports in the literature of MCR-transfected cells, many of them using transient transfections. I did establish permanently transfected lines, but it was unfortunate that they were not useful for protein detection using available commercial antibodies for human MC3R and MC5R. I note that the majority of these cell transfection papers study the presence of the receptor at the cell surface by using either a tagged construct (hence endpoint protein) or functional readouts detecting cAMP production or radio-ligand binding assays looking at displacement of the receptors with different polymorphisms[254, 352, 357]. In my case, I needed detection of the receptor by Western blot or immunofluorescence and this does not seem a common application for the specific receptor

antibodies available. Indeed, there is one study of mouse Mc3r where the Authors criticize the usefulness, and fidelity, of four commercial antibodies[358]. This results in having very few studies with which to compare the data produced in my thesis, particularly for MC3R and MC5R, since there is a greater body of work for MC1R. There are other instances of an HA-tagged transfection of MC3R including the triple HA-tagged construct available from the Missouri cDNA center [352]. However these rely on functional readings to detect the presence of the receptor. Another point to consider is that although HEK293 cells are purported to be melanocortin-free[357], this conclusion results from functional studies and it would be more accurate to propose that HEK293 cells do not have functionally active MCRs as I was able to detect MC1R at mRNA level in HEK293 cells.

My work shows that the vector I used had the correct size insert and this insert was sequenced and found to have the correct sequence for the melanocortin receptors in question. The antibiotic G418 was used to select out the transfected cells at a concentration of 0.625µg/ml, and I validated this concentration of G418 since it caused HEK293 cell death only at ten days. Transfected cells are resistant to G418 as the vector contains a neomycin resistance site. Western blotting data indicated that MC1R was the easiest receptor to find and upon transfection of an HA-tagged construct, antibodies detected an HA-tagged band consistent with the human MC1R. Two antibodies detected 2 bands, one at 38kDA and one at 31kDa (anti-HA clone 4c12 and the anti-HA rabbit polyclonal antibody from Abcam), this dual expression is consistent with the literature pertaining to glycosylated and unglycosylated versions of the HA-tagged MC1R[250]. Two antibodies only detected the larger 38 kDa band consistent with the glycosylated version of HA-tagged MC1R (anti-HA clones 12CA5 and F7). Of interest, HA-tagged MC1R (HA-MC1R) was detected under all incubation conditions whether reduced and denatured at 100°C or reduced and partially denatured at 70°C or room temperature. However I could note that the HA-MC1R tagged bands were more prominent when the sample had been reduced and partially denatured at room temperature than reduced and denatured at 100°C (see figure 3.6 panel c). This suggests there is some destruction of the HA-MC1R product depending on incubation temperature.

In contrast to MC1R, HA-tagged MC3R and HA-tagged MC5R were more elusive and were only detected with one rabbit polyclonal anti-HA antibody. Convincing bands were not seen with any of the mouse monoclonal anti-HA antibodies commercially available, either for HA-MC3R or HA-MC5R. The rabbit polyclonal anti-HA antibody confirmed a band for MC3R just over 38kDA and one for MC5R at 52kDa. There are no studies in the literature with which to compare the HA-tagged MC5R band but it would be consistent with the expected molecular weight according to the manufacturers' literature. Human MC3R is expected to be 40kDa from the manufacturer's literature. By calculation from the individual molecular weights of the amino acids MC3R is 323 amino acids long and should weigh 39kDa with the HA tags added on.

In the literature there is little Western blotting data available for human MC3R. The presence of human MC3R was reported in human bronchial epithelial cells however the size of the protein is not documented in the paper[359]. Rat Mc3r protein has been immunoblotted from neuronal cells showing a molecular weight of 190kDa. The paper suggested that there was significant intracellular processing of the receptor as the right MW was expected to be 66kDa, but only a minor band was seen at this weight [360]. Rat Mc3r has also been immunoblotted from rat chondrocyte cell lines. Here, the Authors found a band between 34 and 43kDa, differing from that observed in neuronal cells [271]. Mc3r has been immunoblotted from mouse peritoneal macrophages using an in-house produced rabbit polyclonal anti-mouse Mc3r, giving a band of 43kDa [340, 361-362]. The same antibody has been used to detect Mc3r from RAW 264.7 cells and mouse brain again with a band at 43kDa[363-364], Mc3r has also been immunoblotted from alveolar macrophages as well as Mc1r, reporting MW of 43kDa and 40 kDa respectively [365]. I note that MCRs have high similarity across species (see Introduction), so it is not unexpected that similar band sizes are observed with human and rodent cells and tissues.

Human MC3R has also been immunoblotted from the human chondrocyte cell line C20/A4. This gave a band of approximately 40kDa[351]. This utilised a rabbit polyclonal antibody. An antibody from

Sigma was used for this paper which was predicted to be specific for rat and mouse Mc3r rather than human MC3R. For this reason this antibody was not tested for the purposes of this thesis.

The size of the HA-MC3R tagged band (just over 37kDa) fits the size expected of human MC3R with 3 HA tags (39kDa) and that seen with human chondrocytes. The pattern of expression in the THP1 cells and brain using the Alomone labs antibody suggests either post-translational modification of the receptor to produce a higher molecular weight protein or dimerisation of the protein giving a band approximately twice the expected weight. I could observe that the Abcam antibody gives a 52kDa band and 102kDa band depending on whether the sample is boiled or left at room temperature. This is somewhat higher than expected but, as discussed above, may be due to posttranslational modification with dimerisation. The manufacturer states that a band at 40kDa is expected. It is difficult to match this size band with the Alomone labs antibody band, bringing into question how accurate the molecular weight marker is or how specific the Abcam antibody is if it gives a band larger than expected. The molecular weight marker was run against β actin and found to be accurate.

Although the anti-HA tag rabbit polyclonal antibody detected a band of greater than 38kDa with the HA-MC3R cell lysate neither the Abcam antibody nor the Alomone labs antibody detected the HA-tagged band. Both these antibodies gave positive bands in brain tissue with different bands being detected depending on whether the sample was incubated at 100°C or at room temperature. Only the Alomone Labs antibody had the immunising peptide available and using pre-absorption experiments, I was able to conclude that the band at 76kDa was specific for human MC3R. The Abcam antibody detected only one band at 52kDa for brain boiled at 100°C and a band at 102kDa for brain incubated at room temperature. It is known that the MCRs can dimerise [250, 366] and that boiling can break the non-covalent bonds between dimers. Therefore, it is likely that dimers are seen at room temperature and monomers when boiled.

Although it is noted in the literature that G protein-coupled receptors -particularly the MCRs- do not pass into the gel as well, depending on the reducing and denaturing nature of the incubation [250], the differences between MC1R, MC3R and MC5R have not previously been documented. With this HA-tagged receptor approach, it appears that whereas MC1R will be visible and enter the gel irrespective of the incubation temperature with DTT-containing reducing loading buffer. MC3R and MC5R are only visible when the sample is reduced and partially denatured e.g. ideally by incubating at room temperature for 15 minutes. I noted that detection of MC3R and MC5R seems to be more heat-sensitive than that for MC1R. From the same gel it appears that the presumed glycosylated MC1R is more heat stable than the smaller band as its density changes with the changing incubation temperature but it does not disappear, whereas the smaller 31kDa band does (Figure 3.6, panel c). It is also possible that more HA-MC1R is produced and therefore there is more target present for labelling than HA-MC3R and HA –MC5R.

Human MC1R is known to transfer as a doublet and this phenomenon is documented in the literature [250] although the sizes of the bands are slightly different (here 38kDa and 31kDa) with bands in the literature being approximately 29 kDa and 34 kDa [367]. According to the antibody manufacturer (Santa Cruz) human MC1R should be 34kDa; my calculation from the amino acid sequence for human MC1R plus the 3 HA tags (each 9 amino acids long), gives a MW of 38kDa. I was pleased to note that the bands I report in this thesis are closer to the expected MW than those reported in the literature. In many studies in the literature, MC1R was tagged using a FLAG tag (an 8 amino acid tag used to label constructs) and were transfected into HEK293T cells thus the proteins were immunoblotted using an anti-FLAG antibody rather than a specific MC1R antibody [250, 253, 368]. These studies indicated that the higher molecular weight band was less intense than the lower molecular weight band which is in opposition to the pattern seen here where the higher molecular weight band is more prominent than the lower molecular weight band. The Santa Cruz sc6875 antibody picked up the higher molecular weight band whereas the Alomone Labs antibody demonstrated both bands of the doublet suggesting that the Alomone Labs antibody is more specific

and should be used to identify human MC1R. The Santa Cruz antibody sc9899 did not pick up any bands from the cell lysate, although there was a 52kDa band seen with skin lysate suggesting that this antibody is less sensitive to MC1R than the other two tested. It may also call into question the specificity of the MC1R antibodies for MC1R from human skin given that 52kDa is larger than expected, however this may be due to undefined post-translational modification.

Sc6875 and sc9899 have been used in the literature for detection of MC1R by immunohistochemistry or immunofluorescence [298, 369]. Other antibodies including Bohm's rabbit polyclonal anti-human MC1R antibody (I refer to Bohm's antibody as to a gifted small aliquot of rabbit antiserum [356]) have also been used to detect human MC1R by immunofluorescence or immunohistochemistry[355-356, 370]. Bohm's antibody has not been used for Western Blotting in the literature. Two monoclonal antibodies have been used to detect MC1R by immunohistochemistry in uveal melanoma but have not been used for Western blotting[371]. Sc6875 has also been used to detect MC1R by Western Blot and here has been confirmed to detect a single band of 38kDa with the HA-tagged cell lysate and a single band at 52kDa with skin lysate. The Alomone labs antibody has not been used in the literature to detect human MC1R but here, using the HA tagged human MC1R cell lysate I could detect with it two bands for MC1R, consistent with both the HA tag antibody but also with the two bands reported in the literature. This antibody also detected a 52kDa band with the skin lysate as does sc6875 and sc9899. It is reassuring that the three MC1R antibodies detect the same size band with the skin lysate, but concerning that not all three antibodies could detect the HA-MC1R. As the immunising peptide was available for both the sc6875 and Alomone labs antibodies, the specificity of these antibodies was further tested by comparing pre-absorbed antibody with non pre-absorbed antibody (see figure 3.6). The immunising peptide should block all specific binding sites and therefore any bands detected with pre-absorbed antibody are non-specific. The bands detected by sc6875 and the Alomone labs antibody disappeared when probed with pre-absorbed antibody. This supports the supposition that these antibodies are indeed specific for human MC1R. In conclusion, given that sc6875 and the Alomone labs antibody both

detected HA-MC1R and a band in skin lysate, and also based on the lack of effect by the experiments of pre-absorption, I then selected these two antibodies for further use in specimen samples.

I also tested a few antibodies for MC5R immunoreactivity but without definitive result since I could not detect any bands with the cell lysate. These antibodies were not tested with any tissue lysates. There is no literature data for MC5R expression by Western blot available for comparison, although some polyclonal antibodies have been used for immunohistochemistry and immunofluorescence in human sebaceous glands or sebocytes[298, 369].

Summary

In summary, stable cell lines were created for HA-tagged MC1R, MC3R and MC5R. These cell lines were used to confirm the specificity of the Applied Biosystem TaqMan® primers and to test commercial anti-human MCR antibodies for Western Blot (and immunofluorescence, data discussed in Chapter 2). TheHA-tagged MC3R and MC5R were shown to be heat-sensitive and only detectable when the samples were reduced and partially denatured at room temperature. This was clear when using the rabbit polyclonal anti-HA antibody. One band was seen for HA-MC3R, and one band was seen for HA-MC5R. Two bands were seen for MC1R. Multiple antibodies were tested for Western Blotting (and immunohistochemistry, data is shown in table 3.1). Two antibodies detected the HA-tagged MC1R as well as a 52kDa band in skin. No antibodies detected the HA-tagged MC3R or MC5R. Two antibodies detected bands in brain tissue. The Alomone labs antibody detected a band at 76kDa. The Abcam antibody detected one band at 52kDa when the sample was boiled and one band at 102kDa when left at room temperature. This could be indirect evidence of dimerisation.

6.2 Optimisation of RNA extraction and Polymerase Chain Reaction

It was also important to determine the efficiency of the Taqman® primers used in the analysis of the synovial samples. Each step of the procedure was tested to make sure that results were as robust and reliable as possible. In fact, as explained in other Chapters, MCRs are intronless genes and thus amplify identically to their cDNA products in RTPCR, therefore water tight protocols had to be devised to monitor gene products in a very robust fashion.

RNA extraction was optimized to a mixture of solvent and column technology which was found to give cleaner results than solvent preparations alone while still maintaining an adequate amount of RNA production. Different forms of DNA treatment were tested to ascertain which gave the cleanest preparation without destroying the RNA in the process. The genomic DNA column of the RNeasy plus kit proved too harsh on the tissue whereas the on-column DNase alone was not adequate treatment. A combination of two DNase treatments were required to adequately exclude genomic DNA contamination (three steps if one allows for the phase separation during chloroform extraction), while allowing good quality RNA of adequate concentration to remain. Random hexamers proved to change the efficiency of the reverse transcription and therefore oligo dTs were optimized as the primer for the reverse transcription. A positive control and calibrator were required for checking that reaction conditions were the same for each experiment. Several tissues were tested for this purpose including placenta, skeletal muscle, tonsil and testes. Testis produced easily obtainable RNA that gave consistent results on testing for the MCRs and the housekeeping gene, GAPDH. This RNA was then taken forward to act as a calibrator. Dilutions of testis RNA were then carried out to ascertain the lowest concentration of cDNA required to give a robust and replicable answer to the question of whether the MCRs were present or not. This was confirmed to be 20ng per reaction of cDNA. MC5R appeared above 35 cycles even at 100ng per reaction leading to the question of whether it was a reliable and robust test for the presence of MC5R.

The efficiency of the primers was also tested and whereas the efficiency of the MC1R primer and GAPDH primer were comparable and reasonably close to 100%, the efficiency of MC3R and particularly MC5R was raised suggesting that these primers were acting at the edge of their detection capabilities. The data for MC3R was therefore used to ascertain the presence or absence of the gene expression rather than quantification of the gene expression. The testing of synovial samples was only carried out once it was known that the technique had been optimised at every step. Due to scarcity of sample (as this was human diseased tissue removed surgically), samples were only tested once in duplicate for the presence of the MCRs.

6.3 Evaluation of Antibodies for Immunohistochemistry

Antibodies were also tested for immunohistochemistry for human MC3R and human MC1R. Although there has been extensive publication with a rabbit polyclonal antibody in skin[355] and other tissues[356, 369] (but not synovium) this antibody could not be used on synovial samples due to the extent of the background staining seen with normal rabbit polyclonal IgG or the normal serum supplied with the antibody. Sc6875 has been used in the immunofluorescence of cells and also for Western blot and although these experiments confirmed its specificity for WB and MC1R, again a similar problem was seen with normal goat polyclonal IgG which despite giving no background staining on tissues such as tonsil, placenta, pituitary and skin gave significant background staining when used with synovium. This was despite using multiple different protein blocking solutions and different conditions. I therefore concluded that despite giving specific staining for WB, sc6875 could not be used to stain for MC1R in synovium. This also applied to ab31309, an anti-human MC3R goat polyclonal antibody, where background staining was seen with normal goat IgG on synovium and staining was seen in MC3R negative samples of melanoma and synovium (by RTPCR). There is a single rabbit monoclonal antibody available for human MC1R, however this gave no staining with normal human skin where one might expect it would pick up melanocytes. In conclusion, none of the current market antibodies for MC1R or MC3R were deemed suitable for testing for the presence of MC1R or MC3R in synovium due to lack of specific staining in immunohistochemistry. This is congruous with a paper which documents the inability of 4 antibodies to detect Mc3r when tested in mice [358].

6.4 Expression of melanocortin receptors in synovial tissue

Although MC1R and MC3R have been shown to be present and functional in mouse models of rheumatoid arthritis[327], there is no current literature pertaining to the gene expression of these receptors in human synovial tissue. My data is the first demonstration of the expression of MCRs in human synovium samples. MC1R was found in all samples whereas MC3R appeared in a dichotomous pattern as being either present or absent. There is no relationship between MC1R level of expression and the presence or absence of MC3R suggesting there is an independence between the expression of these two receptors. The pattern of expression is a robust demonstration of MCR presence as false signals due to inadequate exclusion of genomic DNA were excluded by matched negative controls. This allows me to be very confident of the data produced.

As MC1R can be quantified with real time PCR, it was apparent that MC1R gene expression could be separated by fold change into high and low patterns of expression. This has not previously been shown in the literature. MC1R has been shown in the skin [372] and in cells of skin origin [373] to be up-regulated in the presence of triggers of inflammation, such as UV damage [374] and it may be that MC1R up-regulation is behaving as a “response to” inflammation. Although this pattern was not demonstrated with clinical parameters or cytokine gene expression, there was an association between level of MC1R expression and high macrophage immunohistochemistry scores. This somewhat supports the theoretical premise that MC1R expression may be related to the presence of inflammation, possibly the severity of inflammation. MC1R has been shown to be present in both peripheral blood mononuclear cells[375] and in THP1 cells [376] and so it may be plausible that MC1R is both expressed and up-regulated by the synovial macrophages. It is possible that the higher expression of MC1R could reflect an up-regulation in all immune cells given the distribution of MC1R in multiple cells of immune significance (see table 1.3 in introduction). Macrophages are effector cells in rheumatoid arthritis damage. Macrophage scores have also been shown to change after

clinically effective treatment and so it may be that MC1R correlates with a robust marker of inflammation. However, it is only possible to speculate as to the nature of this association, and it may be that a high macrophage score causes a high MC1R expression level or –equally- it may be that high MC1R expression causes a high macrophage score. It is possible that the higher level of MC1R gene expression is due to an up-regulation in solely the macrophages but the expression is normalized to the total amount of RNA. I would think that high MC1R is consequent to high macrophage numbers, though a UV (ultra-violet) study in humans indicates up-regulation of this receptor on local tissue damage as a whole [372].

The causes of MC1R up-regulation are not well characterized. From human skin and skin keratinocyte experiments it is known that UV irradiation causes the up-regulation of MC1R mRNA and protein [372-373]. Normal human melanocytes up-regulate MC1R in response to UV B irradiation, as well as fibroblast growth factor, endothelin 1, oestrogen and progesterone. MC1R is also up-regulated in response to its own ligand α-MSH[377]. MC1R and αMSH are up-regulated in areas of human burns and in the healing edge of a mouse model of skin trauma[291].

In the mouse, UV A irradiation of the eye induces thickening of the epidermis with induction of pro-inflammatory cytokines in the plasma as well as αMSH. The same stimulus also caused up-regulation of Mc1r in both locally-irradiated and eye-irradiated skin fibroblasts [378]. Trichloroacetic acid is a widely used peeling cosmetic agent for skin. It causes necrosis of the epidermis. Mice treated with trichloroacetic acid to the skin displayed an up-regulation of Mc1r gene expression at 9 hours post-treatment. Mc1r protein was down-regulated at 3 hours, before being the protein being up-regulated at 9 hours [379]. Other models that have shown up-regulation of Mc1r are an ischaemia reperfusion mouse model which showed up-regulation of Mc1r in the mesenteric tissue of Mc3r negative mice [295] and the up-regulation of Mc1r in response to zymosan by Mc3r-negative peritoneal macrophages [329]. Furthermore, the time course of Mc1r induction and Mc3r induction were assessed in the KBxN model of arthritis and found to be independent of each other. Mc1r

expression was greatest at 18 days post-onset whereas Mc3r gene expression started to rise at 6 days post-onset and peaked at 18 days. Interestingly, in Mc3r negative mice, Mc1r was upregulated by day 6 and remained as such through to day 18 [327]. In summary, MC1R and Mc1r are induced by trauma or insult of some sort whether this be irradiation or chemical. This supports my hypothesis that the high expression of MC1R is associated with the degree of inflammation in the tissue (as exemplified by a high macrophage score in RA synovium in my study).

Theoretically, it is possible that these samples represent sampling from a normal distribution of MC1R expression and that sampling error has resulted in two distinct groups. This seems unlikely as all samples were normalized to a consistent calibrator sample. It is also possible that the up-regulation is not related to inflammation itself but the MC1R polymorphism. Loss of function or reduced function MC1R gene polymorphisms can lead to reduced expression of the molecule at the cell surface [353]. The same phenomenon is seen with MC4R loss of function polymorphisms but it is also known that the mRNA expression of these gene polymorphisms is also reduced (Farooqi, S. personal communication). A similar process may be occurring here where the MC1R gene polymorphisms may be dictating the up-regulation or not of MC1R mRNA expression. It could be thought that skin colour may be a way of elucidating this further, but given that this phenotype is also regulated by the POMC gene [380], only MC1R genotyping would truly elucidate this association. One may question whether the grouping is an artificial grouping secondary to the processing of the samples on several PCR plates. However each sample was calibrated to a sample that was consistent throughout plates and each plate was given the same threshold value for MC1R to reduce any variability between plates. Despite these potential confounding factors, I would predict that macrophage infiltration is behind the higher MC1R values in the RA patient synovia *high* samples.

MC3R expression is present in a dichotomous pattern being either present or absent. Again this is the first time that the receptor has been detected in RA synovia. In line with what I observed with

MC1R, MC3R expression is not significantly associated with any clinical parameters, but the pattern of association with ESR and CRP is interesting in that it might seem counterintuitive. MC3R presence is associated with higher levels of ESR and CRP, which suggests that it is associated with higher levels of systemic inflammation, not something that would be expected for an anti-inflammatory target. On the other hand, when inflammation peaks, this is when an endogenous inhibitor would be needed most. This issue is reminiscent of the discovery of cortisol biology, when its' peaking during stress/ disease suggested a potential causal role (the Seyle hypothesis). It is now accepted that cortisol rises to avoid overshooting of the host response[381]. In any case, the pattern that emerged in my 28 samples did not reach significance due to incomplete data and variability within the group.

Thus, MC3R is up-regulated or expressed in response to inflammation a phenomenon that has been seen in data from cell line and mouse models[327] but has never been shown in a human synovial setting. Human MC3R is up-regulated in peripheral blood cells after resistance training of obese human females[382]. This was associated with an increase in IL10 and reduction in monocyte count supporting its anti-inflammatory nature. It may be that the same triggers that mount a pro-inflammatory immune response also initiate an anti-inflammatory response resulting in anti-inflammatory pathways being induced as an attempt (perhaps frustrated in chronic inflammation) to control the overshooting of synovitis. On the other hand, it is also true that RA active phases also resolve to reach a subliminal constant level of inflammation.

It may not be surprising that there were no other correlations with clinical parameters as I would predict the MCRs might be related to markers of inflammation to which medications are loosely related. It might have been thought that there would be a relationship with erosion as a marker of severity of inflammation but the majority of patients had erosive arthritis in this cohort of established disease patient samples. I noted that there was a relationship between low MC1R expression and not being on combination therapy (p=0.07). This might suggest that those patients who did not require combination therapy did not upregulate their MC1R. This may a reflection of a

less inflamed state. It is difficult to know the direction of this association- is the low MC1R expression a result of a less inflamed state resulting in a reduced use of combination therapy or- equally- is the reduced use of combination therapy a result of MC1R expression being low. This is a small cohort of patients and it may be that these relationships simply cannot be demonstrated without using larger numbers of patient samples.

The other association of note is that of a negative association of MC3R positivity with the plasma cell score. If, as discussed in the Introduction, plasma cells are indicative of a more established disease process with local production of auto-antibodies, this would support the idea - again- of MC3R being over-expressed as an anti-inflammatory molecule associated with less advanced disease. Also plasma cells are important in the prediction of response (CD79+ cells) to rituximab. Higher inflammatory scores before treatment predict higher inflammatory scores after treatment [169]. However, there is no causal direction in this association and these data may only be used to generate further hypotheses for testing. The change in plasma cells has been shown to be different in responders and non-responders to rituximab with those having a decrease in plasma cells being more likely to be in the responder group [168]. Linear regression analysis revealed a significant relationship between the decrease in plasma cell numbers and decrease in DAS28 score. This could mean that having a lower plasma cell count would be a marker of responding to rituximab. Extrapolating, this could mean that MC3R is a good prognostic marker for response to this treatment and possibly other treatments. DAS28 scores were not available for this cohort of patients but again extrapolating the low plasma cell score may be reflective of lower clinical inflammatory scores. MC3R may be indicative of lower clinical scores. This should be investigated in a larger, more extensively defined, cohort of patients.

The appearance of MC3R suggests either an up-regulation in a specific cell type to above detectable levels (it is known to be expressed at low levels) or activation of MC3R in all the cells to above the detection threshold. This does lead to the question of why MC3R is present in only some and not

other samples. I could definitively demonstrate this was not associated with the up-regulation or not of MC1R. The same hypothesis may hold true for MC3R as MC1R - there may be MC3R polymorphisms that are expressed at a lower level than others leading to the dichotomous pattern. It may be that some patients have a specific trigger for MC3R expression that is not found in others. It may also be that the genomic DNA treatment of samples was either too harsh or destroyed any MC3R signal from that RNA. It is also possible that there are false negatives in that to ascertain the difference between positive and negative samples a 3 cycle difference was used. If genomic DNA exclusion was not stringent enough there may be a high signal from the negative controls leading to false negative results.

The majority of patients cannot be considered to have early arthritis, given the median duration of disease was 36 months, with a maximum of over 300 months. Only three patients were DMARD naïve and only 2 had disease duration of less than one year. These results should rather be thought of as exploring a cohort of RA patients with established disease. This is important as despite current breakthroughs in treating early arthritis, current referral patterns mean that many people are still referred with established disease and have not been caught in the early arthritis stage. This opens up the MCRs as a receptor that is not only present in early arthritis (MC1R high and MC3R positive in one sample from a disease duration of less than 1 year and MC1R low in a second sample of less than one year) but also in established disease. This should be explored further in a cohort of early arthritis patients as although there was no relationship with duration of disease, it is likely that this sample population was too small to show up any effect.

There is no association demonstrated with cytokine gene expression. However, only a limited panel was examined, it would be interesting to examine cytokines associated with anti-inflammatory actions such as TGFβ or IL10. It may also be interesting to measure other molecules with anti-inflammatory effects such as heme oxygenase 1 (this is known to be induced by activation of MC3r)[383]. No associations were found with clinical parameters or drug exposure in this limited

data set and so no conclusions can be made. The main limitation of this study is that the clinical data was collected retrospectively leading to an incomplete data set for many patients. Also, other markers of clinical activity such as DAS28 or HAQ were not routinely collected and it would have been illuminating to see if these markers may have been associated with melanocortin expression, especially given the preliminary pattern seen with ESR and CRP.

The models to ascertain the covariates of MC1R suggest that CD68lining scores and CD138 scores are predictor variables for MC1R expression level, with CD138 being significantly associated with MC1R expression level even when accounting for age and sex. When including all the CD categories in a model CD68lining and CD138 remained significantly associated with MC1R, and CD3 and CD20 became weak predictor variables. It must be borne in mind when looking at this data that there are multiple correlations within the CD categories on initial univariate evaluation which is why a linear regression model is required to separate the strongly associated variables from the weakly associated variables. There continued to be no association with age and sex in any of the models created which used these variables. This supports the associations found on univariate analysis as discussed above. Since MC3R is a dichotomous variable it cannot be analysed as a continuous variable. PCA analysis did not further elucidate any groupings amongst the data according to immunohistochemical category or cytokine gene expression. This may be due to the small sample size. MC3R was again not analysed in this way.

Thus far, there is no data published on the presence or absence of the melanocortin receptors in human rheumatoid arthritis synovium and therefore it is difficult to put this finding into context with the current literature. However, Mc3r has been shown to be important in mouse models of arthritis (KBxN and gouty arthritis). Patel et al showed that KBxN arthritis was more severe in Mc3r -/- mice than in wild type mice, but also showed that Mc3r increased in wild-type mice during the course of the disease. Interestingly, Mc1r was up-regulated in the Mc3r knockout mice. Mc3r -/- mice had worse clinical scores, and histological scores. Also more osteoclasts were seen in pockets of bone

erosion in the knockout mice than wild-type. Here IL1b, Nos2 and IL6 were induced during the disease and chemokine genes such Cxcl10, ccl2, ccl3 and ccl5 were also upregulated associated with their receptor genes as well. The ameliorating effect of D[Trp^8] γMSH (an Mc3r specific agonist in mice) on the severity of arthritis in wild-type mice was abolished in Mc3r knockout animals. All this together shows that presence and activation of Mc3r in this model is tissue protective[327]. This bodes well for the MC3R positive samples in this human cohort and is suggestive that MC3R could be a biomarker of milder disease (as having low plasma cell scores may attest to). It is also possible that MC3R polymorphisms may mimic the status of Mc3r knockout mice in that some polymorphisms may make the person more susceptible to disease as is the case with SNP rs6127698 in tuberculosis [297]. Patel et al also showed that the Mc3r knockout mice had more osteoclasts with higher responsiveness of harvested bone marrow cells to RANKL and prolonged NFκB binding activity. This suggests that Mc3r modulates the formation of osteoclasts and hence bone erosions. This would suggest that those who are MC3R positive in this human cohort would have less erosive disease. This association may not have been shown because of the size of the sample. It might also suggest that those who are MC3R negative would have a higher erosive load.

In the KBxN model of inflammatory arthritis, cytokines pivotal in inducing arthritis are produced by macrophages[361] and mast cells, both of which express Mc1r and Mc3r[384]. This cohort of patients did not include ascertainment of mast cell status as part of the immunophenotyping of the sample. Some of the increased cellularity seen in those samples with high synovial scores (which correlated well with CD categories) could be due to increased mast cell populations. It would also be interesting to look at markers of activation for macrophages and mast cells, or markers of pro-resolution phenotypes such as CD163 in macrophages [385].

Mc3r has been localized to mouse macrophages both in cell lines [383] and in vivo [338, 365] by Western blotting. It has also been localized to rat synovial macrophages in the diseased joint by in situ hybridization [268]. There is also evidence from models of gouty arthritis that Mc1r is not

required for there to be a response to melancortin agonists [340]. This supports a role for Mc3r in the anti-inflammatory pathway in mice and gouty arthritis. This is suggestive that MC3R may take over when either MC1R is not up-regulated for whatever reason or if MC1R is up-regulated but present with a loss of function polymorphism. This may explain the dichotomy of the MC3R expression. This is not reflected in this set of data but this may be due to the small size of the sample.

Localising the MCRs- MC1R or MC3R- to the joint also provides a target for the treatment of inflammatory arthritis. αMSH has been shown to ameliorate experimental adjuvant-induced arthritis when given to rats [334] with an effect comparing favourably to that elicited by prednisolone. A paper testing a novel method of melanocortin delivery, the latency-associated peptide (LAP) fused to αMSH, reported effective amelioration of collagen-induced arthritis in mice [326]. The same study showed that the LAP containing γMSH was effective at limiting leucocyte influx by 50% in response to urate crystal-induced inflammation compared to approximately 30% by free γ-MSH. It was proposed that increased efficacy was due to the increased stability of the LAP-γ MSH molecule in delivering the molecule to the area of interest. This provides a model for the use of a targeted therapy to humans using a drug delivery system equipped either with αMSH or γMSH. It may be that identifying those patients that are MC3R positive would enable the use of targeted γMSH. I would also suggest that all patients would be responsive to αMSH given that MC1R is present in all samples to a greater or lesser degree. Furthermore, using the LAP protein targets the melanocortin to sites of inflammation thus reducing possible systemic side effects of the drug (e.g. yawning and stretching, melanoma risk, salt-related hypertension and agitation as discussed in the introduction). The pan-melanocortin receptor agonist AP214 (which binds to all MCRs excluding MC2R) has been used to treat KBxN arthritis with reduction in disease score, disease incidence and paw oedema. [329]

Can a melanocortin agonist be a therapeutic for the future? I would note that AP214 is already being evaluated for preventing kidney injury after cardiac surgery and sold for $110M by Action Pharma AS

to Abbott Laboratories. A search on http://www.clinicaltrials.gov, informs us that αMSH is being evaluated for patients with acute renal failure, afamelanotide (a pan melanocortin agonist) is being evaluated for attenuating vitiligo and ACTH (already used in the treatment of gout in America in those patients with comorbidities[236]) is being evaluated in the treatment of diabetic nephropathy and multiple sclerosis.

There is data regarding the anti-inflammatory effect of αMSH in cell lines[350-351], human cells[269] and animal models[327]. αMSH has been found in the synovial fluid of RA patients at a concentration higher than that found in plasma and also compared to OA samples suggesting a role for this melanocortin in rheumatoid arthritis [348]. Treating RA patients with αMSH or a melanocortin agonist would be activating a circuit that modulates inflammation rather than obliterating a certain part of the immune response (e.g. anti-TNF and anti-CD20 drugs). I am confident that identification of MC1R and MC3R in the human synovium is a necessary, albeit preliminary step, to the development of melanocortin agonists in the treatment of human rheumatoid arthritis, or for specific cohorts of patients.

Potential Limitations of this study

In spite of all the novel results produced, I recognize that there are some limitations to this study. For instance, my work necessarily represents a cross-sectional analysis at one time point only: this means that only associations can be seen and causation cannot be inferred from this set of data. Another limitation of this study is its small sample size. It is quite likely that the lack of association between the MCRs and any clinical characteristics were due to, firstly, the clinical characteristics collected and secondly the size of the sample. As the sample data were collected retrospectively there is missing data as is inevitable in a sample of this sort. With such a small sample size it is also important that the samples were correctly classified in terms of immunophenotyping in that reclassifying one sample could have quite a large effect on the data. This can be checked by testing the samples for CD21 long isoform or AID expression and collating this data with the immunohistochemistry phenotype. It is suggested that IL21 and CXCL13 [54] are important in

ectopic lymphoneogenesis. Hence the presence of IL21 and or high CXCL13 would correlate with high CD20 and CD3 scores consistent with lymphoid aggregates. This is not the case in this data set and is not yet established in the literature. Other misclassifications could include the MC1R samples into being falsely high or low due to technical error or MC3R being falsely negative due to inadequate exclusion of genomic DNA from the samples leading to a less than 3 cycle difference between positive samples and the negative controls. There is little evidence of technical error and I am confident that the MC1R and MC3R samples were not misclassified.

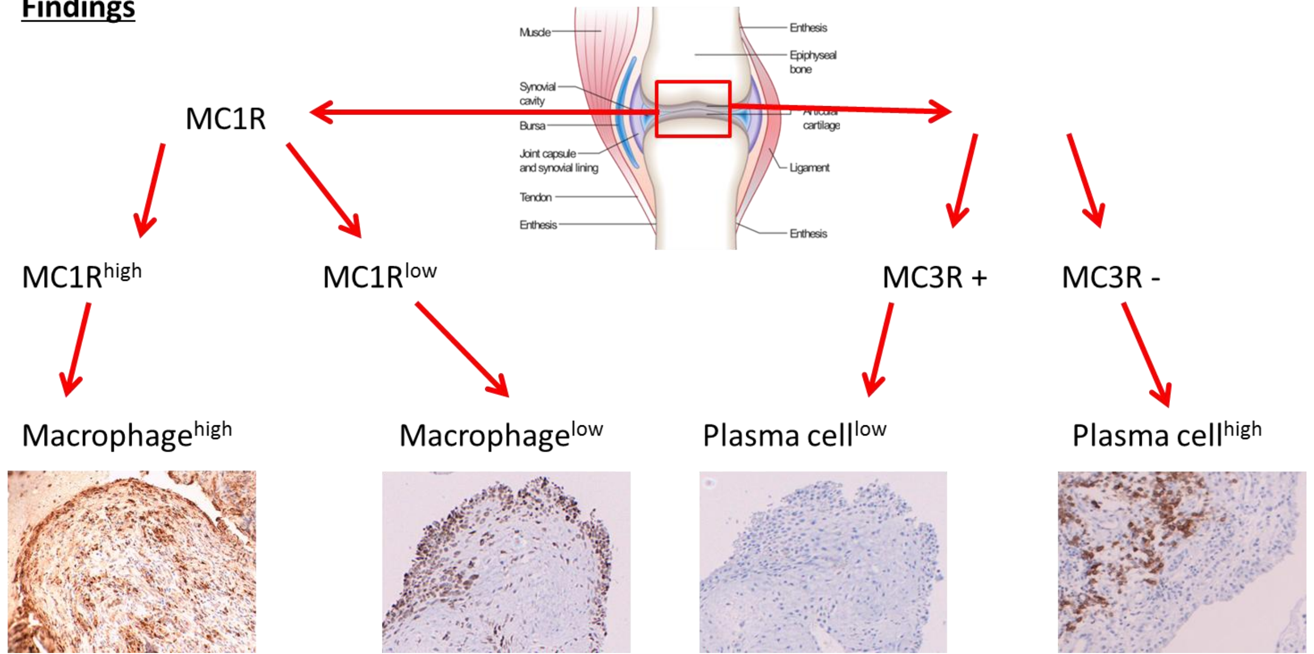
Although MC5R is thought to be expressed in all human peripheral tissues at low levels [243], none was found in 30 samples of human synovium across osteoarthritic and rheumatoid samples. Accompanying this data were the low efficiency of the primer (figure 3.10) and the high C_T values generated by this primer even with the highest amount of substrate (see figure 3.9), it is possible that the Applied Biosystems TaqMan® primer for MC5R was not sensitive enough to pick up very low levels of MC5R expression. As only MC1R and MC3R were subsequently examined, this may be a lost opportunity.

Limitations of Western blots include lack of β actin control and experiments being only performed once due to scarcity of sample (e.g. brain lysate) or lack of repetition. The number of replicates of experiments has been stated in the figure legends. Limitations of the flow cytometry experiments are lack of the use of secondary antibody alone controls and again lack of repetition of certain experiments. Again this is stated in the related figure legend.

Summary

In summary, I show here for the first time that MC1R and MC3R gene products are present in human synovium. MC1R gene expression can be divided into high and low expression groups. MC3R was either present or absent (see figure 6.1). No correlation was found between MCR expression and clinical parameters although an intriguing pattern was seen with ESR, CRP and MC3R. No associations were seen with cytokine gene expression, although again an interesting pattern emerged from the IL21 positive samples.

Of significance, high expression of MC1R was associated with a high macrophage score when assessed by immunohistochemistry and MC3R expression was associated with a low plasma cell score. Therefore peaks of MC1R may be associated with inflammation whereas MC3R presence may be a biomarker for milder disease or less erosive load (see figure 6.1). The identification of the MCRs in rheumatoid synovium indicates presence of a suitable, and novel, target for ACTH (which is already used in the treatment of gout) and other melanocortin peptides. Further studies are required to establish the role of human MC1R and MC3R in the disease course of rheumatoid arthritis. Given that αMSH and related melanocortin peptides are already being tested for safety in other pathologies it may not be a big step to cross disease boundaries for the treatment of RA given the amount of supportive preclinical data available, in combination with the demonstration in this thesis of the relevance of this system in human RA.



Hypothesis

Associations of MCR + disease	**MC1R**		**MC3R**	
	high	low	+	-
Severity	severe	mild	mild	severe
Erosions	more	less	less	more
Response to treatment	changes in response	good	good	poor

Figure 6.1 Summary of findings.
MC1R and MC3R are found in the human synovium. Samples can be grouped into MC1R high and MC1R low which are associated with macrophage lining score high and macrophage lining score low respectively. Samples are independently either MC3R positive or MC3R negative. No MC3R positive samples were plasma cell high. The association with immunohistochemical parameters leads to hypothesis generation regarding severity, erosions and response to treatment. (synovial joint image from en.wikipedia.org.)

Key Findings

- **MC1R and MC3R are present in the human rheumatoid synovium**
- MC1R gene expression can be divided into high and low expression groups.
- MC3R was either present or absent in the synovium.
- High expression of MC1R was associated with a high macrophage score when assessed by immunohistochemistry.
- MC3R expression was associated with a low plasma cell score.
- The identification of the MCRs in rheumatoid synovium indicates presence of a suitable, and novel, target for ACTH.
- No commercial antibodies were found to be of utility in detecting MC1R and MC3R by immunohistochemistry.

Future work

Future lines of experimentation might include investigating RA fibroblast-like synoviocytes or RA synovial cultures for the MCRs and the response of these samples to melanocortin agonists. This would provide further preclinical data and may produce functional information as to which cells are involved in the response to melanocortin agonists. This work has been started with the use of serum amyloid A protein on rheumatoid arthritis tissue cultures with the measurement of the melanocortin receptors and cytokine release after treatment. The data are too preliminary to be included but indicate that pan-agonists to MC receptors can attenuate by 50% the release of CCL2 (monocyte chemoattractant protein 1) and IL6.

Another way of showing the effect of melanocortin agonists on human tissue would be to use the RA-SCID model where the effect of agonists on human tissue could be measured directly. It would also be interesting to note whether MC3R negative samples respond to MC3R agonists to quantify in

a different way whether there was a functional MC3R available that was present at a lower level than detected by PCR.

In any case, it seems to me critically important to generate a reliable monoclonal antibody to either MC1R or MC3R in order to localize the receptor protein from immunohistochemical samples and provide impetus to MCR translational research. This was deemed too expensive and time-consuming at the time of this study and might constitute a project in itself.

Another way of investigating the MCRs further would be to study a different cohort of patients. A cohort of early arthritis patients would be particularly illuminating. More clinical measures would also be useful as there may be an association between presence of the MCRs and markers of clinical activity. It may also be useful to follow the expression of the MCRs over time, for example before and after treatment with combination therapy or biologics.

Another approach would be to look for MCRs by RTPCR in peripheral blood mononuclear cells of RA patients to ascertain whether they could be used as a biomarker documenting change over time and in response to treatment. A biomarker in peripheral blood may be more acceptable to patients.

To complete the characterization of MCRs it may be possible to analyse the receptor in synovial fluid cell pellets and look at CD markers to try and identify which cell type is expressing them (by FACS or RTPCR). If a monoclonal antibody is available then it might be possible to Western blot cell pellets from synovial fluid to confirm expression of receptor at protein level or by FACS for cells in synovial fluid before and after treatment. This would be novel information about the presence or absence of receptors in synovial fluid and would allow the development, perhaps, of novel ways of monitoring response to treatment.Melanocortin drugs are a current reality. This thesis represents a stepping stone in the new arena of translational melanocortin biology to be developed further both for novel medical treatments and identification of novel biomarkers for patient stratification.

Appendix 1

Pearson Correlation Co-efficients for univariate analysis of MC1R as a linear variable

Correlations

		CD138	CD68_L	CD68_subL	CD68
MC1_RQ	Pearson Correlation	.184	.405*	.326	.395
	Sig. (2-tailed)	.389	.045	.112	.051
	N	24	25	25	25
Synovitis_Score	Pearson Correlation	.624**	.535**	.630**	.662**
	Sig. (2-tailed)	.001	.006	.001	.000
	N	24	25	25	25
CD20	Pearson Correlation	.627**	.533**	.441*	.542**
	Sig. (2-tailed)	.001	.006	.027	.005
	N	24	25	25	25
CD3	Pearson Correlation	.689**	.442*	.617**	.602**
	Sig. (2-tailed)	.000	.027	.001	.001
	N	24	25	25	25
CD138	Pearson Correlation	1	.416*	.274	.384
	Sig. (2-tailed)		.043	.196	.064
	N	24	24	24	24
CD68_L	Pearson Correlation	.416*	1	.651**	.890**
	Sig. (2-tailed)	.043		.000	.000
	N	24	25	25	25
CD68_subL	Pearson Correlation	.274	.651**	1	.920**
	Sig. (2-tailed)	.196	.000		.000
	N	24	25	25	25
CD68	Pearson Correlation	.384	.890**	.920**	1
	Sig. (2-tailed)	.064	.000	.000	
	N	24	25	25	25
d_TNF	Pearson Correlation	-.120	.048	-.174	-.058
	Sig. (2-tailed)	.596	.826	.427	.793
	N	22	23	23	23
dIl6	Pearson Correlation	-.051	-.226	-.515*	-.404
	Sig. (2-tailed)	.832	.325	.017	.069
	N	20	21	21	21
dRANKL	Pearson Correlation	-.336	.134	.172	.147
	Sig. (2-tailed)	.126	.543	.431	.503
	N	22	23	23	23
dCXCL13	Pearson Correlation	-.297	.095	-.106	-.002
	Sig. (2-tailed)	.180	.667	.631	.994
	N	22	23	23	23
dIL21	Pearson Correlation	.016	.197	.159	.225
	Sig. (2-tailed)	.943	.367	.468	.301
	N	22	23	23	23

Correlations

		d_TNF	dIl6	dRANKL	dCXCL13
MC1_RQ	Pearson Correlation	.031	.022	-.084	.195
	Sig. (2-tailed)	.882	.921	.689	.361
	N	25	23	25	24
Synovitis_Score	Pearson Correlation	-.234	-.411	-.108	-.119
	Sig. (2-tailed)	.271	.057	.615	.579
	N	24	22	24	24
CD20	Pearson Correlation	-.041	.063	-.258	-.110
	Sig. (2-tailed)	.854	.785	.235	.616
	N	23	21	23	23
CD3	Pearson Correlation	-.156	-.321	-.267	-.254
	Sig. (2-tailed)	.477	.156	.219	.243
	N	23	21	23	23
CD138	Pearson Correlation	-.120	-.051	-.336	-.297
	Sig. (2-tailed)	.596	.832	.126	.180
	N	22	20	22	22
CD68_L	Pearson Correlation	.048	-.226	.134	.095
	Sig. (2-tailed)	.826	.325	.543	.667
	N	23	21	23	23
CD68_subL	Pearson Correlation	-.174	-.515*	.172	-.106
	Sig. (2-tailed)	.427	.017	.431	.631
	N	23	21	23	23
CD68	Pearson Correlation	-.058	-.404	.147	-.002
	Sig. (2-tailed)	.793	.069	.503	.994
	N	23	21	23	23
d_TNF	Pearson Correlation	1	.695**	-.171	.453*
	Sig. (2-tailed)		.000	.414	.026
	N	25	23	25	24
dIl6	Pearson Correlation	.695**	1	-.344	.096
	Sig. (2-tailed)	.000		.108	.672
	N	23	23	23	22
dRANKL	Pearson Correlation	-.171	-.344	1	.262
	Sig. (2-tailed)	.414	.108		.216
	N	25	23	25	24
dCXCL13	Pearson Correlation	.453*	.096	.262	1
	Sig. (2-tailed)	.026	.672	.216	
	N	24	22	24	24
dIL21	Pearson Correlation	-.214	-.061	-.129	-.181
	Sig. (2-tailed)	.304	.780	.538	.397
	N	25	23	25	24
		**	*	*	

Correlations

		dIL21	dIL1	age_	length_of_duration_of_disease_months
MC1_RQ	Pearson Correlation	.384	-.223	-.084	.343
	Sig. (2-tailed)	.058	.332	.670	.275
	N	25	21	28	12
Synovitis_Score	Pearson Correlation	.169	-.015	-.136	-.045
	Sig. (2-tailed)	.429	.949	.506	.889
	N	24	20	26	12
CD20	Pearson Correlation	.127	.172	-.094	-.183
	Sig. (2-tailed)	.564	.481	.657	.591
	N	23	19	25	11
CD3	Pearson Correlation	.158	-.044	-.116	.016
	Sig. (2-tailed)	.470	.858	.580	.962
	N	23	19	25	11
CD138	Pearson Correlation	.016	.128	.136	.465
	Sig. (2-tailed)	.943	.614	.525	.176
	N	22	18	24	10
CD68_L	Pearson Correlation	.197	-.180	-.379	.234
	Sig. (2-tailed)	.367	.461	.062	.490
	N	23	19	25	11
CD68_subL	Pearson Correlation	.159	-.461*	-.297	.251
	Sig. (2-tailed)	.468	.047	.149	.457
	N	23	19	25	11
CD68	Pearson Correlation	.225	-.362	-.376	.250
	Sig. (2-tailed)	.301	.128	.064	.458
	N	23	19	25	11
d_TNF	Pearson Correlation	-.214	.583**	.028	-.260
	Sig. (2-tailed)	.304	.006	.893	.440
	N	25	21	25	11
dIl6	Pearson Correlation	-.061	.459*	.212	-.495
	Sig. (2-tailed)	.780	.042	.331	.146
	N	23	20	23	10
dRANKL	Pearson Correlation	-.129	-.517*	-.595**	.297
	Sig. (2-tailed)	.538	.016	.002	.376
	N	25	21	25	11
dCXCL13	Pearson Correlation	-.181	.320	-.277	.191
	Sig. (2-tailed)	.397	.169	.190	.573
	N	24	20	24	11
dIL21	Pearson Correlation	1	-.182	-.025	-.402
	Sig. (2-tailed)		.430	.904	.221
	N	25	21	25	11

Correlations

		Grade	ESR	CRP
MC1_RQ	Pearson Correlation	-.008	.284	-.083
	Sig. (2-tailed)	.967	.253	.735
	N	28	18	19
Synovitis_Score	Pearson Correlation	-.641**	.026	.301
	Sig. (2-tailed)	.000	.921	.225
	N	26	17	18
CD20	Pearson Correlation	-.339	-.272	-.116
	Sig. (2-tailed)	.097	.308	.657
	N	25	16	17
CD3	Pearson Correlation	-.594**	-.280	.071
	Sig. (2-tailed)	.002	.294	.786
	N	25	16	17
CD138	Pearson Correlation	-.459*	-.378	-.176
	Sig. (2-tailed)	.024	.165	.516
	N	24	15	16
CD68_L	Pearson Correlation	-.303	.106	.258
	Sig. (2-tailed)	.141	.695	.317
	N	25	16	17
CD68_subL	Pearson Correlation	-.462*	-.078	.133
	Sig. (2-tailed)	.020	.774	.612
	N	25	16	17
CD68	Pearson Correlation	-.429*	.024	.211
	Sig. (2-tailed)	.032	.930	.415
	N	25	16	17
d_TNF	Pearson Correlation	.108	-.413	-.362
	Sig. (2-tailed)	.608	.111	.154
	N	25	16	17
dIl6	Pearson Correlation	.442*	-.146	-.379
	Sig. (2-tailed)	.035	.605	.148
	N	23	15	16
dRANKL	Pearson Correlation	-.434*	.456	.446
	Sig. (2-tailed)	.030	.076	.072
	N	25	16	17
dCXCL13	Pearson Correlation	-.136	.170	.274
	Sig. (2-tailed)	.526	.544	.304
	N	24	15	16
dIL21	Pearson Correlation	.152	.571*	.322
	Sig. (2-tailed)	.469	.021	.207
	N	25	16	17

Correlations

		MC1_RQ	Synovitis_Score	CD20	CD3
dIL1	Pearson Correlation	-.223	-.015	.172	-.044
	Sig. (2-tailed)	.332	.949	.481	.858
	N	21	20	19	19
age_	Pearson Correlation	-.084	-.136	-.094	-.116
	Sig. (2-tailed)	.670	.506	.657	.580
	N	28	26	25	25
length_of_duration_of_disease_months	Pearson Correlation	.343	-.045	-.183	.016
	Sig. (2-tailed)	.275	.889	.591	.962
	N	12	12	11	11
Grade	Pearson Correlation	-.008	-.641**	-.339	-.594**
	Sig. (2-tailed)	.967	.000	.097	.002
	N	28	26	25	25
ESR	Pearson Correlation	.284	.026	-.272	-.280
	Sig. (2-tailed)	.253	.921	.308	.294
	N	18	17	16	16
CRP	Pearson Correlation	-.083	.301	-.116	.071
	Sig. (2-tailed)	.735	.225	.657	.786
	N	19	18	17	17

Correlations

		CD138	CD68_L	CD68_subL	CD68
dIL1	Pearson Correlation	.128	-.180	-.461*	-.362
	Sig. (2-tailed)	.614	.461	.047	.128
	N	18	19	19	19
age_	Pearson Correlation	.136	-.379	-.297	-.376
	Sig. (2-tailed)	.525	.062	.149	.064
	N	24	25	25	25
length_of_duration_of_disease_months	Pearson Correlation	.465	.234	.251	.250
	Sig. (2-tailed)	.176	.490	.457	.458
	N	10	11	11	11
Grade	Pearson Correlation	-.459*	-.303	-.462*	-.429*
	Sig. (2-tailed)	.024	.141	.020	.032
	N	24	25	25	25
ESR	Pearson Correlation	-.378	.106	-.078	.024
	Sig. (2-tailed)	.165	.695	.774	.930
	N	15	16	16	16
CRP	Pearson Correlation	-.176	.258	.133	.211
	Sig. (2-tailed)	.516	.317	.612	.415
	N	16	17	17	17

Correlations

		d_TNF	dIl6	dRANKL	dCXCL13
dIL1	Pearson Correlation	.583**	.459*	-.517*	.320
	Sig. (2-tailed)	.006	.042	.016	.169
	N	21	20	21	20
age_	Pearson Correlation	.028	.212	-.595**	-.277
	Sig. (2-tailed)	.893	.331	.002	.190
	N	25	23	25	24
length_of_duration_of_disease_months	Pearson Correlation	-.260	-.495	.297	.191
	Sig. (2-tailed)	.440	.146	.376	.573
	N	11	10	11	11
Grade	Pearson Correlation	.108	.442*	-.434*	-.136
	Sig. (2-tailed)	.608	.035	.030	.526
	N	25	23	25	24
ESR	Pearson Correlation	-.413	-.146	.456	.170
	Sig. (2-tailed)	.111	.605	.076	.544
	N	16	15	16	15
CRP	Pearson Correlation	-.362	-.379	.446	.274
	Sig. (2-tailed)	.154	.148	.072	.304
	N	17	16	17	16

Correlations

		dIL21	dIL1	age_	length_of_duration_of_disease_months
dIL1	Pearson Correlation	-.182	1	.381	-.480
	Sig. (2-tailed)	.430		.088	.190
	N	21	21	21	9
age_	Pearson Correlation	-.025	.381	1	-.436
	Sig. (2-tailed)	.904	.088		.157
	N	25	21	28	12
length_of_duration_of_disease_months	Pearson Correlation	-.402	-.480	-.436	1
	Sig. (2-tailed)	.221	.190	.157	
	N	11	9	12	12
Grade	Pearson Correlation	.152	.087	.212	-.197
	Sig. (2-tailed)	.469	.708	.279	.539
	N	25	21	28	12
ESR	Pearson Correlation	.571*	-.544*	-.250	-.335
	Sig. (2-tailed)	.021	.036	.316	.417
	N	16	15	18	8
CRP	Pearson Correlation	.322	-.423	-.248	-.239
	Sig. (2-tailed)	.207	.116	.306	.568
	N	17	15	19	8

Correlations

		Grade	ESR	CRP
dIL1	Pearson Correlation	.087	-.544*	-.423
	Sig. (2-tailed)	.708	.036	.116
	N	21	15	15
age_	Pearson Correlation	.212	-.250	-.248
	Sig. (2-tailed)	.279	.316	.306
	N	28	18	19
length_of_duration_of_disease_months	Pearson Correlation	-.197	-.335	-.239
	Sig. (2-tailed)	.539	.417	.568
	N	12	8	8
Grade	Pearson Correlation	1	-.014	-.319
	Sig. (2-tailed)		.957	.183
	N	28	18	19
ESR	Pearson Correlation	-.014	1	.690**
	Sig. (2-tailed)	.957		.002
	N	18	18	18
CRP	Pearson Correlation	-.319	.690**	1
	Sig. (2-tailed)	.183	.002	
	N	19	18	19

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 2

Statistical models (developed in R software) for linear regression and PCA analysis

```
Call:
lm(formula = MC1.RQ ~ CD68.subL - 1)

Residuals:
    Min      1Q  Median      3Q     Max
-4.4176 -1.6162 -0.4789  1.8216  5.9647

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
CD68.subL0      3.161      1.571   2.012 0.058633 .
CD68.subL0.5    3.048      2.222   1.372 0.186109
CD68.subL1      1.768      1.405   1.258 0.223772
CD68.subL2      4.582      1.405   3.260 0.004115 **
CD68.subL3      5.681      1.283   4.428 0.000289 ***
CD68.subL4      4.383      1.814   2.416 0.025950 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 3.143 on 19 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.6964,  Adjusted R-squared: 0.6005
F-statistic: 7.264 on 6 and 19 DF,  p-value: 0.0003838
```

```
Call:
lm(formula = MC1.RQ ~ CD68.L - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-4.5224 -2.1092 -0.0933  1.7329  6.5794 

Coefficients:
          Estimate Std. Error t value Pr(>|t|)    
CD68.L0.5    2.549      2.078   1.227 0.234233    
CD68.L1      1.671      1.111   1.504 0.148184    
CD68.L2      5.945      1.697   3.503 0.002237 ** 
CD68.L3      4.671      1.111   4.205 0.000436 ***
CD68.L4      5.067      1.200   4.222 0.000418 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 2.939 on 20 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.7205,  Adjusted R-squared: 0.6506 
F-statistic: 10.31 on 5 and 20 DF,  p-value: 5.324e-05
```

```
Call:
lm(formula = MC1.RQ ~ CD3 - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-4.0088 -2.3399 -0.8879  2.4024  6.3487 

Coefficients:
     Estimate Std. Error t value Pr(>|t|)   
CD30    3.849      1.274   3.020  0.00676 **
CD31    4.002      1.947   2.056  0.05310 . 
CD32    4.001      1.508   2.653  0.01526 * 
CD33    3.270      1.274   2.566  0.01844 * 
CD34    5.297      1.947   2.721  0.01315 * 
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 3.372 on 20 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.6322,  Adjusted R-squared: 0.5402 
F-statistic: 6.875 on 5 and 20 DF,  p-value: 0.0006986
```

```
Call:
lm(formula = MC1.RQ ~ CD20 - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-4.1637 -2.3959 -0.7754  2.3959  6.2574 

Coefficients:
      Estimate Std. Error t value Pr(>|t|)   
CD200    3.399      1.174   2.896  0.00893 **
CD201    4.641      1.660   2.796  0.01115 * 
CD202    3.300      1.255   2.630  0.01604 * 
CD203    3.660      2.347   1.559  0.13466   
CD204    5.389      1.660   3.247  0.00404 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 3.32 on 20 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.6435,  Adjusted R-squared: 0.5543 
F-statistic: 7.219 on 5 and 20 DF,  p-value: 0.0005236
```

```
Call:
lm(formula = MC1.RQ ~ CD138 - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-4.2844 -1.4396 -0.3655  1.0484  3.9695 

Coefficients:
       Estimate Std. Error t value Pr(>|t|)    
CD1380   6.0811     1.4402   4.223 0.000461 ***
CD1381   3.1595     0.8819   3.583 0.001986 ** 
CD1382   1.4778     1.0183   1.451 0.163032    
CD1383   4.1756     1.1155   3.743 0.001377 ** 
CD1384   9.4011     1.7638   5.330 3.82e-05 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 2.494 on 19 degrees of freedom
  (4 observations deleted due to missingness)
Multiple R-squared: 0.7983,  Adjusted R-squared: 0.7452 
F-statistic: 15.04 on 5 and 19 DF,  p-value: 4.865e-06
```

```
> CD68lCD68subllm<-lm(MC1.RQ~CD68.L+CD68.subL-1)
> summary.lm(CD68lCD68subllm)
```

```
> CD68landageandsexm<-lm(MC1.RQ~CD68.L+age+sex-1)
Warning message:
In model.matrix.default(mt, mf, contrasts) :
  variable 'sex' converted to a factor

Call:
lm(formula = MC1.RQ ~ CD68.L + age + sex - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-3.8723 -2.0926 -0.5076  1.3193  5.5541 

Coefficients:
          Estimate Std. Error t value Pr(>|t|)
CD68.L0.5  0.42036    3.98585   0.105    0.917
CD68.L1   -1.21824    3.82203  -0.319    0.754
CD68.L2    3.31580    3.18604   1.041    0.312
CD68.L3    2.35607    3.26225   0.722    0.479
CD68.L4    2.27173    3.11960   0.728    0.476
age        0.04961    0.05131   0.967    0.346
sexm      -2.19202    1.56951  -1.397    0.180

Residual standard error: 2.876 on 18 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.7592,  Adjusted R-squared: 0.6655 
F-statistic: 8.106 on 7 and 18 DF,  p-value: 0.0001681
```

```
> CD3andageandsexlm<-lm(MC1.RQ~CD3+age+sex-1)
Warning message:
In model.matrix.default(mt, mf, contrasts) :
  variable 'sex' converted to a factor
> summary(CD3andageandsexlm)


Call:
lm(formula = MC1.RQ ~ CD3 + age + sex - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-3.8267 -2.5034  0.1415  1.7488  6.5308 

Coefficients:
     Estimate Std. Error t value Pr(>|t|)  
CD30  6.78500    3.80773   1.782   0.0916 .
CD31  5.76204    4.43321   1.300   0.2101  
CD32  5.62394    3.97777   1.414   0.1745  
CD33  5.50485    3.26408   1.686   0.1090  
CD34  7.11846    4.55880   1.561   0.1358  
age  -0.02602    0.05929  -0.439   0.6661  
sexm -3.00986    1.92425  -1.564   0.1352  
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 3.266 on 18 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.6893,  Adjusted R-squared: 0.5685 
F-statistic: 5.706 on 7 and 18 DF,  p-value: 0.001351
```

```
> CD20andageandsexlm<-lm(MC1.RQ~CD20+age+sex-1)
Warning message:
In model.matrix.default(mt, mf, contrasts) :
  variable 'sex' converted to a factor
> summary(CD20andageandsexlm)

Call:
lm(formula = MC1.RQ ~ CD20 + age + sex - 1)

Residuals:
    Min      1Q  Median      3Q     Max
-4.5468 -1.9458 -0.2169  1.7823  6.3724

Coefficients:
      Estimate Std. Error t value Pr(>|t|)
CD200  5.33291    5.13900   1.038    0.313
CD201  6.58854    4.18719   1.573    0.133
CD202  5.80445    4.15058   1.398    0.179
CD203  4.63701    3.88890   1.192    0.249
CD204  7.04451    5.56471   1.266    0.222
age   -0.02300    0.07394  -0.311    0.759
sexm  -2.79998    1.90714  -1.468    0.159

Residual standard error: 3.24 on 18 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.6943,  Adjusted R-squared: 0.5754
F-statistic: 5.841 on 7 and 18 DF,  p-value: 0.001186
```

```
> CD138andageandsexlm<-lm(MC1.RQ~CD138+age+sex-1)
Warning message:
In model.matrix.default(mt, mf, contrasts) :
  variable 'sex' converted to a factor
> summary(CD138andageandsexlm)

Call:
lm(formula = MC1.RQ ~ CD138 + age + sex - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-3.5278 -1.3004 -0.5965  1.2101  4.8491 

Coefficients:
       Estimate Std. Error t value Pr(>|t|)   
CD1380  9.35333    2.82094   3.316  0.00409 **
CD1381  6.75425    2.66562   2.534  0.02141 * 
CD1382  5.29589    2.77114   1.911  0.07301 . 
CD1383  7.43272    2.66175   2.792  0.01250 * 
CD1384 13.48665    3.50495   3.848  0.00129 **
age    -0.05674    0.04216  -1.346  0.19606   
sexm   -0.61884    1.45302  -0.426  0.67552   
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 2.477 on 17 degrees of freedom
  (4 observations deleted due to missingness)
Multiple R-squared: 0.822,	Adjusted R-squared: 0.7487 
F-statistic: 11.22 on 7 and 17 DF,  p-value: 2.777e-05


Call:
lm(formula = MC1.RQ ~ CD68.L + CD68.subL - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-4.3044 -1.2850 -0.4188  1.0425  6.2900 

Coefficients:
             Estimate Std. Error t value Pr(>|t|)  
CD68.L0.5      3.2663     2.8745   1.136   0.2737  
CD68.L1        1.0419     2.6321   0.396   0.6978  
CD68.L2        5.6989     2.9484   1.933   0.0724 .
CD68.L3        4.1686     2.0383   2.045   0.0588 .
CD68.L4        3.9578     2.7824   1.422   0.1754  
CD68.subL0.5  -1.4342     3.3407  -0.429   0.6738  
CD68.subL1     0.7256     3.0195   0.240   0.8133  
CD68.subL2     0.7753     2.4127   0.321   0.7524  
CD68.subL3     1.3982     2.7614   0.506   0.6200  
CD68.subL4     0.2846     2.8208   0.101   0.9210  
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 3.308 on 15 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.7344,	Adjusted R-squared: 0.5573 
F-statistic: 4.147 on 10 and 15 DF,  p-value: 0.006766
```

```
> CD68sublCD68llm<-lm(MC1.RQ~CD68.subL+CD68.L-1)
> summary(CD68sublCD68llm)



Call:
lm(formula = MC1.RQ ~ CD68.subL + CD68.L - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-4.3044 -1.2850 -0.4188  1.0425  6.2900 

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
CD68.subL0     3.2663     2.8745   1.136    0.274
CD68.subL0.5   1.8322     2.8745   0.637    0.533
CD68.subL1     3.9919     3.9941   0.999    0.333
CD68.subL2     4.0416     3.3510   1.206    0.246
CD68.subL3     4.6645     3.5380   1.318    0.207
CD68.subL4     3.5509     3.7241   0.953    0.355
CD68.L1       -2.2244     3.7099  -0.600    0.558
CD68.L2        2.4326     3.3407   0.728    0.478
CD68.L3        0.9023     3.2324   0.279    0.784
CD68.L4        0.6915     3.5903   0.193    0.850

Residual standard error: 3.308 on 15 degrees of freedom
  (3 observations deleted due to missingness)
Multiple R-squared: 0.7344,  Adjusted R-squared: 0.5573 
F-statistic: 4.147 on 10 and 15 DF,  p-value: 0.006766
```

```
> CD68landcd138lm<-lm(MC1.RQ~CD68.L+CD138-1)
> summary(CD68landcd138lm)

Call:
lm(formula = MC1.RQ ~ CD68.L + CD138 - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-3.3415 -0.9467 -0.0599  1.3994  3.1630 

Coefficients:
          Estimate Std. Error t value Pr(>|t|)    
CD68.L0.5    5.173      2.333   2.217 0.042500 *  
CD68.L1      4.484      1.586   2.827 0.012758 *  
CD68.L2      8.705      2.214   3.932 0.001332 ** 
CD68.L3      6.880      1.391   4.946 0.000176 ***
CD68.L4      6.562      2.064   3.179 0.006220 ** 
CD1381      -2.623      1.683  -1.559 0.139901    
CD1382      -4.498      1.718  -2.617 0.019416 *  
CD1383      -2.828      2.051  -1.379 0.188220    
CD1384       2.839      2.621   1.083 0.295917    
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 2.285 on 15 degrees of freedom
  (4 observations deleted due to missingness)
Multiple R-squared: 0.8663,  Adjusted R-squared: 0.7861 
F-statistic:  10.8 on 9 and 15 DF,  p-value: 4.402e-05
```

```
> CD138andCD68Llm<-lm(MC1.RQ~CD138+CD68.L-1)
> summary(CD138andCD68Llm)


Call:
lm(formula = MC1.RQ ~ CD138 + CD68.L - 1)

Residuals:
    Min      1Q  Median      3Q     Max
-3.3415 -0.9467 -0.0599  1.3994  3.1630

Coefficients:
        Estimate Std. Error t value Pr(>|t|)
CD1380    5.1725     2.3332   2.217   0.0425 *
CD1381    2.5492     1.6160   1.577   0.1355
CD1382    0.6750     2.1450   0.315   0.7573
CD1383    2.3444     2.2936   1.022   0.3229
CD1384    8.0114     2.8674   2.794   0.0136 *
CD68.L1  -0.6883     1.9548  -0.352   0.7297
CD68.L2   3.5327     2.3516   1.502   0.1538
CD68.L3   1.7070     2.0556   0.830   0.4193
CD68.L4   1.3897     2.3686   0.587   0.5661
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 2.285 on 15 degrees of freedom
  (4 observations deleted due to missingness)
Multiple R-squared: 0.8663,  Adjusted R-squared: 0.7861
F-statistic:  10.8 on 9 and 15 DF,  p-value: 4.402e-05
```

```
> CD68LandCD138andageandsex<-lm(MC1.RQ~CD68.L+CD138+age+sex-1)
Warning message:
In model.matrix.default(mt, mf, contrasts) :
  variable 'sex' converted to a factor
> summary(CD68LandCD138andageandsex)


Call:
lm(formula = MC1.RQ ~ CD68.L + CD138 + age + sex - 1)

Residuals:
    Min      1Q  Median      3Q     Max 
-3.5100 -0.9025 -0.2018  1.4290  3.1254 

Coefficients:
           Estimate Std. Error t value Pr(>|t|)  
CD68.L0.5  5.548111   4.647006   1.194   0.2538  
CD68.L1    4.681310   4.317884   1.084   0.2980  
CD68.L2    8.690038   3.629589   2.394   0.0324 *
CD68.L3    7.079088   3.501663   2.022   0.0643 .
CD68.L4    6.403919   3.253872   1.968   0.0708 .
CD1381    -2.485452   1.839990  -1.351   0.1998  
CD1382    -4.074420   2.233177  -1.824   0.0911 .
CD1383    -2.600413   2.348767  -1.107   0.2883  
CD1384     3.245296   3.563383   0.911   0.3790  
age       -0.003446   0.059594  -0.058   0.9548  
sexm      -0.578904   1.624882  -0.356   0.7274  
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 2.443 on 13 degrees of freedom
  (4 observations deleted due to missingness)
Multiple R-squared: 0.8676,  Adjusted R-squared: 0.7556 
F-statistic: 7.745 on 11 and 13 DF,  p-value: 0.0004624
```

```
> CD68LandCD138andCD3andCD20andageandsex<-
lm(MC1.RQ~CD68.L+CD138+CD3+CD20+age+sex-1)
Warning message:
In model.matrix.default(mt, mf, contrasts) :
  variable 'sex' converted to a factor
> summary(CD68LandCD138andCD3andCD20andageandsex)


Call:
lm(formula = MC1.RQ ~ CD68.L + CD138 + CD3 + CD20 + age + sex -
    1)

Residuals:
    Min      1Q  Median      3Q     Max
-2.0325 -0.6098  0.1630  0.6183  1.5712

Coefficients: (1 not defined because of singularities)
          Estimate Std. Error t value Pr(>|t|)
CD68.L0.5 12.45321    5.01834   2.482   0.0477 *
CD68.L1   11.16544    5.94415   1.878   0.1094
CD68.L2   18.68466    6.13672   3.045   0.0227 *
CD68.L3   12.41336    5.54161   2.240   0.0663 .
CD68.L4   10.39337    5.57091   1.866   0.1113
CD1381    -0.91507    1.92677  -0.475   0.6516
CD1382     5.25195    3.19956   1.641   0.1518
CD1383     4.59406    3.21598   1.429   0.2031
CD1384    16.58726    4.76756   3.479   0.0132 *
CD31       1.33105    2.37837   0.560   0.5960
CD32      -7.01578    2.14001  -3.278   0.0169 *
CD33      -1.21820    2.36094  -0.516   0.6243
CD34      -5.35816    2.95174  -1.815   0.1194
CD201      2.14230    2.88068   0.744   0.4852
CD202     -5.12041    2.39802  -2.135   0.0766 .
CD203     -6.61615    3.23522  -2.045   0.0868 .
CD204           NA         NA      NA       NA
age       -0.12267    0.08283  -1.481   0.1891
sexm      -2.03053    1.55692  -1.304   0.2400
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 1.848 on 6 degrees of freedom
  (4 observations deleted due to missingness)
Multiple R-squared: 0.965,   Adjusted R-squared: 0.8601
F-statistic: 9.197 on 18 and 6 DF,  p-value: 0.005715
```

```
> CD68LandCD138andCD3andCD20<-lm(MC1.RQ~CD68.L+CD138+CD3+CD20-1)
> summary(CD68LandCD138andCD3andCD20)


Call:
lm(formula = MC1.RQ ~ CD68.L + CD138 + CD3 + CD20 - 1)

Residuals:
     Min       1Q   Median       3Q      Max 
-2.92452 -0.76527  0.02986  0.90755  1.82543 

Coefficients: (1 not defined because of singularities)
          Estimate Std. Error t value Pr(>|t|)   
CD68.L0.5   5.1024     2.4849   2.053  0.07412 . 
CD68.L1     2.7670     1.9689   1.405  0.19755   
CD68.L2    10.6287     2.9225   3.637  0.00662 **
CD68.L3     5.0576     2.4290   2.082  0.07087 . 
CD68.L4     4.5672     2.8510   1.602  0.14783   
CD1381     -2.5532     1.9839  -1.287  0.23411   
CD1382      0.6571     2.5820   0.254  0.80553   
CD1383      2.2048     3.4448   0.640  0.54004   
CD1384     10.9532     4.4057   2.486  0.03775 * 
CD31        2.1732     2.6756   0.812  0.44016   
CD32       -4.4685     2.0250  -2.207  0.05838 . 
CD33        1.1722     2.1215   0.553  0.59566   
CD34       -3.7907     3.2434  -1.169  0.27615   
CD201       3.1879     2.9602   1.077  0.31292   
CD202      -3.9793     2.3052  -1.726  0.12258   
CD203      -4.1626     3.1875  -1.306  0.22788   
CD204           NA         NA      NA       NA   
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1 

Residual standard error: 2.116 on 8 degrees of freedom
  (4 observations deleted due to missingness)
Multiple R-squared: 0.9389,  Adjusted R-squared: 0.8166 
F-statistic: 7.681 on 16 and 8 DF,  p-value: 0.003249
```

PCA analysis commands

1- Continuous:

```
pc.cr <- princomp(~ MC1.RQ + d.TNF +dCXCL13 + dIl6 + dIL21 + dRANKL + dIL1, data = mcr, na.action = na.exclude, cor = TRUE)
png(file="cont.png",width=580,height=580)
biplot(pc.cr)
dev.off()
```

2.- Discrete:

```
pc.cr <- princomp(~ MC1.RQ + CD20 + CD3 + CD138 + CD68.L + CD68.subL, data =mcr, na.action = na.exclude, cor = TRUE,scale=TRUE)
biplot(pc.cr)
png(file="discrete.png",width=580,height=580)
biplot(pc.cr)
dev.off()
```

3. Both

```
pc.cr <- princomp(~ MC1.RQ + CD20 + CD3 + CD138 + CD68.L + CD68.subL + d.TNF +dCXCL13 + dIl6 + dIL21 + dRANKL + dIL1, data = mcr, na.action = na.exclude, cor = TRUE)
biplot(pc.cr)
png(file="both.png",width=580,height=580)
biplot(pc.cr)
dev.off()
```

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