An immunogenetic study of psoriatic arthritis

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AN IMMUNOGENETIC STUDY

OF

PSORIATIC ARTHRITIS

submitted by

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Summary

Psoriatic arthritis (PsA) is a complex inflammatory arthritis that affects approximately 15% of patients with psoriasis. The aetiology of PsA is poorly understood but there is good evidence from family studies to suggest a strong genetic component. The strongest association is with the MHC region on Chromosome 6 where there are a number of potential candidate genes. One such gene is the MHC Class I Chain related gene, MICA. MICA is a strong candidate gene for both psoriasis and PsA due to its position, function, high degree of polymorphism and expression in the target tissues. It is a stress-inducible molecule which activates the NKG2D receptor on natural killer (NK) and T cells, cells which are known to be important in both psoriasis and PsA.

Patients with PsA and psoriasis were recruited to study the associations of polymorphisms of MICA. Alleles of MICA which carry 9 'GCT' repeats in exon 5 (A9) were significantly associated with both PsA (p_{corr}<0.0005, OR 2.4) and psoriasis (p_{corr}<0.0005, OR 2.5). Further analysis of the extensive polymorphism within exons 2,3 and 4 of MICA revealed a strong association between the MICA *002 allele and PsA (p_{corr}<0.05, OR 2.3) and the MICA *017 allele and psoriasis (p_{corr}<0.0015, OR 5.01). Immunohistochemistry with antibodies to MICA demonstrated upregulated expression of MICA in inflamed synovium and downregulated expression of MICA in psoriatic skin.

Further studies were performed to investigate whether there was any association between PsA and the HLA-DRB1 'shared epitope' (SE) that is so strongly associated with rheumatoid arthritis. This revealed no overall association but the SE was more common in patients who developed erosive disease (p_{corr}<0.05, OR 2.11). In addition, a study was performed to examine the prevalence of anti-cyclic citrullinated peptide antibodies in PsA. Although anti-CCP antibodies were not significantly increased in PsA, their presence was associated with markers of disease severity.

In summary, this thesis examines the immunogenetic factors which contribute to the susceptibility to and severity of PsA.

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Abbreviations

α1	Alpha 1 chain
AH	Ancestral haplotype
AFA	Anti-filaggrin antibodies
AIDS	Acquired Immunodeficiency syndrome
4AOH	4th Asia-Oceania histocompatibility workshop
AS	Ankylosing spondylitis
β2m	Beta-2 microglobulin
°C	Degrees Celsius
CCP	Cyclic citrullinated peptide
CDSN	Corneodesmosin
CLA	Cutaneous lymphocyte antigen
CMV	Cytomegalovirus
CRP	C reactive protein
ddH_2O	Double distilled water
DIP	Distal interphalangeal joint
DMARD	Disease modifying anti-rheumatic drug
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ELISA	Enzyme linked immunosorbent assay
EMCIF	Extended MHC Class I family
FcRN	Neonatal Fc receptor
HAQ	Health assessment questionnaire
HGH	Human growth hormone
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
IBD	Inflammatory bowel disease
IDDM	Insulin dependent diabetes mellitus
IF	Immunofluorescence
IFNγ	Interferon gamma
IgM	Immunoglobulin M

IHC	Immunohistochemistry
IHW	International histocompatibility workshop
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-10	Interleukin-10
IU	International units
JIA	Juvenile idiopathic arthritis
kb	Kilobases
kDa	Kilodaltons
KIR	Killer immunoglobulin-type receptor
LD	Linkage disequilibrium
M	Molar
MCP	Metacarpophalangeal
MEGA	Molecular evolutionary genetics analysis
µmol	Micromoles
MHC	Major histocompatibility complex
MICA	MHC Class I chain related antigen A
MICB	MHC Class I chain related antigen B
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NK	Natural killer
nmol	Nanomoles
NS	Not significant
OA	Osteoarthritis
OR	Odds ratio
PAD	Peptidylarginine deiminase
PASI	Psoriasis area severity index
PCR	Polymerase chain reaction
pmol	Picomoles
Ps	Psoriasis
PsA	Psoriatic arthritis
PSORS1	Psoriasis susceptibility locus 1

PV	Plasma viscosity
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNHRD	Royal National Hospital for Rheumatic diseases
RPM	Revolutions per minute
RR	Relative risk
SCID	Severe combined immunodeficiency
SD	Standard deviation
SE	Shared epitope
SIJ	Sacroiliac joints
SLE	Systemic lupus erythematosis
SNP	Single nucleotide polymorphism
SSP	Sequence specific primers
T cells	Thymocyte cells
TH1	Thymocyte helper cells, type 1
TM	Transmembrane
T_m	Melting temperature
TNF	Tumour necrosis factor
UV	Ultraviolet
VAS	Visual analogue scale

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Expression patterns of extended class I family products (PERB11, MIC) in the skin and synovium in psoriasis and psoriatic arthritis. **E Korendowych**, P Hollingsworth, B De Boer, M Platten, S Gaudieri, JF Williamson, RL Dawkins. J Hansen and B Dupont, eds. *HLA 2004 : Immunobiology of the Human MHC. In press*

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Conference contributions arising from the work of this thesis

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The highly polymorphic MHC Class I related gene MICA is expressed in the synovium in psoriatic and rheumatoid arthritis. **E Korendowych**, NJ McHugh, JF Williamson, S Gaudieri, P Hollingsworth, RL Dawkins. *British Society for Rheumatology,* (Poster session, Seronegative Spondyloarthropathies), Manchester, April 2003

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CHAPTER 1 INTRODUCTION

1.1 Introduction

The aim of this thesis is to study the immunogenetic factors that contribute to the development of psoriatic arthritis (PsA). There are three main themes that will be explored. Firstly, the genetic and immunological features that aid the *diagnosis* of psoriatic arthritis (PsA) and distinguish it from other inflammatory arthritides such as rheumatoid arthritis (RA) and ankylosing spondylitis (AS) will be examined. Secondly, the genetic factors primarily associated with *psoriasis itself* will be distinguished from those that contribute to the development of the inflammatory arthritis. Finally, any genetic or immunological features that may be associated with the *classification and severity* of PsA will be explored.

1.2 PsA – background

The association between psoriasis and arthritis was first recognised by Alibert in 1822 and was later described as 'Psoriasis Arthritique' by Bazin in 1860. However, it was not until the discovery of rheumatoid factor (RF) and its absence in the majority of patients with PsA that the condition was recognised by the Arthritis and Rheumatism Association in 1964 (1). The original classification of PsA (2) defined five subgroups comprising asymmetrical oligoarthritis, symmetrical polyarthritis, predominant distal interphalangeal (DIP) joint disease, predominant spondyloarthopathy and arthritis mutilans. This classification has since been challenged and modifications of the criteria have been proposed (3-6). Long term follow-up studies suggest that at least two major groups exist - those with predominant peripheral joint disease and those with predominant axial disease (7). The precise definition and classification of PsA remains controversial and internationally agreed diagnostic criteria have yet to be developed and applied.

Psoriasis itself is a common inflammatory hyperproliferative skin disease which affects approximately 2% of the Caucasian population (8). It has been classified into two major categories : Type I (early onset of disease before 40 years of age) and Type II (late onset of disease after 40 years of age). The former has been shown to have the strongest genetic

predisposition, whereas late onset disease is thought to be predominantly sporadic (9). The prevalence of PsA is somewhat harder to ascertain as it depends on the population studied and the techniques used for its diagnosis. The prevalence of inflammatory arthritis in patients with psoriasis has been shown to vary between 5% and 42% (10). Therefore the overall prevalence of PsA may approach that of rheumatoid arthritis (RA) and ankylosing spondylitis (AS).

An additional confounding factor in population studies of PsA is the variable temporal relationship between the onset of the skin and the joint disease. In the majority of patients (approximately 80%), the onset of psoriasis precedes the onset of arthritis. However, in some 20% of patients the arthritis appears before there is any evidence of skin disease.

Clues to the correct diagnosis can sometimes be elicited if there is a positive family history of psoriasis or in the presence of nail disease. The diagnosis may well be suspected on the basis of clinical and radiological features, and indeed, such a condition has been coined psoriatic arthritis 'sine psoriasis'. Interestingly, approximately 35% of patients exhibit clear simultaneous exacerbations of their skin and joint disease (4), whereas others show no such correlation. It is possible that closer analysis of such patients may reveal some genetic or immunological factors that are common to both the skin and the joint disease and others that are unique. There may also be a disparity between the severity of the skin disease and the severity of the arthritis. Some patients have severe psoriasis but never develop arthritis whilst others have a small patch of psoriasis and develop a disabling and deforming arthritis. However, in general terms, it is more common to develop arthritis if the psoriasis is severe (11).

The acceptance of PsA as a distinct disorder, rather than the fortuitous association of two common disorders (psoriasis and arthritis) is supported by epidemiological studies. The observed prevalence of PsA is much higher than the combined prevalence of psoriasis and inflammatory arthritis (12). The most likely explanation for this is that PsA exists as a distinct entity with unique clinical characteristics. An alternative explanation could be that psoriasis acts as a risk factor for a non-specific inflammatory arthritis or that psoriasis and arthritis share a common genetic or environmental trigger. Since psoriasis is a common

condition, there will be patients with psoriasis who develop RA or AS. Separating those patients with true PsA from those who have psoriasis and a co-existing inflammatory arthritis is clearly important for diagnosis, prognosis and appropriate treatment of patients. It is also vital for those involved in the study of the genetic contribution to PsA to be able to distinguish it from other inflammatory arthritides. The lack of consensus on diagnosis and classification has been a key contributory factor to the relative lack of research into the aetiology of PsA.

Taxonomically, PsA belongs to the spondyloarthropathies. This family of conditions share several characteristics such as predominant axial and asymmetrical peripheral joint involvement, association with HLA-B27, negative RF and enthesopathy (13). Recent studies using magnetic resonance imaging have led to the proposal that the synovitis associated with PsA is secondary to entheseal inflammation (14). This could be a common thread with the other spondyloarthropathies and appears to be a distinguishing feature from RA where the synovitis is a primary event. However, the enthesis is a very avascular structure. It is possible therefore that the inflammation actually starts in the adjacent bone. Clearly, there are additional and more complex factors involved in the aetiology of PsA that influence the diversity of joint involvement, erosion, ankylosis and destruction.

There are certain features that help distinguish PsA from the other spondyloarthropathies and RA. Clinical features include involvement of the DIP joints, nail disease, asymmetry, the distribution of affected joints in a 'ray' rather than a 'row', enthesitis, dactylitis and axial disease. There are also radiological features that can aid diagnosis such as erosion with new bone formation, asymmetrical sacroiliitis and 'chunky' syndesmophytes. Clearly, some of the clinical and radiological features are more diagnostically helpful than others. A prospective study of patients in the Norfolk arthritis register suggested that patients who had psoriasis and early inflammatory arthritis had a similar degree of symmetry and metacarpophalangeal (MCP) joint involvement to those patients without psoriasis (15). They found a slight increase in DIP joint involvement, significant reduction in RF and fewer radiological erosions in the group of patients who had co-existing psoriasis.

A further study suggested that the one feature that distinguished PsA from other forms of polyarthritis was DIP joint involvement, although this was not frequent enough to be a sensitive diagnostic marker (16). Population studies suggest a different demographic profile for patients with PsA, RA and AS. Firstly, there is an equal sex incidence in PsA in contrast to RA which is significantly more common in females and AS which is more common in men. The peak incidence of PsA is in the 5^{th} and 6^{th} decades of life as opposed to RA which increases in frequency with age and AS which rarely presents after the 5^{th} decade.

With regards to pathogenesis, several studies of synovial fluid cytokine profiles suggest that any differences between PsA and RA are largely quantitative rather than qualitative (17; 18). For example, levels of tumour necrosis factor alpha (TNFα) are highest in the synovial fluid of late RA, followed by early RA, then late PsA and finally early PsA which has the lowest levels. This might reflect differences in the intensity of the inflammatory response rather than any distinct pathogenetic mechanisms. However, others have demonstrated some differences in cytokine and growth factor profiles on synovial biopsies of patients with PsA and RA (19). For example, interleukin 10 (IL-10) is reduced in the synovial fluid of patients with late PsA compared with RA and osteoarthritis (OA) where the levels of IL-10 are increased (20).

There is also some evidence that psoriatic arthritis synovium exhibits distinct vascular patterns with more blood vessels and an increased tortuosity compared with RA (21). The lining layer of the joint is not as hyperplastic in PsA as it is in RA. Studies of angiogenesis factors have shown that levels of vascular endothelial growth factor (VEGF) and the angiogenesis factors 1 and 2 are increased in the synovial membrane and skin of patients with PsA and psoriasis (22). The differential expression of these angiogenesis factors may explain some of the differences in vascular pattern observed in PsA.

In PsA, there tend to be fewer macrophages compared with RA but equal numbers of lymphocytes staining for CD3, CD4 and CD8. However, patients with RA have twice as many CD4 positive T cells in the peripheral blood as patients with PsA. There is also evidence that the CD4:CD8 ratio is reversed in PsA compared with RA (23) (24). The

ratio of CD4:CD8 is 0.7 : 1 in PsA but 1.1 : 1 in RA. Most of the CD8 positive T cells in PsA carry DR4 and CD45RO suggesting that they are activated and mature. Thus CD8 positive T cells may be the main effector T cell in PsA leading to the release of cytokines, upregulation of adhesion molecules, recruitment of further inflammatory cells and perpetuation of the inflammatory response. The role for natural killer (NK) cells in PsA as well as the immunological differences between PsA and psoriasis will be discussed later in the chapter.

Genetic studies of PsA are confounded by diagnostic and classification difficulties both within the disease itself and in relation to the other inflammatory arthritides. Misclassification of clinical phenotypes, incomplete penetrance, variable expression and genetic heterogeneity are likely to complicate the detection of a gene(s) for PsA. Interestingly, there appears to be differential expression of PsA according to the sex of the disease-transmitting parent, with excessive paternal transmission (25). Another key issue is whether any genetic susceptibility to PsA is beyond that which predisposes to psoriasis itself. Relatively few genetic studies of PsA contain an adequate control group of patients who have psoriasis but no arthritis. The presence of occult arthritis in patients with psoriasis is well described and failure to account for this will weaken any apparent genetic association. Conversely, almost without exception, genetic studies of psoriasis ignore the possibility of co-existent arthritis. Failure to recognise the different phenotypes within psoriasis will certainly limit the interpretation of such genetic studies.

1.3 Psoriasis - background

Psoriasis is a chronic inflammatory hyperproliferative skin disease that affects approximately 2% of the Caucasian population. It is rarely fatal but leads to significant physical impairment and psychological distress. It is a complex disease in terms of its aetiology and phenotypic spectrum. The most common phenotype is plaque psoriasis but there is a spectrum of presentations, including pustular psoriasis and erythroderma. A proportion of patients will also develop psoriasis of the nails with nail pitting, ridging and onycholysis. Involvement of the nails is often associated with an accompanying arthritis, particularly in the DIP joints. Susceptibility to psoriasis, like PsA, is likely to involve

environmental triggers and a particular genetic and immunological background. Some of these will be common to both psoriasis and PsA, whilst others will be unique to each condition.

1.4 Genetics of psoriasis

A significant genetic contribution to psoriasis is supported by familial clustering of disease and increased concordance rates in monozygotic twins. There are numerous reports of disease association with the HLA-Cw6 antigen, particularly with early onset (Type I) psoriasis. However, the percentage of patients with psoriasis who are HLA-Cw6 positive is relatively low, depending on the population studied. The prevalence is approximately 45% in Caucasians but only 10% in the Japanese population (26) (27) (28). In addition, psoriasis only appears to run in families approximately one third of the time. Psoriasis does not follow simple patterns of dominant or recessive inheritance implying that several genes at several different loci are required for the disease to develop. It is likely that these genes will affect susceptibility to psoriasis, severity of the disease and the variety of its many clinical manifestations. Some of these genes will be unique to psoriasis whilst others will be common to other inflammatory skin diseases perhaps via more generalised regulation of the immune response.

Twin studies offer some of the most compelling evidence for a strong genetic component to psoriasis. Concordance rates are three times greater in monozygotic than dizygotic twins, although these levels differ according to the population studied (29-31). These studies can be used to calculate the *heritability* of the condition (the proportion of variability of a trait that is due to genetic factors). Estimates of the heritability of psoriasis range from 60-90%, placing it amongst the highest of the multifactorial genetic disorders (32). Family studies offer further support for the genetic contribution to psoriasis. The risk ratio (prevalence of disease in a given relative divided by the prevalence of disease in the general population) for first degree relatives of a patient with Type I psoriasis is approximately 10 (8). This would certainly make psoriasis amenable to genetic linkage studies.

Several candidate loci for psoriasis have been identified using genetic linkage. The major susceptibility locus appears to be within the MHC on Chromosome 6 and has been termed 'PSORS1'. An increasing number of other loci outside the MHC have been suggested as additional contributors to the development of disease (*Table 1.1.*). Some of these susceptibility loci may not be disease specific. For example, there is some overlap with the susceptibility regions defined for atopic dermatitis and psoriasis (PSORS2, PSORS4, PSORS5 and chromosome 20p) suggesting a common pathway for the development of inflammatory skin disorders. Most work has concentrated on the association with the MHC in an attempt to further refine the PSORS1 region and identify the major psoriasis gene.

1.4.1 The PSORS1 locus

The association between psoriasis and the MHC has been recognised for over 30 years (33). This association is stronger with Type I psoriasis than Type II psoriasis (8). Case control studies have shown the strongest associations are with haplotypes containing HLA-Cw6 (ancestral haplotypes AH13.1, AH57.1 and AH37.1) (32). In northern Europe and the United States, approximately two thirds of patients with psoriasis carry HLA-Cw6 (34). This is much lower in other populations in particular Oriental populations where HLA-Cw7 plays a more prominent role.

A number of independent groups have presented robust data linking psoriasis to the MHC region with the use of whole genome scans (a technique using a collection of polymorphic markers distributed evenly throughout the genome to test for genetic linkage with a particular trait) (*Table 1.1*). Linkage was detected with HLA-C with a lod score of nearly 10 (35). A lod score is a measure of genetic linkage that refers to the logarithm of the odds ratio. The odds ratio is defined as the likelihood of encountering the observed outcome if the marker and disease are linked, divided by the likelihood of the same outcome if the marker and disease are unlinked. Linkage between HLA-C and psoriasis does not mean that HLA-Cw6 is PSORS1. There is no clear direct pathological role for HLA-Cw6 in psoriasis. The MHC region is nearly 3.5 million base pairs long and contains over 200 genes including several strong functional candidate genes in close proximity to HLA-C. In

addition, not all HLA-Cw6 bearing haplotypes confer equal risk for psoriasis suggesting that HLA-Cw6 itself is not the psoriasis gene (35).

Further definition of the PSORS1 locus was achieved following the analysis of extended or ancestral haplotypes. Founder population haplotypes will recombine through successive generations (36). Analysis of the minimal conserved haplotype that segregates with disease can help determine the segment of the MHC region that is likely to carry the 'risk allele'. Two major studies (35; 37) have demonstrated that the strongest link with psoriasis is with the 57.1 ancestral haplotype (Cw6-B57-DR7-DQ3). This is consistent with serological studies that have demonstrated associations with individual components of this haplotype (32).

The PSORS1 region has been further refined using linkage disequilibrium (LD) fine mapping (38). Three independent studies (39-41) used distinct statistical approaches leading to differing conclusions as to the location and extent of the PSORS1 region (*Figure 1.1*). It should be noted that these studies involved different ethnic populations from northern Europe, USA and Japan. This may account for some of the differences in the localisation of PSORS1. Psoriasis does seem to be associated with different haplotypes in the Japanese, where those containing HLA-Cw7 predominate. It has been postulated, however, that a more lenient interpretation of the data may result in a 150kb region for PSORS1 that is common to all ethnic groups (42).

1.4.2 Candidate gene analysis in psoriasis

A number of candidate genes that lie within or close to the PSORS1 region have been investigated. HLA-Cw*0602 is an obvious candidate that has been consistently shown to be the most significant marker for psoriasis (39; 43; 44). However, the variable disease risks conferred by the differing haplotypes bearing HLA-Cw6 together with the lack of a clear functional role and its apparent location outside the PSORS1 region make it a less attractive candidate gene. Other candidates that have been proposed include corneodesmosin (CDSN) (43-46), HCR (47-49), MICA (50-52), OTF3 (53) and TCF19 (54). Several of these candidates have shown significant associations with psoriasis, but

the presence of these alleles along the same disease associated haplotype hampers the ability to dissect out which gene is primarily responsible. Clearly, it is possible that several genes along a disease-associated haplotype contribute to the development of psoriasis and need not, therefore, be mutually exclusive. Some of the candidate genes have a more plausible functional role in psoriasis than others. This, together with expression and functional studies of the gene products in the skin, may help delineate which genes are the most important for future study.

1.5 Immunology of psoriasis

Psoriasis is associated with keratinocyte hyperproliferation and a dermal and epidermal infiltration of activated T lymphocytes (9). At the cellular level, psoriasis is characterised by epidermal proliferation and abnormal differentiation, dilatation and elongation of capillaries and a mixed inflammatory cell infiltrate.

In addition to T cells, NK cells are also present in psoriatic plaques although their exact role in the pathogenesis of the condition remains unclear. It would appear that certain phenotypes of NK and NK-T cells are present in psoriasis more often than others. One study has demonstrated increased numbers of CD16, CD57, CD94 and CD158 NK cell markers as well as CD161 (found predominantly on NK-T cells) in psoriatic plaques compared with adjacent normal skin (55). Reduced numbers of circulating NK cells (CD16+, CD56+, CD94+, CD158+) have been reported in the peripheral blood of patients with psoriasis compared with controls (56). Reduced numbers of NK-T cells (CD3+, CD56+) have also been described in association with psoriasis (57). The number of these cells does rise following specific anti-psoriatic therapies, but it never reaches a level seen in the control population, suggesting some intrinsic deficiency in the patients who develop psoriasis. This suggests a possible role for NK cells in the immunopathology of psoriasis.

One of the key cytokines in psoriasis appears to be IL-18. It plays an important role in the TH1 response primarily via its ability to induce interferon-gamma production in T cells and NK cells. IL-18 is produced by keratinocytes as well as monocytes and macrophages, therefore local production in the skin may enhance the cutaneous immune response.

Immunohistochemical analysis has demonstrated enhanced expression of IL-18 in lesional psoriatic skin (58). In addition, levels of IL-18 in the plasma of patients with psoriasis correlates with the severity of their psoriasis as judged by the psoriasis area severity index (PASI) (59).

1.6 Genetics of PsA

Unfortunately, there is no data available from twin studies in PsA. However, family studies do show that first degree relatives have an increased risk of developing the disease. The original family studies of Moll and Wright suggested a much higher risk for siblings (λ_s) developing PsA than psoriasis itself (2). The familial recurrence risk for PsA appears to be about ten times greater than for psoriasis alone or RA (60). This would certainly suggest that the genetic contribution to PsA is even greater than that to psoriasis alone.

1.6.1 HLA studies in PsA

Case control studies have demonstrated associations between PsA and a variety of HLA antigens. The strongest association has repeatedly been shown to be with HLA-Cw6 (61), although this association would appear to be stronger with psoriasis itself than with PsA. The reported associations with HLA-B13, B57 and DR7 are likely to be secondary to their presence on recognised ancestral haplotypes, AH 13.1 and AH 57.1, which both contain HLA-Cw6 (36).

Some of the HLA antigens have been linked with certain clinical manifestations of PsA. For example, HLA-B38 and B39 have been associated with a peripheral arthritis (62). The association with HLA-B27 remains controversial, with differing conclusions as to the strength of the link with psoriatic spondyloarthritis and sacroiliitis (63). HLA-B27 has also been reported in association with DIP joint disease, suggesting that the link represents more than simply the co-existence of AS and psoriasis (61). HLA-DR4 has been associated with a peripheral symmetrical polyarthritis resembling RA (28), although this has not been replicated by others. It is worth noting that in the studies where psoriasis

controls were used as well as population controls, many of these HLA associations were much weaker with PsA than with psoriasis alone (63; 64).

One study has attempted to map the disease susceptibility locus in PsA (65). However, the sample size used for the study was small (n=95) and the results must therefore be interpreted with caution. It was suggested that the susceptibility region for Spanish patients with psoriasis was telomeric to HLA-C, in a region containing the microsatellite marker C1_4_4, OTF3 and HCR. The region for PsA was delimited by HLA-C and the C1_4_4 marker, thus providing an overlapping interval of 100kb between HLA-C and OTF3 which might contain the PsA gene. Clearly more powerful studies are needed in order to better refine the susceptibility region for PsA.

1.6.2 Candidate gene analysis in PsA

A small number of studies have examined potential contributions to PsA from non-HLA genes within the MHC region, with particular emphasis on the region neighbouring HLA-B and HLA-C. The MHC Class I Chain related antigen A (MICA) is one such candidate which has been studied in two relatively small populations with PsA (66-68). The role for MICA in PsA will be explored in Chapters 3-5, where the associations of this fascinating and highly polymorphic gene within our PsA population will be presented.

Another gene which has generated a significant level of interest is the tumour necrosis factor (TNF) gene which lies just centromeric to MICA (20; 69-71). Associations have been reported between the '–308' polymorphism and PsA, although these studies do not include an adequate control group with psoriasis and no arthritis. It remains unclear whether this association is primarily with psoriasis, or whether a separate risk is associated with PsA. It is equally difficult to establish whether the association is secondary to a stronger link with another gene along a disease-associated haplotype. This problem continues to confound most genetic case-control association studies within the MHC region due to the highly conserved nature of the genetic sequence along ancestral haplotypes. Several other neighbouring candidates have been examined, but none as yet have shown any convincing association over and above the known associations with the

HLA antigens themselves. The majority of the studies have only examined the neighbouring genes in psoriasis (where no reproducible major candidate genes have been located). Therefore more studies will be needed to examine the MHC region in PsA.

There has been some interest in genes which lie outside the MHC region for their potential role in PsA. A recent study suggested an association between the CARD15 gene on chromosome 16q and PsA in a Canadian population (72). This is interesting as the CARD15 gene has been shown to be strongly associated with Crohns disease (73) which shares some features in common with the seronegative arthritides. In addition, 16q has been reported as one of the potential susceptibility regions for psoriasis (74). However, more studies are needed to confirm this association between CARD15 and PsA as some studies have failed to replicate the results (75).

Another region of interest is the IL-1 gene cluster on chromosome 2q, particularly as expression of IL-1 is upregulated in the serum and synovium of patients with PsA (76) (77). Associations between polymorphisms of the IL-1 gene cluster have been made with RA, psoriasis, ankylosing spondylitis and ulcerative colitis (78). A recent publication has confirmed an association with the IL-1 alpha 889C polymorphism and PsA (78).

1.7 Environmental factors in PsA

1.7.1 Infection

The theory that infectious agents have a role in the aetiology of both psoriasis and PsA remains a contentious one. It is possible that certain infections may be a common aetological factor for both psoriasis and PsA. Alternatively, infectious agents may act to increase the susceptibility of patients with psoriasis to develop arthritis. There is certainly good evidence to support a role for streptococcal infection in guttate psoriasis. There is also some evidence to support a role for streptococcal infection in PsA. Antibodies to Group A streptococcal endotoxins have been demonstrated in the serum of 50% of patients with PsA, a level which is approximately double that seen in either RA or psoriasis (79). There may also be a role for viral infection in PsA. Circulating levels of 2-

5A, an adenylic acid polymer thought to be a marker of viral replication, are increased in PsA compared with RA and controls (80). Patients with PsA also have an increased frequency of Hepatitis C infection, which is double that seen in uncomplicated psoriasis (81).

The evidence for HIV infection is more compelling; a recent study from Zambia found 94% of consecutive patients with PsA were HIV positive compared with 30% of the general population (82). This contrasts with RA which is not increased in HIV infected individuals. HIV infection may be directly involved in the triggering of the disease or it may herald the co-existence of other infectious triggers. Equally, it may alter the T cell balance in favour of developing PsA. It is also interesting that certain HLA haplotypes are known to alter the expression of HIV: the 57.1 ancestral haplotype (which has been linked with PsA) is associated with long term non-progression to AIDS (83; 84).

The association between the infection and the arthritis may therefore not be a direct trigger, but simply a reflection of a common HLA susceptibility. It is possible that certain haplotypes are associated with disease through differing immunological responses to infection. No one organism would appear to be responsible for triggering PsA. It is likely that a variety of infectious organisms can trigger the onset of PsA, depending on the environment the individual is exposed to and their particular genetic background.

1.7.2 Trauma

There have been several reports of trauma inducing PsA, leading to speculation that there may be a 'deep Koebner' phenomenon occurring within the joints which mirrors the superficial response known to occur in the skin with psoriasis. Trauma certainly seems to be a relatively common feature prior to the development of PsA compared with RA; 8-9% of patients with PsA report a clear acute traumatic event compared with only 1-2% of patients who develop RA (85; 86). It is intriguing that some of the clinical features of PsA, such as DIP joint involvement and plantar fasciitis, may be a response to chronic trauma. However, the diverse nature of the types of trauma thought to predate PsA, probably suggests more of a role for psychological stress than direct physical contact. This may

well be a feature in common with psoriasis which is frequently exacerbated by psychological stress.

1.8 Immunology of PsA

There are some similarities between PsA and psoriasis in terms of their immunological basis. In histological sections, both psoriatic skin and synovium have dilated blood vessels and a perivascular inflammatory infiltrate consisting largely of mononuclear cells, predominantly CD8 and CD4 positive T cell clonal expansions (23) (24). Both diseases are commonly regarded as T cell mediated conditions – the lymphocyte infiltrate is an early event and both conditions respond to treatment with anti-T cell therapies such as ciclosporin (87) and alefacept (88). Equally, the occurrence of both psoriasis and PsA after bone marrow transplantation from a donor who has psoriasis suggests at least some common immunological mechanism. However, there are important differences between the skin and the joint that suggest unique mechanisms are involved. Firstly, T cells derived from psoriatic skin injected into severe combined immunodeficient (SCID) mice can induce *psoriasis* in normal human grafted skin. However, the same T cells do not induce an *arthritis* in the SCID mice. Secondly, the cytokine profile differs between the two conditions: the production of TH1-type cytokines, such as IL-2 and interferon-γ, is increased in psoriatic skin but not in the synovium. Thirdly, T cell receptor oligoclonal patterns from paired skin and synovial samples are not the same, suggesting different roles for the T cells in the two compartments. Finally, T cells positive for the skin-homing receptor cutaneous lymphocyte antigen (CLA) accumulate in the skin but not in paired synovium (89).

Some of the T cells that are detected in the synovium and serum of patients with inflammatory arthritis possess receptors that are also found on NK cells. A cytometric analysis of T cell receptor subsets in RA found that the expanded population of T cells were CD4+CD28- and possessed CD161, one of the receptors usually found on NK cells (90). A further study has demonstrated the presence of NKG2D receptors on this expanded CD4+CD28- T cell population in RA (91). In addition to the presence of NK cell receptors on T cells, NK cells themselves are expanded within inflamed joints,

particularly those bearing CD56 (92). A further study has confirmed an increased number of CD56 positive NK cells in RA synovium (93). In this study, the absolute number of NK cells was not increased but the phenotype of cells differed between RA and healthy synovium, with an increase in CD56 and CD94 and a reduction of CD158. The functional significance of this phenotype has yet to be confirmed. The role of NK cells in PsA has been less explored. There is some evidence that NK cells and NKT cells are increased in the synovial fluid of patients with PsA (94). Levels of the activatory NK cell receptor, CD69, are upregulated on NK and NKT cells in synovial fluid from patients with PsA. In addition, the levels of CD57, a receptor associated with apoptosis, are reduced. This suggests that those NK cells that are present in the PsA synovium are not only activated but also protected from apoptosis and therefore able to persist within the joint.

1.9 Immunogenetic studies of PsA

1.9.1 Patient selection

Psoriatic arthritis is a heterogeneous condition where patients not only exhibit a wide variety of different phenotypes but also have the capacity to move between subsets (95). Therefore accurate clinical assessment and documentation is imperative in any study which attempts to examine the genetic and immunological background of such patients. The patients with PsA who participated in the research outlined in this thesis attended a specialist clinic at the RNHRD in Bath, where clinical, laboratory and radiological data were collected prospectively on an annual basis over many years. Some patients have now been followed up for over 15 years. The carefully documented clinical details should ensure a high level of quality for subsequent genetic or immunological studies.

Many genetic studies of PsA do not contain an adequate control group of patients with psoriasis but no arthritis. Thus it is impossible to demonstrate which associations are truly with PsA and which are accounted for by the psoriasis alone. Equally, almost all studies of psoriasis do not control for the presence of a complicating inflammatory arthritis. In the search for specific genetic or immunological features of PsA, it is therefore necessary to examine a group of patients with a similar severity and duration of psoriasis but have not

developed an arthritis. We have therefore recruited patients from a Dermatology clinic and a General Practice and screened them for the presence of arthritis with a questionnaire and full clinical examination. Due to the high prevalence of asymptomatic sacroiliitis in patients with psoriasis (96), all patients also underwent sacroiliac joint radiographs and any patients with sacroiliitis were excluded from the control group. The availability of such a control group not only allows accurate distinction from patients with PsA, but also provides a unique group in which to study the genetic basis of psoriasis.

1.9.2 The MICA gene

A major part of this thesis has been devoted to the study of the MHC Class I Chain related gene, MICA. This fascinating gene is a strong candidate gene for PsA by virtue of its position within the MHC, its high level of polymorphism and its putative function. It is expressed on a wide variety of epithelial cells and activates T and NK cells via the NKG2D receptor. Expression of NKG2D can be induced by TNFα and IL-15, two cytokines found in abundance in the inflamed synovium and serum in both RA and PsA. The synoviocytes in RA express MICA, which, via an interaction with the NKG2D receptors, were capable of stimulating the T cell cytokine and proliferative responses. Peripheral blood serum samples from RA patients were found to contain substantial amounts of synoviocyte-derived soluble MICA. This soluble MICA was unable to down-modulate the NKG2D receptors due to the opposing activity of TNFα and IL-15.

Therefore, a dysregulation of the MICA / NKG2D interaction may be a factor in auto-reactive T cell stimulation and may play a role in the self-perpetuating pathology of inflammatory arthritis. Equivalent experiments in PsA have yet to be done but would be likely to exhibit very similar mechanisms. The role of polymorphisms within the transmembrane and extracellular segments of MICA in PsA is explored in Chapters 3 and 4, respectively. An investigation into the expression of MICA in the skin in psoriasis and the synovium in PsA has been undertaken in Chapter 5.

1.9.3 HLA associations of PsA

The sixth chapter has been dedicated to the study of associations between the HLA antigens themselves and PsA. Particular emphasis has been given to the role of HLA-Cw6 and HLA-B27 as well as the HLA Class II HLA-DRB1 antigens. The results were analysed for any clinical correlations, in particular any associations with disease phenotype and subset. An assessment was also made as to whether there were any associations with markers of disease *severity* such as the presence of erosive disease on radiographs. In addition, the role of a subset of HLA-DRB1 alleles bearing the 'shared epitope' (SE) was explored. This group of alleles, predominantly HLA-DRB1*0101, *0401 and *0404 have been strongly associated with RA (97). A study was therefore undertaken to investigate whether the 'shared epitope' alleles are associated with any particular subset of PsA, perhaps those resembling RA or with a more severe disease presentation.

1.9.4 Anti-CCP antibodies

Antibodies to cyclic citrullinated peptides (anti-CCP) have been shown to be highly specific for RA (98). The test is now being used in laboratories to help establish a diagnosis of RA in preference to RF due to its high specificity and sensitivity. The presence of anti-CCP antibodies in RA is associated with more severe erosive disease. In addition, they are a useful diagnostic test in the early arthritis setting as they can be present up to several years in advance of the onset of clinical symptoms.

The link between anti-CCP antibodies and the SE is intriguing and may provide some more insight into the precise nature of the autoantigens involved in the antibody response. It has been demonstrated that the SE alleles such as HLA-DR*0401 present peptides containing citrulline but not arginine (99). Therefore citrullination of proteins, a process which often results from hypoxia or other tissue damage, appears to be a crucial step in the generation of these antibodies and the initiation of the inflammatory response.

The prevalence and prognostic significance of anti-CCP antibodies in PsA has never been adequately investigated. The final chapter has therefore been dedicated to the assessment

of the role of anti-CCP antibodies in PsA. Particular emphasis was placed on the associated clinical features, such as disease subset or markers of disease severity. In addition, the association between the presence of anti-CCP antibodies and the presence of the SE was examined.

1.10 Aims

The aims of this thesis are:

- To obtain a well characterised population of patients with
  - PsA
  - Type I Psoriasis screened for the presence of arthritis

- To explore the role of the highly polymorphic gene MICA in PsA and Psoriasis
  - the role of the microsatellite polymorphism in the transmembrane region
  - the role of the extracellular region polymorphism
  - the expression patterns of MICA in the synovium and skin

- To examine the role of the HLA Class I and II antigens in PsA with regards to
  - Disease subset and classification
  - Disease severity and progression

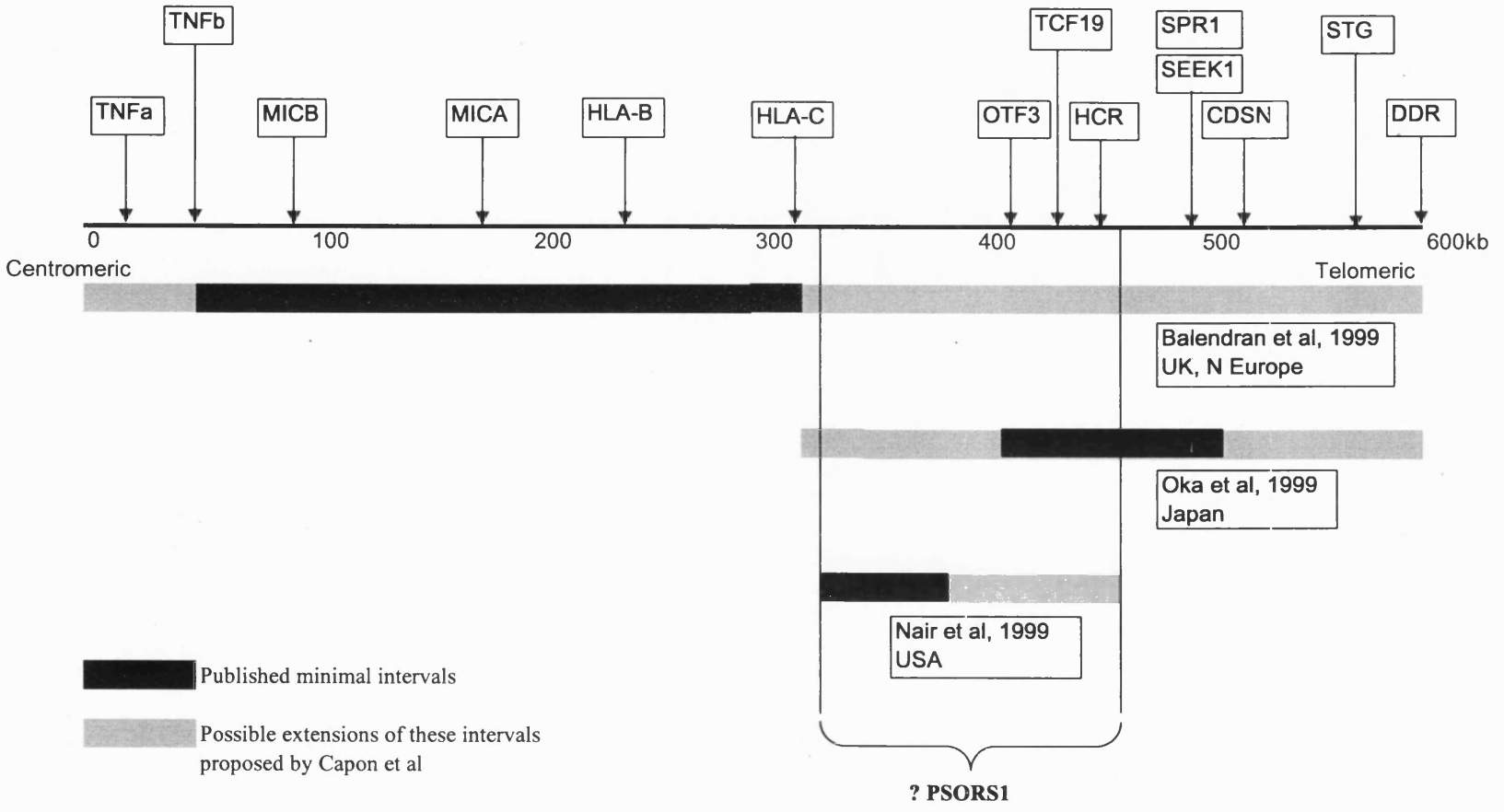
- To investigate the prevalence and associations of the 'shared epitope' in PsA

- To examine the prevalence of anti-CCP antibodies in PsA

Table 1.1. **Published Psoriasis susceptibility loci**

Locus Name	Chromosomal location	Sources
PSORS1	6p21.3	Trembath et al (100) Nair et al (39; 74) Oka et al (40) Balendran et al (41) Veal et al (101) Enlund et al (102) Samuelsson et al (103) Capon et al (104) Jenisch et al (35) Burden et al (34)
PSORS2	17q25	Nair et al (74) Samuelsson et al (103) Tomfohrde et al (105) Enlund et al (102)
PSORS3	4q	Matthews et al (106)
PSORS4	1q21	Bhalerao and Bowcock (107) Capon et al (108)
PSORS5	3q	Samuelsson et al (103) Enlund et al (109)
PSORS6	19p13-q13	Lee et al (110) Veal et al (101)
PSORS7	1p35-p34	Veal et al (101)
unnamed	2p	Veal et al (101) Bhalereo and Bowcock (107)
unnamed	2q	Trembath et al (100)
unnamed	4q13	Bhalerao and Bowcock (107) Samuelsson et al (103)
unnamed	7	Veal et al (101)
unnamed	8q	Trembath et al (100)
unnamed	14q	Bhalerao et al (107) Veal et al (101)
unnamed	15	Samuelsson et al (103)
unnamed	16q	Nair et al (74)
unnamed	20p	Nair et al (74) Trembath et al (100)

Figure 1.1 **Possible location of the PSORS1 locus. Adapted from Capon et al, J Invest Derm 2002, 118:745-751**



CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical Reagents

Name of reagent	Supplier
Agarose	Seakem, Scot Scientific, USA
Alconox	Sigma, USA
Ammonium Persulfate	Amresco, USA
Boric acid	Sigma, USA
Bromophenol blue	Sigma, USA
Copper Sulphate	Sigma, USA
EDTA	Sigma, USA
Ethidium Bromide	Sigma, USA
Hydrogen Peroxide	Sigma, USA
Sodium Chloride	Sigma, USA
Sodium Citrate	Sigma, USA
Sodium Hydroxide	Sigma, USA
Sucrose	Boehringer Mannheim, USA
Tris base	Sigma, USA
Trizma Base	Sigma, USA

2.1.2 Enzymes

Taq DNA Polymerase 250units (5.5U/μl)	Fisher Biotec, Australia

2.1.3 Primers

All primers were supplied by Geneworks, Australia or ABgene, UK. Each primer was supplied as a dry powder containing a given amount in nmol. This was diluted with double distilled water (ddH_2O) to give a stock concentration of 250pmol/μl. A working dilution of 25pmol/μl was made by diluting the stock 1:10 with ddH_2O. The primers used for the MICA typing are listed in *Table 2.1*. The primers used for HLA-B27 and HLA-Cw6 typing are listed in *Table 2.2*. The primers used for HLA-DRB1 typing are listed in *Table 2.3*.

2.1.4 Antibodies

The antibodies used for the immunofluorescence and immunohistochemistry studies are listed in *Table 2.4.*

2.1.5 Buffers and Solutions

1% Agarose solution with ethidium bromide

4g	Agarose (SeaKem, Scot Scientific)
400mls	0.5 x TBE
20µl	Ethidium bromide stock solution (10mg/ml)

Add the agarose to the 0.5 x TBE in a sterile 500ml glass bottle. Microwave on high without a lid for 2-3 minutes until all the agarose is dissolved and large bubbles form when the gel is gently agitated. When cooled slightly, add the ethidium bromide and agitate until dispersed. Use immediately or, if allowed to cool and set, reheat in the microwave as above.

2.5% Agarose solution with ethidium bromide

10g	Agarose
400mls	0.5 x TBE
20µl	Ethidium bromide stock solution (10mg/ml)

The gel is prepared as detailed for the 1% agarose gel.

1% Alconox solution (for cleaning Gene Scanner 2000 glass plates)

5g	Alconox
500mls	ddH_2O

10% Ammonium Persulfate solution (APS)

50 mg	Ammonium Persulfate (pro-pure proteomics grade)
500µl	ddH_2O

Store the APS in a desiccater in aliquots to avoid excess exposure to moisture.

Antibody diluent solution DAKO

Supplied ready for use

Bind Silane solution Lois and Keith, USA

Supplied ready for use

Chloroform Sigma, USA

Supplied ready for use

Copper sulphate solution

4g	CuSO4
6.4g	NaCl
800mls	ddH_2O

10 x DNA Polymerase Reaction Buffer Fisher Biotec, Australia

Supplied ready for use

Dideoxynucleotide triphosphates (dNTPs) Fisher Biotec, Australia

Supplied as a highly purified nuclease free solution of each dNTP (dATP, dCTP, dGTP, dTTP). Each dNTP was supplied at a concentration of 100mM, pH 7.5 and diluted with ddH_2O to a working concentration of 10mM.

100% Ethanol solution Fisher Biotec, Australia

Supplied ready for use

Haematoxylin solution Fisher Biotec, Australia

Supplied ready for use

3% Hydrogen Peroxide Sigma, USA

3g	Hydrogen Peroxide
100mls	ddH_2O

6 x Loading buffer for agarose gels

40 %	Sucrose	Boehringer Mannheim,USA
0.25%	Bromophenol blue	Sigma

Use neat to load samples into the agarose gel wells at a ratio of 2µl loading buffer: 5µl PCR product.

4 x Loading buffer for Gene Scanner

6.5mls	Ficoll Type 400 (300mg/ml)	Pharmacia, Sweden
3.5mls	Blue Dextran (100mg/ml)	Sigma, USA
3.5mls	Dextran Sulphate (20mg/ml)	Sigma, USA
4mls	20 x TBE	
2.5mls	ddH_2O	

Filter the stock using 5µm pore sized filters, followed by 0.45µm filters. Aliquot and store at -20°C.

Loading Buffer for Gene Scanner

300µl	4x Loading Buffer
125µl	6x TBE
150µl	59 bp internal marker
25µl	555bp internal marker

Mix 1 µl loading buffer: 1µl PCR sample prior to loading samples into the wells.

Long Ranger solution (polyacrylamide gel) AT Biochem, USA

Supplied and used as a 50% concentrate solution. Store at 4°C protected from light.

Magnesium Chloride Solution 25mM Fisher Biotec, Australia

Supplied ready for use

Mid 100bp marker (for agarose gels) Fisher Biotec, Australia

Molecular weight marker with 30 bands every 100bp (100-3000bp) and brighter bands at 1000bp and 3000bp. Supplied as 150µl of 200µg/ml solution. Dilute as follows:

150µl	200µg/ml marker solution

375µl 6 x loading buffer

225µl ddH_2O

Use 3µl as a size marker for PCR products on agarose gel electrophoresis. Store at -20°C.

Molecular Weight Marker (for Gene Scanner 2000) Bresatec, Australia

pUC-19 DNA supplied ready digested with the restriction enzyme HpaII (band sizes 26, 34, 68, 117, 120, 147, 190, 242, 331, 404, 489, 501 base pairs). The internal 59 and 555 base pair markers are made in house at CMII, Perth, Australia

75µl pUC-19

150 µl 0.6 x TBE

150µl 4 x LB

50µl 555bp marker

150µl 59bp marker

Load 1 µl into a well of the polyacrylamide gel as a size marker for the PCR products.

Nuclei Lysis Buffer

10mM Tris HCl (pH8.2)

0.4M NaCl

2mM Na_2EDTA (pH8)

Phenol

Supplied ready for use

Polyacrylamide gel (for Gene Scanner 2000)

For 8% long gel (total volume 40mls)

6.4ml 50% Long Ranger

2.4ml 10x TBE

31.2ml ddH_2O

200µl Ammonium Persulfate Solution (APS)

20µl TEMED

Gently mix the Long Ranger, TBE and ddH_2O in a 50ml syringe. Degas the syringe by creating a vacuum in the syringe and tapping the syringe on the workbench several times. Release the gas from the syringe. Quickly add the APS and TEMED, mixing gently, taking care not to introduce any air bubbles. Pour the gel mixture as per instructions.

Proteinase K

10mg/ml in buffer

2mM	Na_2EDTA (pH8)
1%	w/v SDS

Red Cell Lysis Buffer

0.144M	NH_4Cl
10mM	$NaHCO_3$

20% SDS

Supplied ready for use

3M Sodium Acetate

Supplied ready for use

5M Sodium Chloride

14.62g	NaCl
up to 50mls	ddH_2O

Gradually add NaCl to ddH_2O, mixing thoroughly until all NaCl is dissolved.

Store at room temperature.

1% Sodium Hydroxide (for glass plate cleaning)

5g	NaOH
500mls	ddH_2O

20 x TBE

216g	Trizma Base	Sigma, USA
110g	Boric Acid	Sigma, USA
80 ml	0.5M Disodium EDTA pH8	Sigma, USA

Adjust pH to 8.3. Add sterile ddH_2O to 1000mls. Aliquot, autoclave and store at room temperature in the dark. Dilute appropriately to provide concentration of TBE required with ddH_2O.

10 x TBE

500mls	20 x TBE
500mls	ddH_2O

0.6 x TBE

60mls	10 x TBE
940mls	ddH_2O

0.6 x TBE with Ethidium Bromide

1000mls	0.6 x TBE
3µl	Ethidium bromide (10mg/ml stock solution)

0.5 x TBE

50mls	10 x TBE
950mls	ddH_2O

TBS Buffer

121g	Tris base
170g	NaCl
70mls	Concentrated HCl
4mls	Tween 20

Make up to 20 litres with ddH_2O. Adjust pH to 7.6.

TEMED solution (N,N.N',N'-Tetramethylethylenediamine) Amresco, Ohio

Supplied ready for use. Store at 4°C.

10 x TRIS/EDTA buffer solution (for antigen retrieval / immunohistochemistry)

2.5g	Tris base
5g	EDTA
3.2g	Sodium Citrate
1000mls	ddH_2O

Adjust pH to 8. Dilute 1:10 with ddH_2O prior to use.

Xylene solution (for rinsing slides)

Supplied ready to use

2.1.6 Equipment

Spectrophotometer	Beckman DU 640, Australia
Palm-Cycler Version 2.0 Thermocycler	Fisher Biotec, Australia
PTC-200 DNA Engine	MJ Research, UK
Gene Scanner GS 2000	Corbett Research, Australia
Agarose electrophoresis tank with 'Powerpac 300'	Biorad, Australia
Wellwash 4 Mark 2 plate washer	Labsystems, Sweden
Multiskan Ascent plate reader	Labsystems, Sweden

2.1.7 Commercial kits

LSAB+ peroxidase kit	DAKO
Anti-CCP ELISA kit	Axis-Shield, UK
RF ELISA kit	Sigma, USA

2.2 Patient recruitment and clinical data

2.2.1 Ethics approval

Approval was sought from the Local Research Ethics Council (LREC) in Bath prior to the initiation of this study. Full approval was given for the collection of the clinical and radiological data detailed herein. Approval was also granted for the collection of blood samples for the extraction and storage of serum and DNA for use in the immunogenetic analysis described in this thesis. Approval was provided for the DNA samples to be transported to Perth in Australia for the continuation of this work.

2.2.2 Recruitment of subjects

2.2.2.1 Controls

DNA samples from random blood donors were provided by the Tissue Typing centre, Southmead Hospital, Bristol. Although screened for the presence of many conditions, psoriasis was not actively screened for and therefore the presence of background psoriasis in the controls cannot be excluded. The controls were matched for sex (70 male: 78 female) but the age of the donors was not known.

2.2.2.2 Patients with Psoriatic Arthritis (PsA)

Patients with PsA were recruited from a specialist clinic at the RNHRD in Bath. Patients from this clinic have their clinical and radiological details recorded prospectively by a rheumatologist or allied health professional at least once a year. These details are then stored on an Access (Microsoft) database together with background demographic information and details of previous genetic and epidemiological studies. Some patients have been followed for over 15 years.

Patients were invited to participate in this research programme whilst attending the PsA clinic for routine follow-up. All patients were given an information sheet (Appendix 1) to read prior to the consultation. If they agreed to participate they were asked to sign an

informed consent (Appendix 2). The original consultation was either extended or they were examined separately after the routine clinic appointment. Clinical and demographic information was recorded on a defined proforma (Appendix 3) and background information was documented if the patient was new to the clinic (Appendix 4 and 5). The data was then inputted into the PsA Access database. The clinical information that was collected included a psoriasis area severity score (PASI), nail score, modified Ritchie tender and swollen joint count (111), Health Assessment Questionnaire (HAQ) (112) and a visual analogue scale (VAS) for pain (Appendix 6).

2.2.2.3 Patients with Psoriasis

Ethical approval was given to recruit subjects with psoriasis who did not have a complicating inflammatory arthritis. These subjects were recruited from a specialist Dermatology clinic at the Royal United Hospital (RUH), Bath and from the Number 18 Surgery General Practice in Oldfield Park, Bath. The differing methods of recruitment were intended to capture a broad spectrum of severity of psoriasis. This would enable the patients with PsA who had varying severities of psoriasis to be well matched with the psoriasis-only population for subsequent immunogenetic studies.

Patients with psoriasis attending the department of dermatology of the RUH, Bath were targeted by attendance of speciality psoriasis clinics by a member of the research team. Patients were offered an information sheet inviting them to take part in the study (Appendix 7). If they agreed to participate they were interviewed and examined in the dermatology department. Patients with psoriasis in General Practice were targeted by searching the practice database for all patients with a known diagnosis of psoriasis. A letter of invitation (Appendix 8) was then sent to these patients together with the information sheet and a stamped addressed envelope. Any patients who replied were contacted and invited to attend the RNHRD, Bath for a consultation.

Once a patient decided to participate they were asked to sign an informed consent form (Appendix 9). They were then asked to complete a short screening questionnaire (Appendix 10) intended to gather some demographic information and to screen for the

presence of an inflammatory arthritis. The questionnaire was designed by a group of rheumatologists working at the RNHRD in Bath in consultation with Prof D Symmonds from the ARC Epidemiology Institute in Manchester. The patients were then examined by a member of the research team (rheumatologist or allied health professional). The three elements of the examination included a tender, swollen and damaged joint score, a PASI score and a nail involvement and severity score (Appendix 3). After informed consent a blood sample was taken for serum and DNA extraction. If consent was given for radiographs to be taken and there were no contraindications (eg pregnancy, age<16), all subjects attended for radiographs of the sacroiliac joints regardless of the findings of the examination and questionnaire. If the assessing health professional felt there was clinical evidence to suggest an underlying arthritis, further radiographs (eg. hands and feet) were requested with the patient's permission.

13th International Histocompatibility Workshop

The Centre for Molecular Immunology and Instrumentation (CMII) in Perth was chosen to coordinate the psoriasis component of the 13th International Histocompatibility Workshop (13IHW) held in Seattle, USA in May 2002. This workshop meets every four years and acts as a forum for international researchers to meet and discuss the current thinking on the immunobiology of the MHC. A large and active component of the workshop is that interested in MHC and disease. Participating laboratories collaborate to investigate areas of interest in different ethnic populations.

The psoriasis workshop involved collaborations between clinicians and laboratories in the UK, Belgium, Germany, Italy and Australia. Samples of DNA from patients with psoriasis were sent to CMII, Perth for investigation of polymorphisms of MICA. The details of the participating laboratories and the number of samples received is given in *Table 2.5.*

2.2.2.4 Reference samples

A well characterised reference panel of DNA extracted from cells which formed part of the 4th Asia-Oceania Histocompatibility Workshop (4AOH) and the 10th International Histocompatibility Workshop (10IHW) was available for analysis. These cell panels had

been sequenced along HLA Class I and II as well as several genes in the Class III region including MICA. Cells containing examples of the different alleles of MICA could therefore be selected for use as reference alleles for the development and optimisation of MICA typing techniques. Two examples of each MICA transmembrane TM allele were used for reference whilst developing and performing the TM typing. The cell panel did not contain examples of all of the described alleles of MICA based on the extracellular sequence. Therefore, during the development of this technique, some alleles were tested without the benefit of a positive control.

2.2.3 The Psoriasis Area Severity Index

The PASI is a measure of the extent and severity of psoriasis (Appendix 3) (11). It is commonly used for research purposes as well as monitoring the response of the skin to treatments. It examines the degree of erythema, infiltration and desquamation of the skin in 4 main areas (head, arms, trunk and legs). The PASI is then calculated as the total extent multiplied by the total severity of the skin disease (maximum score = 72).

2.2.4 Psoriasis Nail Score

Involvement of the nails by psoriasis is relatively common. A score which records the presence or absence of nail disease in each of the fingernails has been developed (Appendix 3). The feet are also commonly involved but not included in the score as fungal infections of the toenails can be virtually indistinguishable. The presence of three key features is recorded: pitting (small pits on the surface of the nail), onycholysis (lifting of the thickened nail from the nail bed) and hyperkeratosis (thickened, often yellow nails). The presence of severe nail deformity is recorded separately. The presence of all four of these features in ten fingernails gives a maximum nail score of 40.

2.2.5 Modified Ritchie tender and swollen joint counts

The widely used Ritchie joint scoring system (111) was modified for use in PsA by inclusion of the distal interphalangeal joints. A total of 71 tender joints and 62 swollen

joints were recorded prospectively every year on all patients attending the PsA clinic (Appendix 3). Where serial measurements were available, a median PASI score was calculated and used for the purposes of the genetic analysis. A measure of the destruction caused by the arthritis was also obtained by recording which of the joints showed signs of damage (eg. fixed flexion deformities, valgus deformities). If examination of the spine suggested the presence of a spondyloarthritis, the patients were referred to the physiotherapy department for formal metrology. Following joint examination, the patient was assigned to a subgroup of PsA as originally defined by Moll and Wright (2), ie. DIP disease only, oligoarthritis (<4 involved joints), polyarthritis ($\geq$ 4 involved joints), spondyloarthritis (predominant spinal involvement with sacroiliitis +/- syndesmophyte formation), monoarthritis or arthritis mutilans. These details were all recorded on the database. For the purposes of the analysis, the most recently allocated clinical subgroup from the latest clinical assessment was used.

2.2.6 Health Assessment Questionnaire

The HAQ is a functional index designed to measure any difficulties with activities of daily living experienced by a patient (112). The questionnaire was completed by the patients on an annual basis and the information recorded on the database. A visual analogue scale (VAS) asking the patient to score the amount of pain (on a scale of 0-10) related to their arthritis that they have suffered in the last week was also recorded (Appendix 6).

2.2.7 Radiology

Plain anteroposterior radiographs of the hands, feet and sacroiliac joints were taken on all patients at baseline. Radiographs of the hands and feet were updated every two years. The radiographs were reported by a radiologist. Some of the radiographs from patients who had participated in previous research studies had been scored using a modified Sharp score (113). The majority of the patients were simply recorded on the database as "erosive" or "non-erosive" depending on the results of the most recent radiographs. The presence of sacroiliitis or other radiological features of spondyloarthritis were also recorded.

2.2.8 Laboratory tests

Routine blood tests were taken following the clinic appointment as judged necessary by the clinician. At baseline all patients were screened with a rheumatoid factor (RF). Inflammatory markers (C Reactive Protein and plasma viscosity), full blood count, renal and liver function tests were checked at least at every annual appointment, if not more frequently. Following informed consent, blood was taken for serum and DNA extraction.

2.3 Serum and DNA collection

2.3.1 Collection of whole blood

Following informed consent, 20mls of peripheral blood was collected from the patient into a 10ml plain tube and a 10ml EDTA tube. It was then taken at room temperature to the laboratory for extraction of the serum and DNA.

2.3.2 Extraction of serum

The serum was separated from whole blood following centrifugation at 1200 r.p.m. for 10 minutes. The serum was then removed with a pipette into a clean tube and stored at -20°C until required.

2.3.3 Extraction of DNA

1. Centrifuge the 10ml tubes of whole blood at 1200 r.p.m. for 10 minutes
2. Carefully pipette the "buffy coat" (containing the white blood cells) into a clean tube
3. Add 8ml red cell lysis buffer to the buffy coat, mix gently and leave for 20 minutes
4. Centrifuge at 1200 r.p.m. for 10 minutes
5. Pour off the supernatant (red cell lysate) and resuspend the pellet in 3ml nuclei lysis buffer, 100µl proteinase K and 100µl 20% SDS
6. Incubate in a water bath at 55°C for 3 hours
7. Add 1ml of 5M NaCl and shake the tube vigorously for 20 seconds
8. Centrifuge at 1200 r.p.m. for 10 minutes
9. Collect the supernatant into a universal vial, taking care not to disturb the pellet which contains the precipitated proteins
10. Add 8ml of 100% ethanol and mix by gently inverting the tube several times

11. Remove the precipitated DNA by winding onto the end of a sealed Pasteur pipette, squeezing the excess alcohol out onto the side of the tube
12. Transfer the DNA to a 0.5ml Eppendorf tube and dissolve the DNA in 100µl ddH_2O
13. Quantify the amount of DNA using a spectrophotometer and store at -20°C

2.3.4 Labelling and storage of the DNA samples

All DNA samples were labelled with a unique identifier and anonymised for storage and transport in accordance with the requirements of the Ethics committee. The patient details relating to the DNA samples were recorded on a password protected Microsoft Access database. An aliquot of each sample was transported to Australia for genetic analysis using a recognised freight company. All samples were passed through the Australian Quarantine and Inspection Service (AQIS) on Permit number 200118650. The samples were then quantified and a working dilution of 20ng/µl was made. All samples were then stored at -20°C.

2.3.5 Quantification of DNA by spectrophotometry

Nucleotides in solution absorb light in the ultraviolet (UV) region of the spectrum, with a maximum absorption at a wavelength of 260nm. Proteins also absorb UV light but maximally at 280nm wavelength. Therefore, DNA samples can be quantified and the purity of the preparation can be estimated by measuring the absorption of UV light at 260 and 280nm in a spectrophotometer.

The stock solution of DNA was thoroughly mixed on a vortex to ensure the DNA was resuspended. The sample to be tested was then diluted 1:50 by diluting 2µl of the DNA sample in 98µl ddH_2O and mixing thoroughly. The Beckman Spectrophotometer was switched on at least 5 minutes before use to allow the UV lamp to warm up. The machine was set to read the samples at a 1:50 dilution. The machine was blanked by placing 100µl of the ddH_2O that was used to dilute the DNA samples into the cuvette. One hundred microlitres of the 1:50 diluted DNA samples was then placed in turn into the cuvette and a reading taken. The cuvette was washed out between samples with ddH_2O. The readings

were obtained for the absorbency of UV light at 260nm and 280nm wavelengths. The software attached to the spectrophotometer then calculated the concentration of DNA in ng/µl and the concentration of protein in ng/µl. The equation utilised to calculate the concentration is :

DNA concentration (ng/µl) = Absorbance at 260nm x 50 x dilution factor (50)
Protein concentration (ng/µl) = Absorbance at 280nm x 50 x dilution factor (50)

The ratio of absorbancy at 260nm/280nm gave an indication of the purity of the sample. A ratio of 1.8 indicated pure samples. Any samples with a ratio below 1.7 were purified according to the method given below.

2.3.6 Purification of DNA

1. Add an equal volume of phenol to the DNA suspension
2. Shake in a closed tube to form an emulsion of the denatured protein
3. Centrifuge at 1300 r.p.m. for 15 seconds
4. Transfer the 'upper' phase into a fresh Eppendorf
5. Add an equal volume of 1:1 mixture of phenol and chloroform and mix, centrifuge and separate as before, retaining the 'upper' phase
6. Add a 1/10 volume of 3M sodium acetate, followed by 2 volumes of 100% ethanol
7. Mix thoroughly and centrifuge as before
8. Take off as much fluid as possible taking care not to disturb the DNA pellet
9. Wash the pellet twice more with ethanol and leave to airdry
10. Resuspend in 100µl ddH_2O and re-quantify by spectrophotometry

2.4 Genetic analysis

2.4.1 MICA exon 5 microsatellite typing

2.4.1.1 PCR Conditions

All PCR reactions were performed in a total volume of 20µl. A master mix of all reagents except the DNA and the Taq DNA polymerase was made by multiplying the volumes below by the number of reactions to be performed. Each 20ng/µl DNA dilution was mixed thoroughly and 5 µl added directly to one of the wells of the PCR strip. Each PCR run contained the test DNA, a number of positive DNA reference samples and a control well (ddH_2O instead of DNA) to check for contamination. The PCR reactions were set up on ice in sterile conditions in a laminar flow hood. The Taq DNA polymerase was added to the master mix at the last moment and mixed thoroughly. Fifteen µl of the master mix was then added to the DNA in each well and gently mixed with the pipette. The wells were sealed with a tight-fitting lid and placed into the heating plate of the thermocycler. The reactions were carried out in a Palm-cycler Version 2.0 (Fisher Biotec, WA). Once the cycles were completed the samples were stored at 4°C until needed.

The following concentrations of reagents were found to yield optimal results in terms of the intensity, clarity and specificity of the PCR product by experimentation with differing concentrations of the individual reagents.

Reagent	Volume added	Concentration
10 x PCR Buffer	2µl	1 x PCR Buffer
10mM dNTP mix	1µl	500µM
25mM $MgCl_2$	1.2µl	1.5mM
Taq DNA polymerase	0.2µl	1 Unit
Forward Primer (25pmol/µl)	1µl	1.25µM
Reverse Primer (25pmol/µl)	1µl	1.25µM
DNA (20ng/µl)	5µl	100ng
Double distilled water	8.6µl	

Cycling conditions

The annealing temperature was adjusted until the intensity and specificity of the product bands was maximised. The following conditions were found to be optimal:

PERTMF + PERTMR

Cycle	Step	Temp (°C)	Time	Number of cycles
1	1	96	60 sec	1
2	1	96	25 sec	
	2	70	45 sec	5
	3	72	30 sec	
3	1	96	25 sec	
	2	65	45 sec	21
	3	72	30 sec	
4	1	96	25 sec	
	2	55	60 sec	4
	3	72	120 sec	
5	1	72	10 mins	1

PERTM100F + PERTM100R

Cycle	Step	Temp(°C)	Time	Number of cycles
1	1	95	1 min	
	2	66	1 min	30
	3	75	1 min	

2.4.1.2 Preparation of the gel

The polyacrylamide gel was prepared in a 50ml syringe as described in Chapter 2.1.5. The lower glass plate was placed horizontally in the plate holder. A strip of 0.1μm spacer tape was placed on the long edges of the lower glass plate to allow the formation of a 0.1μm thick gel between the glass plates. The upper, inner edge of the top glass plate was wiped with bind silane solution to aid in the formation of the wells. The top plate was placed on top of the lower plate, with the front of the top plate elevated so that the gel can be

squeezed gently from the syringe between the plates. The top plate was then gently lowered onto the bottom plate, forming the gel, ensuring no air bubbles appeared in the gel. A 48 well comb was placed between the upper edges of the plates to form the wells. The plate holder was then screwed down to ensure equal pressure between the plates to ensure the gel set uniformly. The gel was allowed to set for 1 hour at room temperature before removal of the 48 well comb. The outer surfaces of the glass plates were checked to ensure no gel had spilled onto the outside. Any excess gel was wiped off with 100% ethanol and lint free paper. The plates were now ready to be placed vertically into the Gene Scanner 2000.

Placing the gel into the Gene Scanner 2000

The lower chamber was placed in situ and the electrical connections were established. The plates were placed vertically into the lower chamber with the lower glass plate at the back, flush with the vertical surface of the Gene Scanner. The lower chamber was then filled to the designated line with 0.6x TBE + 3µl/litre Ethidium Bromide buffer. The upper chamber was then attached over the plates onto the Gene Scanner and the electrical connections were established. The upper chamber is then filled with 0.6x TBE (*no* Ethidium Bromide). The wells were washed out with 0.6x TBE using a fine gauge needle and syringe to ensure no air bubbles remain in the wells. The door is then closed over the chambers containing the gel and the power is switched on. The buffers were then pre-run through the gel by selecting "pre-run" at 1000V and 25°C for 30 minutes. The wells were then washed out as before.

Loading the gel

The samples were then loaded into the wells by mixing 1µl of PCR product with 1µl of Gene Scanner Loading Buffer on a piece of parafilm. A maximum of five samples were loaded at a time to ensure the samples did not evaporate. 1µl of this mixture was then loaded into each well following a designated proforma. 1µl of molecular weight marker for the Gene Scanner was loaded at the beginning, end and between every five samples.

Running the gel

The samples were then "pulsed" into the gel by selecting "pulse" at 1000V for 60 seconds. The excess sample in the wells was washed out as before. The gel was then run at 1000V for 4 hours until the product and marker had run through the gel. The marker and products were scanned by a laser as they ran through the gel, to produce an image which was stored on the computer as a '.flf' file.

2.4.1.3 Analysis of the gel

The resultant '.flf' file was converted by the Genescanner 2000 software programme into a '.tif' file for analysis. The lanes of the gel were centralised through the product bands correcting for any irregularity in the gel. The resultant file was then saved as a '.txt' file and opened using Microsoft Excel. A line graph was then plotted for each sample, with the intensity of the band(s) on the 'y' axis against time on the 'x' axis. Each graph was corrected for 'smile' in the gel due to slight variations in current by aligning the internal markers. The sample graphs were then compared with the graphs obtained for the known MICA alleles from reference DNA samples. Each sample graph was aligned with each reference graph using the Genescanner 2000 software. The MICA TM alleles for each patient sample could therefore be assigned.

2.4.2 Typing exons 2,3 and 4 of MICA

2.4.2.1 Background

The rationale for the PCR-SSP technique employed for the typing of the alleles of MICA defined by the single nucleotide polymorphisms (SNPs) in exons 2-4 is detailed in Chapter 4.

2.4.2.2 Phylogenetic analysis

The nucleotide sequence of the currently described alleles of MICA was downloaded from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/) in FASTA format (114). This was then imported into the Molecular Evolutionary Genetics Analysis software programme, MEGA (115). This programme uses the nucleotide sequence of the alleles of

a gene to generate a 'family tree' by comparing similarities in sequence. An analysis can also be performed on the amino acid sequence to compare similarities between alleles on a protein level. Since the analysis was performed in order to generate primers for subsequent analysis, the nucleotide sequence was used.

The full nucleotide sequence of exons 5 and 6 of MICA has not yet been published for approximately 50% of the MICA alleles. The absence of full data for these alleles could influence the phylogenetic analysis. The analysis was therefore performed solely on the nucleotide sequence of exons 2,3 and 4 with exclusion of the incomplete data. The resultant phylogenetic tree was then utilised to select group-specific primers for the PCR-SSP technique.

2.4.2.3 Selection of Primers

The details of the primers used for the typing of exons 2,3 and 4 of MICA are given in Table 2.1. The majority of the primers were those used in an earlier PCR-SSP technique to define the alleles of MICA when there were only 16 alleles described in the literature (116). There are currently 54 alleles of MICA whose sequences have been (at least partially) sequenced. Therefore, there are now many more alleles of MICA that are recognised by these original primers.

The nucleotide sequences of the 54 alleles of MICA were downloaded from the IMGT/HLA database as before. The sequences were then imported into a Microsoft Excel spreadsheet and aligned. All non-polymorphic nucleotide positions were deleted. The polymorphic positions of all of the currently described 54 alleles were then labelled according to the nucleotide position starting from the beginning of exon 1. The alleles were then arranged in phylogenetic order according to the nucleotide sequence without exons 5-6 (as detailed above).

A column for each forward and each reverse primer was made and each allele that was recognised by that particular primer was marked with a "+". A separate table listing all potential primer pair combinations was formulated. Any combinations that resulted in a PCR product less than 100bp or more than 1500bp were discarded as these would be

difficult to detect on simultaneous agarose gel electrophoresis. Products that would be close to the size of the internal control (480bp) were also discarded. All potential primer combinations were then examined for those that divided the alleles into equal-sized, mutually exclusive groups. Five primer pairs were selected which could separate the 54 alleles into 5 groups that were all mutually exclusive. There was only one rare allele (MICA *044) which was recognised by two of the primer pair groups (Groups 2 and 3). The segregation of the alleles of MICA largely followed the phylogenetic analysis barring a few exceptions (*Figure 4.3*)

The 2nd stage of the PCR-SSP procedure required the definition of alleles known to be present at a frequency of $\geq 1\%$ (117; 118). Although some of the original primers could be used, some novel primers were required to recognise the more common newly described alleles. The novel primers were designed to be compatible with the existing primers in terms of length, GC content and melting temperatures (T_m). They were designed to recognise the required polymorphic position and checked against all known human sequences to ensure there was no cross-reactivity using the programme 'BLAST' (ncbi.nlm.nih.gov/BLAST/).

2.4.2.4 PCR conditions

The PCR reactions were set up in exactly the same way as described for the MICA TM typing. Successful amplification of each reaction was confirmed with the presence of a pair of internal control primers specific for a conserved region of the Human Growth Hormone (HGH) gene. The PCR was found to be optimal with the following reaction mix:

Reagent	**Volume**	**Concentration**
10 x PCR Buffer	2µl	1 x PCR Buffer
10mM dNTP mix	1µl	500µM
25mM $MgCl_2$	1.2µl	1.5mM
Taq DNA polymerase	0.2µl	1 Unit
Forward *Specific* Primer(25pmol/µl)	1µl	1.25µM
Reverse *Specific* Primer (25pmol/µl)	1µl	1.25µM
Forward HGH Control Primer (2.5pmol/µl)	1µl	0.125µM

Reverse HGH Control Primer (2.5pmol/µl)	1µl	0.125µM
DNA (20ng/µl)	5µl	100ng
Double distilled water	6.6µl	

Cycling Parameters

The original PCR-SSP method described for MICA (116) used the conditions detailed below. These conditions worked well for most primer pairs in this updated PCR-SSP method but some PCR reactions were sub-optimal. Adjustments to the annealing temperatures of these primer pairs yielded better results (see below).

Cycle	Step	Temp(°C)	Time	Number of cycles
1	1	95	5 min	1
2	1	95	20 sec	
	2	65*	50 sec	30
	3	72	30 sec	

*The following primer pairs have a different annealing temperature:
F10+R17 (62°C), F14+R2 (66°C), F4+R6 (67°C) and F7+R7 (69°C)

Each sample was tested with five initial (1st stage) primer pairs. The alleles were then further defined using the 2nd stage primer pairs appropriate to each group following the typing algorithm given in *Table 2.6.* and *Figure 2.1.*

2.4.2.5 Agarose Gel Electrophoresis

The PCR products were then identified by agarose gel electrophoresis. The majority of the PCR products were identified using 1% agarose gels but 2.5% agarose gels are used if the product sizes were less than 250 bp.

2.4.2.6 Preparation of the gel

The agarose gel was prepared following the instructions given in Section 2.1.5. The plastic gel mould was placed on a level surface and the comb(s) for the number of wells required was inserted into the mould. The gel was microwaved for 2-3 minutes until fully dissolved and large bubbles formed when the hot gel was gently agitated. The gel was then poured

into the mould until the gel was approximately 7mm thick and left to set for 30 minutes. The comb(s) was then removed and the gel is placed into the electrophoresis tank.

2.4.2.7 Loading the gel

The gel was covered with 0.5x TBE. 5µl of Mid-100 marker was pipetted into the first well and between every 8 samples. 2µl of loading buffer and 5µl of PCR product were mixed on a piece of parafilm and the total volume (7µl) was then loaded into each well until all the samples had been loaded.

2.4.2.8 Running the gel

The positive and negative terminals were then connected to the power pack. The controls were set at 200V and 400mA. The gel was allowed to run for 20 minutes.

2.4.2.9 Interpretation of the gel

The gel was then removed from the tank and the bands visualised on an ultraviolet light box and photographed. The size of the product band was compared with the 100-3000bp molecular weight marker. Successful PCR amplification was verified with a 480bp band resulting from the internal control primers (HGHF + HGHR). The appearance of a specific band of the expected size confirmed the presence of an allele recognised by the primer pair used in the reaction. The expected product sizes are given in *Table 2.7*. The absence of a specific band but presence of a control band confirmed that the sample tested did not possess an allele recognised by that primer pair. The absence of a control and specific band was regarded as a failed PCR reaction and was repeated.

2.4.3 HLA-DRB1 'shared epitope' typing

2.4.3.1 Primers

The primers used for the HLA-DRB1 typing and HLA-DRB1*01 and HLA-DRB1*04 subtyping are given in *Table 2.3*. Initial HLA-DRB1 typing was performed using 10 forward primers and 16 reverse primers to form 20 primer pairs (*Table 2.8*) (119). Each reaction also included a primer pair specific for a conserved sequence in the 3rd intron of HLA-DRB1 (C3+C5) to act as an internal amplification control. Specific amplification of

the individual HLA-DRB1*01 and HLA-DRB1*04 alleles was performed with an additional 4 and 8 primer pairs, respectively (120; 121). The primer mixes used for the HLA-DRB1*01 and HLA-DRB1*04 subtyping are listed in *Tables 2.9 and 2.10*, respectively.

2.4.3.2 PCR conditions

The primer mixes were prepared to give a final concentration of 0.25mM of each specific primer and 0.05mM of each control primer within each reaction. Five µl of the primer mix was added to 5µl of reaction mix in each well of the PCR plate. The reaction mix was prepared depending on the number of primers to be used, eg. for 20 primer pairs (initial HLA-DRB1 screening), 105µl of reaction mix was prepared:

22µl reaction buffer (750mM Tris HCl, pH8.8),200mM $(NH_4)_2SO_4$,0.1% Tween 20)
22µl dNTP (dATP, dGTP, dTTP, dCTP) (125µM)
12µl Magnesium Chloride (25µM)
41µl sucrose/cresol red (60% sucrose, 1mM cresol red, (Sigma))
8 µl DNA (200ng/µl)
1.1µl Taq polymerase (added just prior to use)
(All PCR reagents were supplied by ABgene, UK)

The PCR amplifications were carried out in a M J Research PTC-200 DNA Engine using the following cycling parameters:

Cycling parameters

1. 96°C for 2 minutes
2. 94°C for 25 seconds
   65°C for 50 seconds
   75°C for 30 seconds
   } 10 cycles
3. 96°C for 10 seconds
   61°C for 50 seconds
   72°C for 30 seconds
   } 20 cycles
3. 72°C for 10 minutes

The PCR products were visualised following electrophoresis on a 2% agarose gel as before and the HLA-DRB1 alleles were allocated by reference to the table of primer pair specificities (*Table 2.8*).

2.4.4 HLA Class I typing

All samples (PsA, Type I Psoriasis and controls) were sent to the 13th International Histocompatibilty Workshop coordinating laboratory (J. Hansen, Seattle, USA) for full sequence-based HLA typing at the HLA-A, B, C, DR, DQ and DP loci. This work is still awaiting completion. The PsA samples were therefore typed for HLA-B27 and HLA-Cw6 using PCR-SSP until the full HLA Class I typing was available. The primers (*Table 2.2*), reaction mixes and cycling parameters used were based on the published technique by Bunce et al (122).

2.4.5 Analysis of Genetic data

2.4.5.1 Basic statistics

The allele frequencies were determined by direct counting using the filter facility on Microsoft Excel. The MICA alleles were examined for the presence of Hardy-Weinberg equilibrium by comparing the observed with the expected genotype frequencies. These were then compared using the Chi-squared test with the appropriate degrees of freedom. Allele frequencies were expressed as a fraction (0-1) by dividing the total number present of any given allele by the total number of alleles within the population (n x 2). The results were also expressed as phenotype frequencies (the number of individuals within the population who express at least one copy of the allele).

Differences between patient groups and controls were determined using the Chi-squared test with Fisher's Exact test if the sum of the numbers within a 2 x 2 table were less than 20. All statistical tests were performed using Microsoft Excel and SPSS for Windows (Microsoft). Continuous variables that were not normally distributed (eg. PASI, swollen and tender joint counts) were compared using the Mann Whitney U test. It should be noted that ethnically matched controls were not available for the European patients that were participants in the 13IHW. Comparison with the UK controls is not statistically valid,

however, the values will be reported to illustrate trends. Further work with ethnically matched controls would need to be undertaken.

The *p values* were considered to be significant if less than 0.05. The *p values* were adjusted using the Bonferroni correction for the number of alleles observed. For example, the *p values* comparing patient and control groups for the MICA TM alleles would be multiplied by a factor of 5 for the 5 recognised alleles. Thus, an initial *p value* of 0.01 would be needed for the final result to obtain significance. When a significant result was observed, the odds ratio was calculated together with 95% confidence intervals. The relatively small sample sizes of the populations studies will lead to a lack of power within the study. The results should therefore be interpreted with due caution.

2.4.5.2 Haplotype analysis

Some of the data from the patient samples was available for haplotype analysis. The DNA samples from the patients with PsA had been typed at the HLA Class II loci, HLA-DRB1 and HLA-DQB1. The HLA Class I typing in the PsA group was not sufficient for haplotype analysis as samples had only been examined for the presence or absence of HLA-B27 and HLA-Cw6. The Type I psoriasis patients from the 13IHW had full HLA typing at the HLA Class I and II loci and were therefore available for haplotype analysis.

The MICA data was examined using the population genetics data analysis software package 'Arlequin' (123), available for download at www.lgb.unige.ch/arlequin/. Arlequin is an exploratory population genetics software programme able to handle large samples of molecular data (RFLPs, DNA sequences, microsatellites), while retaining the capacity of analyzing conventional genetic data (standard multi-locus data or mere allele frequency data). It has the ability to compare genetic similarities between sequences and calculate the most likely haplotype sequences and frequencies. The likelihood of the predicted haplotypes is provided by reference to a Chi-squared value. The patient and control data was imported from an Excel database and converted into the correct format using the progamme PantelidisP5mod (personal communication). Haplotype combinations and frequencies were determined by the Arlequin programme. The linkage disequilibrium between individual polymorphic loci was calculated to obtain D values (a measure of

deviation from random association between alleles at different loci) (117). The D' values were then calculated (the D value standardised by the maximum value it can take given the allele frequencies). A D' value of 0 suggests independent association of two alleles, whereas a D' value of 1 suggests strong linkage disequilibrium between loci.

2.5 Expression work

2.5.1 Antibodies

The antibodies used for immunofluorescence and immunohistochemistry are detailed in *Table 2.4.* All antibodies were used at a working dilution of 1:50 except huMICA M673 which was used at a dilution of 1:10.

2.5.2 Immunofluorescence

1. Remove frozen sections of skin from the freezer and allow to thaw
2. Dilute primary antibody with antibody diluent (DAKO) using differing dilutions between 1:10 and 1:100
3. Place several drops of diluted primary antibody onto each section and leave for 15mins
4. Wash with PBS (pH 7.6) for 10 mins
5. Dilute appropriate secondary antibody (eg swine anti-rabbit or rabbit anti-mouse) 1:20 with antibody diluent (DAKO)
6. Add a few drops of diluted secondary antibody and leave for 15 minutes
7. Wash 3 times with PBS
8. Mount the sections with mounting solution and a cover slip
9. Examine slides for presence of fluoresence with dark background microscopy
10. Photograph slides using microscope camera to produce projection slides
11. Scan the projection slides using a slide scanner and download the images onto a CD-ROM.

2.5.3 Immunohistochemistry

1. Label formalin fixed, paraffin-embedded skin sections on glass slides for the antibody to be tested
2. Make dilutions of the primary antibodies with antibody diluent (DAKO) (between 1:20 and 1:100) if optimal dilution is not yet known
3. Place slides in a vertical rack
4. Dewax and rehydrate the slides by placing the rack sequentially in 4 chambers of xylene and 2 chambers of alcohol, leaving the rack in each chamber for 5 minutes
5. Whilst the slide rack is in the 3rd chamber of xylene, fill a buffer chamber with 500mls of TRIS/ EDTA pH 8 and place a lid on top of the chamber. Place 300mls of ddH_2O in the bottom of a pressure cooker. Place the buffer chamber inside the pressure cooker and microwave (without pressure cooker lid) on high for 15 minutes
6. Rinse the slide rack under running water
7. Place the slide rack in the hot buffer solution, put a lid over the buffer chamber and microwave on high in the water-filled pressure cooker (without lid) for a further 10 minutes. This is the antigen retrieval step.
8. Rinse in cold water for 30 minutes until the slides are cool
9. Place the slide rack in 3% hydrogen peroxide for 5 minutes to block endogenous peroxide production
10. Rinse the slide rack with running water
11. Place the slides onto a horizontal rack over a container with some ddH_2O placed in the bottom to lessen evaporation. Add 2 drops of diluted primary antibody solution to each slide. Cover the container and leave for 30 minutes
12. Place the slides back in the vertical rack and rinse with TBS by placing the rack into a TBS-filled chamber and agitating the slides gently for 1 minute. Place the slides back on the horizontal rack.
13. Add 2 drops of secondary antibody solution (biotinylated anti-mouse, rabbit and goat antibodies from LSAB+ peroxidase kit, DAKO). Leave for 30 minutes
14. Rinse with TBS in the vertical rack as before and then replace on the horizontal rack.
15. Add 2 drops of streptavidin conjugated with peroxidase from the LSAB+kit (DAKO). Leave for 30 minutes
16. Rinse with TBS in the vertical rack as before and replace on the horizontal rack.

17. Add 2 drops of the substrate solution, Diaminobenzidine. Leave for 5 minutes for the brown colour to develop
18. Immediately place the slides in the vertical rack and rinse with TBS as before
19. Place the slide rack in copper sulphate solution for 2 minutes, haemotoxylin solution for 8 seconds then rinse thoroughly under running water
20. Place the slide rack in 2 chambers of alcohol and then 4 chambers of xylene, for approximately 1 minute each, gently agitating the slide rack to rinse the slides.
21. Place the slides into a mounting machine for the mounting solution and cover slips to be added
22. Examine slides on a light microscope
23. Photograph the slides using a digital camera attachment and download the images onto a CD-ROM

2.6 Anti-CCP antibodies

2.6.1 Collection of serum

Blood samples were collected from patients following informed consent into plain tubes. Serum was extracted following centrifugation to remove the cellular pellet. The samples were given a unique identifier and stored at -20°C until use.

2.6.2 Anti-CCP ELISA

The presence of anti-CCP antibodies was tested using a commercial ELISA plate (Axis-Shield, UK). The second generation kit (CCP2) was used to provide higher sensitivity and specificity. All samples were diluted according to the manufacturer's instructions and tested in duplicate together with calibrating positive and negative controls. Several samples were tested on consecutive plates to ensure good inter-assay reliability. All plates were washed thoroughly between the stages of the ELISA with a Wellwash 4 Mark 2 plate washer (Labsystems, Sweden). On completion of the ELISA, the plates were read using a Multiskan Ascent plate reader (Labsystems, Sweden). The optical density for each sample was converted by the integral software into IU/ml. A cut-off value of >6IU/ml was chosen to represent a positive result.

2.6.3 Rheumatoid Factor ELISA

A commercial ELISA plate (SIGMA, St Louis, USA) was used to test the serum samples for the presence of IgM RF. All samples were diluted according to the manufacturer's instructions and tested in duplicate together with calibrating positive and negative controls. The plates were read with the Multiskan Ascent plate reader (Labsystems, Sweden) and the optical density of each sample was converted into the corresponding IU/ml by the attached software. A positive value was regarded as >6IU/ml.

Table 2.1. **Primers used for MICA typing**

Primer name	**Sequence (5'-3')**	**Target**	
PERTMF	TCACCTGCTACATGGAACACAGCG	MICA Exon 5	
PERTMR	CTTTTCTCACCTGGACCCTCTGCA	MICA Exon 5	
PERTM100F	ACATTCCATGTTTCTGCTGTTG	MICA Exon 5	
PERTM100R	TCACCTGGACCCTCTGCAG	MICA Exon 5	
Primer name	**Sequence (5'-3')**	**Target**	**Nucleotide Position**
F1	TAACCTCACGGTGCTGTCCT	MICA Exon 2	109T
F2	CCTCACGGTGCTGTCCG	MICA Exon 2	109G
F3	GGAACTACGGCGATATCTAG	MICA Exon 3	586G
F4	TCAGCCCTTCCTGCGCTA	MICA Exon 2	176A
F5	AGGAACTACGGCGATATCTAA	MICA Exon 3	586A
F7	TGTGCAGTCAGGGTTTCTCG	MICA Exon 2	139CG
F8	ACGGCGATATCTAGAATCCG	MICA Exon 3	592G
F10	CAGAGCCCCACAGTCTTCC	MICA Exon 2	86C
F11	TTTCTTGAAGGAAGATGCCG	MICA Exon 3	520G
F14	CCCAGCATTTCTACTACGATA	MICA Exon 3	409A
F18	CTGTGCAGTCAGGGTTTCTCA	MICA Exon 2	139CA
F20	GGCTTGCATTCCCTCCG	MICA Exon 3	341G
F25	GCTCTTCCTCTCCCAAAACG	MICA Exon 3	433G
R1	TGGGGCATTGTCCATTCCTT	MICA Exon 3	442T
R2	CTCAGGACTACGCCGGATTT	MICA Exon 3	586T
R4	GGCCAGCGTCCGTACCTC	MICA Exon 3	611C
R5	AACCTCTGCTCCTCTCCTC	MICA Exon 4	821C
R6	CCTGTTCTCCTCAGGACTAT	MICA Exon 3	595T
R7	CTCTGGAGGACTGGGGCAT	MICA Exon 3	454T
R8	CCTGACGCCAGGTCAGTA	MICA Exon 4	707A
R12	CTGCATGCATAGCGTGATAGA	MICA Exon 3	536A
R13	TCTGGAGGACTGGGGCAC	MICA Exon 3	454C
R16	GTGTCGTGGCTCAAAGATAG	MICA Exon 4	730G
R17	GGCCAGCGTCCGTACCTG	MICA Exon 3	611G
R22	GCTCACCAGAGGGCACAGC	MICA Exon 4	880C
R29	TGAACCTCTGCTCCTCTCCTT	MICA Exon 4	821T
HGH F	CAGTGCCTTCCCAACCATTCCCTTA	HGH Gene	
HGH R	ATCCACTCACGGATTTCTGTTGTGT	HGH Gene	

Table 2.2. **Primers used for HLA B27 and HLA Cw6 typing**

Name	Primer sequence	Target allele
280 (sense)	GCTACGTGGACGACACGCT	HLA-B*2701-9
281(antisense)	CTCGGTCAGTCTGTGCCTT	HLA-B*2701-9
367(sense)	TACTACAACCAGAGCGAGG	HLA-Cw0602
127(antisense)	GGTCGCAGCCATACATCCA	HLA-Cw0602

Table 2.3. **Primers used for HLA-DRB1 typing**

Name	Forward Primer Sequence	Name	Reverse Primer Sequence
5'01	TTGTGGCAGCTTAAGTTTGAA	3'01	CCGCGCCTGCTCCAGGAT
5'02	TCCTGTGGCAGCCTAAGAG	3'02	AGGTGTCCACCGCGGCG
5'03	TACTTCCATAACCAGGAGGAG	3'03	TGCAGTAGTTGTCCACCC
5'04	GTTCTTGGAGCAGGTTAAACA	3'06	CTGGCTGTTCCAGTACTC
5'05	GTTTCTTGGAGTACTCTACGT	3'08	CACTGTGAAGCTCTCCAC
5'06	GACGGAGCGGGTGCGGTA	3'10	CCCGCTCGTCTTCCAGGA
5'07	CCTGTGGCAGGGTAAGTATA	3'11	TCTGCAATAGGTGTCCAC
5'08	AGTACTCTACGGGTGAGTGTT	3'12	TCCACCGCGGCCCGCC
5'09	GTTTCTTGAAGCAGGATAAGT	3'15	CCGCCTCTGCTCCAGGAG
5'10	CGGTTGCTGGAAAGACGCG	3'17	CCCGCCTGTCTTCCAGGA
		3'18	GCTGTTCCAGTACTCGGC
Name	**Control Primers**	3'19	CTGTTCCAGTGCTCCGCA
C5	TGCCAAGTGGAGCACCCAA	3'041	GTCCACCGCGGCCCGCT
C3	GCATCTTGCTCTGTGCAGAT	3'042	CGCGGCCCGCTCGTCT
		3'043	TGCAGTAGGTGTCCACCT
		3'044	CTGCAGTAGGTGTCCACC
		3'045	TGTTCCAGTACTCGGCGC
		3'046	CGCTGTCGAAGCGCACGG
		3'047	CTGCACTGTAAGCTCTCA
		3'048	CTGCACTGTGAAGCTCTC
		3'079	CCCGTAGTTGTGTCTGCA
		3'A	ACTGTGAAGCTCTCACCA
		3'B	TGTGAAGCTCTCCACCGC
		3'C	GGCCCGCCTCTGCTCCA
		3'D	CGGCCCGCTCGTCTTCC

Table 2.4. **Antibodies used for immunofluorescence and immunohistochemistry**

Name	Type	Target	Source
PERB11.1 204	Rabbit Polyclonal	MICA (+MICB)	CMII, Perth
PERB11.1 205	Rabbit Polyclonal	MICA (+MICB)	CMII, Perth
huMICA-M673	Mouse Monoclonal	MICA	Immunex, USA
W6/32	Mouse Monoclonal	HLA Class I	DAKO
Swine anti-rabbit conjugated with FITC	Swine Polyclonal	Rabbit immunoglobulin	DAKO
Rabbit anti-mouse conjugated with FITC	Rabbit polyclonal	Mouse immunoglobulin	DAKO

Table 2.5. **Laboratories contributing samples to the 13th IHW on psoriasis**

Country	Lead Researcher	Psoriasis samples	Psoriatic Arthritis samples
UK	Dr NJ McHugh	90	158
Belgium	Dr Dupont	102	-
Germany	Dr T Hohler	37	23
Italy	Dr G Novelli	50	-

Table 2.6. **MICA PCR-SSP typing algorithm**

Group	1st stage Primer Pair	Alleles recognised	2nd stage Primer Pairs	Alleles recognised
1	F1+R8	*008,*010,*016*,019, *027, *033,*048	F8+R8 F10+R17 F3+R16	*008,*027,*048 *010 *016
2	F3+R29	*004,*006,*009,*014, *024,*028,*036,*044, *049	F25+R17 F25+R4 F4+R6	*006,*009,*049 *004,*044 *006
3	F2+R13	*013, *022, *044		
4	F1+R2	*001,*005,*007,*012, *018,*021,*025,*026, *029,*031,*032,*037, *038,*039,*040,*042, *043,*045	F18+R1 F1+R12 F5+R5 F7+R7	*001 *012, *021, *032 *005, *035, *037, *039, *042 *007, *026, *029, *037, *038, *039, *040, *043, *045 (+ all Group 5 alleles)
5	F2+R2	*002,*011,*015,*017, *020,*023,*030,*034, *035,*041, *046,*047	F11+R22 F14+R2 F20+R7	*011 *015 *017

Table 2.7. **Expected product sizes following amplification with MICA PCR-SSP typing primer pairs**

Group	1st stage primer pair	Amplicon size (bp)	Annealing Temp °C	2nd stage primer pair	Amplicon size (bp)	Annealing Temp °C
1	F1 + R8	1525	65	F8 + R8	739	65
				F10 + R17	841	62
				F3 + R16	770	65
2	F3 + R29	861	65	F25 + R17	215	65
				F4 + R6	730	67
				F25 + R4	215	65
				F2 + R13	653	65
3	F2 + R13	653	65			
4	F1 + R2	780	65	F18 + R1	617	65
				F1 + R12	741	65
				F5 + R5	861	65
				F7 + R7	628	69
5	F2 + R2	777	65	F14 + R2	218	66
				F20 + R7	148	65
				F11 + R22	979	65
Internal	HGH F + R	485	62-69			

Table 2.8. **Primer mixes used for initial HLA-DRB1 typing**

Primer Mix	5' primer	3' primer	Amplified DRB1 alleles
1	5'01	3'15	*0101, *0102
2	5'01	3'10	*0103
3	5'02	3'01	*1501-1502
4	5'02	3'02	*1601-1602
5	5'03	3'03	*0301-0302
6	5'06	3'048	*0301
7	5'03	3'047	*0302,*1302,*1305, *1402-1403
8	5'04	3'047	*0401-0411
9	5'07	3'079	*0701-0702
10	5'08	3'045	*0801-0805
11	5'09	3'079	*0901
12	5'10	3'047	*1001
13	5'05	3'06	*1101-1104
14	5'08	3'08	*1201-1202
15	5'03	3'10	*1301-1302
16	5'05	3'045	*1303-1304
17	5'03	3'17	*1305
18	5'05	3'11	*1401, *1404-1405
19	5'03	3'12	*1305, *1402-1403
20	5'04	3'19	*1410

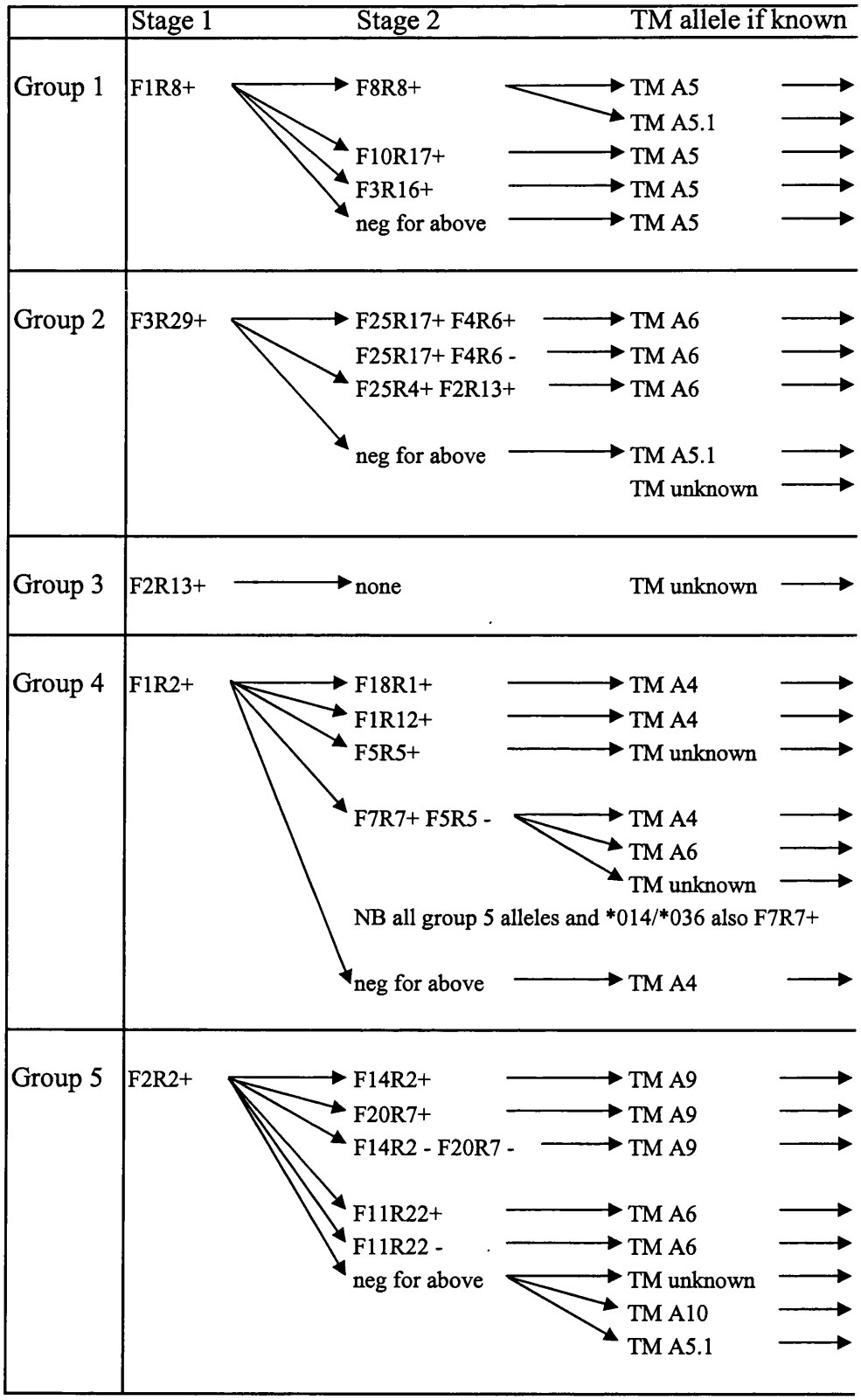
Table 2.9. **Primer mixes used for HLA-DR*01 subtyping**

Primer mix	5' primer	3' primer	Amplified DRB1*01 alleles
1	5'01	3'A	*0101, *0103
2	5'01	3'B	*0102
3	5'01	3'C	*0101, *0102
4	5'01	3'D	*0103

Table 2.10. **Primer mixes used for HLA-DR*04 subtyping**

Primer	5' primer	3' primer	Amplified DRB1*04 alleles
1	5'04	3'041	*0401, *0402, *0409
2	5'04	3'042	*0402
3	5'04	3'043	*0403, *0406, *0407, *0411
4	5'04	3'044	*0401, *0402, *0404, *0405, *0408, *0409, *0410
5	5'04	3'045	*0405, *0409, *0410, *0411
6	5'04	3'046	*0406
7	5'04	3'047	*0401, *0405, *0407, *0408, *0409
8	5'04	3'048	*0402, *0403, *0404, *0406, *0410, *0411

Figure 2.1. **Algorithm for MICA typing**



CHAPTER 3 MICROSATELLITE POLYMORPHISM OF THE MICA GENE

3.1 Introduction

3.1.1 Genomic structure of MICA

The MHC Class I Chain related Gene A (MICA) is one of the most polymorphic non-HLA genes in the human genome. It lies 46kb centromeric to HLA-B within the heart of the MHC complex. The MICA gene was discovered in 1994 by two independent laboratories (124; 125). Since then, the closely related MICB gene has been identified 83kb centromeric to MICA. Subsequently, five related pseudogenes, MICC, MICD, MICE, MICF and MICG have been discovered (126). So far, none of the pseudogenes appear to be functional, although it is possible that MICE may serve some function in certain haplotypes.

Both MICA and MICB are unusually large genes: MICA is 11.7kb and MICB is 12.9kb (127; 128). This compares with the average length of the MHC Class I genes which is 3.5kb. The overall genomic structure of MICA and MICB mirrors that of the MHC Class I genes with distinct functional domains (3 extracellular, a transmembrane and a cytoplasmic domain) each encoded by separate exons. The extracellular domains of MICA and MICB (α1, α2 and α3) show an average 21%, 19% and 34% homology, respectively, to the MHC Class I genes. There is a striking difference in the size of the first intron of the MIC genes compared with the MHC Class I genes: 6.8kb in MICA, 7.4kb in MICB and less than 200bp in the Class I genes. In addition, MICA and MICB possess unique exons that encode the cytoplasmic tail and 3′ untranslated segment. The major discernible difference between MICA and MICB lies in the presence of a microsatellite repeat in the transmembrane segment encoded by exon 5 of MICA which is absent in MICB. The significance of these genomic features remains uncertain.

There are a number of genes which share some homology with both the MIC genes and the Class I MHC genes. These genes collectively form a family known as the 'Extended MHC Class I Family" or EMCIF. The genes within this family serve a wide variety of functions including iron haemostasis, the transfer of maternal immunity and interaction with bacterial and viral ligands. The genes are dispersed throughout the genome, with only HFE having a place within the MHC complex. The additional members of the EMCIF

include CD1, MR1, Zinc α2 glycoprotein, neonatal F_c receptor, HFE and the CMV binding proteins, the ULBPs (129).

There is clear evolutionary conservation of the MIC genes across mammalian species. The apparent exception to this is rodents where degenerate PCR has failed to find any remotely homologous sequences. The region of the mouse H_2 complex where the MIC genes would be expected to be located has been truncated from 173 to 40kb. Intriguingly, deletion of the same region of the human MHC has resulted in a haplotype found in some southeast Asians. This haplotype, the MIC 'null' haplotype (AH48.1) carries complete deletion of the MIC genes (130). This haplotype is extremely uncommon outside southeast Asia with the exception of some Amerindians. In southeast Asia, the AH48.1 is relatively common with a frequency of 3.2% and an expected homozygous carriage of 0.1%. So far, 8 individuals homozygous for the MIC null haplotype have been reported in the literature. Ostensibly, these individuals have no clinical symptoms and a limited exploration of their NK and T cell repertoires has proved unremarkable. This would suggest that either the MIC genes are not essential for life, which seems unlikely in view of the high level of polymorphism, or that they are replaced in these individuals by functional homologues.

3.1.2 Expression of MICA

In contrast to classical MHC genes, MIC genes are not ubiquitously expressed. Initially, expression of MICA was reported to be limited to the intestinal epithelium (131). However, it has since been confirmed that MICA is present in epithelial cells from a large variety of tissues, including the skin, liver and kidney. Notable exceptions to expression include the central nervous system and the spleen. MIC transcripts are not detectable in cells of lympho-haematopoietic lineage.

Transcription of the MIC genes is not enhanced by interferons and the gene sequence does not contain interferon response elements. However, it does contain sequences with significant homology to promoters of the heat shock protein gene (131). These sequences contain duplications of the heat shock response element (HRE) pentanucleotide motif common to a number of stress-induced genes such as the HSP70 gene. Transcription of

MIC mRNA can be enhanced and cell surface expression increased by stressing epithelial cells at a variety of temperatures (131; 132). The physiological relevance of these in vitro experiments remains unclear. Equally, the effects of some of the stressful challenges experienced by epithelial cells *in vivo* such as hypoxia or infection have not yet been directly investigated.

MICA and MICB glycoproteins can be detected in a variety of transfected cells of diverse lineages (131). The presence of MICA mRNA would appear to be sufficient for expression. However, monoclonal and polyclonal antibodies to MICA do show tissue and site specific staining. There are several possible explanations for this. Differential expression may be related to the polymorphism within the gene. Another explanation may be the high level of glycosylation within the MIC genes. MICA has 8 N-linked glycosylation sites compared with only one in classical Class I proteins. Thus tissue or site specific glycosylation may cause differences in cell surface expression.

The native MICA molecule has been shown to localise to the basolateral surface of the intestinal epithelium. This intracellular targeting of the glycoprotein appears to be dependent on the cytoplasmic tail. This has been demonstrated by analysis of both a tail-less construct and a construct containing the MICA A5.1 allele that has no cytoplasmic tail. The latter results in faulty location of the MICA molecule onto the apical surface of the cell, which may well lead to a loss of interaction with its receptor (133).

3.1.3 The MICA receptor

The receptor for MICA and MICB was originally thought to be the Vδ1 receptor on γ/δ T cells (134). However, there were several reasons to suggest that this was not the true receptor. Firstly, MIC expression patterns do not match Vδ1 expression patterns. Secondly, direct physical contact between MICA and the Vδ1 receptor has never been proven. Thirdly, direct Vδ1 interaction has been shown more recently with another contender, CD1c. Finally, direct involvement of MICA with another receptor found ubiquitously on NK and T cells has now been demonstrated.

MICA has been shown to interact directly with the NKG2D receptor found on NK and T cells (135). NKG2D belongs to the lectin family of NK cell receptors encoded by a cluster of genes on chromosome 12 (12p12-13). NKG2D is the most genetically divergent of this family. NKG2D receptors are expressed on most αβ and γδ CD8+ T cells and NK cells in association with a signal-transducing unit called DAP10 (136). In NK cells, MICA can lead to direct stimulation via the NKG2D receptor. In contrast, the mechanism of activation in T cells, is indirect and co-stimulatory by amplifying signals mediated through the T cell receptor.

Interestingly, unlike MICA, there is a murine orthologue of NKG2D. This would suggest that MIC molecules are not the sole occupier of these receptors. Indeed, soluble NKG2D binds to HLA Class I molecules with considerable avidity (137). In addition, more recently, a distinct set of ligands for NKG2D has been identified during a search for molecules which interact with the cytomegalovirus UL16 glycoproteins (129). These ULBPs are absent from most adult tissues but appear to be selectively expressed by tumour cells. They may well be a major signal for NK cell-mediated killing of tumour cells.

3.1.4 Function of MICA

It is possible that the function of MICA in health differs from its function in disease. Although MICA is widely expressed in a variety of tissues there is some evidence for tissue and site specific expression. In addition, expression may be upregulated by cell 'stress'. When MICA is expressed on the cell surface of epithelial cells it binds to the NKG2D receptor on NK and T cells leading to cell activation. Activatory signals within NK and T cells then lead to the generation of specific cytokines that can further activate the immune response. MICA may therefore play a role in the response of the host to any stress, perhaps such as cell damage, hypoxia or infection. It may act as an important co-stimulator of T cells in addition to Class I HLA molecules. The activation of NK cells leads to targeted cell destruction through the release of lytic particles. However, the NKG2D receptor is not the only receptor controlling the activation of NK cells. Any one

NK cell has many receptors, including a number of the 'immunoglobulin-like' inhibitory receptors or KIRs. Thus, the profile of receptors on an individual's NK cells (determined by the Leucocyte Receptor Complex on chromosome 19q) may also influence the ability to respond to signals of activation by MICA.

The strength of the immune response may well be related to the affinity of binding of MICA to NKG2D. Some polymorphisms have been shown to directly affect the binding affinity of MICA to its receptor by up to 50 fold, although the functional consequences of this are not known (138). This may be one of the driving forces for the extensive degree of polymorphism within the MICA gene.

It has been demonstrated that the MIC molecules certainly play a role in the recognition of self. They appear to be involved in the rejection of HLA-matched kidney transplants, where specific antibodies to MIC have been isolated (139). In addition, MIC seems to be a component of the evasion of NK cell immunity by tumour cells. Several epithelial tumours have been shown to over-express MIC molecules. Recently, it has been demonstrated that the NK cells of patients with adenocarcinoma of the colon lack expression of NKG2D receptors which have been internalised and down-modulated by high levels of soluble MIC in the serum (140). Thus tumours can evade the host immune response by deactivating NKG2D receptors by the overproduction of MIC. It is possible that a similar dysregulation of the MIC / NKG2D system may play a part in an individual's response to viral or other infections and triggers. Equally, continued stimulation of the NK and T cell response may promote autoreactivity and perpetuation of an immune response leading to the development of an inflammatory arthritis.

3.1.5 Structure of the MICA glycoprotein

The crystal structure of a soluble MICA glycoprotein (allele *001) has been analysed (141). It is a 383 amino acid polypeptide with a molecular weight of 43kDa. The 3 dimensional structure closely resembles that of MHC Class I with the membrane-proximal

α3 domain and the α1 and α2 domains forming a distal eight stranded, antiparallel, β pleated sheet topped by two largely parallel α helices. Unlike MHC Class I, MICA is not associated with β_2-microglobulin (β_2m). The reason for this is unclear, although the large number of glycosylation sites and the lack of well-documented β_2m contact points are possible explanations. It may also be related to the remarkable flexibility of the MICA molecule due to a long linker peptide between the α3 domain and the α1α2 platform. In contrast to Class I molecules, there is no intimate contact between these two sections of the protein, indeed they are separated by a deflection of 113.5 degrees.

The putative binding cleft of the MICA molecule is significantly smaller than that seen in MHC Class I molecules. The distance between the two α helices is much narrower (7–10 Å) than Class I molecules (>18 Å). Indeed, it is even narrower than related molecules (CD1 and FcRn) that have been shown not to bind to a peptide. Multiple attempts to detect a peptide that associates with MICA have failed. In addition, there is no unaccounted for electron density within the crystal structure (141). Thus far, it would appear that MICA does not require any additional cargo to interact with its receptor. This feature is apparently out of keeping with the high degree of polymorphism detected within the MICA gene.

3.1.6 Polymorphism of the MICA gene

The MICA gene displays a very high level of polymorphism. To date, 54 alleles have been described (http://www.anthonynolan.org.uk). There are three main mechanisms of polymorphism: single nucleotide polymorphism (SNP), insertion/deletion and triplet repeat microsatellite polymorphism.

3.1.6.1 Single nucleotide polymorphism

There are a total of 40 SNPs in the MICA gene which are located predominantly in exons 2,3 and 4 which encode the extracellular domains. There is a very high level of non-synonymous substitution (75%), particularly in exon 3 where 15 out of 16 SNPs result in a

change of amino acid (*Figure 3.1*). Another interesting feature of the SNPs within MICA is the strictly biallelic nucleotide substitution, that is, there is only ever one alternative nucleotide at each position. This contrasts with MHC molecules where any nucleotide can be substituted at any position.

The nature of the selective forces maintaining this polymorphism is not known. In classical MHC molecules, much of this selective pressure is thought to derive from ever-changing infectious agents generating an increasingly diverse range of peptides. The fact that the majority of polymorphism in Class I MHC molecules is located near the peptide binding cleft supports this theory. However, virtually none of the polymorphic positions found in Class I MHC molecules are mirrored in the MICA molecule. In MICA, many of the polymorphic sites are in positions where they can influence the NKG2D receptor binding site (*Figure 3.1*). Thus, the driving force for the polymorphism appears to be related to changes in the avidity of receptor binding. The reason for this has not yet been discovered.

MICB seems to be less polymorphic than MICA although this may reflect the fact that it has been less thoroughly studied. There are 15 SNPs, 12 of which are non-synonymous, which define the 16 currently recognised alleles (MICB001 – MICB016) (142). One of these alleles, MICB010, has a stop codon in exon 3. This allele is always associated with a 100kb deletion rendering it a MICB and MICA null allele. Between the MICB and MICA genes is a 1000 fold 'TA' repeat which may represent a hot spot for recombination, although some alleles do appear to be maintained along ancestral haplotypes.

3.1.6.2 Insertion / deletion

There are several examples of the insertion or deletion of a single nucleotide within the MICA gene. This seemingly trivial event can trigger a frame shift, truncation of the polypeptide and potentially dramatic effects on protein function. An insertion of a guanine within the triplet microsatellite repeat in exon 5 results in the most commonly occuring allele, A5.1. The resultant protein lacks a cytoplasmic tail which affects the localisation of the protein on the cell surface (133). Deletion of a guanine at the end of the 4th exon

occurs in the MICA *015 and *017 alleles. This changes the reading frame for the 5th exon resulting in a totally different TM segment with a long, hydrophobic polyleucine region and a shortened cytoplasmic tail. The functional consequences of this deletion are not yet known.

3.1.6.3 Microsatellite polymorphism

The microsatellite 'GCT' repeat in exon 5 is another interesting contributor to the genetic diversity of MICA (143). It is one of the features of the polymorphism of MICA not shared by MICB. The microsatellite encodes 4, 5, 6, 9 or 10 alanines producing the alleles A4, A5, A6, A9 and A10, respectively (143; 144). It is curious why there appears to be no A7 or A8 allele. One of these alleles (A5) has an additional insertion of a guanine producing the A5.1 allele.

3.1.7 Nomenclature

The nomenclature of MICA can be confusing. There are two main groups of alleles that are commonly referred to in clinical studies. Firstly, the alleles can be divided into just 6 groups by virtue of the microsatellite repeat in exon 5. Thus, this nomenclature comprises the A4, A5, A5.1, A6, A9 and A10 alleles. However, these alleles can differ in exons 2,3 and 4 due to single nucleotide polymorphisms. Therefore there are in fact several alleles that are 'A4', ie. that have 4 GCT repeats in exon 5. These include MICA *001, *007, *012 and *018. The more correct and official nomenclature therefore labels these alleles MICA *001- *049 (114). Some of the alleles are very closely related and only differ by one SNP that does not result in an amino acid change. These alleles are labelled as subdivisions of a single allele, for example, *00201, *00202, *00701, *00702, *00801, *00802, *00803, *00901, *00902, *01201, *01202. Historically, there is no *003 allele as the allele that was originally thought to be *003 was not correctly sequenced.

3.1.8 Frequency of the MICA alleles in control populations

There have been several studies of the frequency of the alleles of MICA in a variety of ethnic populations (118; 145). There are some differences in allele frequencies, particularly between Caucasian and non-Caucasian populations. The best characterised populations come from the USA, UK and Japan. By far the most common allele in all studied populations is MICA*008. This allele has a TM region containing 5 GCT repeats and a guanine insertion and is thus one of the A5.1 alleles. The next most common alleles are MICA*002, *004, *009 and *010. A recent study of inflammatory bowel disease in the UK examined a large number of controls and patients for the presence of all 54 alleles (117). Only 13 of the 54 alleles were present at a frequency of greater than 1%, suggesting the more recent evolutionary generation of new alleles from a series of more conserved ancestor alleles.

3.1.9 Disease associations

Due to the proximity of MICA to the HLA Class I genes, a growing number of studies have examined associations between polymorphisms of MICA and disease. Although most studies have involved the diseases classically associated with Class I HLA genes (Behcets, Psoriasis, AS, Inflammatory Bowel Disease) many other diseases have been investigated. The majority of studies have concentrated on the microsatellite repeat in exon 5. Fewer studies have attempted to address the more complex polymorphism within the 2nd, 3rd and 4th exons. The disease associations that have been reported are outlined in *Table 3.1.*

3.1.10 Studies of MICA in Psoriasis and PsA

Associations of the polymorphism within the transmembrane region of MICA have been studied in Type I psoriasis. One study in Australian patients found an association between Type I psoriasis and the MICA allele which has 9 GCT repeats (A9) and a guanine deletion at the beginning of intron 4 (50). The allele with the same number of GCT repeats (also A9), but no deletion, was not associated. Other studies in Chinese and Korean patients have shown an association between the truncated form of the protein, A5.1, and

Type I psoriasis (51; 52). None of these studies looked for the presence of arthritis complicating the psoriasis.

Three studies have found an association between PsA and the MICA A9 allele but relatively small numbers of patients have been studied considering the complexity of the condition. The first study examined 45 patients with psoriasis and 65 patients with PsA from Spain (67). The patients with psoriasis were not specifically screened for the presence of arthritis or silent radiographic sacroiliitis. The frequency of the A9 allele was significantly higher in the PsA group, but the frequency in psoriasis was no different from controls. These findings were later confirmed in a slightly larger group of 81 patients with PsA (68). The third study to find a positive association between the A9 allele and PsA was in a Jewish population of 52 patients but there was no psoriasis control group (66).

3.1.11 MICA in RA, AS and JIA

MICA has also been investigated in a number of other rheumatological conditions including RA, AS and JIA (146). The A6 allele has been shown to be protective for RA (147). The frequency of the A4 allele is increased in AS (148-151), although it would appear that this is due to its close association with HLA-B27. The A4 allele has also been associated with Juvenile Idiopathic Arthritis (152).

3.2 Aims of the study

The aim of this study was to investigate whether there was any association between the microsatellite polymorphism of MICA and PsA. Patients with well documented PsA, patients with Type I psoriasis screened for the presence of arthritis and controls were recruited for this study.

3.3 Methods

3.3.1 Patients

Three major groups of patients were included in this study:

(i) The first group consisted of patients with well-documented PsA who were recruited from a specialist Rheumatology outpatient clinic at the RNHRD in Bath (n=158), as described in Chapter 2.2.2.

(ii) The second group of patients had psoriasis. They were recruited from a specialist dermatology clinic and GP practice in Bath, UK, as described in Chapter 2.2.2. A total of 90 patients were recruited, of whom 49 were found to have Type I psoriasis uncomplicated by arthritis, 15 had Type II psoriasis and 26 had Type I/II psoriasis together with clinical or radiological evidence for an inflammatory arthritis. For the purposes of the analysis, only the 49 patients with uncomplicated Type I psoriasis were included. The remaining patients were analysed separately.

(iii) The third group of patients were recruited from the 13th International Histocompatibility Workshop on psoriasis. Samples from patients with Type I psoriasis were contributed from laboratories in Belgium (n=72), Germany (n=33) and Italy (n=26). None of the patients were screened for the presence of a complicating arthritis.

3.3.2 Controls

The control samples (n=148) were recruited from random blood donors in the UK as described in Section 2.2.2. All controls underwent appropriate screening but the presence of mild background psoriasis can not be excluded. It should be noted that no ethnically matched controls were available for the 13IHW patients.

3.3.3 Reference Samples

Samples from the 4AOH and 10th IHW panels with well documented MICA alleles were used for reference (*Table 3.2*).

3.3.4 Preparation of DNA samples

DNA was extracted from blood samples following the procedure outlined in Section 2.3.3. The concentration of DNA in each sample was then measured on a spectrophotometer and any samples with a high protein content were purified accordingly (Section 2.3.6). Working dilutions of 20ng/μl were made by diluting the DNA with ddH_2O. The stock solutions and diluted DNA samples were then frozen until needed.

3.3.5 Development of a technique for distinguishing the microsatellite alleles of MICA

There have been a number of techniques published for the separation of the MICA microsatellite alleles. The basis for all these techniques is the separation of the alleles by size. Most of the alleles differ by 3 base pairs, but one (A5.1) only differs by 1 base pair. Therefore a high resolution separation technique is needed. It was decided to utilise a *Gene Scanner 2000* (Corbett Research) which separates PCR products on the basis of their size and can be adapted for high resolution. There are several advantages of this machine over some of the other techniques used to separate different sized products. Firstly, it is able to detect *double stranded* PCR products thus avoiding the need for a denaturing gel. Secondly, the products can be detected by ethidium bromide using a laser, therefore no extra labelling of the sample is required. Thirdly, the resolution of the technique has been shown to be high, with the ability to discriminate products that differ in size by just 1 base pair.

3.3.6 Optimisation of the technique

Initially, previously published primers were selected to amplify a region of exon 5 containing the GCT repeat microsatellite (50). These primers, called PERTMF and PERTMR (*Table 2.1*) yielded products varying in size according to their allele: 297bp(A4), 300bp(A5), 301bp(A5.1), 303bp(A6) and 312bp(A9). In order to achieve maximum resolution of the products, the concentration of polyacrylamide in the gel was increased gradually from 4% to 10%. At the higher concentrations, there was some loss of

clarity of the gel with blurring of the products and increased "smile" in the gel due to uneven distribution of current. The optimal concentration was found to be 8%. However, resolution of the products was not high enough, even with the use of a longer gel (30cm). Thus a smaller product size was sought to maximise the ability to detect small differences in size.

The primer pair, PERTM100F and PERTM100R (67) yields product sizes of just over 100bp. The sizes of the products for the different alleles are: 118bp(A4), 121bp(A5), 122bp(A5.1), 124bp(A6) and 133bp(A9).

3.3.7 Typing for MICA microsatellite alleles

Details of the reaction mix and PCR conditions used for the MICA microsatellite typing are given in Chapter 2.4.1.1. The PCR samples were then analysed using the Gene Scanner 2000 using the method described in Section 2.4.1. The resultant image of the gel produced by the laser scanning the PCR products is stored as a tif image. An example of a gel is given in *Figure 3.2*. Molecular weight marker is loaded at regular intervals between groups of eight samples. The MICA alleles are assigned for each PCR product after conversion of the tif image into a text file which is then imported into an excel spreadsheet. Each product is then plotted as a line graph with intensity of the product on the 'y' axis and time on the 'x' axis. Examples of the resultant graphs for varying combinations of alleles are given in *Figures 3.3-3.12*. The results were then inputted into an excel database containing all demographic and clinical information on the patients.

3.3.8 HLA typing

All PsA, Type I psoriasis (UK) and control samples were typed for HLA-Cw6 and HLA-B27 using sequence-specific primers (122) (Chapter 2.4.4). Some of the 13IHW samples (Belgian, n=72) were provided with full Class I and Class II HLA typing. The PsA samples were also typed for the HLA-DRB1 SE as described in Chapter 2.4.3.

3.3.9 Statistics

The frequencies of the MICA alleles were determined by direct counting. Results were expressed as phenotype frequencies (the number of patients with at least one allele) and allele frequencies (the total number of alleles present). Differences between patient groups and controls were determined using the Chi-squared test with Fisher's Exact test where necessary. No ethnically matched controls were available for the 13IHW patients. The results were therefore compared with the UK controls but the results should therefore be interpreted with caution. For continuous variables, such as tender and swollen joint counts and PASI, the Mann Whitney U test was applied. The *p values* were considered to be significant if <0.05. The *p values* were corrected using the Bonferroni correction for the number of alleles observed. Odds ratios with 95% confidence intervals were also calculated.

3.4 Results

3.4.1 Clinical characteristics of the disease groups

The demographic and clinical information on the patients with PsA and Type I psoriasis from the UK are given in *Table 3.3.* There was very limited clinical information available for the patients from Belgium, Italy and Germany who took part in the 13th IHW. Due to the methods of recruitment, there were some inherent differences between the patients with PsA and the patients with psoriasis. The patients with uncomplicated Type I psoriasis were significantly younger and had a significantly younger age of onset of psoriasis. Some of these differences were overcome by stratifying the patients with PsA for the age of onset of their psoriasis. Although some patients with psoriasis were recruited from general practice, the majority of patients were recruited from a specialist dermatology clinic. This, not surprisingly, resulted in a higher median PASI score for the psoriasis only group. It is interesting to note that the incidence and severity of nail disease was very similar in the two groups.

3.4.2 Frequencies of the MICA microsatellite alleles in the patient populations

The frequencies of the MICA microsatellite alleles in the patient populations have been expressed as both phenotype frequencies and allele frequencies. The phenotype frequencies in PsA, Type I psoriasis (UK), Type I psoriasis (13IHW) and controls are given in *Table 3.4.* The MICA A9 allele was significantly increased in the PsA (p_{corr}<0.0005, OR 2.4 (1.48-3.88) and 13IHW (p_{corr}<0.0005, OR 2.5 (1.52-4.15)) populations. A similar increase was not observed in the UK Type I psoriasis population. MICA A4 was increased in the PsA population, but this did not reach significance. The majority of the increase in MICA A9 was accounted for by a decrease in MICA A5.1, but this did not reach significance. There were no significant differences in the other MICA phenotype frequencies.

If the MICA microsatellite alleles were expressed as allele frequencies, once again MICA A9 was significantly increased in the PsA (p_{corr}=0.0025, OR 2.31) and 13IHW psoriasis (p_{corr}=0.0005, OR 2.31) populations (*Table 3.5*). The allele frequencies of MICA A5.1 now become significantly reduced in the PsA (p_{corr}=0.008, OR 0.59) and 13IHW (p_{corr}<0.0005, OR 0.55) populations compared with controls.

The phenotype frequencies of the MICA microsatellite alleles in the different populations of Type I psoriasis are illustrated in *Table 3.6.* The A9 allele was significantly increased in the Belgian (p_{corr}=0.005, OR 2.8 (1.55-5.04)) and German populations (p_{corr}=0.05, OR 2.63 (1.21-5.71)), but not in the UK or Italian populations. If all of the Type I psoriasis populations were grouped together, A9 was significantly increased (p_{corr}=0.005, OR 2.8 (1.55-5.04)). If the results were expressed as allele frequencies, only the Belgian and total psoriasis groups reached significance (*Table 3.7*). Once again the reduction in MICA A5.1 became significant if the total number of alleles was considered. The significance of these results cannot be guaranteed due to the lack of an ethnically matched control group for the European patients.

3.4.3 Associations of the MICA microsatellite alleles with clinical subgroups of PsA

The phenotype frequencies of the MICA microsatellite alleles in the subgroups of PsA are expressed in *Table 3.8*. The main clinical subgroups were polyarthritis, oligoarthritis and spondyloarthritis. Patients with spondyloarthritis could also have peripheral joint involvement. Patients in the UK psoriasis group who were screened and found to have occult arthritis were also included in this analysis. The MICA A9 allele was increased in all groups, although, perhaps due to sample size, this only reached significance in the polyarthritis and oligoarthritis groups. The frequency of MICA A5.1 was significantly reduced in the polyarthritis group. The frequency of MICA A4 was increased in all groups but this failed to reach significance.

3.4.4 Associations of the MICA microsatellite alleles with clinical manifestations of PsA

There were no significant associations between the MICA microsatellite alleles and any of the clinical parameters of disease severity measured, including tender and swollen joint counts, PASI, nail score, DMARD use or HAQ. There was no association between the age of onset of psoriasis or the age of onset of PsA and any of the MICA alleles. There was also no association between any of the MICA alleles and positive family history of either psoriasis or PsA. The MICA alleles were present at equal frequencies between those PsA patients that had erosive disease and those that had not developed erosions (*Table 3.9*). In particular, MICA A9 was present in 47% of erosive patients and 47% of non-erosive patients.

3.4.5 Stratification for age of onset of psoriasis

If the patients with PsA were stratified according to the age of onset of their psoriasis (<40 and >40 years of age), the A9 allele was still significantly increased in both groups. Marginally more of the patients with PsA with early onset psoriasis were positive for the MICA A9 allele compared with those with a later onset of their skin disease (49% vs 40%, NS).

3.4.6 Associations of the MICA microsatellite alleles with HLA-Cw6 and HLA-B27

The frequency of HLA-Cw6 was significantly higher in the PsA than the control population (65/151 (43%) HLA-Cw6 positive PsA patients compared with only 26/131 (20%) controls, p<0.0001, OR 3.05 (1.79-5.22)). HLA-Cw6 was also highly significantly associated with Type I psoriasis (33/46 (72%) Type I psoriasis (UK) compared with 26/131 (20%) controls (p<0.0001, OR 10.25 (4.74-22.19)). The MICA A9 allele is significantly associated with HLA-Cw6 (p_{corr}<0.0005, OR 3.36 (1.71-6.59)) (*Table 3.10.*).

The MICA A4 allele was significantly associated with HLA-B27 (88% of A4 alleles were associated with HLA-B27 (p_{corr}<0.0005, OR 47 (13-173)) (*Table 3.11*). MICA A9 alleles were observed more frequently in HLA-B27 negative patients, although this significance disappeared on correction for multiple testing. There were no other associations between the MICA alleles and HLA-B27.

3.4.7 Stratification for HLA-Cw6

The patients with PsA were analysed separately according to their HLA-Cw6 status. If all HLA-Cw6 patients and controls were removed, 34% of the remaining patients were positive for MICA A9 compared with 19% of controls (p_{uncorr}=0.015, p_{corr}=0.075, OR 2.24 (1.16-4.32). Conversely, if the MICA A9 positive patients were removed from the analysis, HLA Cw6 was present in 30% of the patients with PsA compared with 11% of controls (p_{uncorr}=0.002, p_{corr}=0.01, OR 3.25 (1.48-7.16)).

3.4.8 Associations of the MICA microsatellite alleles with the HLA-DRB1 'shared epitope' (SE) and HLA-Class II alleles

Overall, there was no association between any of the MICA alleles and the HLA-DRB1 SE alleles (HLA-DRB1*0101, *0102, *0401, *0404, *0405, *0408, *1001) (*Table 3.12*). MICA A9 was more likely to be associated with SE negative alleles, but this significance was no longer present after correction for multiple testing. Comparison of the MICA alleles was also made with HLA-DR1, HLA-DR4 and HLA-DR7. There was a significant association between the MICA A4 allele and HLA-DR1 (p_{uncorr} = 0.006, p_{corr} = 0.03, OR

2.8 (1.33-5.88)) (*Table 3.13*). On further analysis, this association was primarily due to HLA-DRB1* 0101 (p_{uncorr} =0.003, p_{corr} =0.015, OR 3.25 (1.47-7.19)) (*Table 3.14*). There were no further associations between any of the MICA alleles and HLA-DR4 or HLA-DR7.

3.5 Discussion

Patients who develop PsA have a significantly higher proportion of MICA alleles that contain 9 GCT repeats in exon 5 than controls. In fact nearly 50% of patients with PsA possess at least one MICA A9 allele compared with only 26% of controls. This finding is highly significant whether one considers the number of patients with a particular allele (phenotype frequency) or the absolute number of alleles (allele frequency). The increase in MICA A9 seems to be largely at the expense of the MICA 5.1 allele. This reaches significance if the absolute numbers of alleles is taken into account. The MICA A5.1 allele is the commonest allele in most populations and could therefore be assumed to be associated with 'health'. The MICA A9 allele has been previously described in association with both psoriasis and PsA (50; 66)(67) albeit in much smaller populations than described here. There have been no reports of the association of MICA A9 with any other diseases except Insulin Dependent Diabetes Mellitus in a Taiwanese population (153). It would appear therefore that there is something unique about this allele that contributes to the development of PsA. A key question, however, is whether the association is predominantly with PsA or whether MICA A9 is more important in the development of psoriasis.

In order to address this question, a control group of patients with Type I psoriasis was recruited who were screened for the presence of arthritis. Interestingly, MICA A9 was not increased in frequency in this population. However, MICA A9 was increased in the population of patients with Type I psoriasis who were involved in the 13IHW, particularly the Belgian and German patients. There could be several explanations for this. Firstly, the 13IHW psoriasis populations were not specifically screened for the presence of arthritis. Therefore the increase in the frequency of MICA A9 in this group could be explained by a high prevalence of either overt or occult arthritis. This explanation assumes that the primary association with A9 is with psoriatic arthritis rather than psoriasis. Secondly, the

UK psoriasis population is relatively small (n=49), therefore the figures may fail to reach significance because of the sample size. Thirdly, MICA A9 may be important in the development of psoriasis in some European populations but not in others. This final argument would be supported by differences in the MICA allele frequencies between the psoriasis populations. In particular, the Italian psoriasis population would appear to be different to the others with a lower frequency of MICA A9 and a higher frequency of MICA A4. The 13IHW results must be interpreted with caution due to the lack of an ethnically matched control group.

Another potential problem when comparing the allele frequencies between the PsA and psoriasis groups was the difference in the onset and severity of the psoriasis. The patients with PsA had a significantly later age of onset of their psoriasis compared with the patients with Type I psoriasis recruited in the dermatology and GP clinics in the UK. Therefore, some of the patients who were classified as psoriasis without arthritis could potentially still develop arthritis in the future. Indeed the patients in the psoriasis group were significantly younger than the patients with PsA. However, the overall duration of psoriasis between the two groups was similar (25 and 28 years). It is known that most patients with PsA develop the arthritis after the psoriasis, but it would be unusual for many patients to develop arthritis after having psoriasis for at least 25 years. The patients with PsA were stratified according to the age of onset of their psoriasis and MICA A9 was found to be equally significant in patients with PsA with both early and late onset psoriasis.

The patients with Type I psoriasis also had a higher PASI score suggesting that their psoriasis was in general more severe than the patients with PsA. This is not entirely surprising in view of the recruitment strategy used. The majority of patients were recruited from a hospital Dermatology clinic and therefore would have more severe skin disease than those recruited from general practice or indeed the community. These differences must be taken into account when directly comparing the results between PsA and psoriasis. Ideally the groups should be better matched for age, age of onset of psoriasis and severity of psoriasis. However, it is still helpful to have a psoriasis only control group and despite these clinical and demographic differences it will certainly help distinguish

those genes that are important for the skin disease from those that are important in arthritis.

The distribution of the MICA alleles between the different clinical subgroups of PsA was fairly even. MICA A9 was globally increased in all groups, including those with occult arthritis recruited in the dermatology clinic. The increase in MICA A9 only reached significance in the polyarthritis and oligoarthritis groups, probably due to sample size. The increase in MICA A4 just failed to reach significance once the statistics were corrected for multiple testing.

None of the MICA alleles, including A9, were associated with any of the clinical or radiological parameters of disease severity such as tender and swollen joint counts, PASI, DMARD use or the development of erosions. This was also the case in the psoriasis population, where there were no associations between the MICA alleles and the PASI. This would suggest that MICA A9 is a *susceptibility* and not a *severity* gene. This would be supported by the lack of association between MICA A9 and the HLA-DRB1 SE. It will be demonstrated later that the SE is associated with the development of erosive disease in PsA and may therefore be a '*severity*' gene. MICA A9 is negatively associated with the SE alleles (although not significantly so), suggesting that they are independent of one another, perhaps one acting to increase susceptibiblity to PsA and the other modifying its presentation.

There were some associations between the MICA alleles and HLA-B27 and HLA-Cw6. MICA A4 was strongly associated with HLA-B27 suggesting strong linkage disequilibrium between the two alleles. This is corroborated by the findings of several studies that have demonstrated an association between MICA A4 and ankylosing spondylitis (148; 150; 151). MICA A4 is also associated with HLA-DR1, particularly the HLA-DRB1 *0101 allele, due to its presence on the same ancestral haplotype. MICA A9 was found to be strongly associated with HLA-Cw6. Clearly HLA-Cw6 is known to be associated with psoriasis and to a lesser degree PsA and the relationhip between HLA-Cw6 and MICA A9 requires further examination.

A major problem encountered during the interpretation of significant associations between alleles within the MHC region and disease is whether the association is indeed primarily with the gene of interest or whether the association is secondary to a stronger association with a neighbouring gene. In the case of MICA, it is possible that the association with MICA A9 and PsA is all due to the presence of MICA A9 on haplotypes containing HLA-Cw6. To explore this possibility, the patients with PsA were stratified and analysed according to their HLA-Cw6 status. Removal of all HLA-Cw6 positive patients and controls resulted in a loss of significance for MICA A9 after correction for multiple testing ((p_{uncorr}=0.015, p_{corr}=0.075, OR 2.24 (1.16-4.32)). Thus it would appear that a major reason for the association of MICA A9 with PsA is due to its linkage with HLA-Cw6. However, a component of the MICA association is independent of HLA-Cw6. This component just failed to reach significance perhaps due to the smaller numbers in the groups once HLA-Cw6 patients had been eliminated. There are several MICA alleles that have 9 GCT repeats in exon 5 (A9 alleles). Some of these are known to be present on HLA-Cw6 bearing haplotypes (such as MICA *017), whereas others (such as MICA *002) are more independent of HLA-Cw6. Further analysis of the MICA alleles that are associated with PsA would clarify the situation.

The technique utilised for the identification of the microsatellite alleles of MICA was easy to use and relatively quick to interpret. There were some inherent problems with the technique. One problem encountered was uneven current running through the gel. This resulted in 'smile' across the gel where the products in the centre of the gel progressed more rapidly than the products on the edges of the gel. Care needed to be taken when pouring and setting the gel to ensure an even gel was produced. Molecular weight marker was loaded at regular intervals to provide accurate reference points for the interpretation of the products. The use of internal markers within the loading buffer for each sample also gave a static reference point. The vast majority of allele combinations were easy to distinguish. However, homozygous samples and samples that were heterozygous for A5 and A5.1 required some caution in their interpretation since the level of discrimination was down to one base pair. It is possible that some errors occurred. Further exploration of the MICA alleles could be used to confirm the allele designation based on the microsatellite repeat.

It is unclear why a repeat of the GCT microsatellite in exon 5 of the MICA gene should be associated with disease. The MICA A9 polymorphism results in 9 alanines in the transmembrane region. This would result in a slightly longer TM segment but the functional consequences of this are not known. It is possible that the polymorphism may affect the transportation to or stability of the molecule at the cell surface. It may have some effect on the interaction with the NKG2D receptor but this would seem unlikely since there is no direct contact between the TM region of the protein and the receptor. It would seem more likely that a polymorphism within the α1 or α2 domains where the platform for receptor binding is formed would be responsible for an association with disease. There are several alleles of MICA that have 9 GCT repeats in the 5^{th} exon. A more detailed exploration of the polymorphisms within the 2^{nd}, 3^{rd} and 4^{th} exons of the MICA gene would clarify which of the A9 alleles are associated with PsA and which, if any, are associated with psoriasis.

Table 3.1. **Disease associations with the MICA alleles**

Disease	MICA TM allele association	MICA allele association	Ethnic group	Reference
Psoriatic arthritis	A9	*002	Caucasian (Spain)	(67)
	A9	-	Caucasian (Israel)	(66)
Psoriasis	PERB11.1 *06 (A9)	-	Caucasian (Australia)	(50)
	A5.1	-	Oriental (Korea)	(52)
	A5.1	-	Oriental (China)	(51)
Ankylosing Spondylitis	A4	-	Caucasian (Europe)	(148)
	A4	-	Caucasian (Europe)	(150)
	-	*007 and *010	Oriental (Japan)	(149)
	A4	-	Caucasian (Italy)	(151)
	NS (HLA-B27)	NS (HLA-B27)	Caucasian, African and Asian	(154)
Rheumatoid Arthritis	A6 (protective)	-	Caucasian (Europe)	(147)
Juvenile Idiopathic Arthritis	A4	-	Caucasian (Latvia)	(152)
Inflammatory Bowel Disease	-	NS	Caucasian (UK)	(117)
	A6	-	Oriental (Japan)	(155)
	NS (HLA-B52)	-	Oriental (Japan)	(156)
	-	*007	Caucasian (UK)	(157)
IBD + peripheral arthropathy	-	*00801	Caucasian (UK)	(157)
Behcets	A6	-	Oriental (Japan)	(143)
	A6	*009	Middle Eastern (Palestinian)	(158)
	-	*009	Oriental (Japan)	(159)
	A6	-	Caucasian (Greece)	(160)
	NS	-	Caucasian (Spain)	(161)
	NS	-	Oriental (Japan) and Caucasian (Greece and Spain)	(162)

Disease	TM allele association	EC allele association	Ethnic group	Reference
Insulin dependent diabetes mellitus	A4 + A5.1	-	Oriental (Japan)	(163)
	A6 (protective)	-	Oriental (Japan)	(163)
	A5	-	Caucasian (Italy)	(164)
	A9	-	Oriental (Taiwan)	(153)
Addison's disease	A5.1	-	Caucasian (Italy)	(165)
	A6 (protective)	-	Caucasian (Italy)	
Primary sclerosing cholangitis	-	*002 (protective)	Caucasian (UK)	(166)
Kawasaki disease	A5	-	Oriental (Taiwan)	(167)
	A4 + A5.1 (protective)	-		
Leprosy	A5 (protective)	-	Oriental (China)	(168)
Cervical neoplasia	NS	-	Caucasian (Sweden)	(169)

Table 3.2. **Reference DNA panel for MICA microsatellite typing**

MICA TM allele	MICA allele	Cell ID	4AOH number	10 IHW number	Ancestral haplotype
A4	*001	EVA SP	100051Y	9135	18.2
A4	*00701	BM92	100201F	9092	51
A5	*010	BSM	100073N	9032	62.1
A5	*016	JO528239	100019U	9041	35.1
A5.1	*00801	REE,GD	100044V	9132	8.1
A5.1	*00801	PLH	100064P	9047	47.1
A6	*004	RSH	100007B	9021	42.1
A6	*006	KAS116	100154N	9003	51
A9	*00201	YAR	100009X	9026	38.1
A9	*017	DBB	100084G	9052	57.1

Table 3.3. **Demographic and clinical characteristics of the PsA and psoriasis populations**

Characteristic	Psoriatic arthritis (n=158)	Type I psoriasis (UK) (n=49)
Sex M:F	79F: 79M	22F: 27M
Median age (yrs) (range)	60 (33-92)	41 (17-80)[1]
Median age of onset of psoriasis (yrs) (range)	31 (3-70)	19 (3-39)[2]
Median duration of psoriasis (yrs) (range)	28 (7-79)	25 (2-67)
Median age of onset of arthritis (yrs) (range)	37 (10-71)	-
Median duration of arthritis (yrs) (range)	19 (7-67)	-
Nail involvement	71%	59%
Median Nail score	1.5 (0-13.5)	2.9 (0-23)
Median PASI (range)	1 (0-27)	2.9 (0.2-16.5) [3]

[1] $p<0.0001$ comparing median age of PsA and Type I Ps patients

[2] $p<0.0001$ comparing median age of onset of psoriasis of PsA and Type I Ps patients

[3] $p<0.0001$ comparing median PASI of PsA and Type I Ps patients

Table 3.4. **Phenotype frequencies of the MICA microsatellite alleles in the different disease groups**

MICA allele	PsA (n=158)	Type I Psoriasis (UK) (n=49)	Type I Psoriasis (13IHW) (n=131)	Controls (n=148)
A4	46 (29%)	7 (14%)	37 (28%)	26 (18%)
A5	17 (11%)	5 (10%)	22 (17%)	28 (19%)
A5.1	97 (61%)	37 (76%)	80 (61%)	110 (74%)
A6	48 (30%)	16 (33%)	39 (30%)	50 (34%)
A9	**73 (46%)**[1]	16 (33%)	**62 (47%)**[2]	39 (26%)

[1] p_{uncorr} <0.0001, p_{corr}<0.0005, OR 2.4 (1.48-3.88)

[2] p_{uncorr} <0.0001, p_{corr}<0.0005, OR 2.51 (1.52-4.15) (compared with UK controls)

NB. No ethnically matched control group was available for the 13IHW patients

Table 3.5. **Allele frequencies of the MICA microsatellite alleles in the different disease groups**

MICA allele	PsA (n=316)	Type I Psoriasis (UK) (n=98)	Type I Psoriasis (13IHW) (n=262)	Controls (n=296)
A4	0.158	0.071	0.145	0.091
A5	0.054	0.051	0.084	0.101
A5.1	**0.371**[1]	0.510	**0.351**[2]	0.497
A6	0.158	0.184	0.160	0.179
A9	**0.259**[3]	0.184	**0.260**[4]	0.132
Total	1	1	1	1

[1] $p_{uncorr} = 0.0016$, $p_{corr} = 0.008$, OR 0.59

[2] $p_{uncorr} < 0.0001$, $p_{corr} < 0.0005$, OR 0.55 (compared with UK controls)

[3] $p_{uncorr} = 0.0005$, $p_{corr} = 0.0025$, OR 2.31

[4] $p_{uncorr} = 0.0001$, $p_{corr} = 0.0005$, OR 2.31 (compared with UK controls)

NB. No ethnically matched control group was available for the 13IHW patients

Table 3.6. **Phenotype frequencies of the MICA microsatellite alleles in the patients with Type I psoriasis from different populations**

MICA allele	Type I Psoriasis (UK) (n=49)	Type I Psoriasis (Belgium) (n=72)	Type I Psoriasis (Germany) (n=33)	Type I Psoriasis (Italy) (n=26)	Type I Psoriasis (Total) (n=180)	Controls (n=148)
A4	7 (14%)	19 (26%)	9 (27%)	9 (35%)	44 (24%)	26 (18%)
A5	5 (10%)	17 (24%)	2 (6%)	3 (12%)	27 (15%)	28 (19%)
A5.1	37 (76%)	43 (60%)	21 (64%)	16 (62%)	117 (65%)	110 (74%)
A6	16 (33%)	19 (26%)	11 (33%)	9 (35%)	55 (31%)	50 (34%)
A9	16 (33%)	**36 (50%)**[1]	**16 (48%)**[2]	10 (38%)	**78 (43%)**[3]	39 (26%)

[1] $p_{uncorr} = 0.001$, $p_{corr} = 0.005$, OR 2.80 (1.55-5.04) compared with UK controls

[2] $p_{uncorr} = 0.01$, $p_{corr} = 0.05$, OR 2.63 (1.21-5.71) compared with UK controls

[3] $p_{uncorr} = 0.001$, $p_{corr} = 0.005$, OR 2.80 (1.55-5.04) compared with UK controls

NB. No ethnically matched control group was available

Table 3.7. **Allele frequencies of the MICA microsatellite alleles in the patients with Type I psoriasis from different populations**

MICA allele	Type I Psoriasis (UK) (n=98)	Type I Psoriasis (Belgium) (n=144)	Type I Psoriasis (Germany) (n=66)	Type I Psoriasis (Italy) (n=52)	Type I Psoriasis (Total) (n=360)	Controls (n=296)
A4	0.071	0.132	0.136	0.192	0.125	0.091
A5	0.051	0.118	0.030	0.058	0.075	0.101
A5.1	0.510	**0.340**[1]	0.379	0.346	**0.394**[2]	0.497
A6	0.184	0.132	0.197	0.192	0.167	0.179
A9	0.184	**0.278**[3]	0.258	0.212	**0.239**[4]	0.132
Total	1	1	1	1	1	1

[1] p_{uncorr}= 0.002, p_{corr}= 0.01, OR 0.523 compared with UK controls

[2] p_{uncorr}= 0.0002, p_{corr}= 0.001, OR 0.660 compared with UK controls

[3] p_{uncorr}= 0.009, p_{corr}= 0.045, OR 2.53 compared with UK controls

[4] p_{uncorr}= 0.0005, p_{corr}= 0.0025, OR 2.07 compared with UK controls

NB. No ethnically matched control group was available for the 13IHW patients

Table 3.8. **Frequencies of the MICA microsatellite alleles in different subgroups of PsA**

MICA allele	Polyarthritis (n=86)	Oligoarthritis (n=66)	Spondyloarthritis (+/- peripheral joint disease) (n=38)	Occult arthritis in psoriasis (n=26)	Controls (n=148)
A4	26 (30%)	16 (24%)	11 (29%)	9 (35%)	26 (18%)
A5	10 (12%)	5 (8%)	5 (13%)	5 (19%)	28 (19%)
A5.1	**50 (58%)**[1]	44 (67%)	25 (66%)	16 (62%)	111 (75%)
A6	25 (29%)	21 (32%)	11 (29%)	5 (19%)	50 (34%)
A9	**40 (47%)**[2]	**32 (48%)**[3]	17 (45%)	12 (46%)	39 (26%)

[1] $p_{uncorr} = 0.01, p_{corr} = 0.05$, OR 0.48 (0.27-0.84) compared with controls

[2] $p_{uncorr} = 0.002$, $p_{corr} = 0.01$, OR 2.43 (1.39-4.25) compared with controls

[3] $p_{uncorr} = 0.001$, $p_{corr} = 0.005$, OR 2.63 (1.44-4.82) compared with controls

Table 3.9. **Frequencies of the MICA microsatellite alleles in erosive and non-erosive PsA**

MICA allele	Erosive PsA (n=97)	Non-erosive PsA (n=58)	*p* value
A4	26 (27%)	17 (29%)	NS
A5	13 (13%)	4 (7%)	NS
A5.1	55 (57%)	39 (67%)	NS
A6	32 (33%)	16 (28%)	NS
A9	46 (47%)	27 (47%)	NS

Table 3.10. **Associations of the MICA microsatellite alleles with HLA Cw6 in the PsA population**

MICA allele	HLA-Cw6 positive (n=65)	HLA-Cw6 negative (n=86)	p_{uncorr}	p_{corr}	OR (95% CI)
A4	15 (23%)	28 (33%)	NS	NS	-
A5	3 (5%)	14 (16%)	NS	NS	-
A5.1	40 (62%)	52 (60%)	NS	NS	-
A6	20 (31%)	27 (31%)	NS	NS	-
A9	**41 (63%)**	29 (34%)	**p<0.0001**	**p<0.0005**	**3.36 (1.71-6.59)**

Table 3.11. **Associations of the MICA microsatellite alleles with HLA B27 in the PsA population**

MICA allele	HLA-B27 positive (n=27)	HLA-B27 negative (n=124)	p_{uncorr}	p_{corr}	OR (95% CI)
A4	**24 (88%)**	18 (14%)	**p<0.0001**	**p<0.0005**	**OR 47 (13-173)**
A5	2 (7%)	15 (12%)	NS	NS	-
A5.1	13 (48%)	81 (65%)	NS	NS	-
A6	6 (22%)	12 (9%)	NS	NS	-
A9	7 (25%)	63 (50%)	p=0.02	NS	-

Table 3.12. **Associations of the MICA microsatellite alleles and the presence or absence of the HLA-DRB1 shared epitope**

MICA allele	SE positive (n=70)	SE negative (n=83)	p_{uncorr}	p_{corr}
A4	25 (35%)	20 (24%)	NS	
A5	9 (13%)	8 (9%)	NS	
A5.1	44 (63%)	48 (58%)	NS	
A6	20 (29%)	27 (33%)	NS	
A9	27 (37%)	45 (54%)	0.05	0.25

Table 3.13. **Associations of the MICA microsatellite alleles and the presence of HLA-DR1**

MICA allele	DR1 positive (n=44)	DR1 negative (n=109)	p_{uncorr}	p_{corr}	OR (95%CI)
A4	**20 (45%)**	25 (23%)	**0.006**	**0.03**	**2.8 (1.33-5.88)**
A5	7 (16%)	10 (9%)	NS	-	-
A5.1	20 (45%)	72 (66%)	0.02	0.1	0.43 (0.21-0.87)
A6	11 (25%)	36 (33%)	NS	-	-
A9	22 (50%)	50 (46%)	NS	-	-

Table 3.14. **Associations of the MICA microsatellite alleles and the presence of HLA-DRB1 *0101**

MICA allele	DRB1*0101 positive (n=34)	DRB1*0101 negative(n=119)	p_{uncorr}	p_{corr}	OR (95%CI)
A4	**17 (50%)**	28 (24%)	**0.003**	**0.015**	**3.25 (1.49-7.19)**
A5	4 (12%)	13 (11%)	NS	-	-
A5.1	17 (50%)	75 (63%)	NS	-	-
A6	9 (26%)	38 (32%)	NS	-	-
A9	16 (47%)	56 (47%)	NS	-	-

Table 3.15. **Control population genotypes to illustrate observed and expected genotypes and their compliance with Hardy Weinberg equilibrium**

Genotype	OBSERVED number of controls with this genotype	EXPECTED number of controls with this genotype
A4 A4	1	1.2
A4 A5	4	2.7
A4 A5.1	12	13.4
A4 A6	7	4.8
A4 A9	2	3.6
A5 A5	2	1.5
A5 A5.1	11	14.9
A5 A6	7	5.4
A5 A9	4	4
A5.1 A5.1	37	36.5
A5.1 A6	25	26.3
A5.1 A9	25	19.4
A6 A6	3	4.7
A6 A9	8	7
A9 A9	0	2.6
Total	148	148

CHI-SQUARE = 5.86
14 degrees of freedom
p value= 0.969788

Figure 3.1. **Synonymous and non-synonymous amino acid substitutions in Exons 2,3 and 4 of MICA and their positions on the receptor binding platform**

Exon 2 (α1)

Position				
6	CGT	CCT	**Arg**	**Pro**
14	TGG	GGG	**Trp**	**Gly**
23	CTC	CTT	Leu	Leu
24	ACT	GCT	**Thr**	**Ala**
26	GTA	GGA	**Val**	**Gly**
36	TGT	TAT	**Cys**	**Tyr**
56	AAT	AAC	Asn	Asn
64	AGA	AGG	Arg	Arg

Exon 3 (α2)

Position				
91	CAG	CGG	**Gln**	**Arg**
105	AGG	AAG	**Arg**	**Lys**
112	TAC	TAT	Tyr	Tyr
114	GGG	CGG	**Gly**	**Arg**
122	CTG	GTG	**Leu**	**Val**
124	ACT	TCT	**Thr**	**Ser**
125	AAG	GAG	**Lys**	**Glu**
129	ATG	GTG	**Met**	**Val**
142	GTC	ATC	**Val**	**Ile**
151	ATG	GTG	**Met**	**Val**
156	CAC	CTC	**His**	**Leu**
156	CAC	CGC	**His**	**Arg**
173	AAA	GAA	**Lys**	**Glu**
175	GGC	AGC	**Gly**	**Ser**
176	GTA	ATA	**Val**	**Ile**
181	ACA	AGA	**Thr**	**Arg**

Exon 4 (α3)

Position				
191	AGC	AGT	Ser	Ser
193	GCC	GCA	Ala	Ala
198	ATT	ATC	Ile	Ile
205	TCT	TCC	Ser	Ser
206	GGC	AGC	**Gly**	**Ser**
210	TGG	CGG	**Trp**	**Arg**
213	ACA	ATA	**Thr**	**Ile**
215	AGC	ACC	**Ser**	**Thr**
221	GTA	CTA	**Val**	**Leu**
247	ACC	ACT	Thr	Thr
251	CAA	GAA	**Gln**	**Glu**
251	CAA	CGA	**Gln**	**Arg**
255	CAG	CAA	Gln	Gln
256	AGG	AGT	**Arg**	**Ser**
268	AGC	GGC	**Ser**	**Gly**
271	CCT	GCT	**Pro**	**Ala**

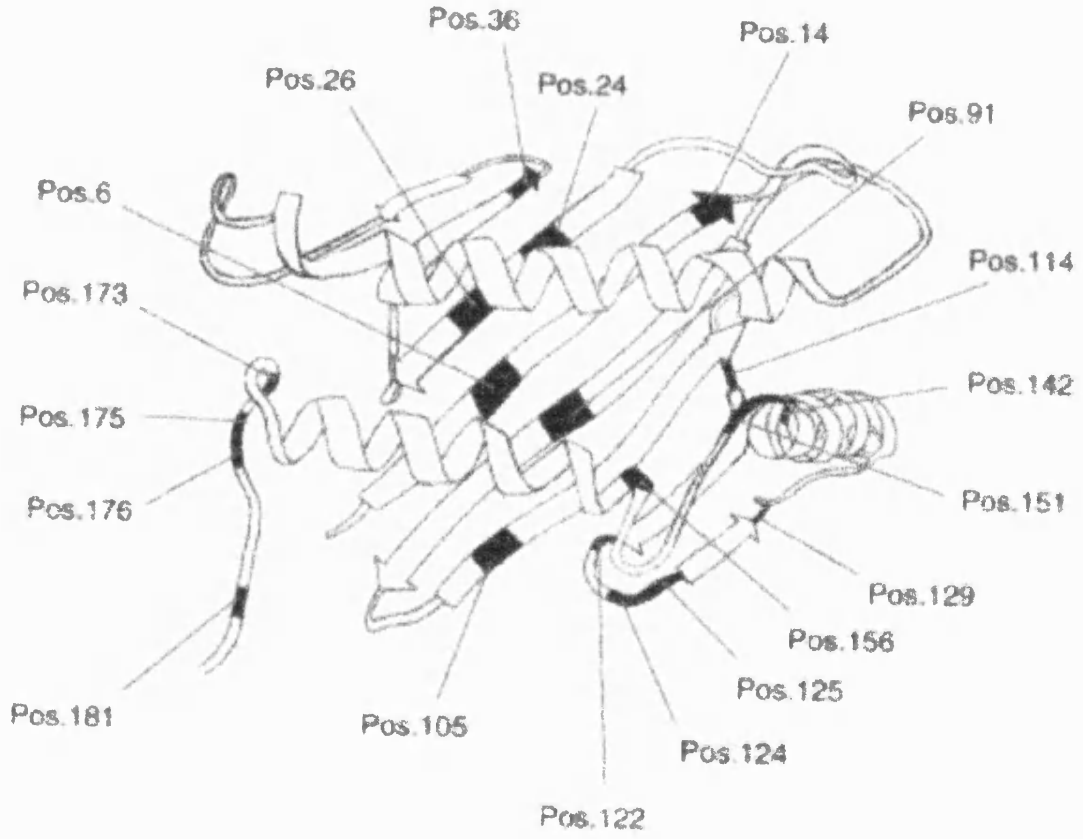
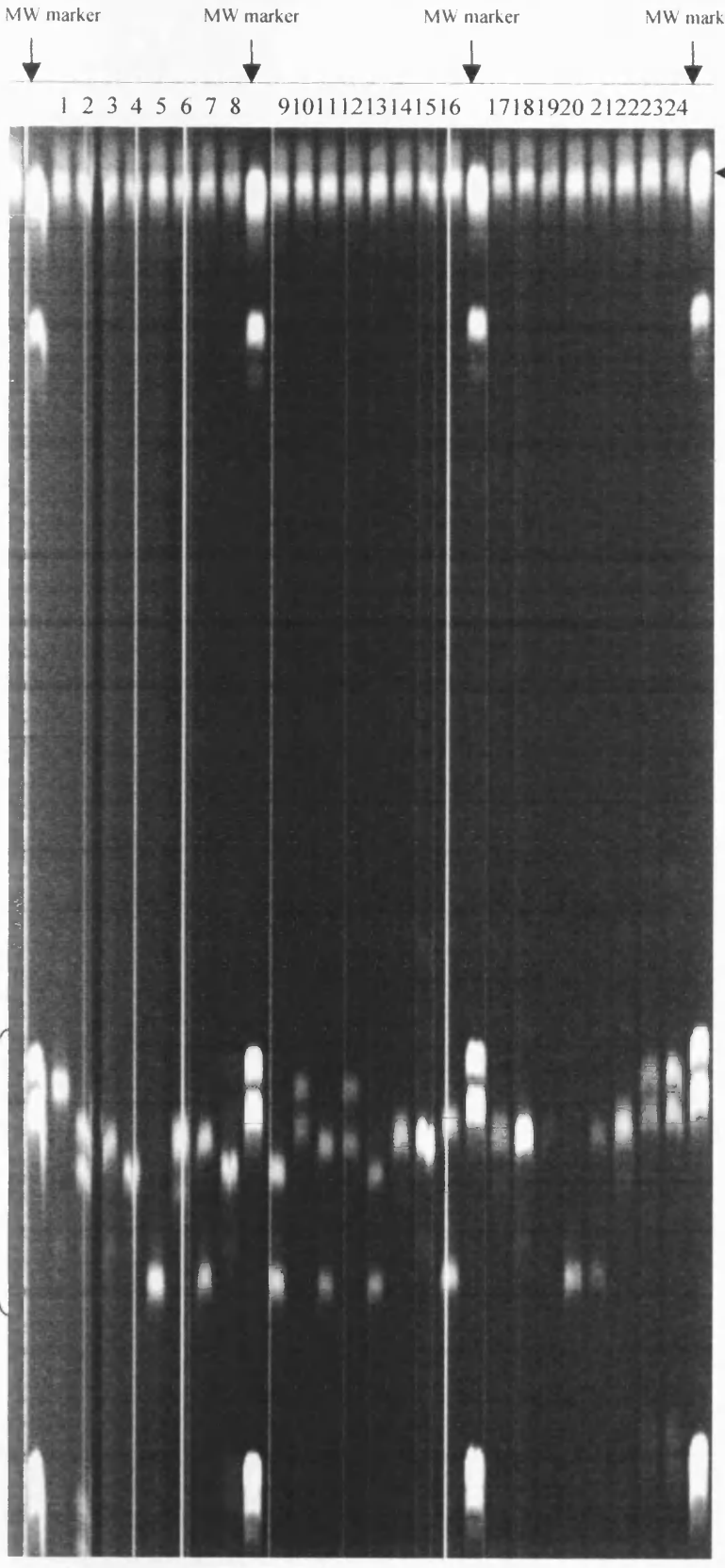


Figure.3.2. Genescanner 'tif' image collected by laser scanning the polyacrylamide gel



Lane	MICA allele
Lane 1	A4 homo
Lane 2	A5 + A6
Lane 3	A5.1 homo
Lane 4	A6 homo
Lane 5	A9 homo
Lane 6	A5 + A5.1
Lane 7	A5.1 + A9
Lane 8	A6 homo
Lane 9	A6 + A9
Lane 10	A4 + A5.1
Lane 11	A5.1 + A9
Lane 12	A4 + A5.1
Lane 13	A6 + A9
Lane 14	A5 + A5.1
Lane 15	A5.1 homo
Lane 16	A5 + A9
Lane 17	A5 + A5.1
Lane 18	A5.1 homo
Lane 19	A5.1 homo
Lane 20	A9 homo
Lane 21	A5.1 + A9
Lane 22	A5 + A5.1
Lane 23	A4 + A5
Lane 24	A4 + A5

Figure 3.3. **Genescanner graph showing a patient who is a heterozygote for the MICA A4 and A5.1 alleles**

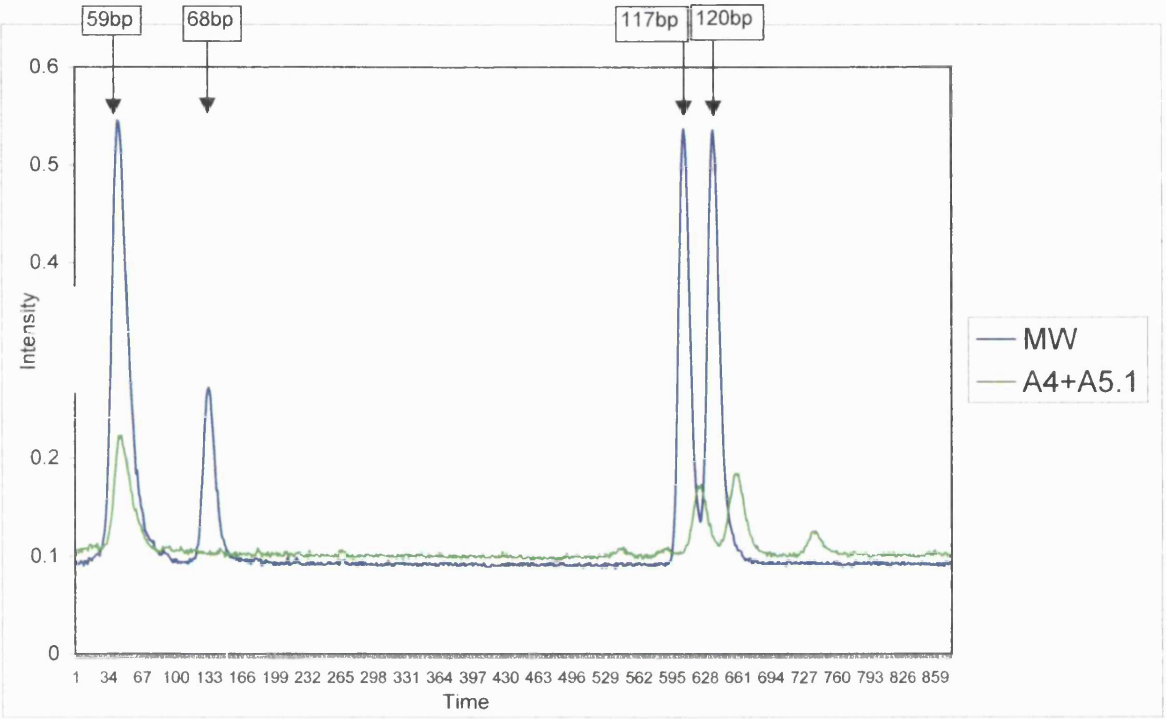


Figure 3.4. **Genescanner graph showing a patient who is a heterozygote for the MICA A4 and A6 alleles**

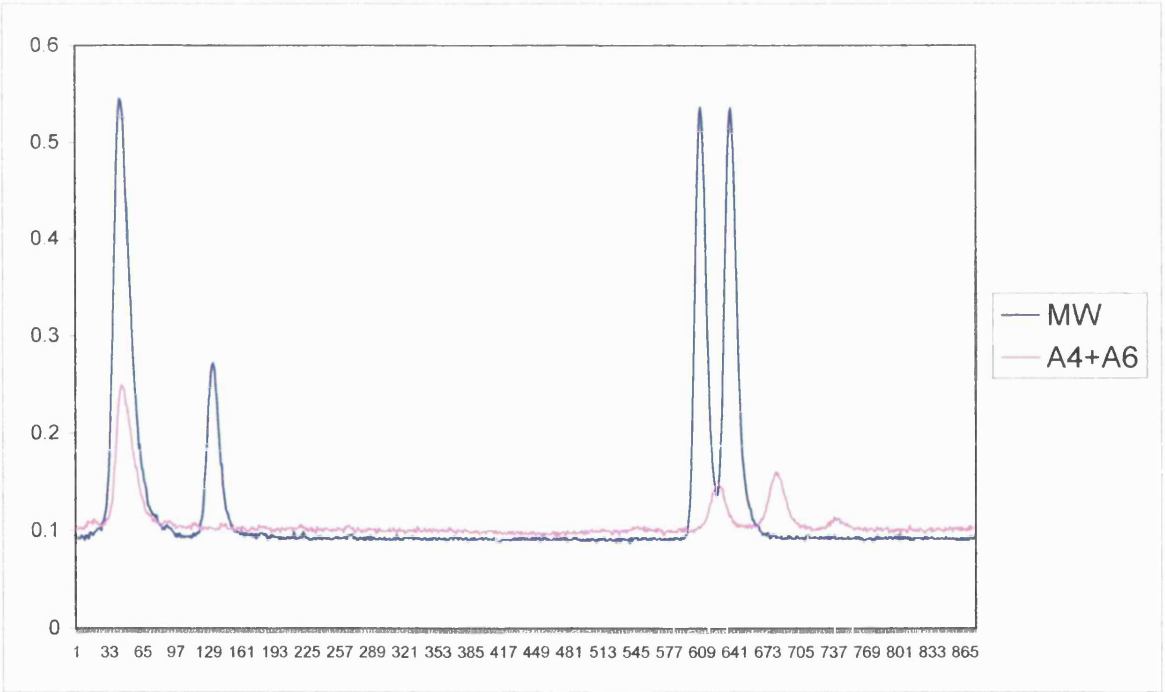


Figure 3.5. **Genescanner graph showing a patient who is a heterozygote for the MICA A5 and A5.1 alleles**

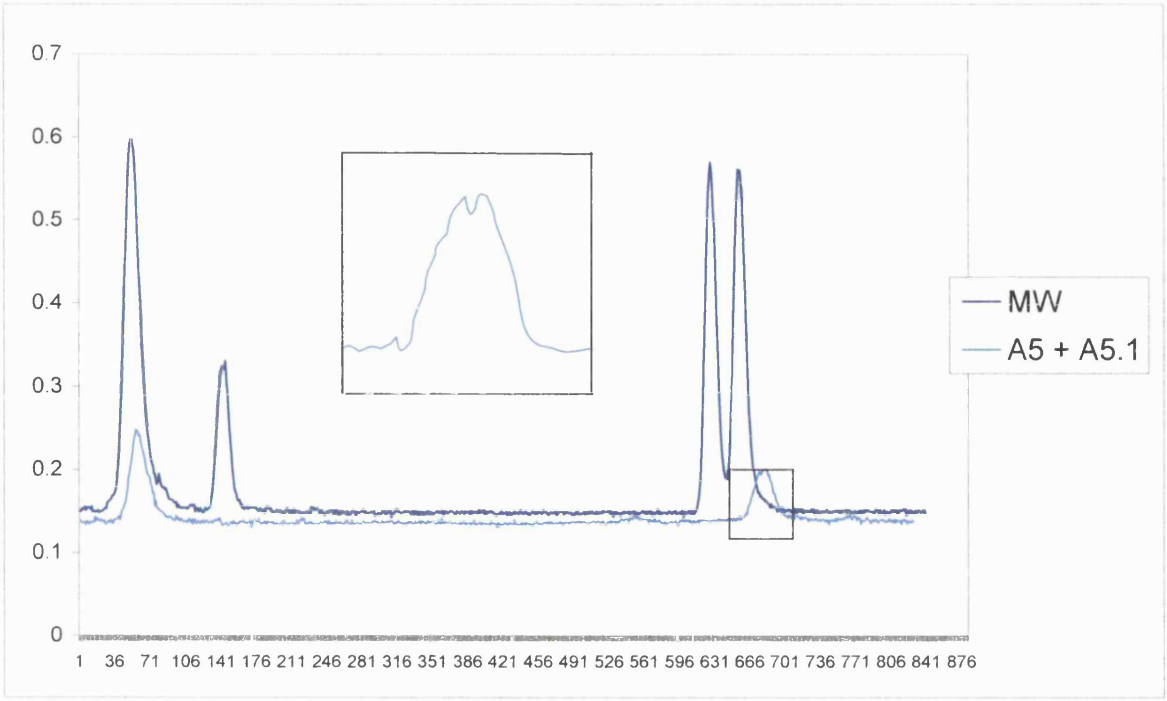


Figure 3.6. **Genescanner graph showing a patient who is a heterozygote for the MICA A5 and A6 alleles**

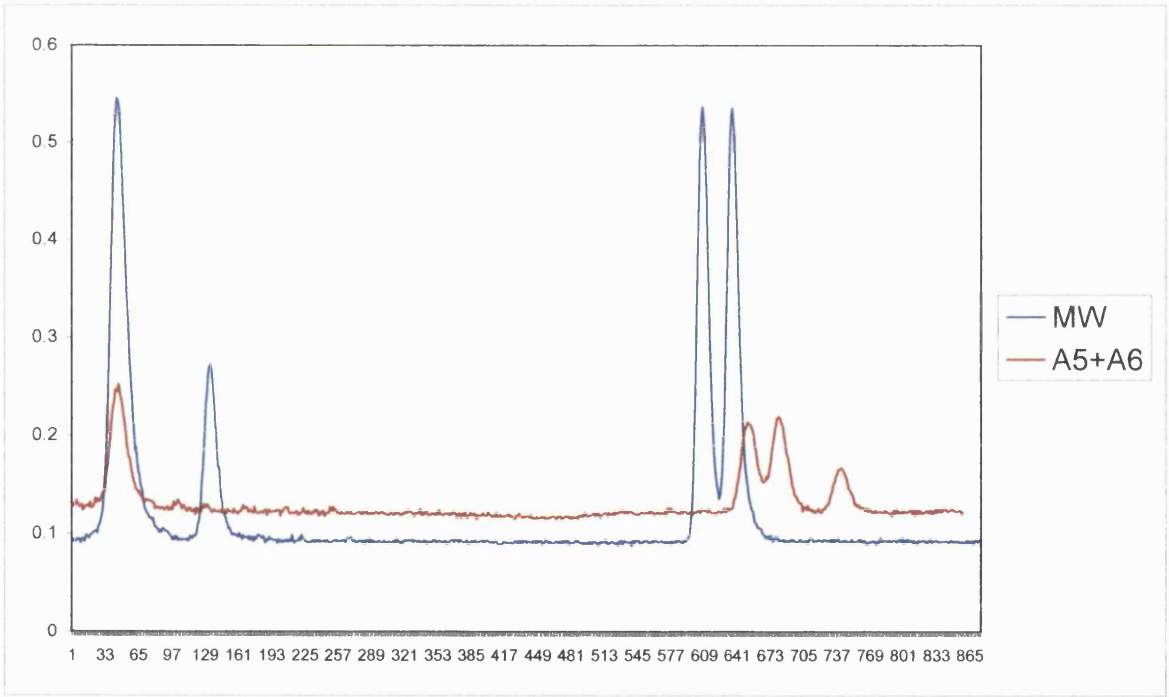


Figure 3.7. **Genescanner graph showing a patient who is a heterozygote for the MICA A5.1 and A9 alleles**

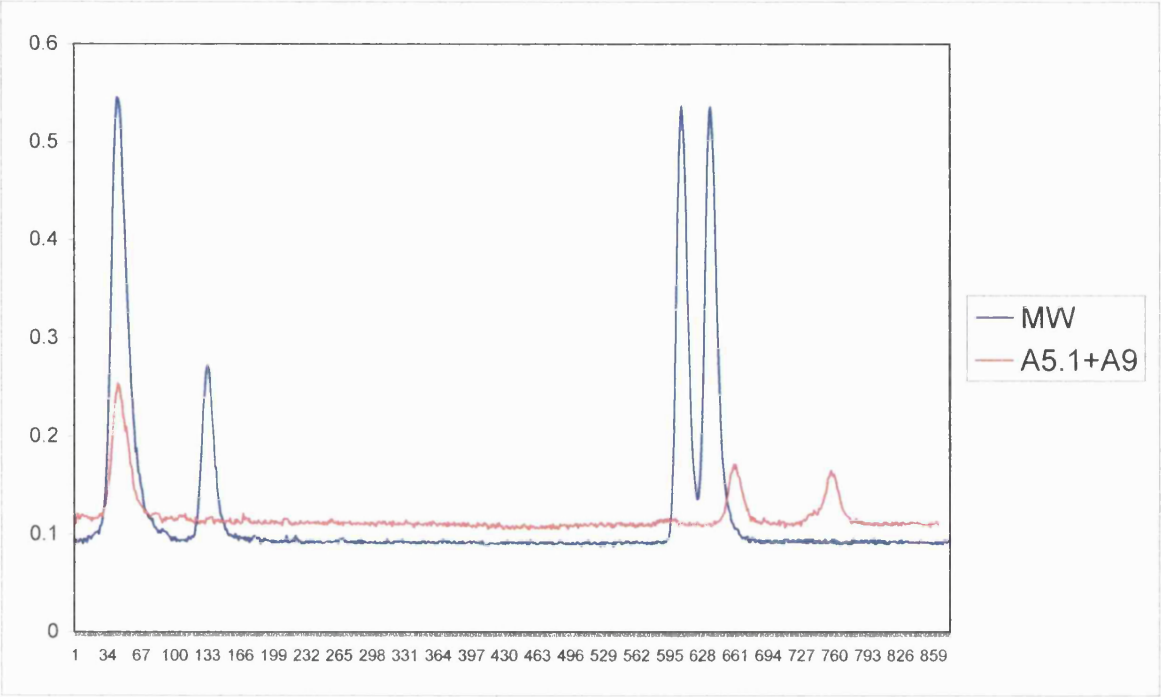


Figure 3.8. **Genescanner graph showing a patient who is a heterozygote for the MICA A6 and A9 alleles**

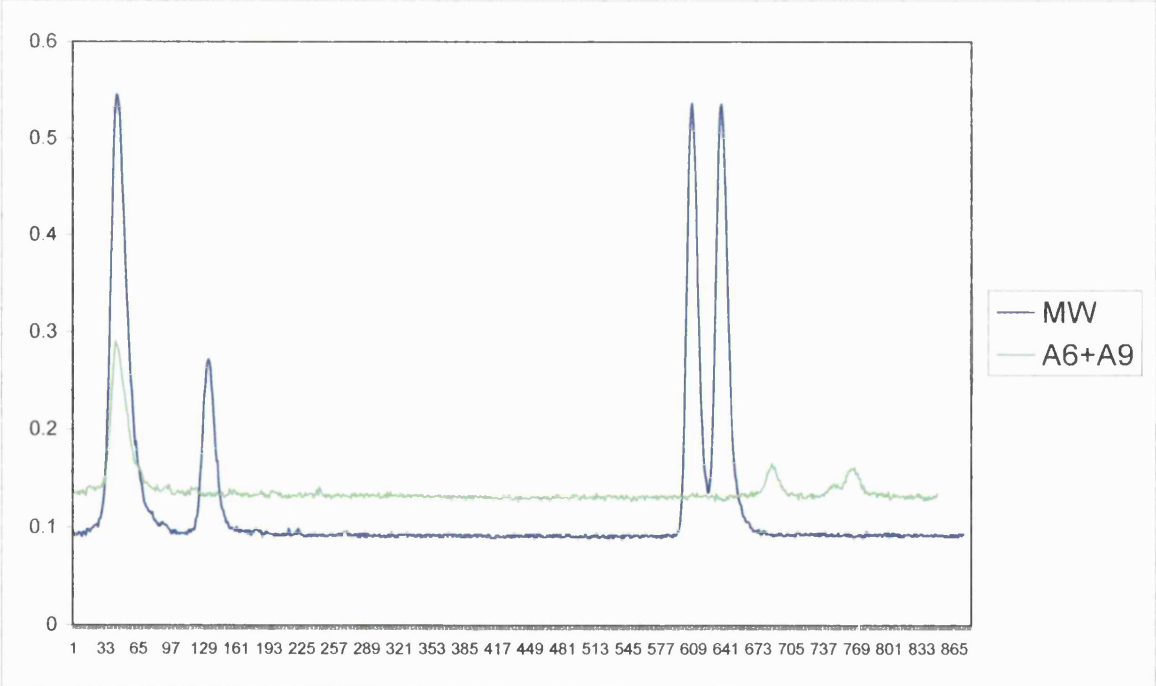


Figure 3.9. **Genescanner graph showing 2 patients who is are heterozygote for MICA A4/A6 and A5/A6**

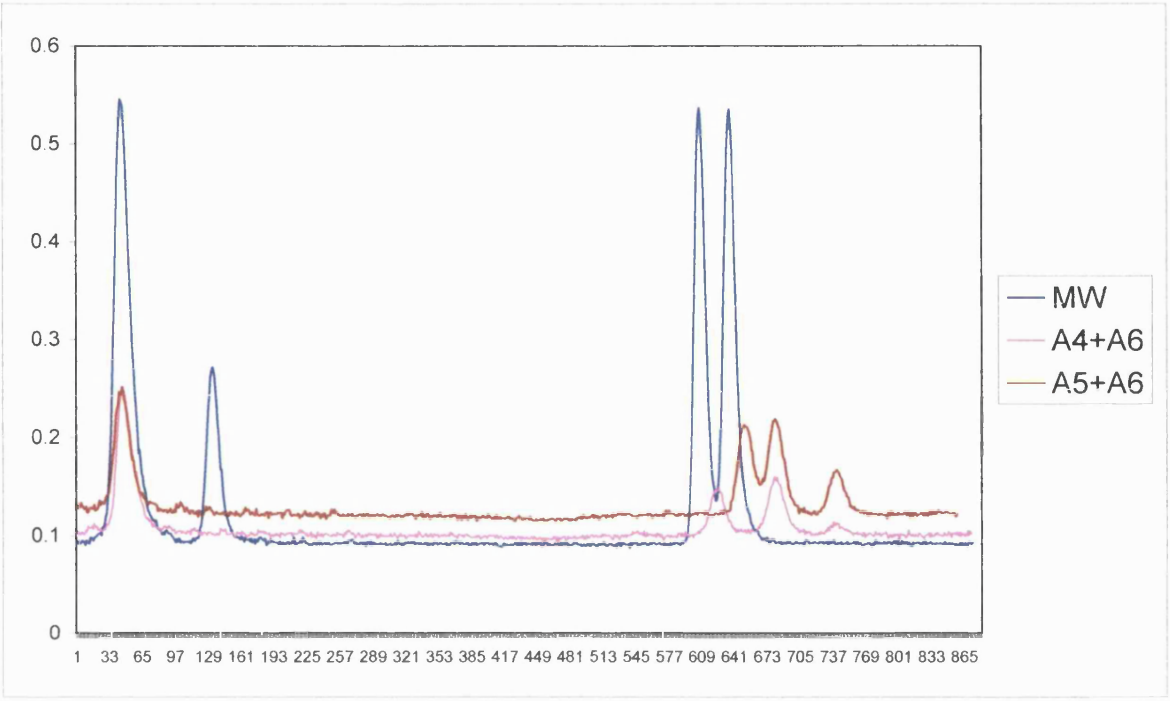


Figure 3.10. **Genescanner graph showing 2 patients who are heterozygote for MICA A4/A5.1 and A4/A6**

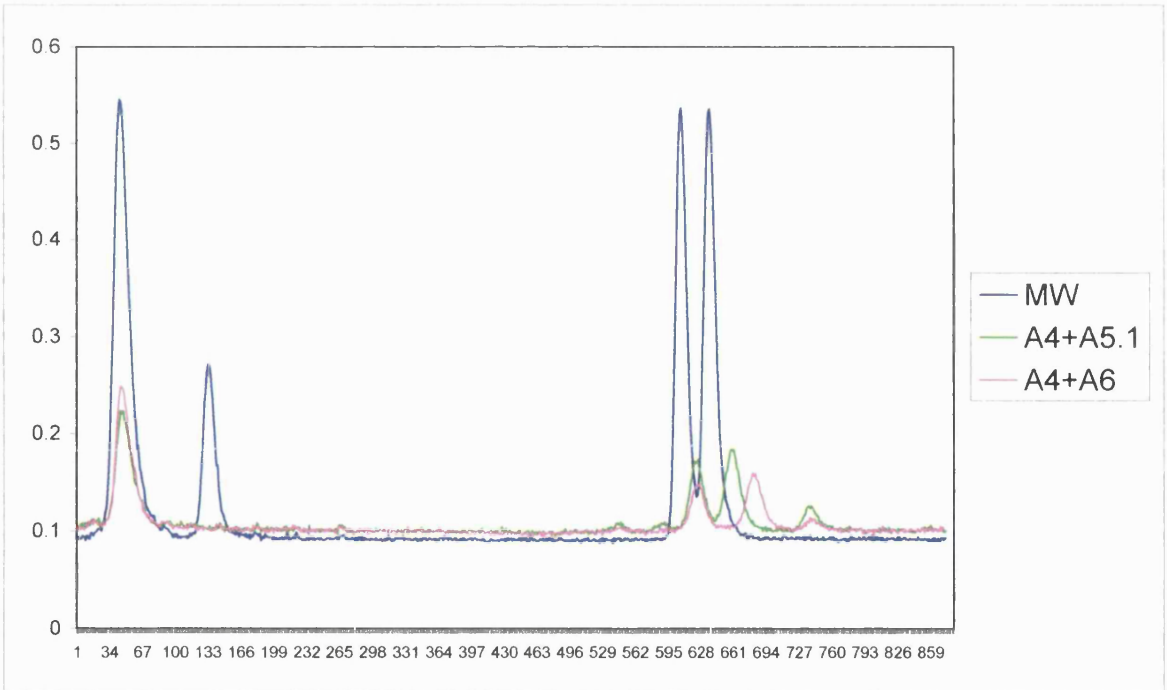


Figure 3.11. **Genescanner graph showing 2 patients who are heterozygote for MICA A4/A5.1 and A5.1/A9**

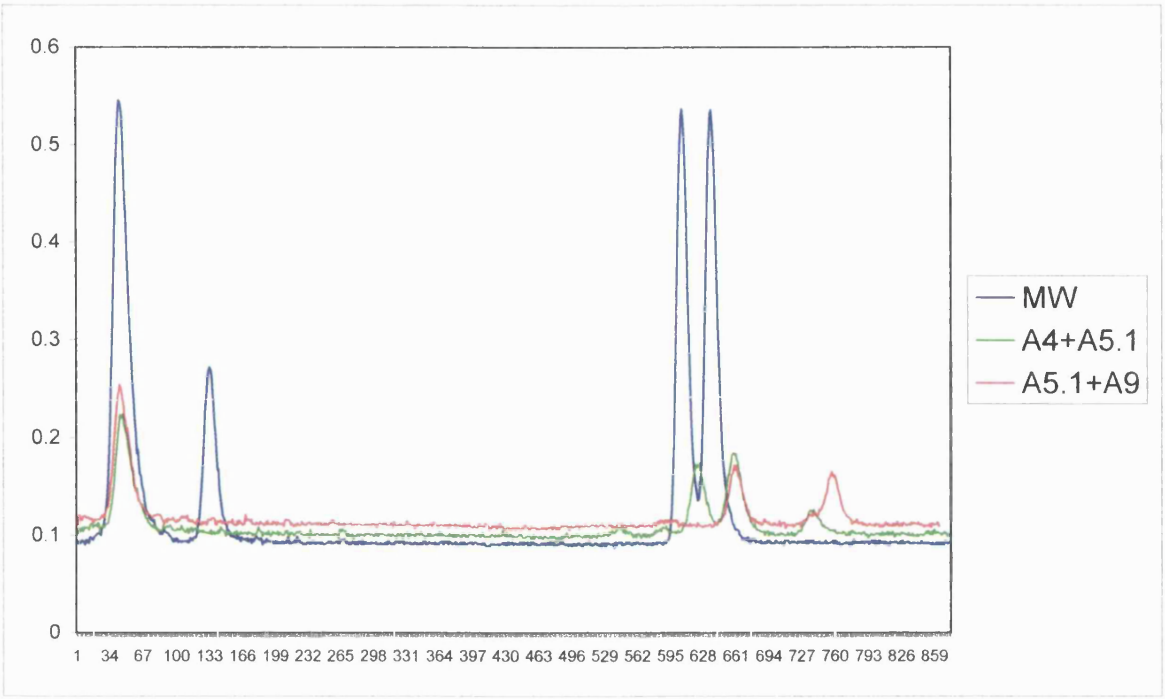
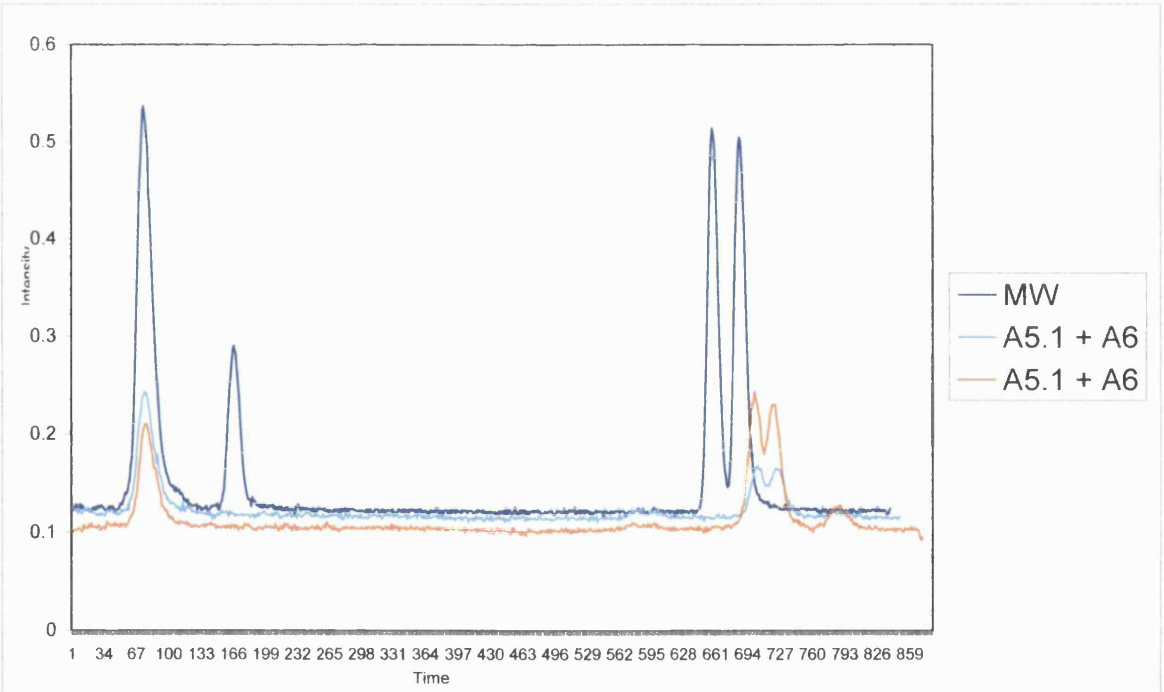


Figure 3.12. **Genescanner graph showing 2 patients who are both heterozygotes for MICA A5.1 and A6**



CHAPTER 4 POLYMORPHISMS OF EXONS 2,3 AND 4 OF MICA

4.1 Introduction

The major contribution to the high level of polymorphism within the MICA gene is from single nucleotide polymorphisms within exons 2,3 and 4 which encode the extracellular portion of the molecule. It is this part of the molecule which directly interacts with the NKG2D receptor on NK and T cells to influence the immune response. It is not known why such a high degree of polymorphism exists since, unlike classical MHC molecules, MICA does not appear to bind to a ligand. An ever increasing diversity of ligands that need to be presented to T cells is the clear driving force behind the extensive polymorphism within the MHC. The situation with MICA is less certain. It is known that certain polymorphisms within the region of MICA that binds to its receptor can influence the affinity of binding by up to 50 fold (138). It would not be unreasonable to assume that the affinity of binding may influence the ability of MICA to activate NK or T cells. Thus polymorphisms within the extracellular part of the molecule are potentially of great interest in disease association studies.

4.1.1 Polymorphisms of MICA

In the previous chapter it has been demonstrated that alleles of MICA that contain 9 GCT repeats in exon 5 are strongly associated with PsA and possibly psoriasis. However, there are several alleles of MICA that possess 9 GCT repeats in exon 5 which differ due to polymorphisms within exons 2, 3 and 4. The aim of this study is to devise a technique for the identification of the polymorphism within these extracellular exons and use this to identify any associations with PsA and psoriasis.

There are a total of 40 SNPs within exons 2,3 and 4 of MICA. The majority of these are non-synonymous, resulting in a change of amino acid. These SNPs, together with the microsatellite polymorphism in exon 5, define the 54 currently recognised MICA alleles. Recently, a number of new SNPs have been identified by nucleotide sequencing of subjects participating in genetic studies. However, the application of sequencing to a large number of samples in a case-control study is both time consuming and expensive. The

development of a technique using sequence-specific primers (PCR-SSP) would therefore be potentially advantageous.

4.1.2 PCR-SSP for MICA typing

Two publications have independently described methods for typing the 54 currently recognised alleles of MICA using PCR-SSP (117; 170). Both methods require all samples within the test population to be screened with a large number of primers before the appropriate MICA allele can be assigned. One of these studies is an update on a previously published method for typing the 16 alleles of MICA that were recognised at the time (116). The study utilised the 10^{th} International Histocompatibility Workshop cell panel (171) to verify accurate amplification with the newly designed primer pairs. However, it should be noted that positive examples of 22 of the 54 alleles were not available for analysis. Furthermore, the technique was not applied to a sample control or disease population. A large number of the primer pairs used in the technique therefore remain theoretical rather than proven identifiers of certain alleles. The described method uses a total of 71 sequence specific primers and 2 internal control primers to make 68 primer pair combinations that are used to test each sample. This allows the independent identification of all 54 MICA alleles. Primers were also used to identify the MICA/B deletion on the B*4801 haplotype and the presence of methionine at position 129 which is known to enhance binding affinity with the NKG2D receptor. Some heterozygote combinations would not be able to be distinguished with this technique, including MICA*002/*020, MICA*007/*026 and MICA*009/*049.

The second publication on high resolution MICA typing uses PCR-SSP to examine a population with inflammatory bowel disease (IBD) (117). The method was designed to investigate a possible association with the extracellular polymorphism of MICA and IBD as an association with the TM A4 allele of MICA had previously been demonstrated (157). The technique was applied to a large Caucasian population (577 patients with IBD and 354 controls). A total of 64 specific primers forming 56 primer pair combinations were used. These included one primer pair specific for the 100kb deletion on the B*4801 haplotype. Only 23 of the 54 alleles were actually detected in the patient and control

populations and, of these, only 13 occurred at a frequency of more than 1%. This represents one of the largest studies of MICA allele frequency in Caucasian populations ever published. It suggests that the majority of the more recently described alleles, many of which have only been reported in Japanese populations, are either not present or present at a very low frequency in Caucasians. Large population studies have yet to confirm the frequency of the MICA alleles in other populations.

The two methods described above provide invaluable reference material and are clearly necessary if an unusual allele is discovered. However, when studying complex genetic conditions such as IBD it is unlikely that any of the alleles occurring at a frequency less than 1% will be relevant. The majority of the more common alleles are those which can be detected by the simpler original PCR-SSP technique by Stephens et al (116), with the notable exceptions of MICA*017, *018 and *019. The presence of only a minority of the 54 possible MICA alleles in the Caucasian population vindicates the use of a simpler, less laborious system. Clearly, the presence of some of the rarer alleles could not be specifically excluded with a simplified scheme. However, the yield from routinely checking every sample in case control studies with such a large number of primer pairs is likely to be very low. It is also very time consuming, expensive and potentially wasteful of limited DNA resources. Since over 50% of the Caucasian population are known to possess at least one copy of MICA*008, it would make sense to approach the typing of MICA in a logical, step-wise fashion rather than routinely testing all samples for all possible alleles irrespective of how rare they may be.

4.2 Aims of the study

The aim of the current study was to produce a simple, more logical technique for typing MICA that could be applied to a large number of patient samples. A group-based, step-wise PCR-SSP method was developed. The groups were based on a phylogenetic analysis of the available nucleotide sequence of the MICA alleles. This segregated the alleles into evolutionarily similar groups enhancing the significance of alleles present on disease-associated haplotypes. Alleles within each group could then be further defined utilising a second stage PCR. Rarer alleles within each group would not be specifically excluded

with these primer combinations. However, a third stage PCR using the published primers that specifically recognise these rarer alleles could be applied if desired (117; 170).

4.3 Methods

The method utilised for the PCR-SSP technique is given in Chapter 2.4.2. A summary of the rationale for the method is presented here.

4.3.1 Phylogenetic analysis

The nucleotide sequence of the currently recognised alleles of MICA published in the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla/) was imported into the phylogenetic analysis programme MEGA (Molecular Evolutionary Genetic Analysis) (115). The analysis was performed on the sequence of exons 2,3 and 4 of MICA. The sequence of exon 5 (TM region) was omitted in the analysis as approximately half of the MICA alleles have not been sequenced along this exon. The defining polymorphic positions of the MICA alleles in exons 2,3,4 and 5 are illustrated in numerical order in *Figure 4.1.* This order reflects the current nomenclature of MICA but does not reflect the structural similarity of the alleles. The alleles are presented in phylogenetic order in *Figure 4.2.* following analysis of the alleles with the programme MEGA. The alleles of MICA then become arranged in an order which better reflects their structural and evolutionary similarity. The resultant amino acid sequence of the alleles once they are arranged in phylogenetic order is demonstrated in *Figure 4.4.*

4.3.2 Primer Selection

The phylogenetic order of the alleles defined by the MEGA analysis was then used to form the basis for the PCR-SSP typing system. The MICA alleles defined by the primers originally designed by Stephens et al were updated to take account of the new alleles that have now been described in the literature. The new primer specificities were then tabulated onto an excel spreadsheet (116). All possible primer combinations were then calculated, choosing those primer combinations that resulted in a PCR product between 100 and 1500 bp where possible to simplify future gel electrophoresis. Five primer pairs

that defined mutually exclusive phylogenetic groups of MICA alleles were chosen to form the initial PCR-SSP groups. Only one allele, MICA*044, was present in more than one group. Within each PCR-SSP group there were a few deviations from the order defined by the phylogenetic analysis but the majority were in agreement. The PCR-SSP groups (Groups 1-5) are shown in combination with the phylogenetic analysis of the MICA alleles in *Figure* 4.3.

4.3.3 PCR-SSP analysis

Stage One

All patient samples were screened in a first stage PCR with all 5 of the group-specific primer pairs. Full details of the primers used are given in *Table 2.1.* A sample could then be classified as containing two alleles from two different groups (eg. PCR products from Group 1 and Group 5) or two alleles from the same group (eg. PCR product only from Group 5). In the latter case, this could represent homozygosity for the same allele within Group 5 (eg. MICA *002 / *002) or heterozygosity for different alleles within Group 5 (eg. MICA *002/*017).

Stage Two

A second stage PCR was then designed to define the most common alleles within each primary group. The primer pairs used in this second stage were limited to those recognising alleles present at an expected frequency of >1%. Some of the rarer alleles within each group were therefore not specifically targeted (eg. MICA*041, MICA*042 etc). In the majority of cases, permutations of existing published primers could be used (116). Additional primers were designed to recognise the more frequent newly described alleles (MICA *017, *018, *019, *023, *027). These primers were designed to have similar annealing temperatures to the existing primers so that they could all be used in combination. Full details of the primers used are given in *Table 2.1.*

The presence of a particular allele was identified by the appearance of a PCR product band of the correct size (*Table 2.7*) using agarose gel electrophoresis as described in Chapter

2.4.2. All reactions were verified with the use of internal control primers that recognise a conserved sequence within the human growth hormone (HGH) gene. An algorithm for the MICA PCR-SSP typing scheme is given in *Figure 2.1.*

4.3.4 DNA for analysis

Genomic DNA was available from the 10th International Histocompatibility Workshop (IHW) cell reference panel. This DNA was used to verify the MICA primer specificities. Examples of the representatives of the MICA alleles from the 10th IHW are given in *Table 4.1.*

The PCR-SSP technique was applied to the same patient samples used for the analysis of the exon 5 microsatellite polymorphism: Type I Psoriasis (n=180), Psoriatic Arthritis (PsA) (n=158) and matched controls (n=148). All the Type I Psoriasis samples were from European Caucasians from the UK (n=49), Belgium (n=72), Germany (n=33) and Italy (n=26). The patients with Type I psoriasis from the UK were screened for the presence of occult arthritis as detailed before. The other 13IHW samples were not specifically screened for the presence of arthritis. The samples from patients with PsA and controls were both from the UK.

4.3.5 Statistical Analysis

Full details of the statistical methods used for analysis are given in Chapter 2.4.5. Allele frequencies (the fraction of chromosomes containing each allele) were determined for each disease group and the control group. Phenotype frequencies (the number of patients in possession of at least one allele) were also determined for each group. Associations between the alleles and the disease groups were determined with the Chi squared test with Fishers Exact test for small sample numbers. A Bonferroni correction factor was applied for multiple comparisons according to the number of MICA alleles identified (correction factor = 15). Any significant results were also expressed as Odds Ratios with 95% confidence intervals. Any continuous clinical data, such as PASI or tender and swollen joint counts were compared with the MICA alleles using the Mann Whitney U test. A *p*

value of <0.05 was considered to be statistically significant. Once again, it should be noted that there were no ethnically matched controls for the 13IHW patients from Europe and the results were compared with UK controls. These results should therefore be interpreted with caution.

The HLA associations with the MICA alleles were determined by analysis with the Arlequin programme (Chapter 2.4.5.2). This facilitated the construction of likely combinations of alleles into extended haplotypes. The frequency of the haplotypes within the patient population was also determined. Haplotype analysis could be performed on the 13IHW samples with Type I psoriasis that had full HLA typing. A further analysis of the HLA Class II associations with the MICA alleles could be performed on the patients with PsA who were all typed at the HLA-DRB1 and HLA-DQB1 loci. Unfortunately, full HLA Class I typing was not available for all patient and control groups. In these cases more simplified analysis was performed based on the presence or absence of HLA-Cw6 and HLA-B27. A stratification for the significance of MICA in the absence of HLA-Cw6 gave an indication of the degree of any independent association of the MICA alleles.

4.4 Results

4.4.1 Phylogenetic analysis

The results of the phylogenetic analysis of the MICA alleles are shown in *Figures 4.2 and 4.3*. There is considerable rearrangement of the order of the MICA alleles from the numerical order used in the current nomenclature (*Figure 4.1*). Interestingly, the alleles appear to fall into 2 groups, based largely on the presence of a sequence in exon 4. The significance of this sequence is not known. The amino acid sequences of the alleles in phylogenetic order (*Figure 4.4*) illustrates the dramatic effect on the TM segment of a single base deletion in alleles *015 and *017 and a single base insertion in the A5.1 alleles.

Further distinct groups were identified which were sometimes associated with the same TM region eg. *001, *012, *018 (all A4) but sometimes not, eg. *007 (A4), *045 (A4) and *026 (A6). The majority of the A9 alleles clustered together apart from *015 which appeared in a different group. The alleles containing 6 GCT repeats (A6) were the most diverse, forming 3 separate groups; *004, *006, *009, *044 and *049 all grouped together, whereas *011 and *047 were in a separate group and *026 clustered apart from the other A6 alleles. This would indicate independent evolution of the TM region from the rest of the gene.

4.4.2 Associations of the MICA alleles with the disease groups

Fifteen of the MICA alleles were present in the studied populations. The MICA alleles *005, *006, *013, *014, *015 were not present in any of the patients or controls in the study. Some of the alleles could not be distinguished from each other such as *019/*033, *027/*048, *023/*028 and were therefore each considered as a single allele for the purposes of the analysis. Allele subtypes such as *00201 and *00202 were regarded as one allele (*002).

4.4.3 MICA Phenotype frequencies

The MICA phenotype frequencies in PsA, Type I psoriasis and controls are illustrated in *Table 4.2* . The major results were:

- **MICA *008** was the most common allele in all groups, both disease and control, with phenotype frequencies between 60 and 74%.
- **MICA *002** was significantly associated with PsA (p_{uncorr} = 0.002, p_{corr} = 0.03, OR 2.32 (1.34-4.03)), with approximately twice the frequency in PsA than controls (31% vs 16%).
- **MICA *017** was significantly increased in Type I Psoriasis in the 13IHW population including all the samples from the UK, Belgium, Italy and Germany (p_{uncorr} <0.0001, p_{corr}<0.0015, OR 3.36 (1.74-6.48)) compared with controls.

- A breakdown of the different countries that contributed to the 13IHW on Type I psoriasis is given in *Table 4.3*. This illustrates that **MICA *017** was significantly increased in the screened UK and unscreened Belgian populations, as well as in the group of patients with Type I psoriasis as a whole. The numbers were small in the German and Italian populations, although the German group followed the same trends. The Italian population appeared to be an exception, although the number of patients was too small to draw any firm conclusions. Only 8% of Italian patients with Type I psoriasis were MICA *017 positive compared with 10% of controls.

4.4.4 MICA Allele frequencies

The allele frequencies of MICA in patients with PsA, Type I psoriasis and controls are given in *Table 4.4*. Some of the alleles could not be independently identified in the presence of others, for example, MICA*018 in the presence of MICA*007 or MICA*001. These have therefore been omitted from the analysis and constitute the remaining allele frequencies of 0.003 – 0.04 that are unaccounted for in *Table 4.4*. The MICA allele frequencies for the patients with Type I psoriasis from the UK and other European countries is given in *Table 4.5*.

The major results were:

- **MICA *002** was again found to be significantly increased in PsA (p_{uncorr}=0.0014, p_{corr}=0.021) with a resultant significant decrease in MICA *008 (p_{uncorr}=0.0016, p_{corr}=0.024).
- **MICA *017** was significantly increased in the Type I psoriasis group as a whole (p_{uncorr}<0.0001, p_{corr}<0.0015) as well as individually in the screened UK and unscreened Belgian populations (p_{corr}=0.012 and p_{corr}<0.0015, respectively). In the Belgian group, the increase in MICA *017 resulted in a significant decrease in MICA *008 (p_{corr}=0.02).

4.4.5 Associations of the MICA alleles by phylogenetic group

Examples of the agarose electrophoresis gels from analysis within Groups 1 and 4 are shown in *Figures 4.5 and 4.6.* If the MICA alleles are divided according to the five groups that are defined by the phylogenetic analysis, certain patterns emerge. The distribution of MICA alleles within each patient and phylogenetic group is shown in *Table 4.6.* The *p values* were corrected by a factor of five in view of the five phylogenetic groups.

In both psoriasis and PsA there was an increase in the number of alleles within **Group 5** and a compensatory decrease in the number of alleles in Group 1. This is largely due to an increase in MICA *017 (Group 5) in psoriasis, MICA *002 (Group 5) in PsA and a fall in MICA *008 (Group 1) in both groups.

4.4.6 Associations of the MICA alleles with clinical characteristics

PsA

The increase in MICA *002 that was observed in the PsA group as a whole was also observed in the polyarthritis, oligoarthritis and spondyloarthritis subgroups. There was no significant association between any of the MICA alleles and the clinical subgroup of PsA that a patient belonged to. There was no association between the MICA alleles and the *severity* of PsA as indicated by the presence of erosions, DMARD use, PASI or tender and swollen joint counts in PsA. There were no associations with the presence or severity of nail disease.

Type I psoriasis

MICA *017 was significantly associated with the presence of a high nail score ($p=0.002$). MICA *017 was also significantly associated with a younger age of onset of psoriasis ($p<0.0001$). There were no associations with the severity of psoriasis itself as represented by the PASI score.

4.4.7 Stratification for HLA-Cw6

PsA

In order to test whether the association between MICA *002 and PsA was independent of HLA-Cw6, all HLA-Cw6 patients and controls were eliminated from the analysis. MICA *002 was then present in 32/93 (34%) patients with PsA compared with 22/122 (18%) controls. This result was still significant with a *p value* of 0.006, suggesting that the association between MICA *002 and PsA is independent of HLA-Cw6.

Type I psoriasis

In contrast, if the HLA-Cw6 positive patients were omitted from the analysis of the patients with Type I psoriasis (both UK and Belgian), the association with MICA *017 was no longer significant.

4.4.8 Associations between the MICA alleles and the HLA Class II alleles

Two of the MICA alleles were found to be significantly associated with HLA-DR7 in the patients with PsA. MICA *004 was associated with HLA-DR7 with a *p value* of 0.006, OR 4.3 (1.41-13.1). MICA *017 was strongly associated with HLA-DR7 ($p<0.0001$, OR 10.03 (3.76-26.78), due to its presence on the highly conserved 57.1 ancestral haplotype. There were no associations with the other HLA Class II alleles or the HLA-DRB1 shared epitope.

4.4.9 MICA Haplotypes from 10th workshop / 4AOH cell lines

The data available on the MICA haplotypes of homozygous cell lines from the 10th International Histocompatibilty Workshop (10IHW) was collated from published work (171) and is presented in *Table 4.7*. Many of the MICA alleles were present on conserved ancestral haplotypes or recombinant ancestral haplotypes. Confirmation of the haplotypes was determined by placing the data into the Arlequin programme. These reference haplotypes were utilised to define the haplotypes observed in the psoriasis population.

4.4.10 MICA Haplotypes in Type I Psoriasis

Data was available on the HLA typing at the Class I and II loci for the Type I psoriasis group from Belgium taking part in the 13IHW. HLA typing on the PsA, psoriasis and control samples from the UK and the other workshop samples is awaiting completion by the 13IHW in Seattle.

The MICA haplotypes that were observed in the Belgian population with Type I psoriasis are presented in *Table 4.8.* The frequency of the haplotypes together with estimations of the linkage disequilibrium between the components of the haplotype (D') are given. Some of the MICA alleles dispay a high degree of linkage disequilibrium with the HLA Class I and to a lesser extent Class II alleles indicating conservation of the haplotype through time. Some examples of strong linkage disequilibrium (D'=1) are:

MICA *002 with HLA-B3801, HLA-B3901
MICA *004 with HLA-B4901 and HLA-B4102
MICA *007 with HLA-B2702, HLA-B2705
MICA *008 with HLA-B702, HLA-B1302, HLA-B40011, HLA-B4402
MICA *010 with HLA-B1501
MICA *011 with HLA-B1402
MICA *012 with HLA-B5501
MICA *017 with HLA-B5701, HLA-Cw0602
MICA *018 with HLA-B1801

These MICA haplotypes are represented in diagrammatic form in *Figures 4.7-4.11* which illustrate some of the clear linkage patterns between the MICA, HLA-B and HLA-Cw alleles.

MICA A4 haplotypes (Figure 4.7.)

Fifty percent of the haplotypes containing 4GCT repeats were MICA*007 and all but one of these was found in association with HLA-B27 (the other was found with B51-Cw15). Although the overall increase in the A4 allele in the PsA population just failed to reach

significance, the contribution of *007 was more difficult to elucidate due to some of the difficulties distinguishing *007 and *018. The other haplotypes observed in Type I psoriasis were *018-B18-Cw7, *018-B18-Cw12 (AH18.1), *001-B18-Cw5 (AH18.2),*012-B55-Cw3 (AH55.1), *012-B56-Cw1 and *012-B55-Cw6.

MICA A5 haplotypes (Figure 4.8.)

The majority (10 out of 22) of the haplotypes containing 5GCT repeats were *010-B15-Cw3 (AH62.1). The other haplotypes observed were *016-B35-Cw4 (AH35.4/5), *027/048-B61-Cw2, *027/048-B61-Cw3, *016-B35-Cw6, *019/033-B15-Cw8 and *010-B15-Cw2.

MICA A5.1 haplotypes (Figure 4.9.)

All of the alleles which contained 5GCT repeats with a G insertion were typed as MICA *008. The most common haplotypes were *008-B13-Cw6 (AH13.1), *008-B7-Cw7 (AH7.1), *008-B8-Cw7 (AH8.1), *008-B44-Cw5 (AH44.1), *008-B44-Cw2, *008-B44-Cw7, *008-B37-Cw6 (AH37.1), *008-B47-Cw6 (AH47.1) and *008-B40-Cw3 (AH60.1/3).

MICA A6 haplotypes (Figure 4.10.)

There were fewer clearly dominant haplotypes and fewer recognised ancestral haplotypes seen with the MICA A6 alleles perhaps reflecting the disparate evolutionary distribution seen in the phylogenetic tree. The most frequent alleles were MICA *004 (10/26) and MICA *006 (10/26). Some examples of the haplotypes which emerged were *004-B44-Cw16 (AH44.2/3), *004-B44-Cw4, *004-B49-Cw7, *004-B42-Cw17 (AH42.1), *004-B41-Cw17, *006-B51-Cw15, *006-B35-Cw4, *006-B52-Cw12 (AH52.1), *006-B51-Cw15, *006/9/49-B45-Cw6, *006/9/49-B50-Cw6 (AH50.1) and *011-B14-Cw8 (AH65.1).

MICA A9 haplotypes (Figure 4.11.)

The two MICA alleles with 9GCT repeats (A9 alleles) observed in the Belgian Type I psoriasis population were *002 and *017. The majority of patients who had an A9 allele were typed as MICA*017. This contrasts with the PsA population where the majority of patients with an A9 allele were typed as MICA *002.

In all 25 patients with Type I psoriasis and MICA *017, this allele was present on the AH 57.1 haplotype (HLA-B57, HLA-Cw6) which is reflected in the high D' values between MICA *017, HLA-B5701 and HLA-Cw6. One patient was HLA-B57 positive but had a MICA *002 not a MICA *017 allele. However, this was present on a different haplotype (B57, Cw7) and not the AH57.1 haplotype. Other haplotypes which were seen included *002-B38-Cw12 (AH38.1), *002-B39-Cw12, *002-B35-Cw4, *002-B58-Cw7 and *002-B58-Cw3.

MICA and HLA-DRB1 and HLA-DQB1 haplotypes in the PsA population

The data is not yet available for the HLA Class I typing for the PsA patients and this can therefore not be presented as haplotype data. However, the patients have been fully typed at the HLA-DRB1 and HLA-DQB1 Class II loci. The data was therefore entered into the Arlequin programme to elicit haplotype frequencies. D' values were calculated to obtain an estimate of the degree of linkage disequilibrium between the loci. The data is presented in Table 4.9. As would be expected from the higher genetic distance between the alleles, the D' values are generally lower than those seen with HLA-B and HLA-Cw. Higher numbers of patients within each haplotype would be needed before any definitive conclusions could be made about clear haplotypic patterns.

4.5 Discussion

There are two alleles of MICA that have been shown to be important in PsA and psoriasis. The MICA *002 allele was increased in frequency in PsA, whereas the MICA *017 allele was increased in Type I psoriasis. Both of these alleles contain 9 GCT repeats in their 5th exon and are therefore A9 alleles. The increase in MICA A9 that has been previously

demonstrated is therefore due to these two alleles. Some of the difficulties interpreting the relative contributions of MICA A9 in PsA compared with psoriasis may be explained by the different contributions of these two alleles.

The MICA alleles appear to be responsible for the *susceptibility* to rather than *severity* of PsA. There was no association with disease subgroup, tender and swollen joint counts or PASI.

The differences between the MICA *002 and *017 alleles are two-fold. Firstly a SNP at position 272 results in a change of amino acid at position 91. This is in the centre of the α1α2 platform that interacts with the NKG2D receptor (*Figure 3.1*). A change of amino acid could significantly affect the affinity of the binding of MICA for its receptor, although this has not been formally tested with functional studies. The second change is the deletion of a guanine at position 823, at the very end of exon 4 in the MICA *017 allele that does not occur in MICA *002. This results in a frame shift and a completely different transmembrane region in the MICA *017 allele. Instead of 9 alanines, the MICA *017 molecule has a hydrophobic polyleucine tail with truncation of the cytoplasmic segment. This could have significant effects on the function of the molecule. It could severely hamper the ability of the molecule to be expressed on the cell surface. The true functional effects of these polymorphisms are not known but both of the differences in the genetic sequence between MICA *002 and *017 could potentially result in significant changes of function of the molecule.

The association between MICA *002 and PsA is largely independent of HLA-Cw6. The majority of the haplotypes that contain MICA *002 do not contain HLA-Cw6. It may be that haplotypes containing MICA *002 (B38-Cw12 (AH38.1), B38-Cw6, B39-Cw12, B35-Cw4, B58-Cw6 and B58-Cw3) contain genes that are important in the development of arthritis or that MICA itself is the key gene. It is interesting to note that the frequency of HLA-B38 and HLA-B39 has been reported to be higher in patients with the peripheral polyarthritis subgroup of PsA.

In contrast to MICA *002, there is a strong relationship between MICA *017 and HLA-Cw6, with the vast majority of MICA *017 alleles being present on the 57.1 ancestral haplotype. This would suggest that the association between MICA *017 and Type I psoriasis is not unique to the MICA region of the haplotype, but is an association with the 57.1 ancestral haplotype itself. The true association may therefore be any one of the closely linked neighbouring genes along the haplotype. It is also intriguing that the 57.1 haplotype is frequently found in long term non-progressors in human immunodeficiency virus type I (HIV-I) infection (83; 84). This association may be related to the MICA *017 allele which could alter the immune response to the virus by a change in structure and recognition by NKG2D receptors on NK and T cells. Whether MICA is a strong functional candidate in psoriasis would need to be investigated with further expression and functional studies.

This group-based PCR-SSP method for typing the most commonly occurring alleles of MICA is a rapid and efficient way of screening disease populations for possible associations with the MICA alleles. It is particularly suitable for Caucasian populations, where more is known about the frequencies of the MICA alleles. The study by Ahmad et al (117) demonstrated in a large UK population that only 13 of the currently recognised 54 MICA alleles are present at a frequency greater than 1% and less than half of the alleles were present at all. It would therefore seem reasonable to approach MICA typing, at least in the initial stages, in a more efficient way than routinely testing all samples for the presence of all alleles with up to 70 primer pairs per sample.

The method described here is particularly suited to disease association studies where the transmembrane region (exon 5 microsatellite) has already been typed. Indeed, certain alleles can only be distinguished with knowledge of the TM region. This can also act as a check for the system since knowledge of the TM allele allows one to predict which group an allele is likely to belong to (eg. all A9 alleles are in group 5). The system picks up most of the common alleles by using an average of only 11-13 primer pairs per sample, although certain combinations of alleles cannot be distinguished. It is inexpensive in terms of patient material, reagents, time and equipment. However, as with all step-wise systems, one must be particularly aware of false negatives. The use of internal controls should

prevent this, but there is no doubt that the use of such a simplified system will occasionally produce errors. However, if an allele is truly associated with a disease, this should be strong enough to withstand such potential problems. Knowledge of the TM alleles is particularly helpful in checking the correct allocation of the initial PCR groups, particularly where both alleles appear to be in the same group. The inclusion of known positives and negatives in each PCR run will also ensure good quality control.

The development of a typing technique based on the phylogenetic similarity of the known alleles of MICA provides a more meaningful approach to the definition of disease associated alleles and their haplotypes. The current nomenclature does not provide any insight into which alleles are more closely related through evolution than others. Distinct groups of alleles were identified which were sometimes associated with the same transmembrane region and sometimes not. The majority of the alleles with 9GCT repeats clustered together except *015 which appeared in a separate group. This allele has the same exon 4 deletion as *017 but was not seen in any of the samples studied in this workshop. The alleles with 6GCT repeats were the most disparate, although alleles *004, *006, *009, *044 and *049 were all very closely related.

The phylogenetic clustering of the alleles that were significantly associated with psoriasis (MICA *017) and PsA (MICA *002) suggests a common ancestral link between the two conditions. This is most likely to be a genetic factor that predisposes to psoriasis. This should be common to both haplotypes, either within the MICA gene itself (they both possess 9 GCT repeats in the transmembrane region of the gene) or further along the haplotype in a neighbouring gene. The two diseases, psoriasis and PsA, have then diverged as evidenced by the different alleles of MICA that are associated with each phenotype. MICA is known to play a direct role in the activation of T cells and NK cells via the NKG2D receptor. Both of these immune cells are present in the skin and synovium and appear to play a key role in the inflammatory process. The disease associations with MICA may therefore be primary rather than simply a marker for a disease gene present on the same haplotype.

This method allows the rapid detection of the majority of MICA alleles that are present at a frequency of at least 1% in Caucasian populations. This should be sufficient for screening disease groups to see if any patterns emerge, particularly where an association with the less complex TM region of the gene has already been demonstrated. Further, more detailed, analysis can then be performed if any significant associations appear. Since the technique utilises a phylogenetic analysis, the grouping of the alleles is based more on evolutionary similarity than the current nomenclature of MICA. This may facilitate not only the recognition of disease associated alleles but also potential disease associated haplotypes.

Table 4.1. **Reference DNA panel for MICA typing**

MICA allele	MICA TM allele	Cell ID	4AOH number	10 IHW number	Ancestral haplotype
*001	A4	EVA SP	100051Y	9135	18.2
*001	A4	DUCAF	100161R	9019	18.2
*00201	A9	YAR	100009X	9026	38.1
*004	A6	MOU	100058H	9050	44.3
*004	A6	RSH	100007B	9021	42.1
*006	A6	KAS116	100154N	9003	51
*00701	A4	BM92	100201F	9092	51
*00801	A5.1	REE,GD	100044V	9132	8.1
*00801	A5.1	PLH	100064P	9047	47.1
*00901	A6	HARA	100065M	9142	52.1
*010	A5	BSM	100073N	9032	62.1
*010	A5	BOLETH	100072Q	9031	62.1
*011	A6	LWAGS	100002N	9079	65.1
*01201	A4	HOKKAIDO	100062T	9141	54.1
*015	A9	OMW	100204Z	9058	45
*016	A5	JO528239	100019U	9041	35.1
*017	A9	DBB	100084G	9052	57.1
*018	A4	D0208915	100050A	9008	18.1
*019	A5	CF996	100077D	9094	64.1

Table 4.2. **Phenotype frequencies (%) of the MICA alleles in Type I Psoriasis, PsA and Controls**

Allele	Psoriasis Type I (13 IHW) (n=131)	Psoriasis Type I (UK) (n=49)	PsA (UK) (n=158)	Controls (UK) (n=148)
*001	4 (3%)	1 (2%)	6 (4%)	4 (3%)
*002	29 (22%)	1 (2%)	**49 (31%)**[1]	24 (16%)
*004	14 (11%)	7 (14%)	17 (11%)	23 (16%)
*007	17 (13%)	1 (2%)	16 (10%)	10 (7%)
*007/018	2 (2%)	2 (4%)	11 (7%)	1 (1%)
*008	79 (60%)	35 (71%)	96 (61%)	109 (74%)
*009	24 (18%)	11 (22%)	30 (19%)	30 (20%)
*010	8 (6%)	5 (10%)	8 (5%)	19 (13%)
*011	3 (2%)	-	1 (1%)	-
*012	7 (5%)	2 (4%)	3 (2%)	5 (3%)
*016	10 (8%)	-	3 (2%)	5 (3%)
*017	**36 (27%)**[2]	**15 (31%)**[3]	29 (18%)	15 (10%)
*018	7 (5%)	1 (2%)	13 (8%)	7 (5%)
*019/033	1 (1%)	-	4 (3%)	2 (1%)
*023/028	1 (1%)	1 (2%)	1 (1%)	1 (1%)
*027/048	4 (3%)	-	2 (1%)	3 (2%)

[1] $p_{uncorr}=0.002$, $p_{corr}=0.03$, OR 2.32 (1.34-4.03) compared with controls

[2] $p_{uncorr}<0.0001$, $p_{corr}<0.0015$, OR 3.36 (1.74-6.48) compared with UK controls

[3] $p_{uncorr}=0.001$, $p_{corr}=0.015$, OR 3.91 (1.74-8.78) compared with controls

NB No ethnically matched control group for the 13IHW patients

Table 4.3. **Phenotype frequencies of MICA in Type I psoriasis from the European countries contributing to the 13th IHW**

Allele	Psoriasis Type I (UK) (n=49)	Psoriasis Type I (Belgium) (n=72)	Psoriasis Type I (Germany) (n=33)	Psoriasis Type I (Italy) (n=26)	Psoriasis Type I (Total) (n=180)	Controls (UK) (n=148)
*001	1 (2%)	1 (1%)	-	3 (12%)	5 (3%)	4 (3%)
*002	1 (2%)	13 (18%)	8 (24%)	8 (31%)	30 (17%)	24 (16%)
*004	7 (14%)	6 (8%)	4 (12%)	4 (15%)	21 (12%)	23 (16%)
*007	1 (2%)	8 (11%)	6 (18%)	3 (12%)	18 (10%)	10 (7%)
*007/018	2 (4%)	-	2 (6%)	-	4 (2%)	1 (1%)
*008	35 (71%)	43 (60%)	20 (61%)	16 (62%)	114 (63%)	109 (74%)
*009	11 (22%)	11 (15%)	8 (24%)	5 (19%)	35 (19%)	30 (20%)
*010	5 (10%)	7 (10%)	1 (3%)	-	13 (7%)	19 (13%)
*011	-	2 (3%)	-	1 (4%)	3 (2%)	-
*012	2 (4%)	4 (6%)	1 (3%)	2 (8%)	9 (5%)	5 (3%)
*016	-	6 (8%)	1 (3%)	3 (12%)	10 (6%)	5 (3%)
*017	**15 (31%)[1]**	**26 (36%)[2]**	8 (24%)	2 (8%)	**51 (28%)[3]**	15 (10%)
*018	1 (2%)	6 (8%)	-	1 (4%)	8 (4%)	7 (5%)
*019/033	-	1 (1%)	-	-	1 (1%)	2 (1%)
*023/028	1 (2%)	-	1 (3%)	-	2 (1%)	1 (1%)
*027/048	-	4 (6%)	-	-	4 (2%)	3 (2%)

[1] $p_{uncorr}=0.001$, $p_{corr}=0.015$, OR 3.91 (1.74-8.78) compared with controls

[2] $p_{uncorr}<0.0001$, $p_{corr}<0.0015$, OR 5.01 (2.44-10.28) compared with UK controls

[3] $p_{uncorr}<0.0001$, $p_{corr}<0.0015$, OR 5.01 (2.44-10.28) compared with UK controls

NB. No ethnically matched control group for the 13IHW patients

Table 4.4. **MICA Allele frequencies in Type I Psoriasis, PsA and Controls**

MICA Allele	Type I Psoriasis (13IHW) (n=180)	Psoriatic Arthritis (UK) (n=158)	Controls (UK) (n=148)	Total All samples (n=486)
*008	0.386	**0.367**[1]	0.493	0.413
*002	0.092	**0.168**[2]	0.081	0.113
*017	**0.147**[3]	0.092	0.051	0.100
*009	0.100	0.098	0.101	0.100
*004	0.058	0.057	0.078	0.064
*007	0.050	0.051	0.034	0.045
*010	0.036	0.025	0.064	0.041
*018	0.022	0.044	0.024	0.030
*016	0.028	0.009	0.017	0.019
*012	0.025	0.009	0.017	0.017
*001	0.014	0.019	0.014	0.015
*027 / *048	0.011	0.006	0.010	0.009
*019 / *033	0.003	0.013	0.010	0.008
*023 / *028	0.006	0.003	0.003	0.004
*011	0.008	0.003	-	0.004
Total	0.986	0.960	0.977	0.982

[1] $p_{uncorr}=0.0016$, $p_{corr}=0.024$ compared with controls

[2] $p_{uncorr}=0.0014$, $p_{corr}=0.021$ compared with controls

[3] $p_{uncorr}<0.0001$, $p_{corr}<0.0015$ compared with UK controls

NB. No ethnically matched control group for the 13IHW patients

Table 4.5. **MICA Allele frequencies in Type I Psoriasis from the European countries contributing to the 13th IHW**

MICA Allele	Type I Psoriasis (UK) (n=49)	Type I Psoriasis (Belgium) (n=72)	Type I Psoriasis (Germany) (n=33)	Type I Psoriasis (Italy) (n=26)	Type I Psoriasis (Total) (n=180)
*008	0.500	**0.330**[1]	0.364	0.346	0.386
*017	**0.163**[2]	**0.188**[3]	0.121	0.039	**0.147**[4]
*009	0.112	0.076	0.136	0.096	0.100
*002	0.020	0.090	0.136	0.173	0.092
*004	0.071	0.042	0.061	0.077	0.058
*007	0.010	0.056	0.091	0.058	0.050
*010	0.051	0.049	0.015	-	0.036
*016	-	0.042	0.015	0.058	0.028
*012	0.020	0.028	0.015	0.039	0.025
*018	0.010	0.042	-	0.019	0.022
*001	0.010	0.007	-	0.058	0.014
*027 / *048	-	0.028	-	-	0.011
*011	-	0.014	-	0.019	0.008
*023 / *028	0.010	-	0.015	-	0.006
*019 / *033	-	0.007	-	-	0.003
Total	0.977	0.999	0.969	0.982	0.986

[1] $p_{uncorr}=0.0015$, $p_{corr}=0.02$ compared with UK controls

[2] $p_{uncorr}=0.0008$, $p_{corr}=0.012$ compared with controls

[3] $p_{uncorr}<0.0001$, $p_{corr}<0.0015$ compared with UK controls

[4] $p_{uncorr}<0.0001$, $p_{corr}<0.0015$ compared with UK controls

NB. No ethnically matched control group for the 13IHW patients

Table 4.6. **The number of alleles (%) within each of the phylogenetic groups in Type I Psoriasis, PsA and controls**

	Group 1	Group 2	Group 3	Group 4	Group 5	Total
Type I Psoriasis (n=180)	**167(46%)**[1]	59(16%)	-	45(13%)	**89(25%)**[2]	360
PsA (n=158)	**133(42%)**[3]	50(16%)	-	50(16%)	**83(26%)**[4]	316
Controls (n=148)	176(59%)	54(18%)	-	27(9%)	39(13%)	296

[1] p_{uncorr}=0.0008, p_{corr}= 0.004 compared with UK controls

[2] p_{uncorr}=0.0002, p_{corr}= 0.001 compared with UK controls

[3] p_{uncorr}<0.0001, p_{corr}<0.0005 compared with controls

[4] p_{uncorr}<0.0001, p_{corr}<0.0005 compared with controls

NB No ethnically matched control group for the 13IHW Type I psoriasis patients

Table 4.7. **MICA Haplotypes observed in homozygous cell lines from the 10th International Workshop cells**

MICA TM allele	MICA allele	HLA-B	HLA-C	HLA-A	Ancestral Haplotype	10th IHW Cell
A4	*001	B*1801	Cw*0501	A*3002	18.2	DUCAF
A4	*001	B*1801	Cw*0501	A*0201	18	JVM
A4	*007	B*2705	Cw*0102	A*0201		JESTHOM
A4	*007	B*2705	Cw*0202			
A4	*007	B*5101	Cw*0102		51	BM92
A4	*012	B*5401	Cw*0102	A*2402	54.1	LKT3
A4	*012	B*5501	Cw*0303		55.1	
A4	*012	B*5601	Cw*0102			
A4	*018	B*1801	Cw*0701			BM16
A4	*018	B*1801	Cw*1203	A*2501	18.1	DO208915
A5	*010	B*1501	Cw*0303	A*0101	62.3	CB6B
A5	*010	B*1501	Cw*0303	A*0217	15	AMALA
A5	*010	B*1501	Cw*0304	A*0201	62.1	BOLETH
A5	*010	B*3701	Cw*0602	A*0101	37.1	KAS011
A5	*010	B*4601	Cw*0102	A*0204	46.2	TAB089
A5	*010	B*4601	Cw*0102	A*0207	46.1	T7526
A5	*010	B*5201	Cw*1202	A*0101	52	E4181324
A5	*016	B*3502	Cw*0401	A*0101	35.1	JO528239
A5	*019	B*1401	Cw*0802		64.1	CF996
A5	*019	B*3801	Cw*1203			
A5	*027/*048	B*4002	Cw*0202		40	CALGERO
A5.1	*008	B*0702	Cw*0702	A*0301	7.1	SCHU
A5.1	*008	B*0702	Cw*0702	A*2402	7.2	SA
A5.1	*008	B*0801	Cw*0701	A*0101	8.1	COX
A5.1	*008	B*1302	Cw*0602	A*0201	13	BER
A5.1	*008	B*1302	Cw*0602	A*3001	13.1	LBF
A5.1	*008	B*4001	Cw*0304	A*0201	60.2	SLE005
A5.1	*008	B*4001	Cw*0304	A*2402	60	DKB
A5.1	*008	B*4001	Cw*0304	A*6801	60.3	
A5.1	*008	B*4402	Cw*0501	A*0201	44.1	WELAK
A5.1	*008	B*4701	Cw*0602	A*0301	47.1	PLH
A5.1	*023	B*3801	Cw*1203			
A5.1	*028	B*4001	Cw*0304			

A6	*004	B*4101	Cw*1701	A*0101	41	BM21
A6	*004	B*4201	Cw*1701	A*0101	42.1	RSH
A6	*004	B*4403	Cw*1403	A*3303	44.4	
A6	*004	B*4403	Cw*1601	A*2902	44.2	RITOUT
A6	*004	B*4901	Cw*0701	A*0101	49	BM15
A6	*006	B*5101	Cw*1203	A*2402	51	KAS116
A6	*009	B*3503	Cw*0401			
A6	*009	B*5001	Cw*0602			
A6	*009	B*5101	Cw*1502	A*0204	51	RML
A6	*009	B*5101	Cw*1502	A*3101	51	JHAF
A6	*009	B*5201	Cw*1202	A*0101	52.1	E4181324
A6	*011	B*1402	Cw*0802	A*0301	14	H0301
A6	*011	B*1402	Cw*0802	A*3301	65.1	LWAGS
A6	*026	B*2705	Cw*0102			
A6	*049	B*5101	Cw*1402	A*0201	51	LUY
A9	*002	B*3501	Cw*0401	A*0201	35	BM9
A9	*002	B*3801	Cw*1203	A*2601	38.1	YAR
A9	*002	B*3801	Cw*1203	A*6601	38	TEM
A9	*002	B*5301	Cw*0401	A*6802		AMAI
A9	*002	B*5801	Cw*0701			
A9	*015	B*4501	Cw*1601	A*0201	45	OMW
A9	*017	B*3501	Cw*0401			
A9	*017	B*3508	Cw*0401	A*2402	35.4	TISI
A9	*017	B*5701	Cw*0602	A*0201	57.1	DEM
A9	*017	B*5701	Cw*0701	A*0201	57	WJR076

***Table 4.8*. The MICA haplotypes present within the 131HW Type I Psoriasis population demonstrating the haplotype frequencies and D' values**

	Haplotype Freq	HLA-A	HLA-Cw	HLA-B	MICA	HLA-DRB1	HLA-DQB1
	0.03488	A3	Cw1203	B3801	MICA*002	DR1	DQ501
D'		0.02	0.87	1		0.49	0.39
	0.02326	A2	Cw1203	B3801	MICA*002	DR13	DQ603
D'		0.5	0.87	1		0.33	0.42
	0.01163	A2	Cw1203	B5701	MICA*002	DR1303	DQ301
D'		0.5	0.87	0.14		1	0.11
	0.01163	A2	Cw702	B5701	MICA*002	DR7	DQ301
D'		0.5	0.03	0.14		_	0.11
	0.01163	A2	Cw802	B3901	MICA*002	DR1	DQ402
D'		0.5	_	1		0.49	_
	0.01163	A29	Cw702	B702	MICA*002	DR14	DQ503
D'		0.26	0.03	_		0.07	0.11
	0.01163	A1	Cw702	B4901	MICA*004	DR13	DQ202
D'		0.16	0.23	1		_	1
	0.01163	A24	Cw0701/06	B4901	MICA*004	DR13	DQ604
D'		0.63	0.28	1		_	_
	0.01163	A24	Cw1701/02	B4102	MICA*004	DR7	DQ303
D'		0.63	1	1		1	_
	0.01163	A1	Cw0303/04	B4002	MICA*007	DR13	DQ503
D'		_	0.03	_		_	0.13
	0.01163	A2	Cw0701/06	B2702	MICA*007	DR4	DQ502
D'		0.21	0.09	1		0.68	_
	0.01163	A2	Cw1601	B5701	MICA*007	DR15	DQ301
D'		0.21	1	_		_	0.47
	0.01163	A3	Cw602	B5701	MICA*007	DR4	DQ502
D'		0.03	_	_		0.68	_

	0.01163	A31	Cw602	B2705	MICA*007	DR4	DQ302
D'		0.27	_	1		0.68	0.46
	0.01163	A32	Cw102	B4402	MICA*007	DR1	DQ501
D'		_	_	_		_	_
	0.01163	A32	Cw1502	B3508	MICA*007	DR11	DQ301
D'		_	1	_		_	0.47
	0.03488	A2	Cw602	B1302	MICA*008	DR7	DQ202
D'		0.14	0.14	1		0.15	0.3
	0.01163	A11	Cw0303/04	B40011/12	MICA*008	DR13	DQ603
D'		_	0.19	1		0.3	0.36
	0.01163	A2	Cw202	B4402	MICA*008	DR7	DQ202
D'		0.14	0.12	1		0.15	0.3
	0.01163	A2	Cw702	B5701	MICA*008	DR11	DQ301
D'		0.14	0.45	_		_	_
	0.01163	A2	Cw702	B702	MICA*008	DR15	DQ303
D'		0.14	0.45	1		0.49	_
	0.01163	A2	Cw702	B702	MICA*008	DR15	DQ602
D'		0.14	0.45	1		0.49	0.25
	0.01163	A23	Cw702	B5701	MICA*008	DR16	DQ502
D'		_	0.45	_		0.31	0.21
	0.01163	A24	Cw501	B2705	MICA*008	DR4	DQ301
D'		_	_	_		0.06	_
	0.01163	A24	Cw602	B2705/10	MICA*008	DR14	DQ604
D'		_	0.14	_		_	1
	0.01163	A24	Cw702	B702	MICA*008	DR10	DQ501
D'		_	0.45	1		1	0.07
	0.01163	A25	Cw0701/06	B1801/05	MICA*008	DR15	DQ201
D'		_	_	_		0.49	0.56
	0.01163	A25	Cw102	B1302	MICA*008	DR7	DQ602
D'		_	0.12	1		0.15	0.25

	0.01163	A28	Cw602	B4402	MICA*008	DR16	DQ302
D'		_	0.14	1		0.31	_
	0.01163	A28	Cw602	B4402/03	MICA*008	DR4	DQ302
D'		_	0.14	1		0.06	_
	0.01163	A28	Cw704	B4402	MICA*008	DR16	DQ502
D'		_	1	1		0.31	0.21
	0.01163	A3	Cw1203	B3801	MICA*008	DR15	DQ602
D'		0.58	0.2	_		0.49	0.25
	0.01163	A3	Cw602	B1302	MICA*008	DR13	DQ603
D'		0.58	0.14	1		0.3	0.36
	0.01163	A3	Cw702	B702	MICA*008	DR15	DQ303
D'		0.58	0.45	1		0.49	_
	0.01163	A32	Cw0701/06	B4501	MICA*008	DR4	DQ201
D'		0.56	_	_		0.06	0.56
	0.01163	A1	Cw1203	B1501	MICA*009	DR15	DQ301
D'		0.23	0.02	_		0.14	0.07
	0.01163	A1	Cw602	B801	MICA*009	DR3	DQ301
D'		0.23	_	_		0.27	0.07
	0.01163	A2	Cw501	B4501	MICA*009	DR7	DQ202
D'		0.08	0.46	1		0.27	0.08
	0.01163	A2	Cw702	B51011	MICA*009	DR15	DQ302
D'		0.08	0.17	0.64		0.14	0.18
	0.01163	A24	Cw602	B5701	MICA*009	DR1	DQ303
D'		0.02	_	_		0.07	_
	0.01163	A30	Cw602	B51011	MICA*009	DR15	DQ202
D'		_	_	0.64		0.14	0.08
	0.01163	A31	Cw102	B5001	MICA*009	DR7	DQ501
D'		_	0.64	1		0.27	_
	0.01163	A11	Cw0303/04	B52011	MICA*010	DR8	DQ602
D'		0.47	1	_		0.65	_

	0.01163	A2	Cw0303/04	B1501	MICA*010	DR11	DQ301
D'		0.69	1	1		0.34	0.75
	0.01163	A2	Cw0303/04	B1501	MICA*010	DR4	DQ202
D'		0.69	1	1		0.09	_
	0.01163	A2	Cw0303/04	B1501	MICA*010	DR8	DQ301
D'		0.69	1	1		0.65	0.75
	0.01163	A30	Cw0303/04	B1524	MICA*010	DR11	DQ301
D'		0.03	1	1		0.34	0.75
	0.01163	A26	Cw1203	B1402	MICA*011	DR8	DQ501
D'		0.49	_	1		0.48	_
	0.01163	A33	Cw802	B1402	MICA*011	DR14	DQ503
D'		1	1	1		0.46	0.47
	0.01163	A1	Cw0303/04	B5501	MICA*012	DR14	DQ201
D'		0.37	0.15	1		_	0.3
	0.01163	A2	Cw602	B5601	MICA*012	DR1	DQ202
D'		_	0.25	1		0.18	_
	0.01163	A24	Cw801	B5501/05	MICA*012	DR4	DQ601
D'		_	_	1		0.43	_
	0.01163	A3	Cw0701/06	B5501/05	MICA*012	DR13	DQ501
D'		_	0.19	1		_	0.45
	0.02326	A24	Cw401	B3500	MICA*016	DR11	DQ301
D'		0.63	1	1		1	0.58
	0.01163	A2	Cw401	B51011	MICA*016	DR12	DQ301
D'		_	1	_		_	0.58
	0.04651	A1	Cw602	B5701	MICA*017	DR7	DQ602
D'		0.45	1	1		0.4	0.01
	0.02326	A1	Cw602	B5701	MICA*017	DR7	DQ3032
D'		0.45	1	1		0.4	0.68

	0.02326	A2	Cw602	B5701	MICA*017	DR7	DQ303
D'		0.14	1	1		0.4	0.79
	0.02326	A3	Cw602	B702	MICA*017	DR15	DQ603
D'		_	1	_		0.14	_
	0.01163	A1	Cw602	B3801	MICA*017	DR1301	DQ603
D'		0.45	1	_		1	_
	0.01163	A1	Cw602	B5701	MICA*017	DR14	DQ5031
D'		0.45	1	1		0.16	_
	0.01163	A1	Cw702	B2705/10	MICA*017	DR16	DQ301
D'		0.45	_	_		0.4	0.11
	0.01163	A1	Cw702	B3503	MICA*017	DR7	DQ502
D'		0.45	_	_		0.4	0.24
	0.01163	A2	Cw202	B5701	MICA*017	DR7	DQ3032
D'		0.14	_	1		0.4	0.68
	0.01163	A26	Cw602	B5701	MICA*017	DR11	DQ301
D'		0.37	1	1		0.05	0.11
	0.01163	A29	Cw602	B2705	MICA*017	DR4	DQ602
D'		_	1	_		_	0.01
	0.01163	A29	Cw602	B5701	MICA*017	DR3	DQ503
D'		_	1	1		_	0.01
	0.01163	A1	Cw1203	B804	MICA*018	DR3	DQ601
D'		_	0.24	_		0.31	0.48
	0.01163	A2	Cw0701/06	B1801/05	MICA*018	DR11	DQ301
D'		0.06	0.64	1		0.27	_
	0.01163	A25	Cw1203	B1801/05	MICA*018	DR15	DQ202
D'		0.65	0.24	1		0.2	0.25
	0.01163	A1	Cw602	B1502	MICA*019/*033	DR15	DQ305
D'		_	_	1		1	_

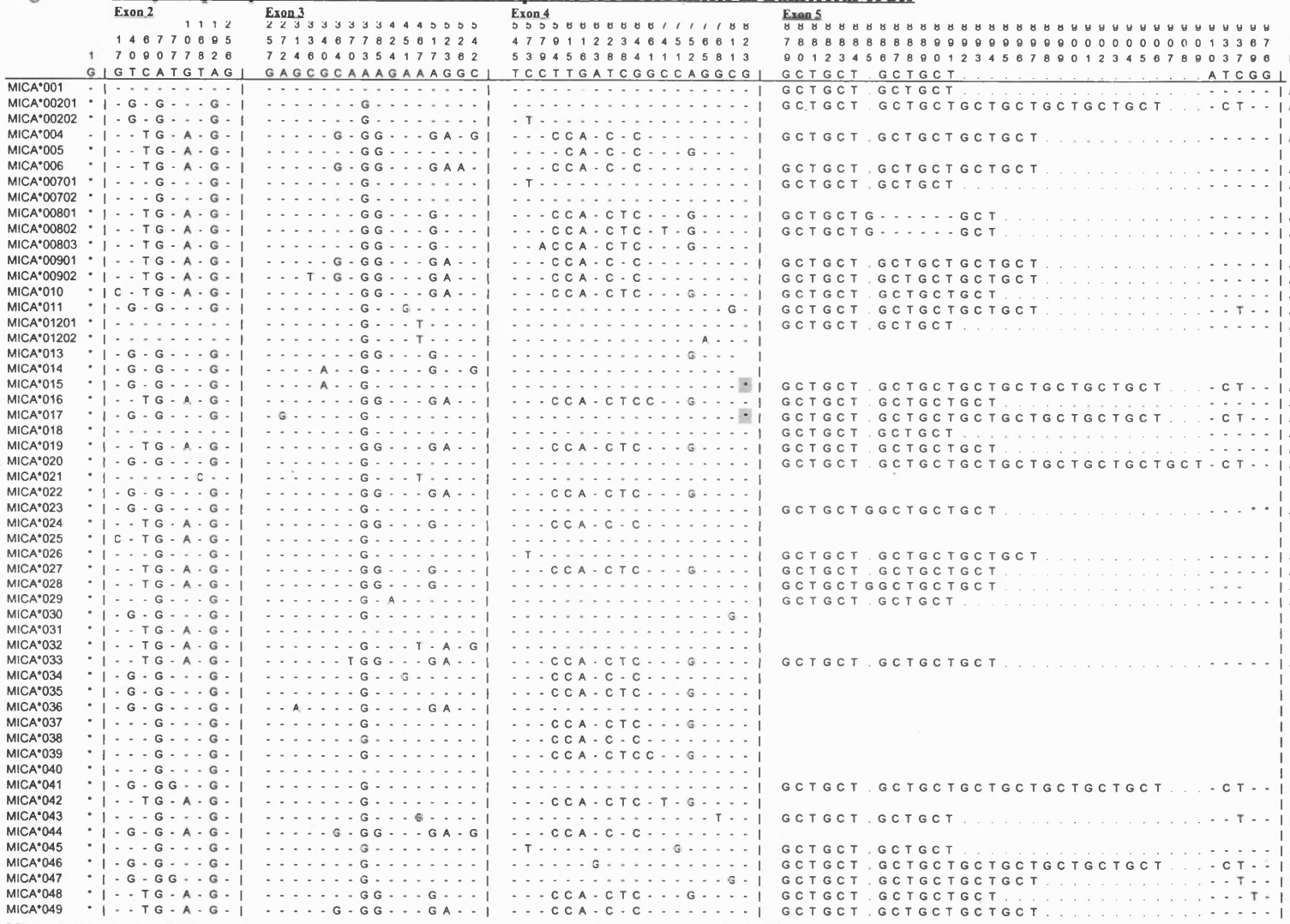
	0.01163	A1	Cw0303/04	B1517	MICA*027/*048	DR4	DQ302
D'		_	0.62	1		_	0.3
	0.01163	A30	Cw202	B4002	MICA*027/*048	DR15	DQ602
D'		0.31	0.31	0.65		_	0.25
	0.01163	A31	Cw0303/4	B4002	MICA*027/*048	DR14	DQ503
D'		0.31	0.62	0.65		0.28	0.29

***Table 4.9*. The MICA haplotypes present within the PsA population at a frequency of at least 1% demonstrating the haplotype frequencies and D' values**

	Haplotype Freq	MICA	HLA-DRB1	HLA-DQB1
	0.01804	MICA*002	DR0101	DQ0501
D'			0.21	0.16
	0.01524	MICA*002	DR0103	DQ0501
D'			0.07	0.16
	0.01494	MICA*002	DR0101-0102	DQ0501
D'			0.17	0.16
	0.01829	MICA*004	DR0701-0702	DQ0202
D'			0.27	0.28
	0.05754	MICA*008	DR0301	DQ0201
D'			0.45	0.22
	0.05491	MICA*008	DR0701	DQ0202
D'			0.04	0.22
	0.01829	MICA*008	DR0401-0411	DQ0301-0304
D'			0.31	0.52
	0.01826	MICA*008	DR1501	DQ0602
D'			0.74	0.64
	0.01524	MICA*008	DR1501-1502	DQ0602
D'			0.07	0.64
	0.01524	MICA*008	DR0401	DQ0301
D'			0.4	0.52
	0.0122	MICA*008	DR13	DQ0603
D'			0.12	0.34
	0.0122	MICA*008	DR1301	DQ0603
D'			0.23	0.34
	0.01171	MICA*008	DR0401-0411	DQ0302
D'			0.31	0.52

	0.02417	MICA*009	DR0301	DQ0201
D'			0.23	0.19
	0.02744	MICA*017	DR0701-0702	DQ0303
D'			0.21	0.44
	0.0122	MICA*017	DR0701	DQ0303
D'			0.38	0.44

Figure 4.1 **. Polymorphic positions of the nucleotide sequence of MICA alleles in numerical order**



"-" indicates identity with reference sequence (*001), "blank" indicates unpublished sequence, " * " with grey fill indicates a deletion.

Figure 4.2a. Phylogenetic tree of the MICA alleles based on the nucleotide sequence of exons 2,3 and 4 using the programme MEGA

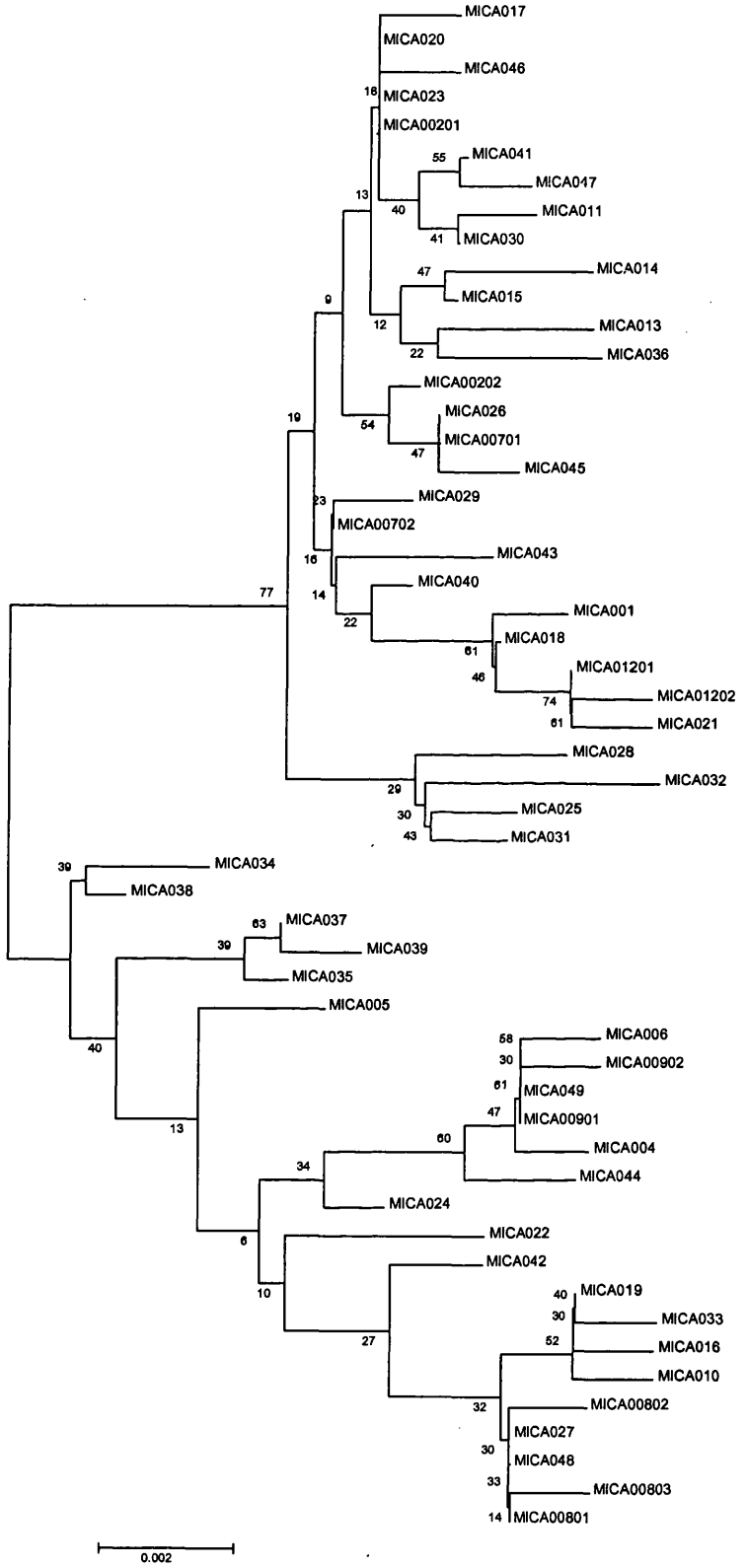
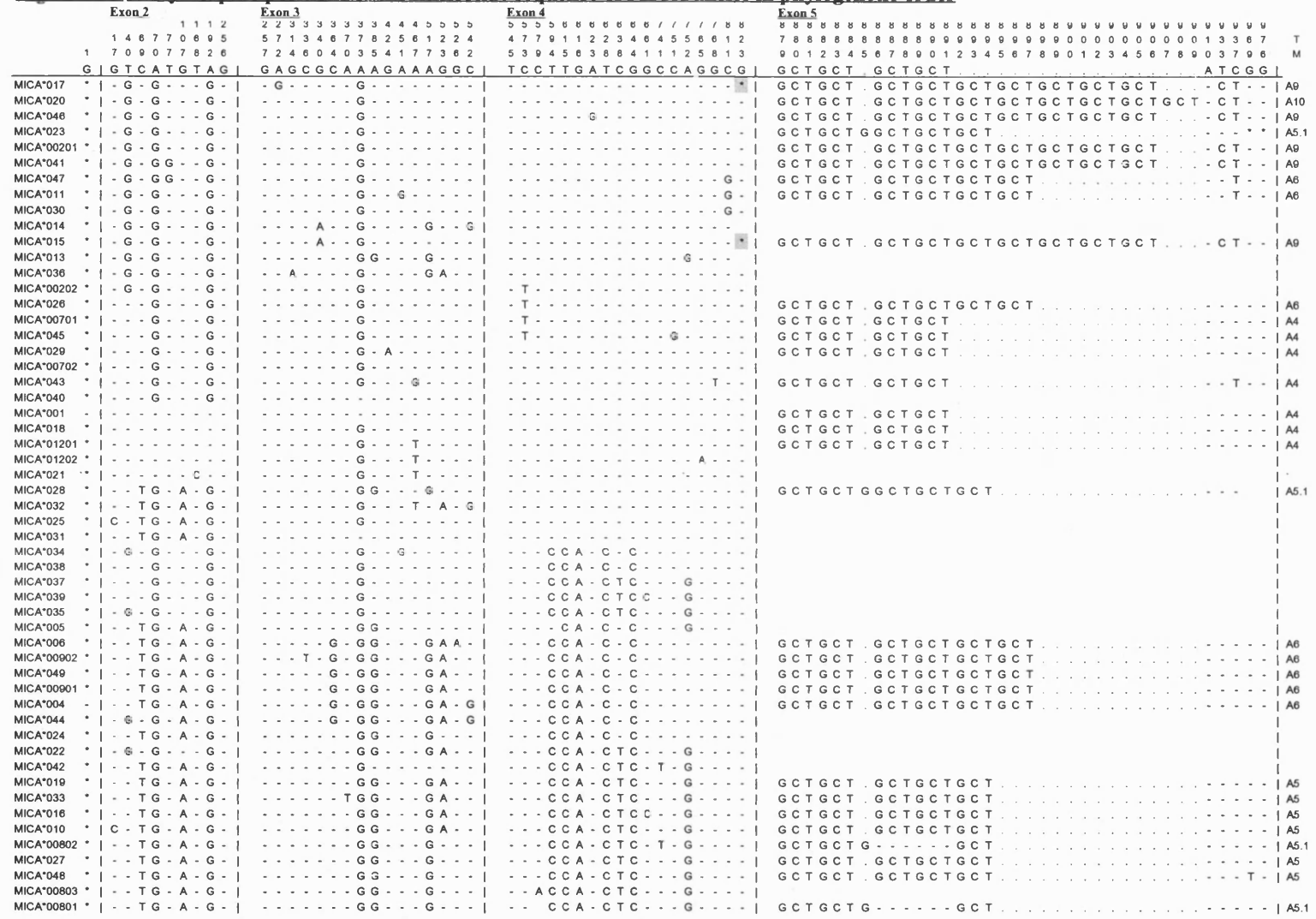
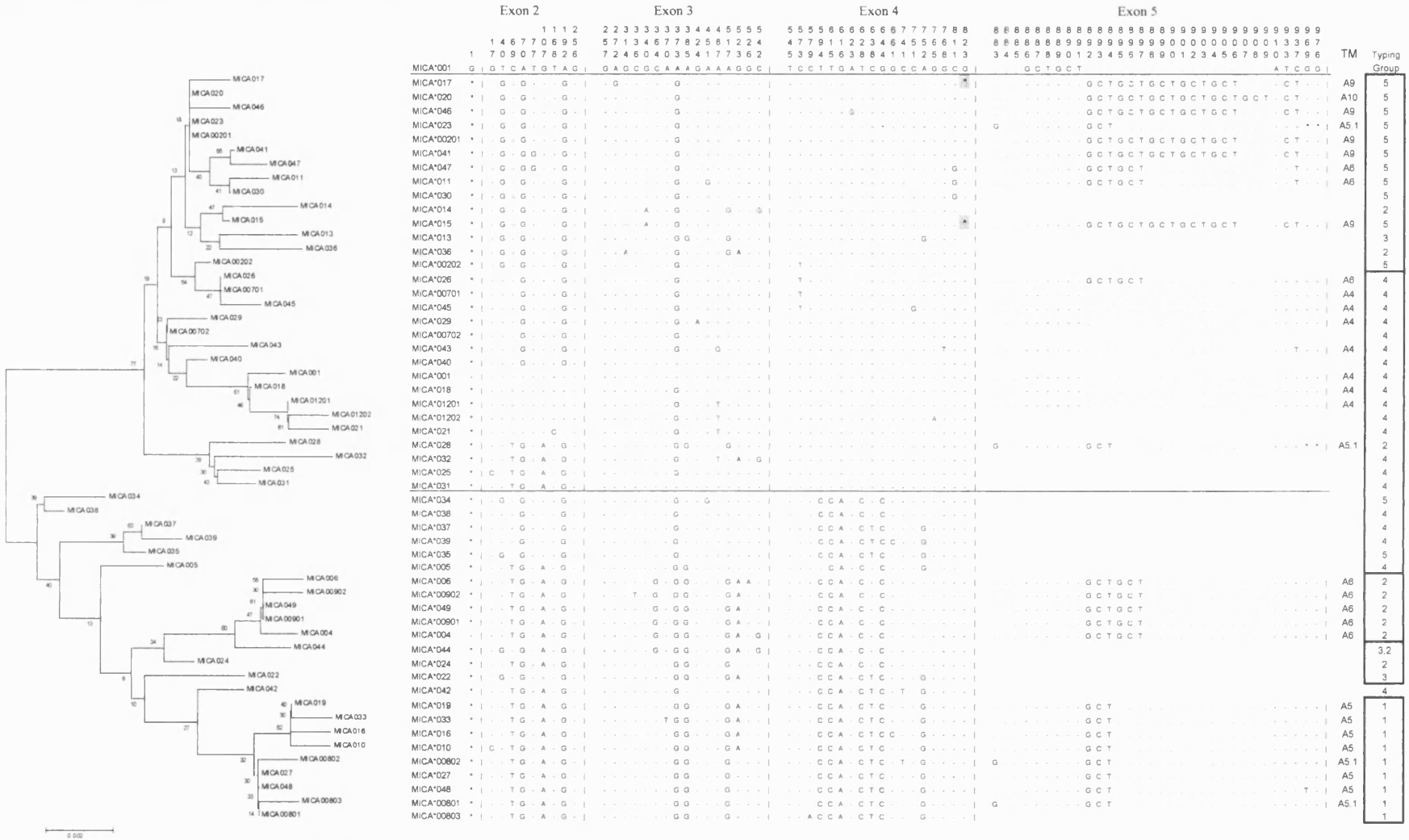


***Figure 4.2b*. Polymorphic positions of the nucleotide sequence of MICA alleles in phylogenetic order**



"-" indicates identity with reference sequence ("001"), "blank" indicates unpublished sequence, " * " with grey fill indicates a deletion.

Figure 4.3. Phylogenetic tree of the MICA alleles and polymorphic positions of exons 2,3,4 and 5 illustrating PCR-SSP Groups



"-" indicates identity with reference sequence (*001), "blank" indicates unpublished sequence, " * " with grey fill indicates a deletion.

Figure 4.4. **Amino acid substitutions at the polymorphic positions in the MICA alleles arranged in phylogenetic order**

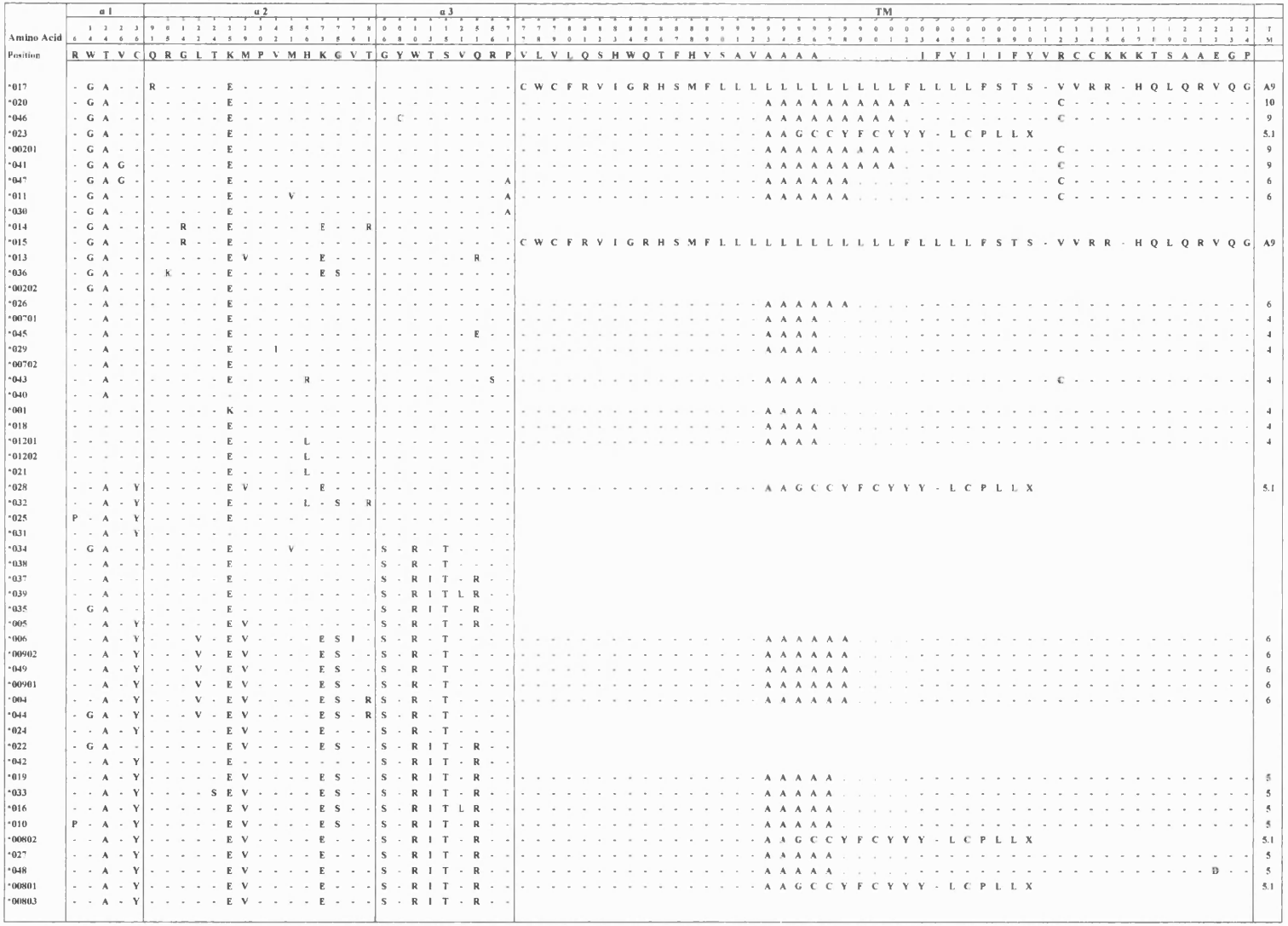
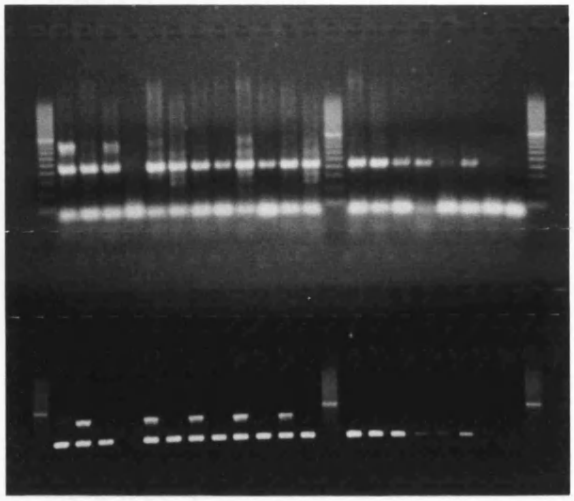

α1
α2
α3
TM
Amino Acid
Position
R W T V C Q R G L T K M P V M H K G V T G Y W T S V Q R P V L V L Q S H W Q T F H V S A V A A A A A I F V I I F Y V R C C K K K T S A A E G P
*017 - G A - - R - - - - E - C W C F R V I G R H S M F L L L L L L L L L L L L L F L L L L F S T S - V V R R - H Q L Q R V Q G A9
*020 - G A - - - - - - - E - A A A A A A A A A A A - - - - - - - - - - C - - - - - - - - - - 10
*046 - G A - - - - - - - E - - - - - - - - - C - - - - - - - - - - - A A A A A A A A A A - - - - - - - - - - C - - - - - - - - - - 9
*023 - G A - - - - - - - E - A A G C C Y F C Y Y Y - L C P L L X 5.1
*00201 - G A - - - - - - - E - A A A A A A A A A A - - - - - - - - - - C - - - - - - - - - - 9
*041 - G A G - - - - - - E - A A A A A A A A A A - - - - - - - - - - C - - - - - - - - - - 9
*047 - G A G - - - - - - E - A A A A A A - - - - - - - - - - - C - - - - - - - - - - 6
*011 - G A - - - - - - - E - - - V - - - - - - - - - - - - - - - - - A A A A A A - - - - - - - - - - - C - - - - - - - - - - 6
*030 - G A - - - - - - - E - A
*014 - G A - - - - R - - E - - - - - E - - R - - - - - - - - -
*015 - G A - - - - R - - E - C W C F R V I G R H S M F L L L L L L L L L L L L L F L L L L F S T S - V V R R - H Q L Q R V Q G A9
*013 - G A - - - - - - - E V - - - - E - - - - - - - - - R - -
*036 - G A - - - K - - - E - - - - - E S - - - - - - - - - - -
*00202 - G A - - - - - - - E - - - - - - - - - - - - - - - - - -
*026 - - A - - - - - - - E - A A A A A A - 6
*00701 - - A - - - - - - - E - A A A A - 4
*045 - - A - - - - - - - E - - - - - - - - - - - - - - - E - - - - - A A A A - 4
*029 - - A - - - - - - - E - - - I - - - - - - - - - - - - - - - - - A A A A - 4
*00702 - - A - - - - - - - E -
*043 - - A - - - - - - - E - - - - - R - - - - - - - - - S - - - - - A A A A - 4
*040 - - A -
*001 - - - - - - - - - - K - A A A A - 4
*018 - - - - - - - - - - E - A A A A - 4
*01201 - - - - - - - - - - E - - - - - L - - - - - - - - - - - - - - A A A A - 4
*01202 - - - - - - - - - - E - - - - - L - - - - - - - - - - - - - -
*021 - - - - - - - - - - E - - - - - L - - - - - - - - - - - - - -
*028 - - A - Y - - - - - E V - - - - E - A A G C C Y F C Y Y Y - L C P L L X 5.1
*032 - - A - Y - - - - - E - - - - L - S - R - - - - - - - - - -
*025 P - A - Y - - - - - E - - - - - - - - - - - - - - - - - - -
*031 - - A - Y -
*034 - G A - - - - - - - E - - - V - - - - - S - R - T - - - - -
*038 - - A - - - - - - - E - - - - - - - - - S - R - T - - - - -
*037 - - A - - - - - - - E - - - - - - - - - S - R I T - R - - -
*039 - - A - - - - - - - E - - - - - - - - - S - R I T L R - - -
*035 - G A - - - - - - - E - - - - - - - - - S - R I T - R - - -
*005 - - A - Y - - - - - E V - - - - - - - - - S - R - T - R - -
*006 - - A - Y - - - V - E V - - - - E S I - - S - R - T - - - - - - - - - - - - - - - - - - -

Figure 4.5. **Agarose gel electrophoresis of two primer pairs within Group 1**



Row 1 : F10 + R17
Product band : 841 base pairs
Control band : 485 base pairs

Row 2 : F3 + R16
Product band : 770 base pairs
Control band 485 base pairs

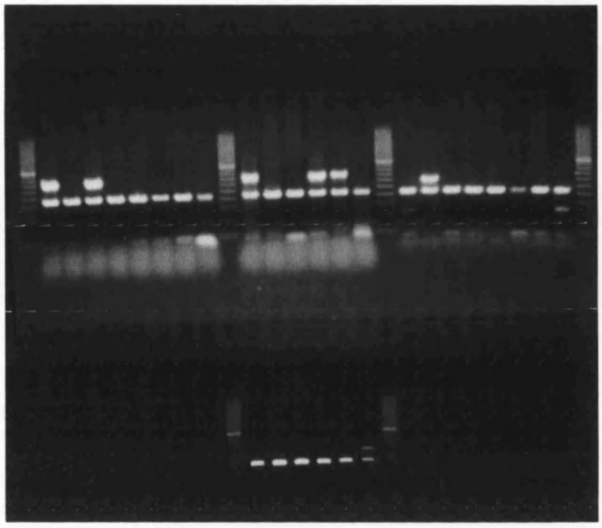
Row 1 : F10 + R17 primer pair (*010)

Lane	Sample	Result
1	*010 control	*010
2	*016 control	neg
3	1	*010
4	2	failed
5	3	neg
6	4	neg
7	5	neg
8	6	neg
9	7	neg
10	8	neg
11	9	neg
12	10	neg
13	MW Marker	
14	11	neg
15	12	neg
16	13	neg
17	14	neg
18	15	weak
19	16	neg
20	17	failed
21	Water	
22	MW Marker	

Row 2 : F3 + R16 primer pair (*016)

Lane	Sample	Result
1	*010 control	neg
2	*016 control	*016
3	1	neg
4	2	failed
5	3	*016
6	4	neg
7	5	*016
8	6	neg
9	7	*016
10	8	neg
11	9	*016
12	10	neg
13	MW Marker	
14	11	neg
15	12	neg
16	13	neg
17	14	neg
18	15	neg
19	16	neg
20	17	failed
21	Water	
22	MW Marker	

Figure 4.6. **Agarose gel electrophoresis of two primer pairs within Group 4**



Lanes 1-16 : F1+R12
Product band: 741 base pairs
Control band : 485 base pairs

Lanes 17-32 : F18+ R1
Product band: 617 base pairs
Control band : 485 base pairs

Lanes 1-16 : F1 + R12 (MICA *012)

Lane	Sample	Result
1	*012 control	*012
2	*001 control	neg
3	1	*012
4	2	neg
5	3	neg
6	4	neg
7	5	neg
8	6	neg
9	MW Marker	
10	7	*012
11	8	neg
12	9	neg
13	10	*012
14	11	*012
15	12	neg
16	MW Marker	

Lanes 17-32 : F18 + R1 (MICA *001)

Lane	Sample	Result
17	*012 control	neg
18	*001 control	*001
19	1	neg
20	2	neg
21	3	neg
22	4	neg
23	5	neg
24	6	neg
25	MW Marker	
26	7	neg
27	8	neg
28	9	neg
29	10	neg
30	11	neg
31	12	*001
32	MW Marker	

Figure 4.7. **MICA A4 allele haplotypes in the 13IHW patients with Type I Psoriasis**

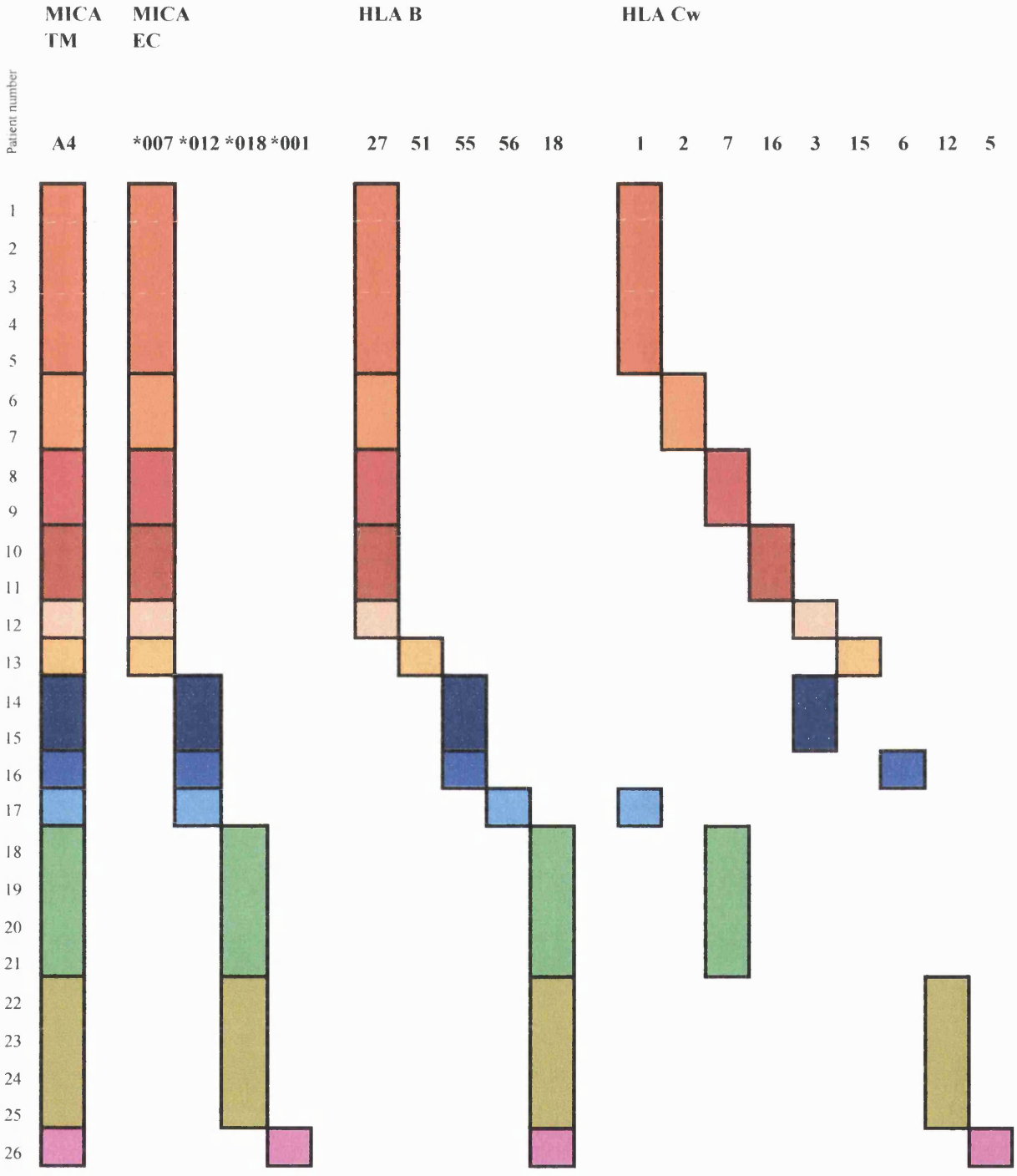


Figure 4.8. **MICA A5 allele haplotypes in the 13IHW patients with Type I Psoriasis**

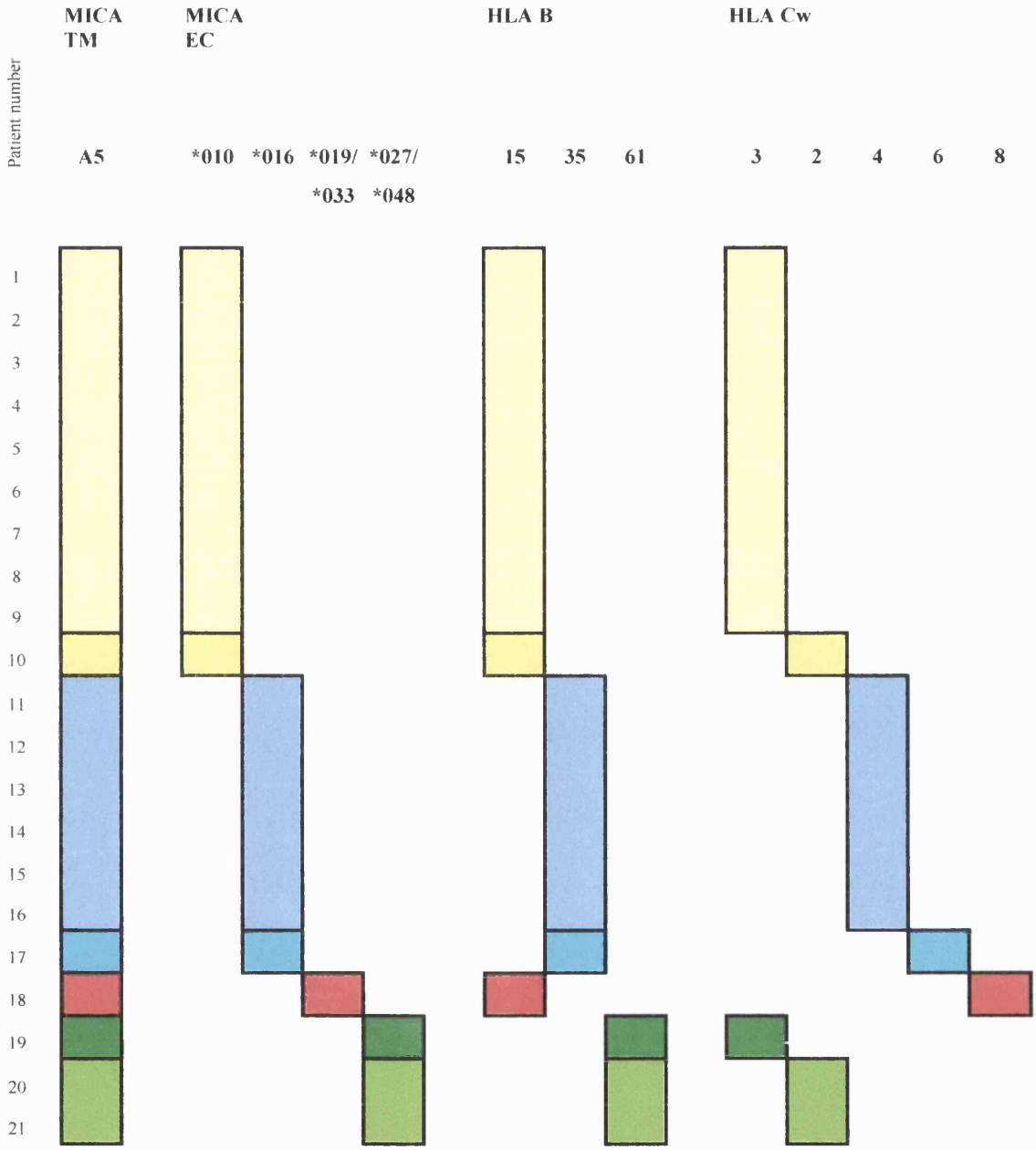


Figure 4.9. **MICA A5.1 allele haplotypes in the 131HW patients with Type I Psoriasis**

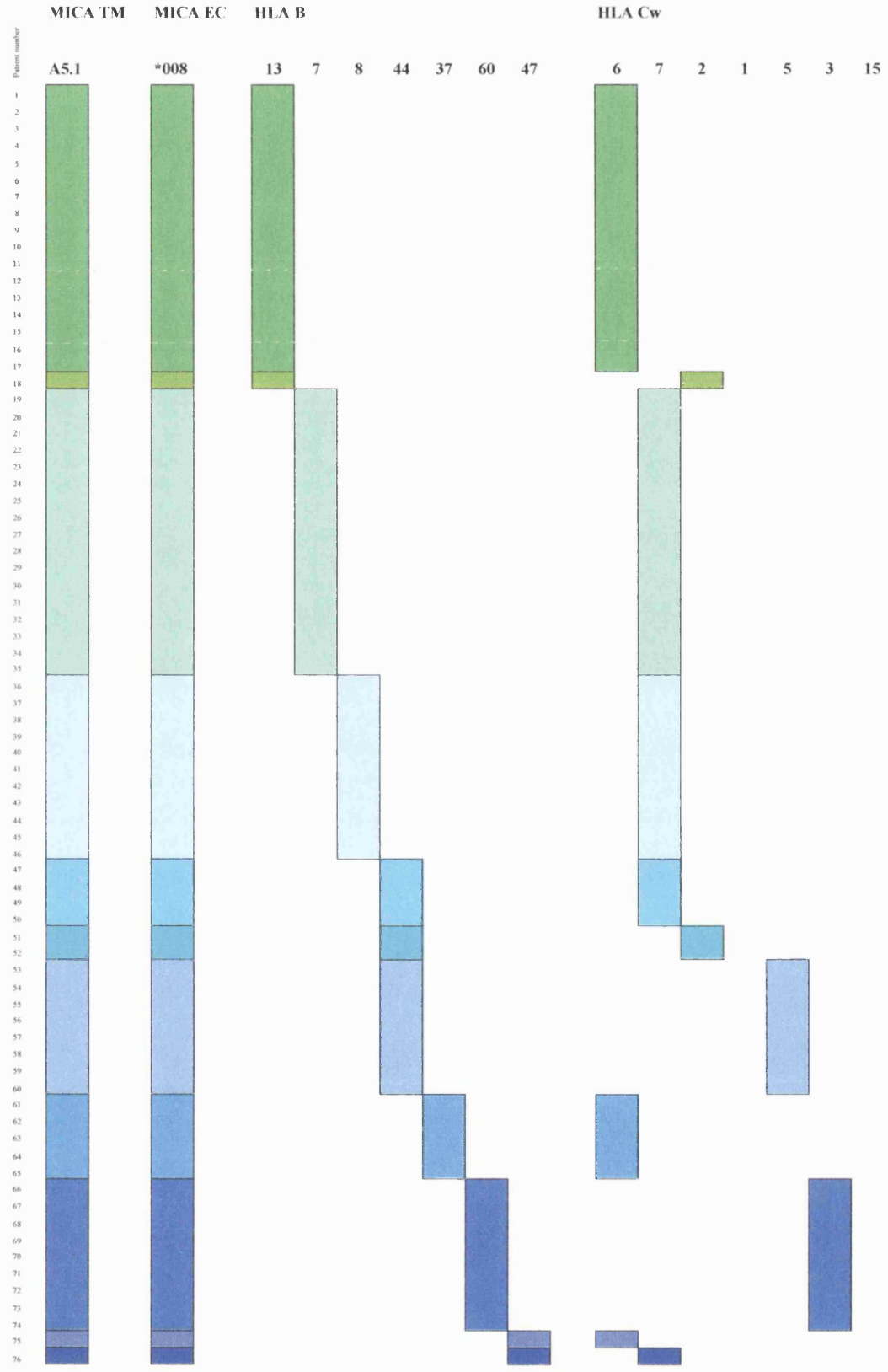


Figure 4.10. **MICA A6 allele haplotypes in the 13IHW patients with Type I Psoriasis**

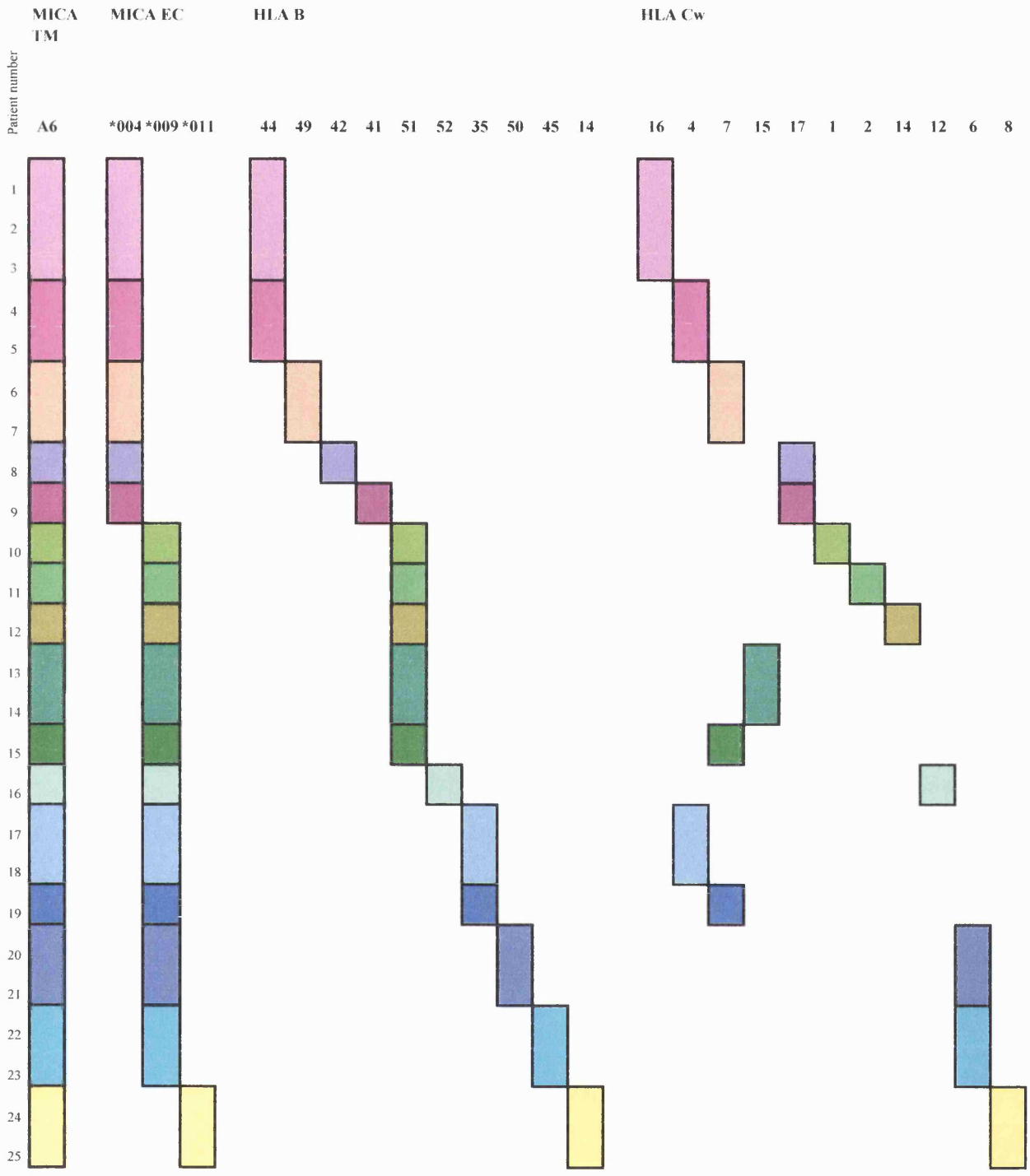
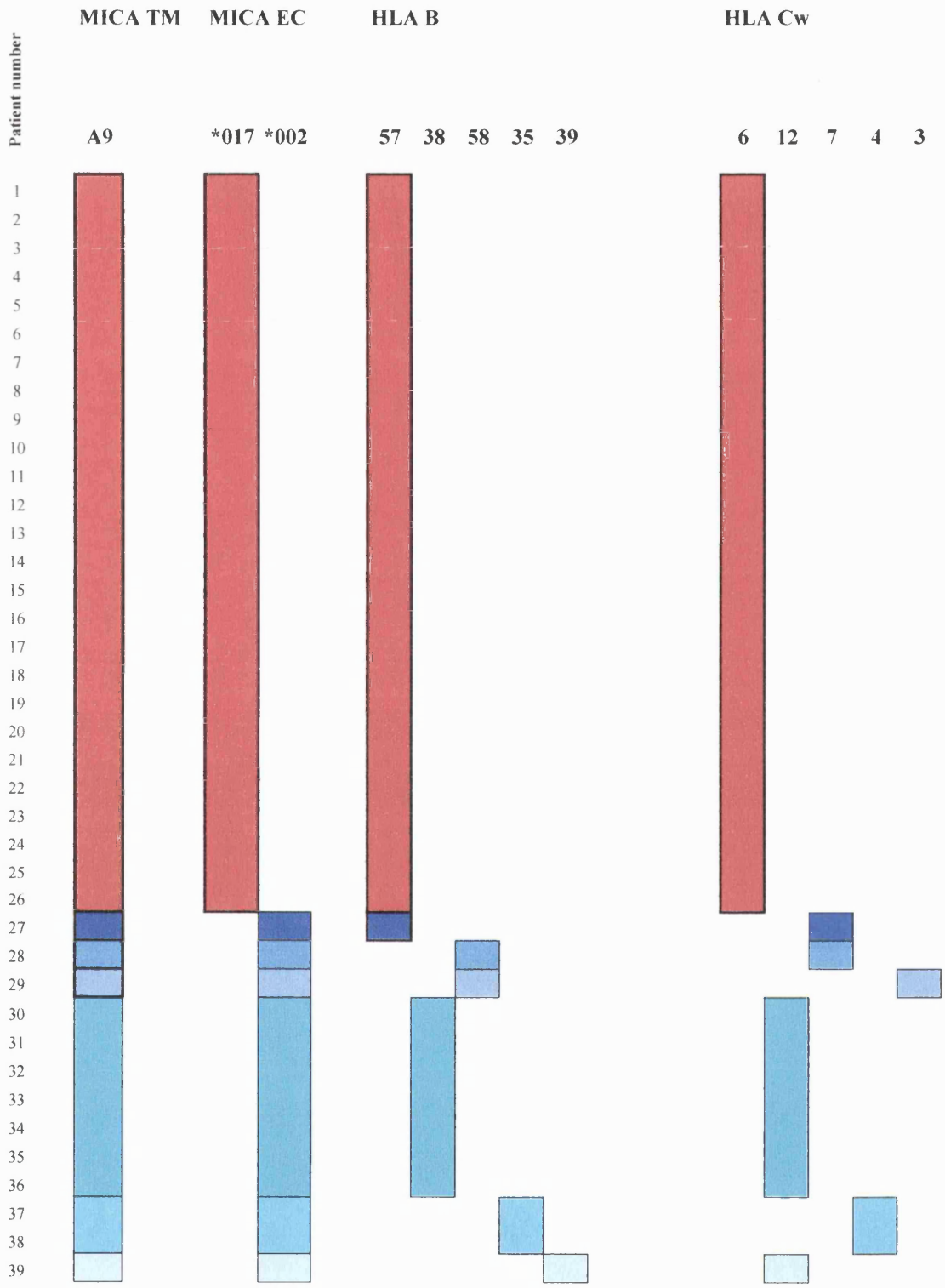


Figure 4.11 . MICA A9 allele haplotypes in the 13IHW patients with Type I psoriasis



CHAPTER 5 EXPRESSION STUDIES OF MICA

5.1 Introduction

The A9 allele of MICA has been associated with psoriasis and psoriatic arthritis in several small studies (50; 52; 67). The genetic study presented here confirms the association of MICA A9 and suggests a role for the *002 allele of MICA in PsA and the *017 allele in psoriasis. It is unclear whether these associations are primary or whether they are due to the presence of more functionally relevant genes along an extended haplotype. There are a number of reasonable functional candidate genes all present within a short distance of the chromosome that display a high degree of linkage disequilibrium to MICA. The task of distinguishing which of these are the key players in the susceptibility to PsA or psoriasis is clearly a difficult one. One factor that should be taken into consideration is the functional relevance of the gene product to the condition. In the case of MICA, its role in the stimulation of an immune response via the activation of a ubiquitous receptor on NK cells, CD8+ $\alpha\beta$ T cells and $\gamma\delta$ T cells makes it a plausible candidate. However, if it could be demonstrated that MICA is expressed in the target tissues, ie. the skin and the synovium, and more specifically that the expression is in some way altered during the disease process, this would add considerable support to MICA being a strong candidate gene for these conditions.

Initial studies on the expression of MICA with monoclonal antibodies suggested specific expression on gastrointestinal epithelium in response to stress, with the only other site of expression being thymic cortical epithelium (131). It was later demonstrated that MICA was also expressed on a wide variety of tumours, including lung, breast, kidney, ovary, prostate and colon carcinomas (172). Recently, a more generalised expression pattern has been described using other monoclonal and polyclonal antibodies, with expression predominantly in epithelial tissues such as the skin and kidney (138).

MICA is not always expressed by the cells in which it is transcribed. It was noted that several tumour cell lines lack surface expression of MICA despite the presence of abundant mRNA (173). Analysis of these cell lines showed that they were homozygous for MICA *010. This allele has a single amino acid substitution (proline for arginine) in the α1 domain that appears to interfere with a stable protein fold. The frequency of MICA *010 in the population has been estimated at 5% in a Caucasian population (118) and 12.5% in a Japanese population (130). To date, no specific disease associations have been described with MICA *010 and these individuals appear to have a fully functional immune system.

Another allele, MICA 5.1, which is the commonest MICA allele in all populations studied, has a truncated cytoplasmic tail which alters expression. The resultant truncated MICA molecules have been shown to translocate to the apical surface of intestinal epithelial cells rather than to the basolateral surface as seen with all other alleles of MICA (133). The pathophysiological significance of this is not known, although several diseases have been reported to show associations with MICA 5.1.

The cytoplasmic tail (encoded by exon 6 of MICA) appears to carry important information for the sorting of MICA molecules and their subsequent transportation to the cell surface. The alleles, MICA *017 and *015, bear a single base deletion at the end of exon 4 that causes a shift in the reading frame. This results, not only in the production of a hydrophobic poly-leucine region, but also in the loss of the cytoplasmic tail. The surface expression of this allele has not been specifically studied, but it could be inferred from the previous studies that these alleles would also result in defective surface expression. The genetic study of MICA presented in this thesis has demonstrated a strong association between the MICA *017 allele and psoriasis. If the above hypothesis is correct, then the expression of MICA in the epithelium in psoriasis may be reduced due to altered transportation to the cell surface.

The MICA allele associated with PsA contains nine GCT repeats (A9) which are translated into nine alanines in the transmembrane region. On further analysis of the

various alleles that contain 9 GCT repeats, the actual allele associated with PsA was MICA *002. This allele is identical to MICA *017 except for two single nucleotide polymorphisms. Firstly, it does not have the single base deletion at the end of exon 4 and therefore has a normal cytoplasmic tail and no frameshift. Secondly, it has a substitution at position 272, which leads to a change of amino acid at position 91. This amino acid is at the heart of the $\alpha 1 \alpha 2$ platform that interacts with the NKG2D receptor. There may therefore be no reason for altered surface expression of MICA in PsA, but its interaction with the NKG2D receptor and therefore its ability to activate the immune response may well be altered. The significance of the number of alanines within the transmembrane region of MICA has not been studied in molecular models and therefore the consequences of the A9 allele on surface expression are not known. It would therefore be reasonable to assume that there is no clear reason why MICA should not be expressed on the cell surface in PsA. However, to date no study has examined whether MICA is expressed in the synovium.

Polyclonal antibodies have been produced which recognise MICA by immunising rabbits with short peptides from the $\alpha 1$ (residues 42-60) and $\alpha 2$ domains (residues 140-160) (174). These residues were chosen as they were in non-polymorphic regions of MICA and did not show appreciable cross-reactivity with the other members of the extended MHC Class I family. These antibodies have been used to immunoprecipitate a 62kDa band which appears to be the native MICA molecule. Western blotting has confirmed the presence of MICA in freshly isolated keratinocytes, monocytes, fibroblasts and umbilical vein endothelial cells. The MICA band was not present in peripheral blood CD4+, CD8+ or CD19+ lymphocytes and was not upregulated by γ-interferon. Flow cytometry has demonstrated that expression in keratinocytes and monocytes is predominantly within the cytoplasm and rarely on the cell surface. This contrasts with endothelial cells and skin-derived fibroblasts which show high levels of expression at the cell surface (175). The reason for this differential surface expression is not clear. It may be related to tissue-specific alternative splicing of the immature MICA RNA, a mechanism that has been described for HLA-G and MR1. It may be that surface expression of MICA is dependent on a tissue-specific transporting

'chaperone' or surface binding ligand. Finally, surface expression may be influenced by the allele of MICA expressed by the cell.

The expression of MICA in a variety of tissues has been studied using indirect immunofluorescence. Several different polyclonal antibodies to MICA were utilised to demonstrate that MICA is expressed in the epithelial layers of the oesophagus, intestinal mucosa, liver, human neutrophils, glomeruli, testis and ovary (176). The strongest pattern of expression was observed with the antibody PERB11-5D. This antibody was produced following immunisation with a peptide from the α1 domain (residues 57-67) of MICA. The same peptide was later used to make the polyclonal antibodies PERB11.1 204 and 205 which were utilised in this study. The PERB11.1 antibodies were used to demonstrate the expression of MICA in the skin of patients with psoriasis in a small study that reported an association between the A9 allele of MICA and psoriasis (50). The expression of MICA was reduced in lesional skin specimens taken from 3 patients with psoriasis using indirect immunofluorescence. To date, there have been no further studies of the expression patterns of MICA in the skin in psoriasis or any other skin pathologies.

5.2 Aims of the study

Since polymorphisms of MICA are associated with psoriasis and psoriatic arthritis, the aim of this study was to evaluate the expression patterns of MICA in the skin and synovium. Disease-specific tissue expression patterns could be examined by immunohistochemistry following antigen retrieval. This technique would enable the use of archived paraffin-embedded, formalin-fixed sections from patients with known diagnoses that demonstrated clear histological patterns. The sections could then undergo antigen retrieval to ensure the MICA molecules became accessible for antibody staining (177). Expression patterns in normal skin and lesional psoriatic skin could then be compared to determine if there are any disease-specific changes. In addition, expression patterns in synovial sections from patients with PsA, RA, non-specific synovitis and

normal synovium could also be examined to elucidate whether MICA is expressed in the synovium and, if so, whether there are any disease-specific patterns.

5.3 Methods

5.3.1 Polyclonal antibody production

The polyclonal antibodies used to detect MICA (PERB11.1 204 and 205) had been produced by the immunisation of different rabbits with a peptide derived from a sequence within the α1 domain. The peptide was synthesised and conjugated to Diptheria toxoid (Chiron Mimotopes Pty Ltd, Victoria, Australia). The region of MICA chosen for the peptide synthesis was a conserved sequence not known to contain any polymorphism (residues 57-67 of the α1 domain, *Table 5.1*). The immunisation procedure involved the injection of rabbits with the peptide-diptheria conjugate using Titremax adjuvant and harvesting serum after 9 and 14 weeks. The sera were tested for reactivity against the peptide using an indirect ELISA (176). The PERB11.1 204 and 205 antibodies would therefore be expected to show the same reactivity patterns as they were both derived from immunisation with the same peptide. However, they may differ in intensity depending on the quality of antibody produced by each rabbit.

5.3.2 Alignment of MICA amino acid sequence with other members of the EMCIF

Since the technique was developed to produce these polyclonal antibodies to MICA, a number of proteins related to MICA have been described in the literature and termed the 'Extended MHC Class I Family' or EMCIF (129; 178). An alignment of the amino acid sequences of the *008 allele of MICA with the other members of the EMCIF is illustrated in *Table 5.1*. The proteins within this family include MICB, HLA Class I, Znα_2 glycoprotein, neonatal F_c receptor, CD1, MR1, HFE and the ULBPs. The MICA peptide used for the immunisation process does share some common residues with the other members of the family. The closest alignment is with MICB which shares 9/11 residues with the peptide in MICA. The PERB11 antibodies would therefore be likely to recognise both MICA and MICB expression, and will hereafter be referred to as 'MIC'. The use of monoclonal antibodies to MICA and MICB may help delineate any

differences in expression. None of the other amino acid sequences share more than 5/11 residues of the PERB11.1 204 and 205 peptides. This would make it unlikely that there would be any significant cross-reactivity with the other members of the family.

5.3.3 Preparation of antibodies

The primary antibodies used for the immunofluorescence and immunohistochemistry studies included two polyclonal antibodies to MIC (PERB11.1 204 and PERB11.1 205), a monoclonal antibody to MICA (huMICA-M673) and an antibody to Class I HLA (W6/32) (*Table 2.4*). All antibodies were stored at 4°C as an undiluted stock solution. The optimal working dilutions of the antibodies was determined by testing sections with differing dilutions between 1:10 and 1:200. The optimal dilution for PERB11.1 204 and 205 and W6/32 was 1:50 for both techniques. The monoclonal antibodies were only used for immunohistochemistry. They showed generally poor reactivity and a dilution of 1:10 was utilised. The antibodies were diluted just prior to use in antibody diluent (DAKO).

5.3.4 Selection of patient material for immunohistochemistry

Skin and synovial sections were obtained from archived, paraffin-embedded, formalin-fixed tissue, stored in the Pathology Department of Sir Charles Gairdner Hospital, Perth, Australia. Permission for the tissue to be used was obtained from the Head of the Pathology Department and was within the remit of their Ethics approval.

5.3.5 Synovium sections

Sections from patients who had PsA, RA, non-specific synovitis and controls were sought. The pathology department database was searched by a Consultant Histopathologist for the words 'psoriatic arthritis', 'rheumatoid arthritis' and 'non-specific synovitis' for appropriate synovial sections. Five sections representative of each diagnosis were retrieved. The diagnosis and histological features were confirmed by review of Haematoxylin and Eosin stained sections by a Consultant Histopathologist. The sections selected to show normal synovium were found to have a mild Grade I non-specific synovitis on review of the histology, but were considered to be 'normal'

controls for comparison with the inflamed synovial sections. Fresh 5μm sections were cut from each paraffin-embedded block and placed onto glass slides and left to air-dry prior to use.

5.3.6 Skin sections

Five sections of skin from patients with psoriasis were obtained by searching the database for the word 'psoriasis'. The histology was reviewed as above. Tissue was also available from normal skin which were used as controls. Fresh 5μm sections were cut from each paraffin-embedded block and placed onto glass slides and left to air-dry prior to use.

5.3.7 Selection of patient material for immunofluorescence

Frozen sections of normal skin were obtained from the Centre for Molecular Immunology and Instrumentation in Perth. The sections had previously been used for localisation of the binding of different polyclonal MICA antibodies for which ethical approval was given. The frozen blocks were cut into 4μm sections and placed on glass slides. They were then left to air-dry for 1 hour and fixed in ice-cold methanol. The slides were stored at –20°C until needed.

5.3.8 Immunofluorescence

The expression patterns of PERB11.1 204, PERB11.1 205 and W6/32 were examined by immunofluorescence on frozen sections of normal skin. The method is described in Chapter 2.5.2. The slides were examined for fluorescence using dark background microscopy. The sections were then photographed on slide film and the slides were scanned into a computer as .tif images.

5.3.9 Immunohistochemistry

The expression patterns of the PERB11.1 204 and PERB11.1 205 polyclonal antibodies to MIC, the MICA monoclonal antibody (huMICA-M673) and W6/32 were examined on the skin and synovial sections described above. The technique of 'antigen retrieval' by super-heating formalin-fixed slides is well described (177). This was achieved by

microwaving the slides on a high power in a buffer solution. The technique had been optimised for routine use in the Immunohistochemistry department at the Sir Charles Gairdner hospital. The method is detailed in Chapter 2.5.3. Photographs of the slides following immunohistochemistry were taken with digital photography and the images were downloaded directly onto a computer.

5.4 Results

5.4.1 Immunofluorescence

Sections of normal skin were examined for the presence of MIC with the polyclonal antibodies PERB11.1 204 and 205 and the presence of HLA Class I with the W6/32 antibody. PERB11.1 204 and 205 showed the same pattern of staining, although the intensity of staining with PERB11.1 205 was more intense. They both demonstrated a granular cytoplasmic pattern of staining of the basal layers of the epidermis. The expression was much less marked in the upper layers of the epidermis. Bright staining of the superficial keratin layer was seen and occasional staining of fibroblast-like cells in the dermis. Examples of the pattern of expression are shown in *Figures 5.1, 5.2 and 5.3.* Comparison of the pattern of staining observed for MIC with the *cell surface* pattern of staining throughout *all* the layers of the epidermis with the antibody to HLA Class I (W6/32) serves to differentiate the two members of the EMCIF (*Figure 5.4.*). Although it is possible that there is some cross-reactivity between the expression of MIC and HLA Class I detected by the polyclonal antibodies, the basal epidermis staining would appear to be specific for MICA.

5.4.2 Immunohistochemistry

5.4.2.1 Normal Skin

There was a granular cytoplasmic pattern of staining throughout the cells of the epidermis. The cornified layer stained strongly. There was no significant difference in the pattern of staining with the PERB11.1 204 or 205 antibody although staining was more prominent in the **basal layer of the epidermis** with PERB11.1 205. In the dermis, there was staining of the vascular endothelium, dermal fibroblasts and sweat ducts (*Figure 5.5.*). Immunohistochemistry using the monoclonal antibody to MICA

(huMICA-M673) demonstrated a granular cytoplasmic staining of the basal layers of the epidermis in normal skin which was markedly reduced in the sections taken from patients with psoriasis (*Figure 5.6.*). There was no staining of the keratin layer or dermis suggesting that the staining observed in these regions with the polyclonal antibodies was non-specific.

5.4.2.2 Skin from patients with Psoriasis

Staining of the expanded epidermal layer and hyperkeratotic cornified layer in skin sections from patients with psoriasis was markedly reduced overall with both PERB11.1 204 and 205 (*Figures 5.7 and 5.8.*). This was most apparent on the sections that contained both lesional and non-lesional skin, where the loss of staining within the pathological skin was striking. The **loss of expression in the basal layers** of the epidermis was the most evident. The *pattern* of staining in lesional skin was also different from that seen in normal skin in some of the sections. In these sections (3 out of 5), the cells of the stratum spinosum of the epidermis exhibited a strong cell membrane pattern not seen in normal skin. The pattern then returned to a predominantly granular cytoplasmic appearance in the upper stratum granulosum of the epidermis. Staining of dermal structures was no different from normal skin although any inflammatory cell infiltrate stained strongly. Staining with the monoclonal antibody to MICA demonstrated loss of expression in lesional psoriatic skin (*Figure 5.9.*).

5.4.2.3 Control synovium

In Grade 0-1 synovitis, PERB11.1 204 and 205 antibodies stained the surface synoviocytes with a granular cytoplasmic pattern. Staining was also seen in the underlying connective tissue predominantly in the vascular endothelium (*Figure 5.10*). The monoclonal antibody to MICA (huMICA-M673) exhibited faint staining of the layer of surface synoviocytes (*Figure 5.11).* There was no staining of the underlying connective tissue or inflammatory infiltrate suggesting that some of the staining seen with the polyclonal antibodies was non-specific.

5.4.2.4 Synovium from patients with PsA

The inflamed synovium was thrown into multiple frond-like villi with an expanded layer of surface synoviocytes which stained strongly with the PERB11.1 antibodies. The underlying connective tissue contained increased amounts of heavily staining vascular tissue. There did appear to be an **increased intensity of staining** in inflamed compared with non-inflamed synovium (*Figure 5.12.*).

5.4.2.5 Synovium from patients with RA

The synovial samples from patients with RA showed similar changes to those seen in the inflamed PsA synovium (*Figure 5.13.*). There were no apparent differences in either the intensity or the pattern of staining with either PERB11.1 204 or 205.

5.4.2.6 Synovium from patients with non-specific synovitis

The non-specifically inflamed synovial samples showed the same distribution and intensity of staining for MIC as the sections from patients where a specific diagnosis of PsA or RA had been made.

5.4.2.7 Control sections

A section of human brain was used as a negative tissue control as MICA is not expressed in the central nervous system. This was confirmed by the lack of staining with the PERB11.1 204 and 205 antibodies (*Figure 5.14.*). An antibody control was also created by staining a synovial section with a polyclonal antibody to glial fibrillary acid protein. There confirmed the expected lack of staining of the section as demonstrated in *Figure 5.15.*

5.5 Discussion

Immunohistochemistry following antigen retrieval has been successfully utilised to evaluate the binding of the PERB11.1 204 and 205 antibodies to the corresponding proteins in the skin and synovium. These antibodies predominantly bind to MICA but

may show some affinity for the gene products of the other members of the Extended MHC Class I Family (EMCIF), particularly MICB which is the most closely related.

This study has shown that expression of MICA was reduced in psoriatic skin and displayed a different pattern of staining from normal skin. Expression of the MICA protein appeared to be almost lost in the basal layers of the skin. In some examples of psoriatic skin, MICA was expressed predominantly on the cell surface of the spiny cells of the stratum spinosum and in a reduced amount within the cytoplasm of the upper stratum granulosum and the cells of the cornified layer. In other examples of psoriatic skin, the staining was reduced in all layers, particularly in the basal layers, but the pattern of staining was predominantly cytoplasmic throughout. This is interesting as it may represent differential expression patterns of the MICA alleles. The two alleles which have been associated with psoriasis are the transmembrane alleles MICA A5.1 and A9 (50; 52). The A5.1 allele has a guanine insertion in exon 5 which causes a frameshift and a truncated protein which may well affect the surface expression. The A9 allele which has been associated with psoriasis in this study (MICA *017) also has a frame shift due to a guanine deletion at the end of exon 4 (179). This causes the production of a hydrophobic, long leucine-rich region and premature termination in the cytoplasmic tail which may in turn lead to altered expression patterns. Typing the MICA allele of the patients from whom the samples were taken would help to clarify the patterns seen and aid in their interpretation. The reason why a reduction in the expression of MICA is seen in psoriatic skin is not clear. It may simply be a function of the altered cell dynamics in psoriasis as the cells differentiate through the layers of the epidermis. The consequences of this apparent reduction of MICA are not known, but it is possible that it may result in a reduced capacity of psoriatic skin epithelial cell MICA molecules to interact with NK cells and T cells. This may ultimately affect the ability of the skin to respond to stresses such as injury or infection.

Expression of MICA in the synovium has not been previously reported. The expression was predominantly seen within the cytoplasm of the surface synoviocytes

but also in the underlying vascular connective tissue. The use of monoclonal antibodies to MICA suggests that the more specific staining is with the surface synoviocytes. There was no clear change in the *pattern* of staining between PsA, RA and non-specific synovitis. All pathologies which resulted in an inflamed synovium caused an increase in the intensity of staining. Thus, MICA expression may be upregulated in inflamed synovium, but, so far, no disease specific patterns have emerged. Further examples of synovium from a number of different pathologies will help to establish whether any disease-specific changes can be found.

The possibility of cross-reactivity of the polyclonal antibodies with other members of the extended MHC Class I family has to be considered. It is very likely that PERB11.1 204 and 205 detect both MICA and MICB since the peptide used for the production of the antibodies share 9/11 residues. Much less is known about the function and expression of MICB than MICA. It does appear to be less polymorphic than MICA and no specific disease associations have been described.

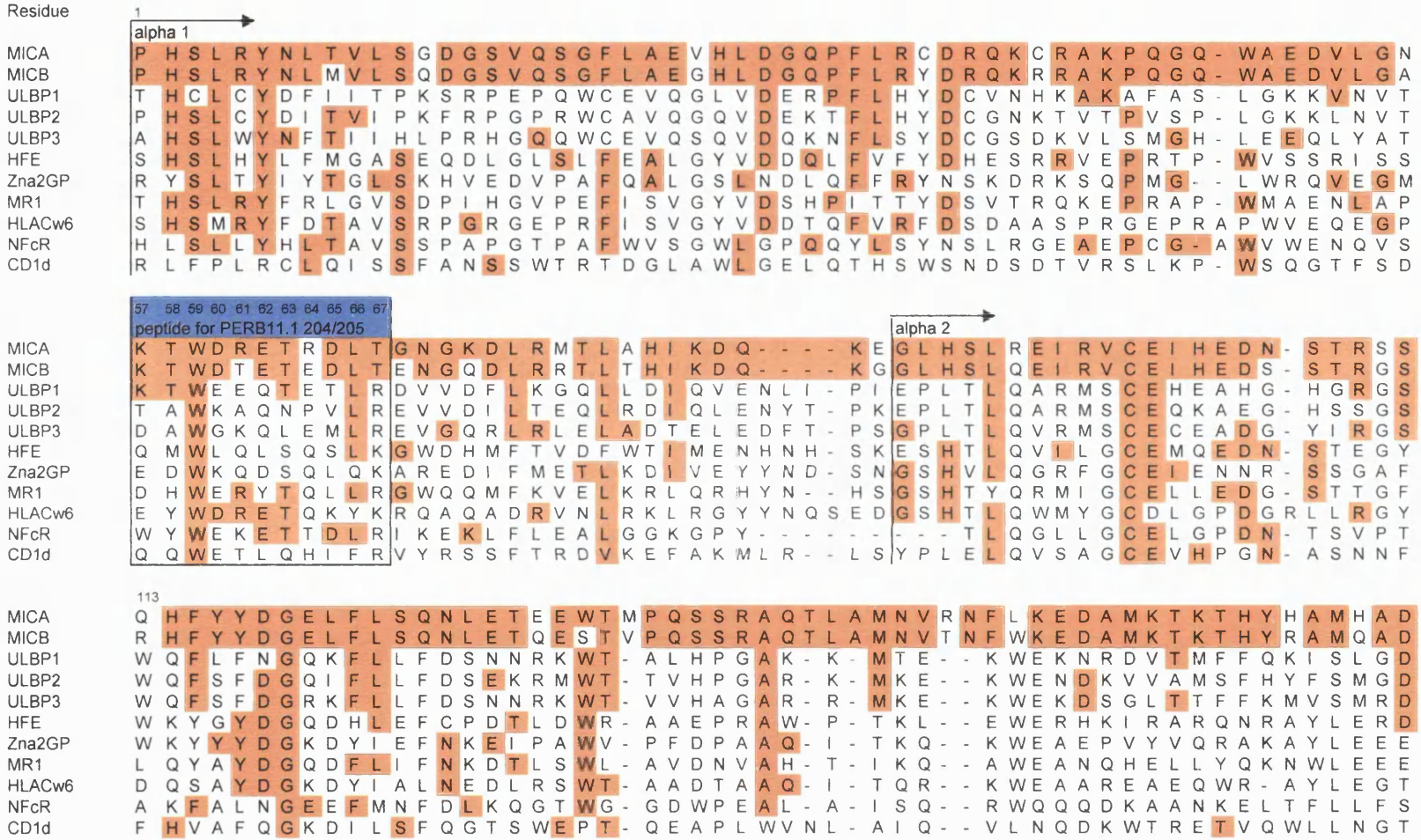
The peptide used for the production of PERB11.1 204 and 205 does share 5/11 residues with HLA-Class I, the neonatal Fc receptor and ULBP1. HLA Class I molecules are ubiquitously expressed in most tissues. However, the pattern of expression obtained using antibodies specific for HLA Class I (W6/32) is clearly different from that obtained with PERB11.1 204 or 205. The neonatal Fc receptor is responsible for the shuttling of maternal IgG from the intestinal lumen into the bloodstream. Although it is possible that it is expressed in the skin or synovium, it would seem unlikely.

Another possible cross-reactivity is with ULBP1. This interesting protein is one of three binding proteins that bind to the human CMV protein UL16. The ULBPs, like MICA, bind to the stimulatory NKG2D receptor that is expressed on NK cells and T cells. It is possible that the CMV protein, UL16, avoids destruction by NK and T cells by binding to the ULBPs, preventing them from activating the host's immune response. The ULBPs are widely expressed on T and B cells, a variety of tissues and

are possibly upregulated in tumour cell lines. Their expression in the skin and synovium has not been reported. The sharing of the NKG2D receptor by MIC molecules and the ULBPs is intriguing and may suggest some complementary role in the regulation of the immune response to viral or other pathogens. A study of the expression patterns of the ULBPs would help to resolve some of these problems.

Further insight into the possibility of a functional role for MICA in the skin and synovium will require the investigation of more histological specimens from differing pathologies. These should include skin sections from other conditions which cause a 'psoriasiform reaction' as well as other inflammatory skin conditions such as eczema. In addition, the utilisation of other antibodies, including monoclonal antibodies, produced by a range of techniques will help delineate which of the patterns of staining are specific and which are due to cross-reactivity or background staining. A better understanding of the functional consequences of the allelic variants of the molecule will help determine whether any of the different expression patterns observed are due to an individual's genetic background. Until more is known of the nature and function of all the members of the EMCIF, it would be appropriate to interpret some of the minor expression patterns seen with these antibodies with caution. However, it would appear that MICA is expressed in the skin and synovium and expression patterns differ between the disease and control samples studied here. Although further work needs to be done, these preliminary findings would support a role for MICA in psoriasis and PsA.

Table 5.1. **Alignment of the amino acid sequence of the alpha 1 and 2 chains of the Extended MHC Class I Family showing the position of the peptide used to make the PERB11.1 204 and 205 antibodies**



Detection of MICA in normal skin by immunofluorescence with the PERB11.1 204 antibody

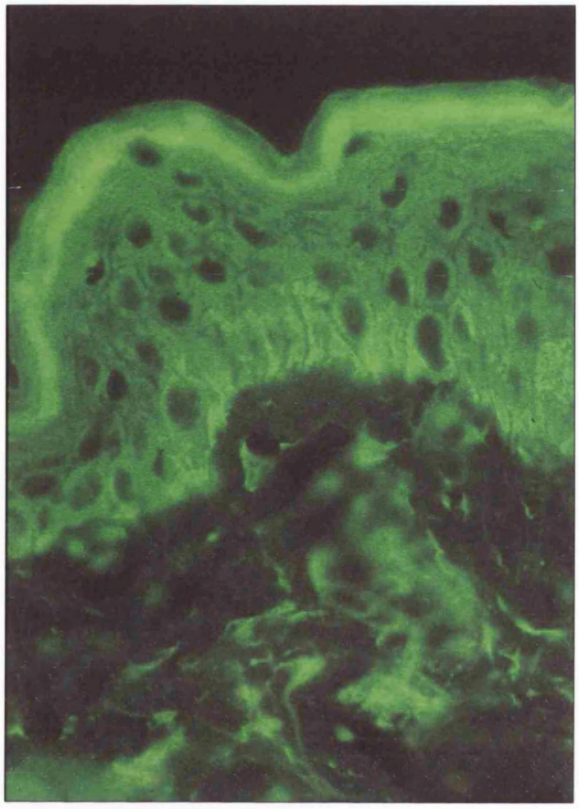


Figure 5.1.

Normal skin

Immunofluorescence with polyclonal antibodies to MICA (PERB11.1 204)

Expression of MICA predominantly in the basal layer of the epidermis

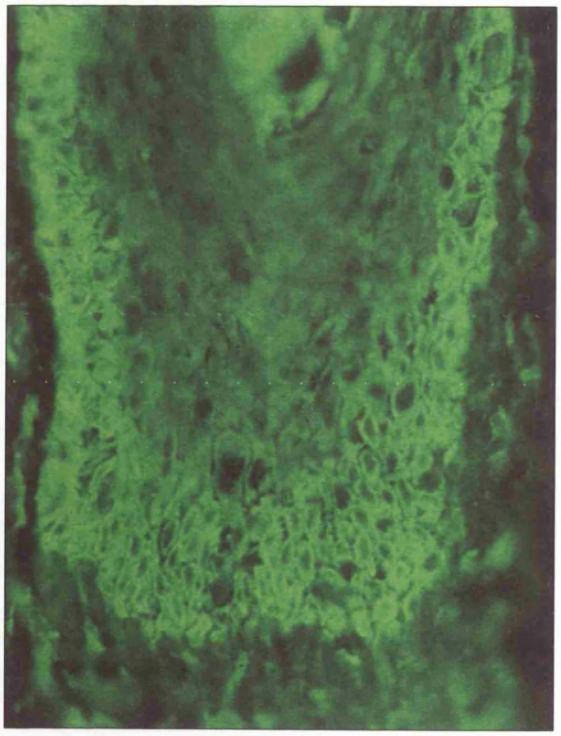


Figure 5.2.

Normal skin

Immunofluorescence with polyclonal antibody to MICA (PERB11.1 204)

Expression of MICA in the basal layers of the epidermis of a hair follicle

Comparison of the patterns of expression of MICA (using PERB11.1 204 antibody) and HLA Class I (using W6/32 antibody)

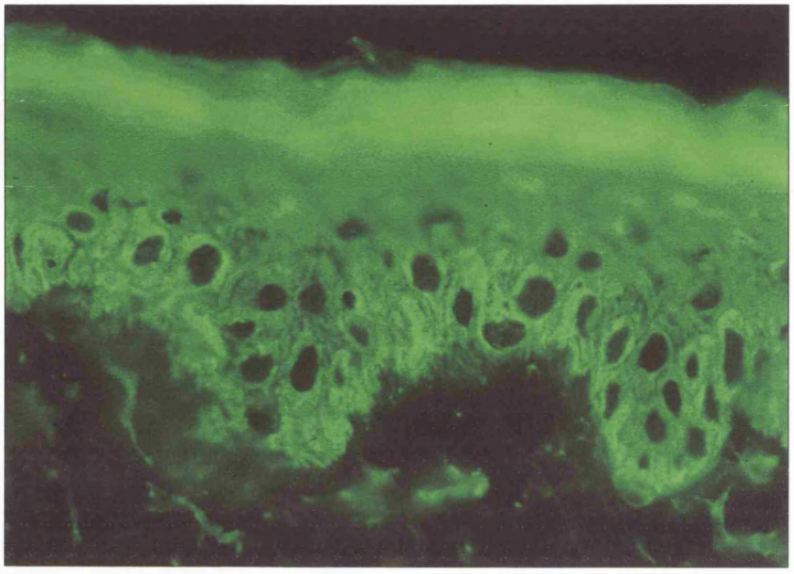


Figure 5.3.

Normal skin

Immunofluorescence with polyclonal antibodies to MICA (PERB11.1 204)

Expression of MICA within the cytoplasm. The expression is predominantly in the basal layers of the epidermis

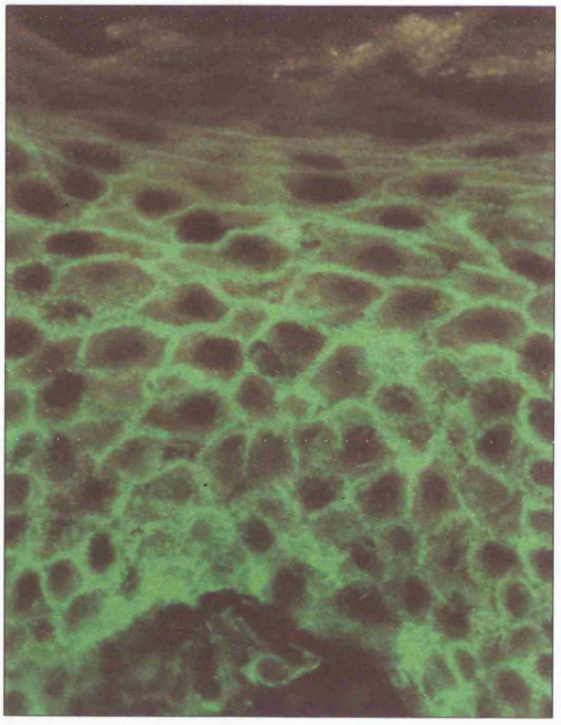


Figure 5.4.

Normal skin

Immunofluorescence with a monoclonal antibody to HLA Class I (W6/32)

Expression of MICA in normal skin demonstrated with a polyclonal antibody to MICA (PERB11.1 204) and a monoclonal antibody to MICA (Immunex)

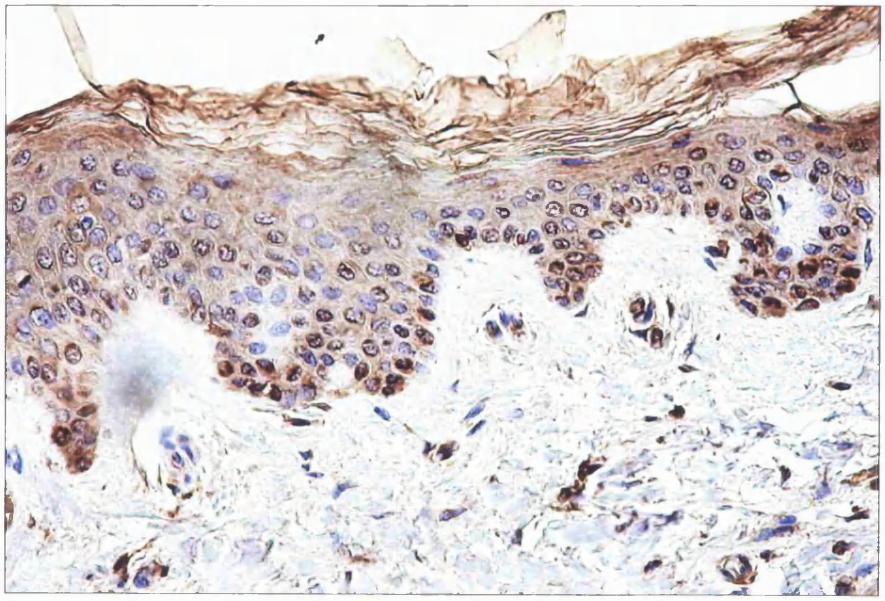


Figure 5.5.

Normal skin.

Immunohistochemistry using polyclonal antibodies to MICA (PERB11.1 204)

Cytoplasmic expression of MICA is seen in the basal layers of the epidermis together with some non-specific background staining

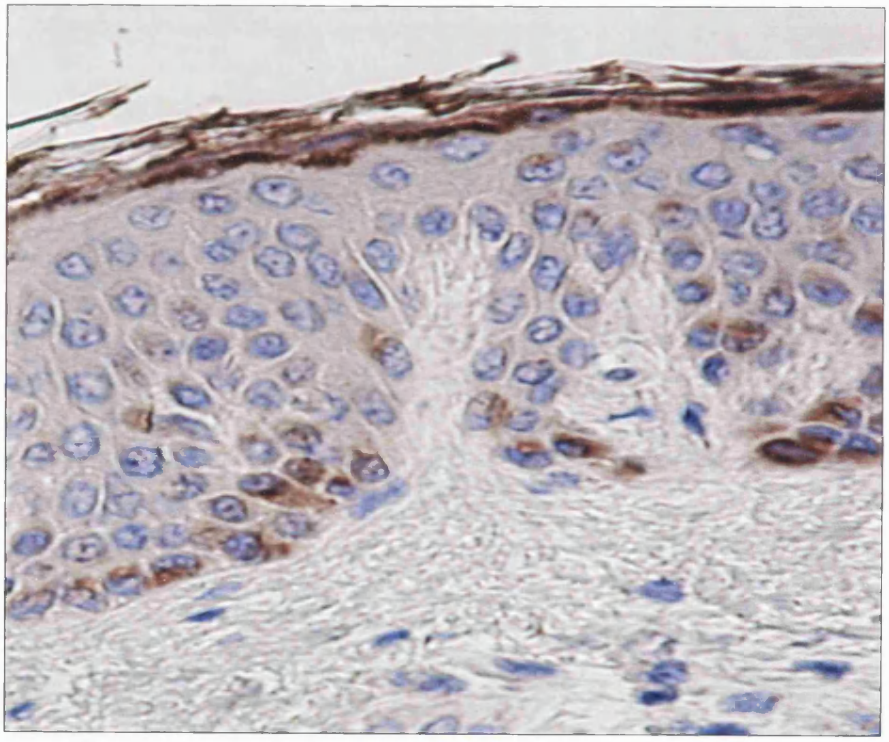


Figure 5.6.
Normal skin.

Immunohistochemistry using monoclonal antibodies to MICA (Immunex)

Cytoplasmic expression of MICA is located in the basal layers of the epidermis.

Non-specific staining of the keratin layer is also seen

Expression of MICA in psoriatic skin demonstrated with a polyclonal (PERB11.1 204) and monoclonal antibody (Immunex) to MICA

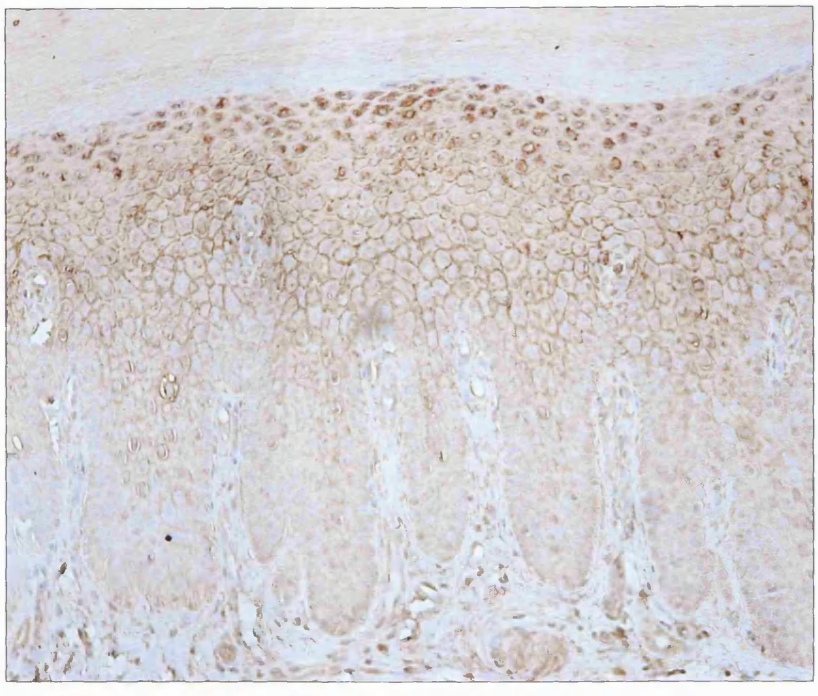


Figure 5.7.
Psoriatic lesional skin, low power view

Immunohistochemistry using PERB11.1 204 antibodies

Loss of expression of MICA in the basal layers of the expanded epidermis.

Cell surface pattern of staining in the central 'stratum spinosum'.

Cytoplasmic expression in the upper layers of the epidermis

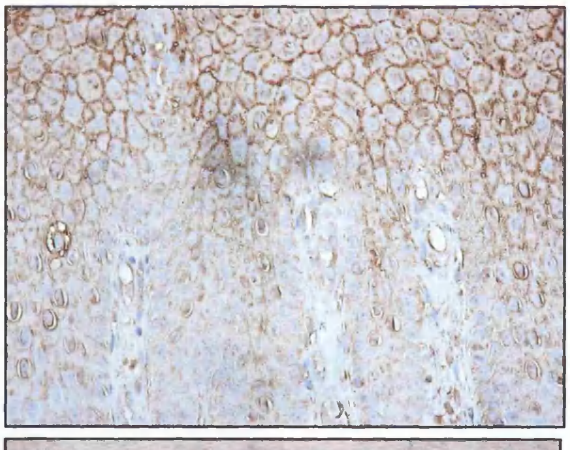


Figure 5.8.
High power view of psoriatic lesional skin stained with polyclonal antibodies to MICA (PERB11.1 204).

Loss of basal layer expression and change of pattern to cell surface expression in the middle layers of the epidermis

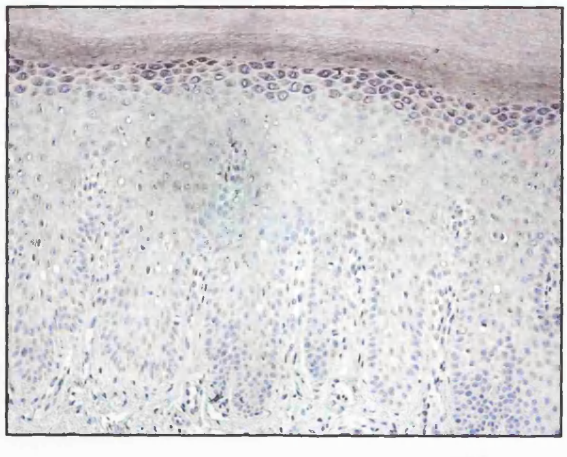


Figure 5.9.
Low power view of psoriatic lesional skin stained with monoclonal antibodies to MICA (Immunex).

Loss of expression of MICA in psoriatic skin in both the basal layer and the keratin layer

Expression of MICA in normal synovium demonstrated with polyclonal (PERB11.1 204) and monoclonal antibodies (Immunex) to MICA

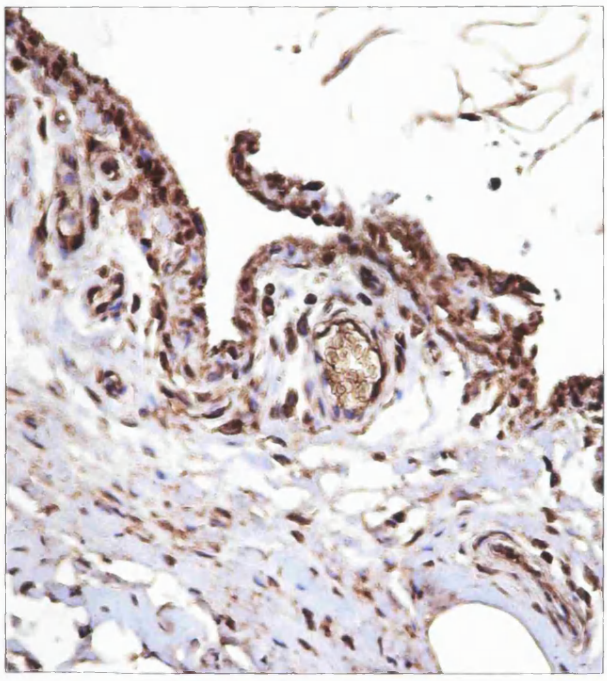


Figure 5.10.

High power view of normal synovium

The section is stained with the polyclonal antibody for MICA, PERB11.1 204.

Expression of MICA is detected in the surface synoviocytes and occasional inflammatory cells

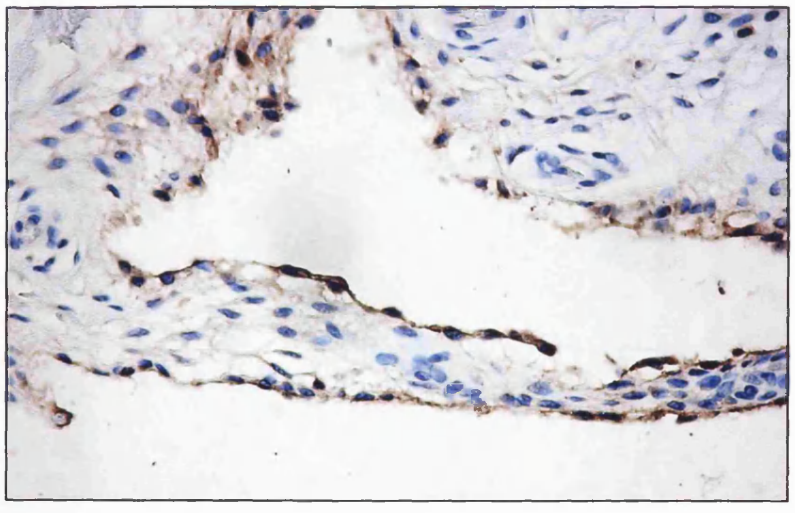


Figure 5.11.

High power view of normal synovium

The section is stained with monoclonal antibody for MICA (Immunex).

Expression of MICA is confined to the surface synovial cell layer

Expression of MICA in synovium taken from one patient with PsA and one patient with RA

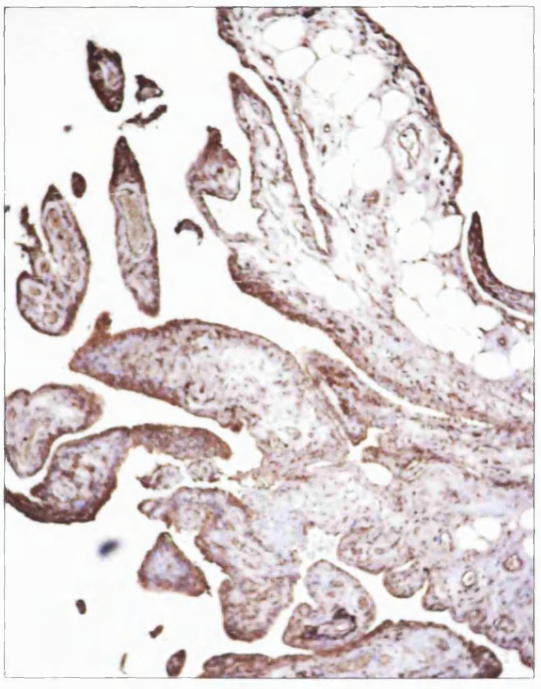


Figure 5.12.

Low power view of inflamed synovium taken from a patient with Psoriatic arthritis.

The section is stained with the polyclonal antibody, PERB11.1 204

Expression of MICA is clearly demonstrated along the surface of the frond-like synovium

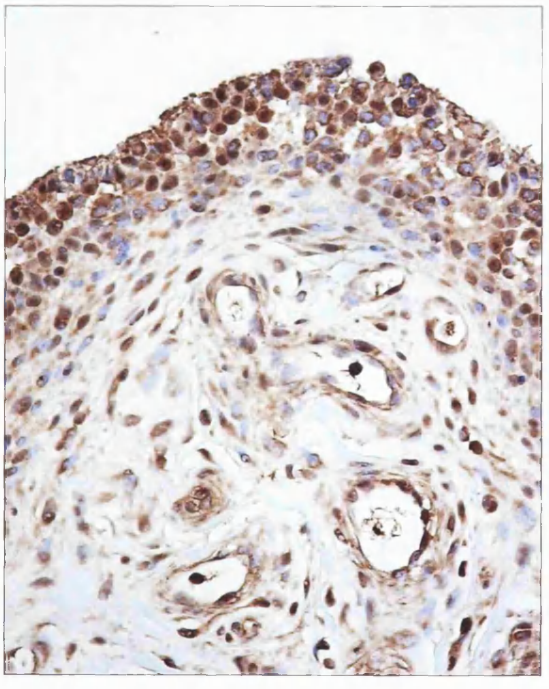


Figure 5.13.

High power view of inflamed synovium taken from a patient with Rheumatoid arthritis.

The synovium is stained with the polyclonal antibody, PERB11.1 204.

There is marked cytoplasmic expression of MICA in the expanded synovial layers and the inflammatory cell infiltrate

Negative controls for immunohistochemistry experiments

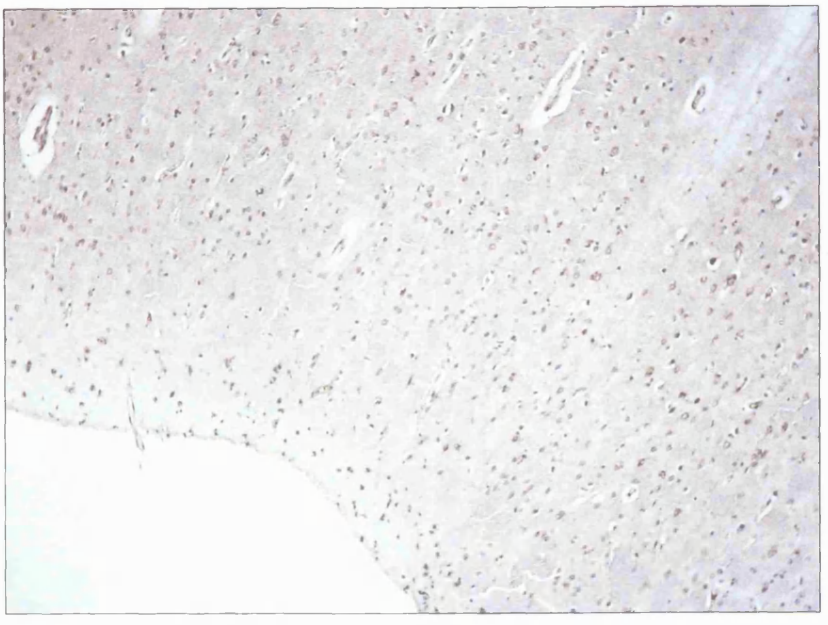


Figure 5.14.

Tissue control

A section of human brain stained with PERB11.1 204 showing no staining

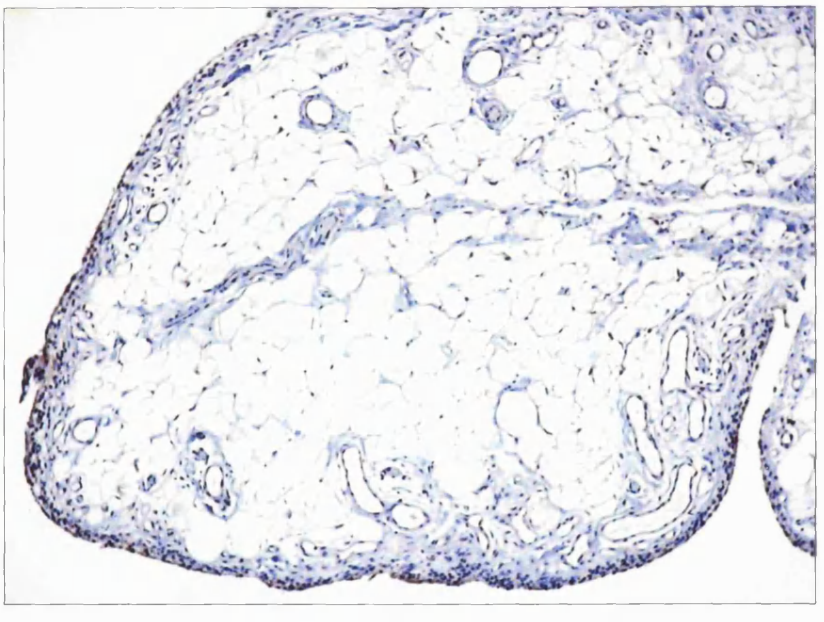


Figure 5.15.

Antibody control

A section of synovium stained with a rabbit polyclonal antibody against glial fibrillary acid protein showing no staining

CHAPTER 6 THE ROLE OF THE HLA-DRB1 'SHARED EPITOPE' ALLELES IN PSA

6.1 Introduction

The role of the MHC Class II loci in PsA has been much less widely studied than the MHC Class I loci. The MHC Class II loci, particularly HLA-DR4, are known to be strongly associated with RA, but their role in PsA remains controversial. The strongest HLA association with PsA does appear to be with HLA-Cw6, although the components of the extended haplotypes containing HLA-Cw6 (AH 13.1, 37.1 and 57.1) have also been implicated (61; 63; 180).

Several MHC Class I alleles have been independently associated with PsA and confer additional contributions to disease susceptibility and presentation. For example, HLA-B16 and its splits HLA-B38 and HLA-B39 have been associated with a peripheral distribution of PsA (62; 63). In contrast, HLA-B27 seems to be associated with sacroiliitis and axial disease (61; 62), DIP joint disease and oligoarthritis (63) and a later age of onset of arthritis (63). However, the correlation between HLA-B27 and spondyloarthritis is far less in PsA than AS. In one study of patients with PsA spondyloarthritis, grade 2 or higher sacroiliitis was present in 38% of HLA-B27 positive patients, but also 27% of HLA-B27 negative patients (63). HLA-B17 has been associated with early age of onset of both psoriasis and PsA(63; 181). HLA-B7 has been correlated with a milder form of PsA with fewer deformed joints (63; 182). There is additional evidence that some of the MHC Class I genes may modulate the severity of PsA. In particular, HLA-B27 and HLA-B39 have been shown to have a role in disease progression (183).

The situation with regards to the role of the MHC Class II locus in PsA is less clear. Most studies have not compared the frequencies of the Class II alleles with uncomplicated psoriasis, thus it is difficult to ascertain the relative contributions to the skin and the joint disease. The frequency of HLA-DR7 has been shown to be increased in both psoriasis and psoriatic arthritis (62; 184). This may well be related to its presence on ancestral haplotypes containing HLA-Cw6 (AH 13.1 and 57.1). One study

suggested an increased association between HLA-DR7 and more severe peripheral disease (62), whereas another has reported an association with milder disease (63). However, in some populations, HLA-DR7 is no more frequent in PsA than in the general population (64). This would suggest different contributions of the MHC to disease in various ethnic populations and confirms the inherent difficulties encountered when studying such a complicated condition.

The role of HLA-DR4 in PsA remains controversial; contradictory associations have been reported in diverse as well as similar ethnic populations. Two initial studies did report an increased frequency of HLA-DR4 in PsA (182; 185), but the numbers of patients studied were small. The more recent and larger studies have failed to find any increase in HLA-DR4 over and above the control population (63; 181; 186-188). HLA-DR4 has been reported to be associated with peripheral arthritis resembling RA by some (63; 63; 182; 185) but not others (64). There is also some evidence to suggest that HLA-DR4 is associated with erosive disease (62) but this has yet to be confirmed (63).

The association between RA and HLA-DR4 has been confirmed in many ethnic populations. Those patients who are not HLA-DR4 positive frequently carry HLA-DR1, HLA-DR6 or HLA-DR10. Closer analysis between these MHC Class II alleles revealed sequence homology between alleles *0101, *0102, *0401, *0404, *0405, *0408, *1001 and *1402 in the area encoding five amino acids at positions 70-74 (QKRAA/QRRAA/RRRAA) on the third hypervariable region of the HLA-DRB1 β chain. Thus the concept of the 'shared epitope' (SE) emerged (97). Furthermore, there is evidence that these shared sequences may be associated with disease *severity* in rheumatoid arthritis (189; 190) rather than disease *susceptibility*. This is particularly seen if the number of copies of a SE allele is considered; the presence of two SE alleles of the same or different type has been linked with more severe erosive disease, inferring a synergistic effect between the alleles (191-193).

The subtypes of HLA-DR4 in PsA have been further explored in a study of 90 patients with PsA, 90 patients with RA and 90 controls who were all HLA-DR4 positive (188).

The HLA-DR4 alleles present in the patients with PsA were less likely to be SE alleles than those present in RA. Patients with PsA were also less likely to carry two copies of a SE allele. HLA-DRB1*0401 was significantly less common in PsA than RA and the non-SE allele, HLA-DRB1*0402 was found significantly more often in PsA than RA. The study did not examine the clinical characteristics of the patients with PsA who were SE positive.

6.2 Aims of the study

A detailed study of HLA-DRB1 alleles in PsA has been undertaken in order to investigate the frequencies of these alleles in the disease group as a whole. The possible associations with clinical subgroup, characteristics and disease severity will also be explored. The role of HLA-DR4 and the SE alleles will be determined to see whether they help define a particular subgroup, perhaps those with more severe, peripheral, erosive disease resembling RA. The role of HLA-Cw6 and HLA-B27 will also be investigated, to examine whether these HLA Class I alleles define any particular clinical subgroup or disease pattern.

6.3 Materials and Methods

6.3.1 Study Population

One hundred and fifty eight patients were recruited from the specialist PsA clinic at the RNHRD in Bath, as detailed in Chapter 2.2. All patients were Caucasian, had psoriasis either at the time of follow-up or in the past confirmed by a physician, an inflammatory arthropathy and were mostly sero-negative for RF at initial assessment. Three patients had low titre positive RF when they were initially seen. There were 78 females and 80 males, the mean age was 56 years and the mean duration of arthritis was 21 years (*Table 6.1*). The patients were divided into disease subgroup as originally described by Moll and Wright (2), except that oligoarthritis was strictly defined as four or less involved joints and polyarthritis as five or more involved joints at the point of assessment. Patients with radiological sacroiliitis and predominant axial symptoms, with or without peripheral arthritis were designated as spondyloarthritis. Patients with classical clinical and radiological features of arthritis mutilans were placed in a separate

group. Two patients had involvement of only one knee and were classified as monoarthritis and one patient had involvement of the distal interphalangeal joints only. Thus, the patient subgroups were as follows: polyarthritis (n=83), oligoarthritis (n=56), spondyloarthritis (n=11), arthritis mutilans (n=5), monoarthritis (n=2) and DIP only (n=1), (*Table 6.1.*). The control population consisted of 250 healthy Caucasian blood donors from the Bath area. Whilst the controls were subject to the routine screen for blood donors, mild psoriasis, which occurs in approximately 2% of the population may have been present.

6.3.2 Outcome Measures

Data was collected annually and recorded on a database as described in Chapter 2.2. The information used for this study included a modified Ritchie tender and swollen joint count (modified to include the terminal interphalangeal joints), the psoriasis area severity index (PASI), a psoriatic nail severity score, the Health Assessment Questionnaire (HAQ) and inflammatory markers (PV or CRP). If the data had been collected on more than one occasion, a mean of the values recorded at different visits was used in this analysis. With regards to subgroup allocation, the subgroup which best categorised the patient on the most recent hospital visit was used. Antero-posterior radiographs of the hands and feet taken at the most recent visit were scored for the presence or absence of erosions and this was used as the main radiological outcome measure.

6.3.3 HLA-DRB1 typing and HLA-DR4 subtyping

Blood samples were collected from the patients following informed consent. DNA was extracted as described in Chapter 2.3.3. The samples were initially typed for the HLA-DRB1 alleles using the method described in Chapter 2.4.3. Any patients who were positive for HLA-DR1 and HLA-DR4 were subtyped for the SE alleles as described in Chapter 2.4.3.

6.3.4 HLA-B27 and HLA-Cw6 typing

The methods used for typing for these MHC Class I alleles is described in Chapter 2.4.4.

6.3.5 Statistical Analysis

The frequencies of the HLA-DRB1 alleles and SE alleles were compared between the patient and control populations using the Chi squared test with Fisher's exact test when necessary. A result was considered to be significant if the *p* value was less than or equal to 0.05. The Bonferroni correction was used to control for multiple comparisons (the *p value* was multiplied by the number of alleles/subgroups tested in each example). The results were also expressed as Odds Ratios. When performing the subgroup analysis, a 3 degrees of freedom (3df) global test was performed in addition to standard pair-wise comparisons between subgroups and controls. The patients from the smaller subgroups were combined to give a third "other" subgroup containing the patients with monoarthritis, DIP joint disease and arthritis mutilans. A 3 degrees of freedom global test was then performed using a 4*2 table and Chi squared test to examine the hypothesis that none of the 3 groups differed from the control population. A further test using a 2*2 table comparing the oligoarthritis subgroup and all other PsA subgroups combined was also performed.

6.4 Results

6.4.1 Associations of HLA-Cw6 with PsA and psoriasis

HLA-Cw6 was significantly associated with PsA (65/151 (43%) HLA-Cw6 positive PsA patients compared with only 26/131 (20%) controls, $p<0.0001$, OR 3.05 (1.79-5.22)). HLA-Cw6 was associated with a younger age of onset of psoriasis ($p=0.003$) and a higher median nail score ($p=0.002$), but there was no difference in the median PASI between HLA-Cw6 positive and HLA-Cw6 negative patients. There were no other clinical associations such as subgroup, DMARD use or presence of erosive disease. There was a significant association between HLA-Cw6 and HLA-DR7 due to their occurrence on the same ancestral haplotypes ($p<0.0001$, OR 6.28 (3.01-13.10)).

There were no associations between HLA-Cw6 and HLA-DR1, DR4 or the shared epitope.

6.4.2 Associations of HLA-B27 with PsA

Overall, the frequency of HLA-B27 was not increased in PsA compared with controls. There was no association between HLA-B27 and any of the clinical subgroups. HLA-B27 was not significantly associated with any other clinical parameters of disease severity. HLA-B27 was significantly associated with HLA-DRB1 *0101 (p=0.006) due to their presence on the same ancestral haplotypes. There were no associations between HLA-B27 and the HLA Class II alleles or the HLA-DRB1 SE.

6.4.3 Associations of HLA-DRB1 alleles with PsA

There was an increased frequency of HLA-DR7 within the PsA population as a whole compared with controls (41% vs 25%, p_{uncorr}=0..001, OR 2.02, p_{corr} =0.01) (*Table 6.2*). There was a trend towards an increased frequency of HLA-DR1 in PsA but this did not reach significance. There was a decreased frequency of HLA-DR2 and HLA-DR4 in PsA with the former only reaching statistical significance if uncorrected for the number of tests performed (p_{uncorr} =0.03, OR 0.59).

6.4.4 Associations of HLA-DRB1 alleles with PsA subgroups

There were no significant associations between disease subgroups and the HLA-DR alleles. HLA-DR4 was equally prevalent in both the polyarthritis and oligoarthritis subgroups (present in 25% of each group). HLA-DR1 was slightly more common in the polyarthritis group than the oligoarthritis group (34% vs 20%). HLA-DR3 was the most common allele in the spondyloarthritis group being present in 5 out of 11 patients (45%) and HLA-DR7 was the most common allele in all subgroups except the spondyloarthritis group.

6.4.5 Associations of the SE alleles with demographic and clinical characteristics

There was no association between the sex of the patients and presence of the SE (50% of the SE positive patients were male and 50% female). There was no difference in the age of onset of psoriasis, age of onset of arthritis, family history of psoriasis/arthritis, Health Assessment Questionnaire, joint score, PASI, nail score or mean plasma viscosity between SE positive and SE negative patients (*Table 6.3*).

6.4.6 Associations of the SE alleles with PsA

All SE alleles were detected in the patient and control populations except *1402 due to its low frequency in Caucasians. Overall there was no difference in the number of patients who were SE positive compared with controls (48% vs 54%, *Table 6.4*). The most frequent SE allele in the PsA group was *0101 (22%) followed by *0401 (17%). The *0404 allele was the only allele to be significantly different in its prevalence between the patient and control populations, present in only 3.8% of PsA patients compared with 10% of controls (p_{uncorr} =0.02, OR 0.34, p_{corr} =NS).

6.4.7 Associations of the SE alleles with PsA subgroups

The SE was present in all disease subgroups although only the oligoarthritis and polyarthritis groups were large enough for analysis. The prevalence of the SE in the polyarthritis subgroup was not increased compared with controls (49% vs 54% in controls, *Table 6.5*). However, there was a reduction in the frequency of the SE in the oligoarthritis subgroup compared with controls (38% vs 54%, p=0.02, OR 0.32 using a pair-wise comparison). This difference failed to reach significance if the oligoarthritis subgroup was compared against all other PsA patients (p=0.202) or if a 3 df test was performed comparing the oligoarthritis group with the polyarthritis group, a group combining the spondyloarthritis, arthritis mutilans, monoarthritis and DIP only patients and controls (p=0.134).

6.4.8 Double dose phenomenon

Two copies of a SE allele were found in 9.6% of the control population. There was no significant difference in the number of PsA patients with a double dose compared with

controls (6.3% vs 9.6%). The double dose phenomenon was seen more often in the polyarthritis subgroup (8.4%) and less often in the oligoarthritis subgroup (3.6%) but the groups were too small to reach significance.

6.4.9 SE and radiological erosions

One hundred and fifty one patients had radiographs of the hands and feet available for scoring for the presence of erosive disease, of whom, 76 had erosions and 75 did not. Erosive disease was most prevalent in the polyarthritis subgroup (73%) compared with 30% of the spondyloarthritis subgroup and 16% of the oligoarthritis subgroup. Those patients who were SE positive were significantly more likely to have erosive disease than those who were SE negative (60% (39/65) vs 43% (37/86), p=0.03, OR 2.11, *Table 6.6*). The presence of erosions was not more strongly associated with any particular SE allele than another. There was no relationship between any overall HLA-DR subtype (including HLA-DR1 and HLA-DR4) and erosive disease. Of the 76 patients with erosions, 39 (51%) were SE positive compared with only 26 out of 75 (33%) of the patients with no erosions.

6.5 Discussion

Since psoriatic arthritis can vary dramatically in its clinical course, any factor which may help identify patients who are more likely to develop severe or erosive disease would be of great benefit in patient management. This study has investigated the influence of the SE on the clinical course of PsA by determining its prevalence in each disease subset and whether it is associated with any particular clinical or radiological characteristic. The study also examined the frequency of HLA-Cw6 and HLA-B27 in PsA and its clinical subgroups.

The associations of HLA Class I antigens with psoriasis and PsA are probably better understood than those with the HLA Class II antigens. In the population studied, the frequency of HLA-Cw6 was significantly increased compared with controls. HLA-Cw6

was associated with a higher nail score but not with a higher skin score. The reasons for this are not clear. As expected HLA-Cw6 was associated with a younger mean age of onset of psoriasis, confirming similar reports in published studies. There were no other clinical or disease subgroup associations with HLA-Cw6. In contrast, HLA-B27 was not found to be increased in the PsA population as a whole, nor was it increased in any of the disease subgroups. Some studies have reported associations between the spondyloarthritis subgroup and HLA-B27, but this was not confirmed here.

More is known about the role of the SE in RA where it has been studied predominantly in early disease. There is mounting evidence that the SE exerts its effect in RA on disease severity as well as disease susceptibility (194). The majority of studies have investigated the role of the SE as a predictor of outcome in patients presenting with early synovitis. These studies have provided somewhat conflicting results (195; 196) but, in general, possession of the SE does appear to be associated with more severe disease particularly in relation to the presence of erosive damage and extra-articular features (190).

In RA, the increased prevalence of HLA-DR4 and HLA-DR1 has been well established and confirmed in many populations. However, a study of HLA-DR4 in newly diagnosed community based patients with RA did not find any association, suggesting that it is more important in the *severity and persistence* of RA than the *susceptibility* (194). There have been several reports of HLA-DR associations with PsA and its subgroups (61). This study has confirmed some of these but did not find some of the associations reported by others. In this group of patients with PsA, there was an increased prevalence of HLA-DR7 compared to a control population. In contrast to some reports of an increased prevalence of HLA-DR4 in psoriatic arthritis, particularly in the polyarthritis subset (63), a non-significant reduction of HLA-DR4 has been demonstrated in this population. The frequency of HLA-DR2 in the PsA population as a whole was reduced. There were no associations of HLA-DR subtypes with any of the disease subsets.

The frequency of the SE in a population with established RA (mean disease duration of 15.4 years) was recently found to be 76% (192). The frequency has been reported to be as high as 95% (197). However, as approximately 50% of the normal population carry at least one SE allele, its use as a predictive test is limited by low specificity and low positive predictive value. The test is probably better applied to patients known to have RA to help identify those at increased risk of severe disease (198). The frequency of the SE in this population of patients with PsA was 48%, with a control frequency of 54%. The most frequent SE allele in the patient population was *0101 followed by *0401. The frequency of *0404 was lower in patients than in controls and clearly different from the frequencies reported in RA where it is often one of the most common alleles. There was no association between different SE alleles and disease subgroup or markers of severity unlike RA where *0401 and *0404 have been linked with more severe disease (199). Another point of interest is that the SE was equally prevalent in male and female patients with PsA, whereas in RA it has been reported to be more frequent in men (200).

It could be hypothesised that the SE may be found at a higher frequency in those patients who develop a polyarthritis resembling RA. However, the frequency of the SE in the polyarthritis subset was found to be no different from the normal population. There was a reduction in the frequency of the SE in patients who remained in the oligoarthritis subset. This may suggest that, in the absence of the SE, patients are less likely to progress to polyarticular disease or that other genetic influences are more important in oligoarthritis. This study is based on patients with well-established disease with a mean duration of arthritis of 21 years. Although it is recognised that patients do move between subsets (95), the majority of patients in this study are likely to be correctly classified in view of the length of their disease process. The genetic associations with disease subsets in this study are therefore less prone to classification difficulties than in studies where the disease duration is shorter.

The influence of the SE on the development of radiological erosions in patients with PsA is another important finding of this study. Seventy-three percent of patients in the polyarthritis group had erosive disease of the hands and feet, compared with only 16%

of the oligoarthritis group and 30% of the spondyloarthritis group. When considering the proportions of patients in each subgroup with erosions, the influence of the SE was not so significant, but looking at the population as a whole, patients who were SE positive were significantly more likely to develop erosions than those who were SE negative (60% vs 43%, p=0.03, OR 2.11). This suggests that the SE may influence disease severity in PsA as well as RA. It is interesting that there is mounting evidence that the predominant influence of the SE on erosive disease in RA appears to be in patients who are seronegative for RF. This has been found in both early synovitis (201; 202) and established RA populations (203). In one of these studies (201), it was found that the SE had no influence on the likelihood of disease persistence in an early synovitis population and only a modest effect on functional disability. The most obvious effect was on the development of erosions (RR 1.9) and restricted to those who were seronegative for RF. No effect on the severity of radiological damage defined by the total Larsen score or number of eroded joints was noted.

It is possible that the SE allele copy number influences disease severity in RA (199; 204). The presence of two identical or two different SE alleles is known as the double dose phenomenon. A study in RA found an increased relative risk for RA in patients who were compound heterozygous for SE alleles (RR 11.7) over those who were homozygous (RR 4.3) for the same SE allele (192). Some 25% of the patients with RA had a double dose of the SE. In this study, only 6.3% of the PsA population as a whole carried two copies of a SE allele compared with 9.6% of the control population. There were no clear associations between the dose of the SE, homozygosity or compound heterozygosity and the disease subset or development of erosions, although fewer patients in the oligoarthritis subgroup had a double dose (3.6% vs 9.6% in controls, NS).

In conclusion, the frequency of SE alleles in this population of patients with PsA was similar to that found in a control population. There was no increase in the frequency of the SE in the polyarthritis subset. There was a lower frequency of the SE in patients who remained in the oligoarthritis subset. As only 16% of the oligoarthritis subset were

erosive, the lower frequency of the SE in this subset may simply reflect the association of the SE with erosive disease. It would seem that the greatest influence of the SE in PsA is in the development of erosions. It is possible the SE influences severity in RA via the development of erosions and this may be a feature in common between the two arthritis populations. However, the clear differences in the prevalence of the SE in our population and its subgroups and published data in RA further delineate the differences between patients with PsA and RA. The data presented here adds to the genetic evidence that PsA is a distinct clinical entity and argues against those who view the polyarthritis subgroup as simply a form of RA with psoriasis. Further genetic studies, both within and outside the HLA region, will hopefully provide more information on genetic classification and severity markers in psoriatic arthritis.

Table 6.1. **Demographic and clinical characteristics of the PsA population**

Characteristic	n or age (yrs)
Number of patients	158
Polyarthritis (≥5 involved joints)	83
Oligoarthritis (≤4 involved joints)	56
Spondyloarthritis	11
Arthritis Mutilans	5
Monoarthritis	2
DIP joint disease only	1
Sex (Male:Female)	80M:78F
Mean age, yrs (+/- SD)	56 (14)
Mean age of onset of psoriasis, yrs (+/- SD)	29 (15)
Mean age of onset of PsA, yrs(+/- SD)	37 (14)
Mean duration of PsA, yrs(+/- SD)	21 (13)

Table 6.2. **Phenotype frequency of HLA-DR alleles in the PsA patient population compared with controls**

HLA-DR	Patients (n=158)	Controls (n=250)	p_{uncorr} value (* = p< 0.05)	OR	p_{corr} value (** = p<0.05)
HLA-DR1	47 (30%)	53 (21%)	0.059	1.57	NS
HLA-DR2	30 (19%)	71 (28%)	**0.034***	0.59	NS
HLA-DR3	36 (23%)	52 (21%)	0.71	1.12	NS
HLA-DR4	44 (28%)	92 (37%)	0.067	0.66	NS
HLA-DR5	23 (15%)	48 (19%)	0.28	0.72	NS
HLA-DR6	35 (22%)	59 (24%)	0.81	0.92	NS
HLA-DR7	64 (41%)	63 (25%)	**0.001***	2.02	**0.01****
HLA-DR8	12 (8%)	13 (5%)	0.40	1.50	NS
HLA-DR9	3 (2%)	4 (2%)	0.99	1.19	NS
HLA-DR10	2 (1%)	2 (1%)	0.64	1.59	NS

Table 6.3. **Clinical and demographic features of PsA patients with and without the SE**

	SE present	SE absent
Females	35	43
Males	35	45
Mean age of onset of psoriasis, yrs (+/-SD)	29 (15.2)	29 (15)
Mean age of onset of PsA, yrs (+/-SD)	36 (14.2)	37(14)
Positive Family History of psoriasis	43%	45%
Mean HAQ at latest follow-up (+/-SD)	10.9 (5)	7.7 (7)
Mean joint score at latest follow-up (+/-SD)	17.5 (16)	15.4 (16)
Mean PASI at latest follow-up (+/-SD)	3.2 (4)	3.4 (3.4)
Mean nail score at latest follow-up (+/-SD)	6.0 (7)	5.4 (5.4)
Mean PV at latest follow-up (+/-SD)	1.81 (0.2)	1.81 (0.2)

Table 6.4. **Phenotype frequency of the SE alleles in the PsA patient population compared with controls**

SE Allele	Patients (n=158)	Controls (n=250)	p_{uncorr} value (* if p<0.05)	OR	p_{corr} value (NS if p>0.05)
*0101	35 (22%)	46 (18.4%)	0.37	1.26	NS
*0102	3 (1.8%)	4 (1.6%)	0.99	1.19	NS
*0401	27 (17%)	54 (21.6%)	0.31	0.75	NS
*0404	6 (3.8%)	25 (10%)	0.02*	0.36	NS
*0405	2 (1.3%)	1 (0.4%)	0.56	3.19	NS
*0408	1 (0.6%)	3 (1.2%)	0.99	0.52	NS
*1001	2 (1.3%)	2 (0.8%)	0.64	1.59	NS
Total	76 (48.1%)	135 (54%)			

Table 6.5. **The association of PsA subgroups with the presence of the SE**

Disease Subgroup	SE present	SE absent
Polyarthritis (n=83)	41 (49%)	42 (51%)
Oligoarthritis (n=56)	21 (38%)*	35 (63%)
Spondyloarthritis (n=11)	5 (45%)	6 (55%)
Arthritis Mutilans (n=5)	1 (20%)	4 (80%)
Monoarthritis (n=2)	1 (50%)	1 (50%)
DIP joint disease only (n=1)	1 (100%)	0 (0%)
Controls	135 (54%)	115 (46%)

* p_{uncorr}=0.027, OR 0.32 following pair-wise comparison between the oligoarthritis group and controls. If the groups are considered as 3 subgroups due to the small sizes of some groups (ie. polyarthritis, oligoarthritis and 'other'), the Bonferroni adjusted p_{corr} value would be 0.081 (3 x 0.027). A global 3df test to test the hypothesis that none of these three groups differs from the control population gives a p value of 0.134. A comparison of the oligoarthritis group with all other PsA patients yields a p value of only 0.202.

Table 6.6. **The association between the presence of the SE and the presence of radiological erosions**

	SE present	SE absent
Erosions present	39 (60%)*	37 (43%)*
Erosions absent	26 (40%)	49 (57%)
Total	65	86

*p=0.03, OR 2.11 when comparing the presence of erosions in SE positive and SE negative patients

CHAPTER 7 ANTI-CYCLIC CITRULLINATED PEPTIDE ANTIBODIES IN PSA

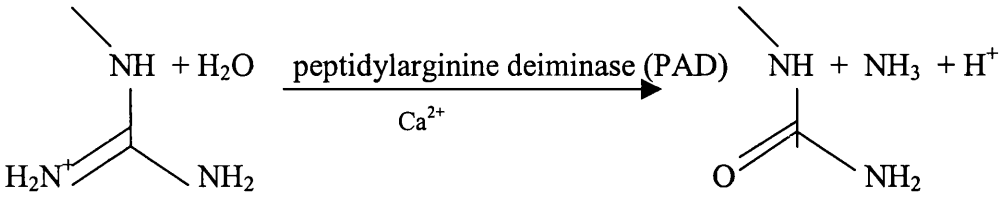
7.1 Introduction

The presence of anti-perinuclear autoantibodies in the sera of patients with RA was first described by Nienhuis and Mandema in 1964 (205). The antibodies were found to stain keratohyaline granules surrounding the nucleus of human buccal mucosal cells (206). These antibodies were not only highly specific for RA, they also seemed to indicate more severe erosive disease, especially in seronegative patients (207). Indeed, several studies have reported that they were more specific for RA than RF (208). They also appear very early in disease, thus aiding the diagnostic process (209). However, difficulties standardising the assay procedure precluded its routine use in clinical practice. The other major problems were related to the subjective and labour-intensive immunofluorescence technique, the availability of suitable buccal cell donors and interlaboratory standardisation (210).

Some of these problems were overcome with the development of an immunoblotting technique (211; 212). However, the quantitative and qualitative interpretation of the results was still reliant on subjective judgment and the sensitivity was poor. The use of native filaggrin extracted from human epidermis as the antigen for the technique provided a poorly standardised substrate due to variations in charge and deimination (213). A technique using synthetic peptides as the antigen for the RA autoantibodies was therefore developed.

The antigen recognised by the anti-perinuclear antibodies was initially thought to be profilaggrin, a structural protein expressed on differentiated epithelial cells (206). However, more recent studies have shown that the antigen is in fact filaggrin, a cytokeratin filament aggregating protein in the human epidermis, rather than its precursor (214). The same target is also recognised by the related autoantibodies originally described as anti-keratin antibodies (209). Anti-perinuclear antibodies and anti-keratin antibodies are therefore more correctly referred to as *anti-filaggrin* antibodies.

Purified human epidermal filaggrin has been used in an Enzyme Linked Immunosorbent Assay (ELISA) to quantify anti-filaggrin antibodies (AFA) in RA sera. The immunodominant epitopes on filaggrin are peptides containing the modified amino acid citrulline (98; 215). This amino acid is believed to arise in native proteins as a result of post-translational deimination of an arginine residue by the enzyme peptidylarginine deiminase (PAD).



Peptidylarginine Peptidylcitrulline

Deimination produces structural changes which lead to the denaturation of proteins (216). Antibodies to several synthetic citrullinated peptides have been described in RA sera with high disease specificity (98). However, an ELISA utilising these citrullinated peptides to recognise AFA (more accurately described as anti-citrullinated peptide antibodies) was not sensitive enough for the development of a routine serological test. The ELISA was therefore adapted to employ a modified peptide variant (*cyclic* citrullinated peptide (CCP)) that was recognised by the RA autoantibodies (anti-CCP antibodies) with relatively high sensitivity in a convenient and reliable test format (217).

Cyclic peptides adopt a more stabilised conformation in solution that is more representative of the antigenic site in the native protein. The use of cyclic (cfc1-cyc) instead of linear (cfc1) citrullinated peptides resulted in an increased sensitivity for RA (49% to 68%) without compromising the specificity (217). Control peptides where the citrulline was substituted with arginine were not reactive with RA sera.

Sequences of synthetic peptides

cfc1	SHQESTXGRSRGRSGRSGS	Linear peptides
cfc0	SHQESTRGRSRGRSGRSGS	
cfc1-**cyc**	HQCHQESTXGRSRGRCGRSGS	Cyclic peptides
cfc0-**cyc**	HQCHQESTRGRSRGRCGRSGS	

X = citrulline

R = arginine

The sensitivity of the test was further increased following the development of the CCP2 ELISA. This test was developed by screening libraries of citrulline-containing peptides for reactivity with RA sera. Novel peptides were then incorporated into the ELISA resulting in an increased sensitivity for RA of 82% (218). This ELISA is now commercially available.

7.1.1 Anti-CCP antibodies in conditions other than RA

The anti-CCP ELISA has been evaluated in a number of rheumatological and non-rheumatological conditions to examine its specificity for RA. Schellekens et al (217) assessed the anti-CCP ELISA in patients with a variety of rheumatological conditions, infectious diseases and healthy individuals. The anti-CCP ELISA was positive in only 2% of patients with rheumatological conditions other than RA. These included primary Sjogrens (1/50), SLE (1/60), systemic sclerosis (3/62), polymyositis/dermatomyositis (0/60), mixed connective tissue disease (1/47), vasculitis (1/30), reactive arthritis (1/40) and osteoarthritis (0/27). These results compared with a relatively high positivity (14%) for an IgM RF ELISA in the same group. Only 2% of the sera from patients with infectious diseases were positive for anti-CCP antibodies compared with 13% positivity for RF. In the healthy control group, less than 1% were positive for the anti-CCP ELISA. Anti-CCP antibodies were not present in ulcerative colitis, Crohns disease or multiple sclerosis. The ELISA was also tested on 19 patients with psoriasis, who were all negative, but not on patients with PsA. The prevalence of anti-perinuclear antibodies

(APF) detected by indirect immunofluorescence has been reported in a small study of patients with PsA (n=76). APF was found to be increased in PsA (7.9%), compared with 2.6% of uncomplicated psoriasis and 1% of healthy controls (219).

Anti-CCP antibodies have also been examined in juvenile idiopathic arthritis (JIA). No evidence of an association was reported: the prevalence of the antibody was between 2% (220) and 5% (221). There was no correlation with anti-nuclear antibodies, inflammatory response, erosions or disease subset. A further study has shown a positive correlation between anti-CCP antibodies and IgM RF positive patients with polyarticular JIA (juvenile RA) (222). These patients had significant radiological damage but the anti-CCP antibodies did not add any further information than the presence of RF.

The prevalence and clinical significance of anti-CCP antibodies has also been evaluated in palindromic arthritis (223). Anti-CCP antibodies were detected in 56% of patients, a similar frequency to that found in their RA population. There were no significant clinical differences between the patients with palindromic arthritis who were positive and those who were negative for anti-CCP antibodies.

7.1.2 The specificity and sensitivity of anti-CCP antibodies

The specificity of the anti-CCP ELISA for RA has been widely reported. One study examined the usefulness of the ELISA in an early arthritis clinic to predict RA (217). After 1 year, 31% of patients had developed RA, 27% undifferentiated arthritis, 10% crystal arthritis, 6% sarcoidosis, 5% psoriatic arthritis and 21% had other causes of an inflammatory arthritis. The specificity of the anti-CCP ELISA for RA was 96% compared with 91% for the IgM RF ELISA. The positive predictive value of the anti-CCP ELISA for RA was 84% compared with 74% for the IgM RF ELISA. The 14 'false positive' anti-CCP patients included 10 with undifferentiated arthritis, 2 with palindromic arthritis, 1 with sarcoidosis and 1 with pseudogout. After 2 years, four of the undifferentiated arthritis patients fulfilled the criteria for RA and three had developed erosive disease.

The sensitivity of the anti-CCP ELISA was found to be quite low: 48% compared with 54% for the IgM RF ELISA. After 1 year of follow-up, the sensitivity of the anti-CCP ELISA for RA increased marginally to 53% and the IgM RF ELISA decreased to 49%. Thus the anti-CCP ELISA changes very little during the disease process and appears to be present early in disease. A small proportion of patients can convert from positive to negative (3%) and from negative to positive (8%).

More recently, the specificity and sensitivity of the CCP2 test for RA has been reported. In a large multicentre trial, 79% of RA patients, 0% of healthy controls and 5% of disease controls were positive for anti-CCP antibodies compared with 78%, 15% and 50%, respectively, for RF (224). This high specificity and sensitivity has been confirmed in two further studies on the CCP2 test, where the sensitivity of the test was comparable to RF (80-82%) but with a much higher specificity; only 1% of healthy controls and 2% of disease controls were positive (218; 225).

There is no evidence that the presence of anti-CCP antibodies increases with age, as seen with the RF test. A recent study has shown that only 1/300 elderly subjects were positive for anti-CCP antibodies and this subject was the one person who also happened to have RA (226).

7.1.3 Anti-CCP antibodies are present early in disease

A further study of 379 patients with inflammatory arthritis of less than 3 years duration confirmed the findings that anti-CCP antibodies are found early in the disease process (227). They noted that in many cases the occurrence of RF and anti-CCP antibodies were independent. The optimal criterion for the distinction of RA from undifferentiated polyarthritis was a combination of the two antibodies, with a combined sensitivity of 55% and specificity of 96.7%. Other studies have confirmed the presence of anti-CCP antibodies early in the disease process, often before disease manifestations become apparent (228-230). A study using the CCP2 test has demonstrated that anti-CCP

antibodies are actually present several years before the onset of RA by the retrospective testing of blood donors (231).

7.1.4 Anti-CCP antibodies predict erosive disease

The association between anti-CCP antibodies and the development of erosive disease in RA has been widely examined. In one study, anti-CCP antibodies were positive in 65% of erosive but only 39% of non-erosive patients with RA at the initial consultation (217). Thus the detection of anti-CCP antibodies at presentation was a significant risk factor for erosive disease (OR 2.95). After 2 years of follow-up, 76% of the patients with RA had erosive disease: the positive predictive value of anti-CCP antibodies for the development of erosions was 91%.

Further studies have shown that anti-CCP antibodies are predictive of erosive disease both in established disease (232) and early in the disease process (233). The latter study demonstrated an increase in total Sharp score (OR 2.5) and higher mean scores for radiographic damage, joint space narrowing and erosions at 0, 3 and 5 years in patients who were anti-CCP positive. Kroot et al found an association between anti-CCP antibodies and erosions at 6 but not 3 years of follow-up (229). Some studies have also demonstrated an increased functional disability and absence of clinical remission in anti-CCP positive patients (234).

7.1.5 Anti-CCP antibodies are associated with the HLA-DRB1 SE

Although the HLA-DRB1 'shared epitope' (SE) is well known to be a major genetic contributer to RA, surprisingly few studies have examined or reported an association between anti-CCP antibodies and the SE. However, two studies have documented an association between these markers of RA. This is particularly interesting in light of the emerging evidence for the presentation of citrullinated peptides by the shared epitopes of HLA DR4 molecules (229; 230).

7.1.6 Possible immunopathological role for anti-CCP antibodies

It remains unclear whether the anti-CCP antibodies have any pathogenic role in the development of inflammatory arthritis. One group has studied the response of peripheral blood mononuclear cells (PBMC) to stimulation with filaggrin and found no response in RA or controls (OA, PsA) (235). This was in contrast to a proliferative response generated by PBMC and synovial T cells from patients with RA after stimulation with A2/RA33, another antigen known to produce autoantibodies in RA.

Immunohistochemistry using polyclonal antibodies has demonstrated the presence of citrullinated proteins in the synovial membranes of patients with RA (236). The same proteins appeared to be absent in control synovium. Interestingly, monoclonal antibodies to filaggrin show that filaggrin is not present in the synovium. However, affinity-purified anti-filaggrin antibody reactivity co-localised with the anti-citrulline activity. This would suggest that citrullinated proteins rather than filaggrin itself are the triggers for the autoimmune response.

Plasma cells actively secreting anti-filaggrin antibodies have been demonstrated in the synovial membrane of patients with RA (237). In addition, anti-CCP antibodies are present and secreted by B cells in the synovium and synovial fluid of patients with RA (238). This active production of IgM anti-CCP antibodies was only seen in patients who had anti-CCP antibodies detectable in their serum. The presence of cells actively secreting these antibodies in the synovium provides some evidence for an antigen-driven maturation of specific B cells at the site of inflammation in RA. The antibodies certainly seem to be locally produced as they constitute a greater proportion of the IgG in synovium than serum. Since filaggrin itself is not found in the synovium, the identity of this citrullinated autoantigen is not yet clear. It should possess deiminated epitopes that are recognised by both anti-CCP and anti-filaggrin antibodies.

One such autoantigen has recently emerged as a strong candidate: deiminated alpha and beta chains of fibrin (239). These two proteins, p64-78 and p55-61, were shown by immunoblotting to be specifically targeted by anti-filaggrin antibodies. Interestingly, it

was demonstrated 40 years ago that intra-articular injection of heterologous fibrin could induce chronic arthritis in a rabbit (240). Thus, autoimmunisation against deiminated fibrin may be a critical step in RA pathogenesis. The question remains, however, whether deimination of synovial fibrin is specific for rheumatoid arthritis or whether it occurs in other inflammatory arthritides.

The enzyme responsible for deimination of proteins is peptidylarginine deiminase or PAD. Deimination is known to interfere with observed in vitro protein-protein interactions and enzymatic processes. Five isotypes of PAD are recognised. PAD2 is found in macrophages and PAD4 in granulocytes. Recently, a particular haplotype of PAD4 was found to be strongly correlated with RA (241). This haplotype may result in functional differences within the enzyme that lead to increased citrullination of proteins.

The PAD enzymes are inactive in normal physiological conditions as intracellular Ca^{2+} concentrations are too low (242). However, following the death of a cell, loss of the cell membrane integrity allows Ca^{2+} to enter the cell. In addition, PAD enzymes may leak out of the cell where the Ca^{2+} concentration is high enough to allow citrullination of extracellular proteins such as fibrin. Citrullination of intracellular proteins such as histones and vimentin has been described in granulocytes and macrophages, respectively (243). Vimentin is certainly present in the synovium and many of the synovial lining cells are macrophages. Thus calcium influx into a dying macrophage may induce citrullination of vimentin. Interestingly, the Sa-antigen appears to be identical to citrullinated vimentin (244). The polyclonal anti-CCP response would suggest that there are additional citrullinated antigens that have yet to be identified in the synovium.

Citrullination of synovial proteins would not appear to be a process specific for RA. It is known to occur in murine models of non-specific inflammatory arthritis (245). Yet, the production of anti-CCP antibodies does seem to be highly specific for human RA. There is some evidence that the antibody response may be genetically determined by HLA subtypes bearing the HLA-DRB1 SE (97). The prevailing subclass of anti-CCP

antibodies is IgG1 (246) suggesting T cell involvement in the antibody production. Recent molecular modeling has shown that peptides containing citrulline but not arginine are bound by HLA-DRB1*0401 molecules (99). HLA-DRB1*0401 transgenic mice produce a proliferative T cell response following stimulation with peptides that contain citrulline but not arginine. This data is supported by the observed co-segregation of the SE and anti-CCP antibodies in clinical studies of RA (229; 230).

Citrullination is one of the post-translational modifications known to occur during apoptosis. Cell death may follow oxidative stress in an inflamed synovium following a relatively minor insult. This can then lead to the release and activation of PAD enzymes and the citrullination of intracellular and extracellular proteins. In the right genetic environment, these modified "self" proteins may then be presented by HLA molecules to T cells causing perpetuation of the initial inflammatory response. The anti-CCP antibodies will also form immune complexes with citrullinated proteins to further stimulate the production of pro-inflammatory cytokines. Thus, in certain pre-disposed individuals, a relatively minor self-limiting trigger can result in a chronic inflammatory disease.

If the process of autoimmune recognition of deiminated proteins is central to the pathogenesis of RA then clearly there is potential for new therapeutic targets. Interestingly, anti-CD20 therapy (rituximab) has been shown to reduce circulating levels of anti-CCP antibodies (247). A better understanding of the immunological and genetic background of individual patients with inflammatory arthritis should allow more appropriate targeting of such therapies. The presence or absence of anti-CCP antibodies may be one such factor.

The prevalence and prognostic value of anti-CCP antibodies in PsA is not known. The aim of this study was therefore to determine whether anti-CCP antibodies defined any particular clinical subgroup of patients with PsA. If anti-CCP antibodies were associated with more severe disease, then the test would be of value in the identification of patients who would benefit from early and aggressive treatment.

7.2 Hypothesis

Anti-CCP antibodies may be present in those patients with PsA who develop a more aggressive, erosive form of arthritis that is phenotypically similar to RA.

7.3 Study design

The study was designed to test a cohort of patients with PsA for the presence of anti-CCP antibodies using the second generation CCP2 ELISA test. Two control groups were used for comparison: normal controls (random blood donors) and patients with seropositive RA. The aims were to assess the prevalence of anti-CCP antibodies in PsA and to evaluate the clinical and genetic characteristics of any anti-CCP positive patients.

7.4 Methods

7.4.1 Patient recruitment

Patients with PsA (n=126) were recruited from the specialist PsA clinic at the RNHRD, Bath. Ethical approval was given by the Bath Local Ethics Council. A blood sample was taken from each patient following informed consent and sent to the laboratory where the serum was extracted and frozen until needed. Clinical and radiological information was collected on the patients participating in the study as described in Chapter 2.2. Control serum samples (n=40) were available from random blood donors and were matched for age and sex (20 male: 20 female, mean age 52 yrs). Serum samples from patients with seropositive RA (n=40) were used as positive controls. The patient and control serum samples were tested for the presence of anti-CCP antibodies using a commercially available ELISA (CCP2, Axis Shield, Scotland).

7.4.2 Statistical analysis

Direct comparisons between patients and controls were made using the Chi-squared test with Fisher's Exact test when necessary. A median of the outcome measures (eg. PASI, tender and swollen joint counts) was calculated from each of the consecutive visits that the score was recorded. These values were compared between groups using the Mann

Whitney U test. A p value <0.05 were considered to represent a significant difference between two groups.

7.4.3 Principle of the Enzyme-linked Immunosorbent assay (ELISA)

The wells of microtitre strips are pre-coated with highly purified specific peptides. During the first incubation, specific autoantibodies in diluted serum bind to the antigen-coated surface. The wells are then washed to remove unbound components. In the second incubation, an enzyme-labelled monoclonal antibody to human IgG binds any surface bound autoantibodies. After further washing, specific autoantibodies are traced by incubation with a substrate. Addition of a stop solution terminates the reaction, resulting in a coloured end product. The amount of autoantibody bound is then quantified by measuring the absorbance of light at a certain wavelength by the coloured solution and a comparison is made with reference controls.

7.4.4 The anti-CCP ELISA

The specific method utilised for the anti-CCP ELISA is given in Chapter 2.6.2. Each 96 well plate provides enough wells to test 40 samples together with the reference controls. All samples and controls were tested in duplicate and some samples were repeated on separate plates to assess intra-assay and inter-assay reliability. The concentration of anti-CCP antibodies present in each sample was calculated directly from the absorbance readings by the software attached to the plate reader (Multiskan Ascent, Labsystems, Finland). A cut off value of >6U/ml was used to indicate a positive result. This slightly higher value was used as 6/40 (15%) of controls had values between 5U/ml and 6U/ml. Using a cut off value of 6U/ml resulted in a control frequency of 0/40 which was a better approximation to the published range for controls of 0-1%.

7.4.5 Rheumatoid Factor ELISA

The samples were also tested in duplicate using a commercial ELISA for Rheumatoid Factor (Sigma Diagnostics, St Louis, USA) as described in Chapter 2.6.3.

7.4.6 HLA-DRB1 shared epitope

All patients were tested for the presence of the HLA-DRB1 shared epitope (HLA-DRB1*0101, *0102, *0401, *0404, *0405, *0408, *1001), as described in Chapter 2.4.3.

7.4.7 HLA-Cw6 and B27 testing

The samples from patients with PsA were tested for the presence of HLA-B27 and HLA-Cw6 as described in Chapter 2.4.4.

7.5 Results

7.5.1 Anti-CCP antibodies in PsA, RA and controls

The number of patients with PsA positive for anti-CCP antibodies did not differ significantly from the control population (7/126 (5.6%) vs 0%, p=0.198). The titres of anti-CCP antibodies found in the 7 positive PsA patients ranged from 6.5U/ml to >100U/ml, with 5/7 patients showing titres of >20U/ml. All but one of the patients with RA were positive for anti-CCP antibodies, with 36/40 (90%) showing a titre of >20U/ml. Rheumatoid factor was positive in 8.7% of patients with PsA, 100% of patients with RA and 5% of controls (*Table 7.1*).

7.5.2 Rheumatoid factor and anti-CCP antibodies

Anti-CCP antibodies were not always associated with the presence of rheumatoid factor. Although both tests are commonly positive in patients with RA, it has been well described that some patients are positive for one but not the other. A positive test for both has a higher predictive value for RA. In the patients with PsA, it was uncommon for both tests to be positive (*Table 7.1*). Only 2 patients with PsA were positive for both anti-CCP antibodies and RF. Neither of the RF positive controls was positive for anti-CCP antibodies. All 40 patients with RA were RF positive and 39/40 were positive for anti-CCP antibodies.

7.5.3 Characteristics of the anti-CCP positive PsA patients

The sex ratio and age of the patients with PsA who were positive for anti-CCP antibodies was no different from the anti-CCP negative patients (*Table 7.2*). The distribution of subgroups was similar between the two groups. Of the 7 positive patients, 4 had a polyarthritis (≥4 involved joints) and 3 had an oligoarthritis (<4 involved joints). One anti-CCP positive patient (14%) had a spondyloarthritis in combination with a polyarthritis. Spondyloarthritis was slightly more frequent in the anti-CCP negative group, where 27 (23%) of the patients had clinical or radiological features of spondyloarthritis (not significant). Of these, only 2 patients had a pure spondyloarthritis and the rest were present in combination with either a polyarthritis (19/27) or oligoarthritis (6/27).

Patients who were anti-CCP positive were more likely to be on a disease modifying drug (DMARD) (100% vs 61%, p=0.049) and had a higher median swollen joint count (p=0.01). There were no other demonstrable differences in the PASI, nail scores or tender joint counts.

7.5.4 Anti-CCP antibodies and the HLA-DRB1 shared epitope in PsA

All 7 patients with PsA who were positive for anti-CCP antibodies possessed at least one copy of the HLA-DRB1 SE (3 patients were HLA-DRB1*0101 positive and 4 patients were HLA-DRB1*0401 positive, including one *0401 homozygote). This was highly significant compared with the anti-CCP negative PsA patients (7/7 vs 48/119, p=0.0036). A comparison of HLA-DR4 between anti-CCP positive and anti-CCP negative PsA patients failed to reach significance (4/7 vs 28/119, p=0.069). HLA-DR1 was present in the same proportion of anti-CCP positive and negative patients (3/7 vs 32/119, p=0.396). There were no associations between Class I HLA antigens (HLA-B27 and Cw6) and anti-CCP antibodies. All 7 anti-CCP positive patients were negative for HLA-B27 (0/7 vs 24/119, p=0.35) and 4/7 were HLA-Cw6 positive (4/7 vs 46/119, p=0.43).

7.5.5 Anti-CCP antibodies and the HLA-DRB1 shared epitope in RA

All but one patient with RA was positive for anti-CCP antibodies. Fifty percent of the patients had titres >100U/ml, 90% had titres >20U/ml and only 3 patients had titres <10Uml. Seventy percent (28/40) of patients carried at least one copy of the HLA-DRB1 SE. The *titre* of anti-CCP antibodies was not associated with the presence of the SE: 65% of those with titres >100 U/ml were SE positive compared with 69% of those with titres of 20-100 U/ml and 100% of those with titres <10 U/ml.

7.5.6 Anti-CCP antibodies and erosive disease in PsA

All of the anti-CCP positive patients had erosive disease on radiographs of the hands and feet (7/7 vs 68/111, p=0.047). Six out of the seven patients also exhibited at least one characteristic radiographic feature of PsA such as new bone formation, DIP joint involvement or sacroiliitis. Review of the radiographs by two independent observers concluded that radiographically these patients had PsA not RA.

A summary of the features of the anti-CCP positive patients is presented in *Table 7.3*.

7.5.7 Characteristics of the RF positive and RF negative patients with PsA

Rheumatoid factor was more common in the older patients with PsA (*Table 7.4*). The sex ratio was the same for RF positive and RF negative patients. There were no differences in the subgroup distribution, with 4/11 RF positive patients presenting as an oligoarthritis and 1/11 with a spondyloarthritis. Unlike anti-CCP antibodies, RF was not associated with the HLA-DRB1 SE (6/10 RF+ SE+ vs 49/109 RF- SE+, p=0.51). There was no association with erosive disease, DMARD use or the number of tender or swollen joints.

7.6 Discussion

To date, there are no serological tests for PsA or the severity of PsA. Most research groups define their PsA cohorts as inflammatory arthritis in the presence of psoriasis and the absence of a RF. The latter is not an absolute criterion as approximately 8% of the general population are RF positive, particularly if >60 years of age. Indeed, most classification criteria for PsA suggest that the majority of patients will be negative for RF but that a diagnosis of PsA can be made in the presence of an incidental RF (2; 63) There are certain clinical and radiological features which are classic for PsA. Therefore, even if the RF is positive, a diagnosis of PsA can be made. For this reason, any RF positive patients with classical PsA have not been excluded from this study.

There is not a strong correlation between the presence of RF and the presence of anti-CCP antibodies in PsA; most patients who are positive for one are negative for the other. This would be in agreement with studies of seronegative RA where a high proportion have anti-CCP antibodies, although the absolute numbers in PsA are lower. The two antibodies probably identify slightly different subgroups of RA patients who may have different prognoses. Both have independently been associated with erosive disease in RA, but this would appear to be stronger with anti-CCP antibodies. In PsA, presence of RF was not associated with any clinical, radiological or genetic characteristics. This suggests that RF is simply an incidental finding of no relevance to the outcome of PsA. In contrast, several correlations between the presence of anti-CCP antibodies and the genotype and phenotype of PsA have been demonstrated.

A strong correlation between the presence of the HLA-DRB1 SE and anti-CCP antibodies has been demonstrated in this study. This has been reported in studies involving patients with RA (229; 230). We have previously shown that presence of the SE in patients with PsA correlates with erosive disease, suggesting that this genetic charcteristic may define a subgroup of patients with a worse prognosis (248). In this study, all 7 anti-CCP positive patients possess at least one copy of either HLA-DRB1*0101 or HLA-DRB1*0401. The mechanism for the association may be related

to the ability of the SE alleles to present peptides containing citrulline but not arginine to T cells. Thus, those patients with this particular genetic background will produce anti-CCP antibodies in response to an injured cell converting its arginine residues to citrulline. The antibodies may then play a direct pathogenic role in the perpetuation of the immune response, leading to the development of a chronic inflammatory disease.

Anti-CCP antibodies were also associated with the presence of radiographic erosions in patients with PsA. This association has been widely reported in RA where anti-CCP antibodies predict the future development of erosive disease. The mechanism for this remains unclear although it is possible that the antibodies, by sustaining the inflammatory response, are directly involved in joint damage.

Patients with PsA who are positive for anti-CCP antibodies also have a higher mean number of swollen joints than anti-CCP negative patients. This would suggest that they may be a marker for or indeed a contributory factor in more severe disease. All anti-CCP positive patients required treatment with a DMARD compared with only 61% of anti-CCP negative patients. It would not be unreasonable to hypothesise that all anti-CCP positive patients should present with a polyarthritis. However, there were in fact equal numbers of patients with an oligoarthritis and a polyarthritis. In view of the higher overall number of swollen joints seen in the positive group, this would suggest that even if <4 joints are involved they are likely to be chronically swollen. In addition, all of the anti-CCP positive patients with oligoarthritis had erosive disease and required treatment with a DMARD. This suggests that these patients fall within the more severe end of the spectrum of oligoarthritis. There was also one patient with features of an associated spondyloarthropathy in the anti-CCP positive group. Therefore, anti-CCP antibodies are present throughout the clinical spectrum of PsA and do not appear to be simply related to a phenotype resembling RA.

The key question here is whether these anti-CCP positive patients truly represent patients with PsA with a more severe prognosis or whether in fact they represent patients who actually have RA. In the latter scenario, the associations with erosive

disease, the SE, DMARD use and higher number of swollen joints may simply reflect the widely accepted view that RA carries a worse prognosis than PsA. There are, however, several reasons for categorising these patients as true PsA. Firstly, only 2 of these patients were also positive for RF and even these 2 patients had clinical and radiological evidence to support PsA. Secondly, 3 patients had a true asymmetrical oligoarthritis and one of the polyarthritis patients had an associated spondyloarthritis with radiological sacroiliitis. Most patients (6/7) had radiological features of PsA such as new bone formation, DIP joint involvement and asymmetry. All patients had confirmed psoriasis of the skin and nails, 50% had a family history of psoriasis but none had a family history of RA and there was an equal sex incidence.

Even if the definitive diagnosis remained in doubt, the evidence presented here suggests any patient with PsA who has anti-CCP antibodies falls into a poor prognostic category. The presence of the antibodies may represent some phenotypic overlap with RA or indeed the antibodies may directly affect the disease process to modify the presentation. We have shown that these patients all develop erosions and all require DMARD therapy. Therefore, if a patient has anti-CCP antibodies, DMARD therapy should be initiated early in the disease process.

There is no evidence from this study that the presence of RF is associated with a more severe outlook. RF was also found more frequently in the older patients, although this did not reach significance. There was no association between RF and erosive disease, DMARD use, subgroup, the number of swollen joints or the presence of the SE. This would suggest that the presence of RF in patients with PsA does not define a group of patients who have RA or indeed more severe disease. In those patients with classical clinical and radiological features of PsA, the presence of RF should not be regarded as a barrier to making the correct diagnosis. For the sake of purity such patients are often excluded from research studies, although one could argue that the evidence presented here suggests that this may not be necessary.

A number of studies have confirmed the presence of anti-CCP antibodies in RA early in the disease process, indeed often preceding the diagnosis by many years. Although they do appear to strongly predict the development of RA, it should be noted that a small proportion will develop PsA. The presence of anti-CCP antibodies does not therefore always mean that a patient with early synovitis will develop RA and can therefore not be used as a test to exclude PsA in diagnostic criteria. If a patient does have PsA, then they are likely to represent the more severe end of the spectrum. This has also been shown in seronegative RA, where the presence of anti-CCP antibodies is strongly associated with the development of erosions.

Although the CCP2 ELISA is an excellent technique, it cannot really be justified as a prognostic test in a PsA population as it will only pick up a small number of the more severe patients. It is probably more useful in a group of seronegative early arthritis patients (that will include some PsA patients) where it can predict those patients most at risk of progressive disease. It should be remembered that many patients with PsA who do not have anti-CCP antibodies will still develop a chronic debilitating arthritis. Thus, there remains a need for a test that will detect the *majority* of patients who will experience a more severe outcome. PsA is no longer regarded as a benign condition: it carries an increased mortality and considerable morbidity with many patients progressing rapidly to a destructive arthropathy. Perhaps clues for a prognostic immunological or genetic marker will come, not from studies based on RA, but from a better understanding of the immunopathology specific to PsA. The development of such a marker will facilitate more targeted and aggressive disease modifying therapy for those patients who will truly benefit.

Table 7.1. **Anti-CCP antibodies and RF in the patient and control populations**

	PsA (n=126)	RA (n=40)	Controls (n=40)
Anti-CCP positive	7 (5.6%)	39 (97.5%)	0 (0%)
RF positive	11 (8.7%)	40 (100%)	2 (5%)

Table 7.2. **Characteristics of anti-CCP positive and anti-CCP negative patients with PsA**

	Anti-CCP positive patients (n=7)	Anti-CCP negative patients (n=119)	*p* value	RR (95% CI)
Female : Male	4 F : 3 M	62F : 57 M	NS	
Age (yrs) (median, range)	59 (55-72)	59 (33-87)	NS	
Polyarthritis	4 (57%)	67 (56%)	NS	
Oligoarthritis	3 (43%)	48 (40%)	NS	
Spondyloarthritis	1 (14%)	27 (23%)	NS	
Median PASI (range)	2.0 (0-7.3)	1.0 (0-13.8)	NS	
Requirement for DMARD	7 (100%)	73 (61%)	<0.05	1.6 (1.4-1.9)
Median number of swollen joints (range)	7.5 (3-16)	2.0 (0-17)	<0.02	
Median number of tender joints (range)	5.5 (0-17)	4.0 (0-34)	NS	
Presence of erosions	7 (100%)	68 (61%)	<0.05	1.6 (1.4-1.9)
HLA-DRB1 SE	7 (100%)	48/112 (43%)	<0.005	2.3 (1.9-2.9)
HLA-DR4	4 (57%)	28/119 (24%)	NS	
HLA-DR1	3 (43%)	32/119 (27%)	NS	

Table 7.3. **Features of the anti-CCP positive patients with PsA**

	Anti-CCP titre	RF titre	HLA-DRB1	Erosions	DMARD	Subgroup
Patient 1	33	<6	*0101	Y	Y	Polyarthritis
Patient 2	90.3	39	*0401	Y	Y	Polyarthritis
Patient 3	48.2	<6	*0401, *0401	Y	Y	Polyarthritis and Spondyloarthritis
Patient 4	>100	<6	*0101	Y	Y	Polyarthritis
Patient 5	>100	15	*0401	Y	Y	Oligoarthritis
Patient 6	9	<6	*0401	Y	Y	Oligoarthritis
Patient 7	6.5	<6	*0101	Y	Y	Oligoarthritis

Table 7.4. **Characteristics of RF positive and RF negative patients with PsA**

	RF positive patients (n=11)	RF negative patients (n=119)	*p* value
Female : Male	6 F : 5 M	60 F : 55 M	
Age (yrs) (median, range)	64 (47-87)	59 (33-85)	<0.06
Polyarthritis	7 (64%)	64 (56%)	NS
Oligoarthritis	4 (36%)	47 (41%)	NS
Spondyloarthritis	1 (9%)	27 (23%)	NS
Median PASI (range)	0.42 (0-6)	1.0 (0-13.8)	NS
Requirement for DMARD	8 (73%)	72 (63%)	NS
Median number swollen joints (range)	5.0 (0-10)	2.2 (0-17)	NS
Median number tender joints (range)	8 (0-33)	4 (0-34)	NS
Erosions	4 (40%)	69 (64%)	NS
HLA-DRB1 SE	6/10 (60%)	49/107 (46%)	NS
HLA-DR4	3/10 (30%)	29/107 (27%)	NS
HLA-DR1	3/10 (30%)	32/107 (30%)	NS

CHAPTER 8 DISCUSSION

8.1 The challenges of researching the MHC in complex diseases

Over the last 30 years, more than 100 diseases have been associated with the HLA complex on chromosome 6. Despite the enormous resource and effort that has been expended in the characterisation of these associations, we are, in most cases, little further on in our understanding of the pathology of these diseases. Two clear exceptions are the HFE gene in haemochromatosis and the 21 hydroxylase genes in adrenal hyperplasia. The majority of HLA-associated conditions are linked to the Class II region (eg. DRB1*04 with rheumatoid arthritis, DRB1*1501 with multiple sclerosis, DQB1*0302 with Type I diabetes). Three notable exceptions are Psoriasis Vulgaris/PsA, ankylosing spondylitis and Behcets disease which are all primarily associated with Class I genes (HLA-Cw6, B27 and B51, respectively). It is interesting that these diseases also break the rule of female preponderance that is such a feature of many of the 'autoimmune' conditions associated with the Class II MHC region. In the absence of a viable explanation for the direct involvement of the Class I alleles in the disease process, the search for the key genes has strayed to those which closely neighbour the associated region.

A major problem with the study of any disease that is associated with the MHC is the strong evolutionary conservation of genes along segments of ancestral haplotypes (36). Large segments of the MHC are therefore inherited "en bloc" through many generations without the occurrence of any recombination events, although there are certain recombination hotspots that must be accounted for within any haplotype analysis. Dissecting out which alleles along an ancestral haplotype are more important than others can be a major challenge. One way is to assess the functional relevance of certain mutations. The impact of a given SNP on secondary protein structure or gene expression levels offers support for the functional relevance of a given allele associated with disease. The gene of interest should therefore be expressed in the target tissue and fulfil a potentially important function within the pathogenesis of the disease.

Some of the difficulties identifying a gene responsible for a complex disorder relate to preconceptions about genetic associations with disease. It is widely assumed that the genetic contribution to complex disease is due to relatively *high* frequency *SNPs* which have subtle effects on protein function (249). This may not always be the case, as has recently been shown in Crohn's disease where a *low* frequency *frameshift mutation* of the CARD15 gene on chromosome 16 is strongly implicated in disease susceptibility (73; 250). CARD15 has also been associated with PsA in one study (72), although this has yet to be confirmed (75). Interestingly, a similar frameshift mutation occurs in the allele of MICA that has been found to be associated with psoriasis, MICA *017, resulting in a polyleucine repeat like CARD15.

It is widely believed that if a gene is truly responsible for disease susceptibility, the association should be replicable in many different populations. However, it is clear that different genes take on different roles in diverse populations. For example, the associations between MICA and the TNF alleles with psoriasis / PsA in Caucasian and Asian populations are very different (51) (52) (67) (69) (70) (251). In addition, HLA-Cw6 bearing haplotypes are associated with psoriasis in Caucasians whereas HLA-Cw7 bearing haplotypes are associated with psoriasis in the Japanese. Similarly, in Type II diabetes, the CAPN10 haplotype confers different risks in different populations (252).

Therefore, the results of genome wide screens and high density mapping of potential disease susceptibility sites from one population can not necessarily be applied to all populations. One of the largest genome-wide screens and fine mapping for the PSORS1 gene was undertaken in the Japanese population (40). Similar techniques applied to a UK population led to the proposal of a different susceptibility region bordering that described in the Japanese population (41), suggesting that adjacent genes could be implicated in the same disease in different populations. Within a certain population or ethnic group these approaches can be extremely helpful in narrowing down the target area. Case control studies investigating genes that are in the correct *position* and have *functional* relevance to a disease remain a very effective tool in the search for candidate

genes. However, it should be remembered that the importance of different candidate genes may well vary between populations.

A further assumption in most association studies is that a single or a few closely related SNPs is responsible for disease. In complex diseases this would seem too simplistic. It is more likely that several SNPs in several genes are important. There is no reason to assume that adjacent genes may not play equal roles in disease susceptibility. Indeed, it may be the combination of a number of alleles along a disease-associated haplotype that places an individual at risk, particularly if these genes potentially interact with each other. In addition, certain genes may be important in disease *susceptibility* whereas others (which may be close-by) could interact in order to affect the *severity* of disease. An example might be the association of a particular haplotype with psoriatic arthritis that contains a certain allele of TNF, MICA and Corneodesmosin. This would be similar to the idea of a 'superlocus' in Insulin Dependent Diabetes Mellitus (IDDM) (253). It should also be noted that the consequences of SNPs may differ according to which *haplotype* they are on due to modulation by intronic sequences. Therefore, it is increasingly recognised that the *disease-associated haplotype* is of paramount importance, rather than the individual SNPs.

8.2 The challenges of studying PsA

There has been a reluctance to study PsA within the research community due to the considerable challenges it presents. There is therefore a relative lack of published research on PsA either from clinical or scientific studies. This contrasts with the wealth of studies investigating the aetiology, treatment and clinical assessment of other conditions with an equivalent prevalence such as systemic lupus erythematosis (SLE) and AS. There are several reasons that PsA is a difficult disease to study.

Firstly, it is a very heterogeneous disease. This has led to difficulties in the classification criteria which have yet to be agreed upon by the international community.

There have been several proposals for the division of patients into subgroups which would appear to have different prognoses, treatments and aetiologies. The most widely used classification follows that originally proposed by Moll and Wright (2), but it is increasingly recognised that patients can move between subgroups, most commonly from oligoarthritis to polyarthritis (95). The numbers of patients with involvement of just the DIP joints appears to be quite rare. Equally, the separation of monoarthritis and arthritis mutilans as distinct groups would be unhelpful for the majority of studies. It is therefore most likely that researchers will classify their patients into those with a peripheral arthritis and those with a spondylarthritis with or without peripheral joint disease. The patients for the studies presented here have been divided into three main groups – polyarthritis, oligoarthritis and spondyloarthritis.

A further difficulty in studying the genetics of PsA is dissecting out which genes are responsible for psoriasis and which for PsA. There will clearly be some genes that are important for both, but there are likely to be others that are more specific for the joints or the skin. The best way to address this is to examine patients who have psoriasis but no arthritis and compare them with patients with PsA. However, there are also some pitfalls in the recruitment of psoriasis controls. Firstly, the arthritis may not develop for many years after the psoriasis, thus some patients who are in the 'no arthritis' group will be bound to develop arthritis at a later date. This effect can be minimised by recruiting patients with a long disease duration. A further factor is the well recognised complication of asymptomatic sacroiliitis. Even though these patients may never complain of any symptoms and indeed may have totally normal spinal movements, they should be excluded from any group claiming to be 'arthritis free' as they are likely to share at least some of the genes that contribute to the inflammatory arthritis. Ethical approval to perform sacroiliac radiographs on asymptomatic patients can sometimes be difficult and therefore not all patients will be adequately screened. We did get ethical approval to perform sacroiliac radiographs on our psoriasis patients and found the incidence of asymptomatic sacroiliitis to be as high as 25% (96).

8.3 The challenges of studying MICA as a candidate gene

The MICA gene lies in the heart of the MHC region between HLA-B and the TNF genes. This places the gene close to the area that has been proposed as a susceptibility region for psoriasis (PSORS1) (39). There have been several reports of associations with PsA and the TNF genes (69) (70) and HLA B alleles (63). Not only does MICA lie in a good position for a candidate gene for psoriasis and PsA but it is also one of the most polymorphic genes in the MHC Class III region. The most extensive polymorphism lies within exons 2-4 that encode the extracellular portion of the molecule. There is a strikingly high level of non-synonymous mutations, a feature reminiscent of the MHC genes themselves. However, the reason for this level of variation between individuals remains unclear as many attempts have failed to detect any ligand binding. However, most of the polymorphism lies within the region of the molecule which directly interacts with its receptor, NKG2D. This lectin-type activatory receptor is found on NK cells as well as CD4 and CD8 positive T cells. Certain polymorphisms have been shown to affect the affinity of binding to the NKG2D receptor by up to 50 fold. It is possible therefore that the variation within MICA results in altered NK and T cell activation which may lay the host open to infection, tumour cell invasion or other attack.

The majority of clinical association studies have examined the microsatellite polymorphism within exon 5 that encodes the TM region of the molecule. There are six microsatellite alleles which vary in the number of GCT repeats encoding up to 10 alanines. However, the functional significance of this elongated TM region is unclear. It would seem unlikely that increasing the number of alanines in the TM region from four to ten would have any significant bearing on the ability of the molecule to interact with its receptor. However, there are some interesting polymorphisms which result in a very different TM region of the protein. This, in turn, may affect the ability of the MICA protein to locate to the cell surface. One such allele is the A5.1 allele which has 5 GCT repeats together with an insertion of an additional guanine causing a frameshift and premature termination of the protein. This results in abnormal localisation of the protein on the cell surface. The full impact of this on the function of the MICA/NKG2D

interaction is not known. However, it is intriguing that it is this allele that is uniformly the most common MICA allele within most populations. Another interesting allele of MICA, MICA *017, has 9 GCT repeats together with a single base deletion at the end of the 4th exon which also results in a frameshift. In this case, the TM region becomes hydrophobic due to the production of a polyleucine rather than a polyalanine repeat. It is this allele that is strongly associated with Type I psoriasis. A similar mechanism is found in the CARD15 allele that is strongly associated with Crohn's disease (72).

A method for the detection of the microsatellite repeat of MICA has been devised for the study of PsA and psoriasis using a Genescanner to separate double stranded PCR products on the basis of their size. The method was simple to use and the results could be obtained rapidly. The technique had the ability to distinguish PCR products that varied by only one base pair. However, this proved to be less reliable in the detection of heterozygotes where each allele only varied by one base pair, such as an A5 and A5.1 allele. This particular combination was sometimes difficult to distinguish with confidence from either A5 or A5.1 homozygotes. This could be resolved by further typing of the extracellular portion of the molecule.

Few disease association studies have examined the more complicated and polymorphic region of MICA encoded by exons 2,3 and 4, the region which makes direct contact with the NKG2D receptor. This is largely because of the number of SNPs involved and the complexity of the alleles. However, it would seem more likely that polymorphisms that affect the extracellular portion of MICA have the potential to cause disease. In order to rationalise the typing of the SNPs, a method using sequence-specific primers (PCR-SSP) was devised which was based on a phylogenetic analysis of the MICA alleles. The primers were then applied in a group-wise fashion to avoid unnecessary testing of every sample with every available primer pair. Although there were some alleles that could not be distinguished with this technique, it proved to be a simple and reproducible method which could easily be applied to other disease association studies.

8.4 The role of MICA in PsA

A major focus of this thesis has been the role of MICA in PsA. There is good evidence for the involvement of T cells and emerging evidence for the involvement of NK cells in both psoriasis (55) (56) (57) and PsA (23) (24) (94). Thus any factor which affects the stimulation of these immune cells within the joint or skin could have a potential role in disease pathogenesis. MICA is therefore a good candidate gene due to its interaction with the NKG2D receptor on T and NK cells leading to cellular activation and cytokine release.

The investigation was divided into three main areas.

- The identification of which microsatellite alleles within exon 5 (encoding the TM region of MICA) were associated with PsA and psoriasis.
- The development of a PCR-SSP method to identify the SNPs within exons 2,3 and 4 of MICA. This technique was then applied to determine the associations of the MICA alleles with PsA and psoriasis.
- The analysis of skin and synovial biopsies from patients with psoriasis and PsA using immunohistochemistry to determine the expression of MICA within the tissues in the presence and absence of disease.

The main conclusion of the microsatellite study was that the MICA A9 allele was strongly associated with PsA.

To determine whether this association was primarily with psoriasis or a true association with PsA, several populations of patients with Type I psoriasis were examined. The patients from the 13IHW from Belgium, Germany and Italy with Type I psoriasis had not been screened for the presence of a complicating arthritis. In this group as a whole, the MICA A9 allele was also significantly increased compared with the control population. However, the UK population with Type I psoriasis that had been fully screened for arthritis failed to show any significant increase in the MICA A9 allele. This may have been because of the smaller population size or because the true association was actually with PsA not psoriasis. The findings of this study confirm the

published smaller studies (67) (66) (68) in Spanish and Jewish populations of an association between MICA A9 and PsA. Interestingly, in one of these studies (67), the association was seen only with PsA and not with psoriasis, although the numbers were small and the psoriasis population unscreened.

The second phase of the investigation was aimed at defining the SNPs within exons 2-4, so that the alleles of MICA associated with disease could be identified. There are several alleles of MICA that carry 9 GCT repeats in exon 5, each defined by unique SNPs within exons 2-4. A method was therefore devised to identify the most common of the 54 known alleles of MICA based on the detection of SNPs with sequence-specific primers.

A method had been published using PCR-SSP to identify the MICA alleles, but at this point only 16 of the now recognised 54 alleles were known (116). The primers were therefore updated and new primers introduced to identify novel alleles. The current nomenclature of MICA is largely based on the time that particular alleles were discovered and bears little relation to sequence similarity. A phylogenetic analysis of the MICA alleles was therefore undertaken in order to identify evolutionarily similar alleles that could be grouped together for the purpose of genetic analysis.

The phylogenetic tree of MICA alleles revealed some interesting features. The alleles fell into 2 groups, based largely on the presence of a particular sequence in exon 4. The significance of this sequence is not known. Further distinct groups were identified which were sometimes associated with the same TM region eg. *001, *012, *018 (all A4) but sometimes not, eg. *007 (A4), *045 (A4) and *026 (A6). The majority of the A9 alleles clustered together apart from *015 which appeared in a different group. The alleles containing 6 GCT repeats (A6) were the most diverse, forming 3 separate groups; *004, *006, *009, *044 and *049 all grouped together, whereas *011 and *047 were in a separate group and *026 clustered apart from the other A6 alleles. This would indicate independent evolution of the TM region from the rest of the gene.

Primer pairs were utilised which recognised the alleles falling into one of five mutually exclusive phylogenetically based groups. A second stage PCR was then performed to elicit the major alleles within each group. The majority of patient samples could be typed for MICA using an average of nine primer pairs per sample. This was clearly an advantage over the two recently published PCR-SSP techniques for MICA (117) (170), both of which use blanket testing of all samples with all primers and need over 50 primer pairs per sample. Indeed, the study which examined the MICA alleles in inflammatory bowel disease tested in excess of 900 samples and found that only 13 of the possible 54 alleles were present at a frequency of >1%, with many not being detected at all. There is therefore a high level of redundancy in testing every sample for every allele.

For this reason a group based approach is clearly preferable and certainly less wasteful of time and resources. However, there are disadvantages to the simpler, step-wise technique employed here. Firstly, some of the alleles could not be adequately distinguished from each other. Secondly, certain heterozygote combinations were unable to be typed with confidence, such as *018 in the presence of *007. Thirdly, since fewer combinations of primers are utilised there are fewer opportunities for double-checking results and errors may therefore occur.

However, as the alleles had also been independently typed for the microsatellite alleles, this provided a method of checking results, particularly with certain heterozygote combinations. It is possible that some of the rarer alleles were present in the patient and control samples, but these were not detectable with the technique that was used. If higher resolution is required, a third stage PCR could be introduced to the groups to exclude the rarer alleles with confidence. However, it was decided for the purposes of this study that medium resolution typing would pick up any major associations with disease.

The major findings of the MICA PCR-SSP study were:

- **MICA *002 was significantly associated with PsA but not Type I psoriasis**
- **MICA *017 was significantly associated with Type I psoriasis but not PsA**

Both of these alleles contain 9 GCT repeats in exon 5. However, the differences between the MICA *002 and *017 alleles are two-fold:

- Firstly a **SNP at position 272** of exon 2 of MICA *017 results in a **change of amino acid from glutamine to arginine at position 91**. This is in the centre of the $\alpha 1 \alpha 2$ platform that interacts with the NKG2D receptor.

- Secondly, the **guanine at position 823**, at the very end of exon 4, of the MICA *017 allele **is deleted**. This deletion does not occur in MICA *002. The deletion results in a **frame shift** and a completely different transmembrane region in the MICA *017 protein. Instead of 9 alanines, the MICA *017 molecule has a **hydrophobic poly-leucine tail with truncation of the cytoplasmic segment.**

Both of these SNPs could therefore have potentially significant effects on the function of the MICA molecule. The alteration of the transmembrane region and truncation of the cytoplasmic segment could severely hamper the ability of the molecule to be expressed on the cell surface. This feature is of interest with regards to the observations from immunohistochemistry using antibodies to MICA on sections of skin with psoriasis. It would appear that the normal pattern of expression of MICA in the basal layer of the epidermis is lost in psoriasis with reduced expression. It is possible that the altered structure of the MICA protein in the *017 allele affects cellular expression. The second SNP is directly in the middle of the MICA platform that interacts with NKG2D. Although the functional consequences of this SNP have not been assessed in terms of

the 3-dimensional structure of MICA and its binding affinity with the receptor, it is certainly of potential importance.

It would appear that MICA *002 is a *susceptibility* rather than a *severity* gene for PsA. A detailed analysis of the prospectively collected clinical and radiological data on the patients with PsA failed to reveal any associations between MICA *002 with disease subset, swollen or tender joint counts, use of DMARDs or the presence of radiological erosions. Therefore the frequency of MICA *002 is markedly increased in PsA as a whole but there would not appear to be any association with markers of disease severity.

It is possible that MICA *002 and *017 share a common evolutionary ancestor. Through time, some individuals have branched off with the development of new mutations which predispose them to develop PsA whereas others follow the branch which leads to the development of uncomplicated psoriasis. The common ancestor may in fact be the A9 allele from which *002 and *017 have diverged.

8.5 MICA Haplotype analysis

The MICA *017 allele is highly conserved on the 57.1 ancestral haplotype and is in strong linkage disequilibrium with HLA-Cw6. If the association between MICA *017 and Type I psoriasis is stratified for HLA-Cw6, the MICA *017 allele is no longer significant. A component of this may be that the numbers involved once all HLA-Cw6 patients have been removed become much smaller. Greater numbers of patients would therefore be needed to demonstrate any independent association of MICA *017 with psoriasis.

However, the association between MICA *002 and PsA is independent of HLA-Cw6. Thus either the MICA gene itself is the key contributor to the association or it is a marker for a further disease-associated haplotype. The latter would seem less likely as *002 exists on many different haplotypes, with no clear alternative haplotype emerging as a strong contender. More information of the other alleles of neighbouring genes that

are found on MICA *002 haplotypes, such as the TNF genes, may help distinguish the importance of the potential candidate genes.

8.6 Studies of the expression of MICA

Although several studies have investigated the role of MICA in a variety of rheumatological conditions, there is no published data concerning the expression of MICA in the synovium. The aims of the expression study were:

- To determine whether MICA was expressed in the synovium
- To establish whether there was any pattern of expression that was specific to PsA by examining synovial biopsies from patients with other inflammatory arthritides
- To extend the limited knowledge of the expression of MICA in the skin and examine whether the pattern of expression differed between psoriatic and normal skin

A technique was developed to enable the use of archived histological material from patients with a clear diagnosis of PsA and psoriasis. Formalin-fixed, paraffin-embedded sections were prepared for immunohistochemical analysis by a process called antigen retrieval. Polyclonal antibodies specific for MICA had been produced by immunising rabbits with a peptide from the extracellular α1 domain of the MICA molecule. Monoclonal antibodies to MICA were also utilised to enhance the specificity for MICA.

One of the problems encountered with the immunohistochemistry analysis was the background staining and potential cross-reactivity with the polyclonal antibodies to MICA. There are a family of proteins which share a proportion of the amino acids with MICA that could potentially be recognised by the polyclonal antibodies. It is likely that the antibodies detect MICB as well as MICA, since the peptide used in the formation of the antibodies shares 9/11 residues between MICA and MICB. The other members of the extended MHC Class I family share much lower numbers of residues and cross-reactivity is therefore possible but not likely. This would be supported by the very different staining pattern observed with antibodies to MHC Class I molecules (W6/32)

compared with that observed with the MICA antibodies, despite MICA and MHC Class I proteins sharing 5/11 residues in the immunisation peptide. Some of the difficulties were addressed by the use of a monoclonal antibody to MICA, provided by a collaborating laboratory. Although the monoclonal antibody did not work optimally with the technique used, it was helpful in the determination of which patterns of staining were likely to be specific to MICA.

The main findings of the immunohistochemistry study were:

- MICA was expressed in the synovium. Expression was predominantly in the cytoplasm of the surface synoviocytes, although some more non-specific staining was also seen in the underlying vascular endothelium and inflammatory cell infiltrate. The monoclonal antibodies confirmed expression in the surface synoviocytes.

- The intensity of the expression was increased during inflammation, but the pattern and intensity of expression did not appear to differ between pathologies (PsA, RA and non-specific synovitis).

- MICA was found to be expressed in the skin, predominantly in the cytoplasm of the cells within the *basal* layer of the epidermis of normal skin. This was confirmed using the monoclonal antibody to MICA. However, in lesional psoriatic skin, the expression of MICA in the basal layer was reduced and in some cases lost.

- There was also a different *pattern* of staining observed in psoriatic skin. In 3 out of 5 sections, the cells of the stratum spinosum of the epidermis exhibited a strong *cell membrane* pattern not observed in normal skin. The pattern then returned to a predominantly granular cytoplasmic appearance in the upper stratum granulosum of the epidermis. Thus MICA expression was lost in the basal layers of the epidermis and then was found on the cell surface in the central epidermal layers. The reason for these changes remains unclear. It is of interest that the MICA allele that is

associated with psoriasis, MICA *017, results in a faulty protein with a hydrophobic transmembrane region and a shortened cytoplasmic tail. If the patients from whom the sections were taken that exhibited these altered staining patterns possessed MICA *017, this could explain some of the findings. Further work using skin sections from patients who had been genotyped for MICA would help to resolve this.

The expression study has provided some interesting results. The confirmation of expression of MICA in the synovium complements the case for the consideration of MICA as a candidate gene in PsA. Further work would need to be performed on a larger number of synovial sections from differing pathologies before any definite conclusions could be drawn with regards to any patterns of expression that may be unique to PsA. There were no obvious differences in this preliminary study. Equally, greater numbers of skin samples would need to be examined to elucidate the true nature of the intriguing changes in expression pattern observed in this study. Some fascinating insights into the expression patterns observed with different MICA genotypes could be gained if DNA and biopsy material were available from the same patient.

8.7 What are the potential functions for MICA?

The potential role of the MIC genes in histocompatibility has recently been explored. HLA compatibility is clearly a key factor in haematopoietic transplants and has been shown to improve survival in kidney transplants. The degree of complication following transplantation is directly related to the degree of histocompatibility. However, it has become clear that matching for alleles other than classical Class I and Class II HLA genes can improve outcome. In bone marrow transplantation, clinical results improve significantly if the donor and recipient are matched for *haplotype* (from a family member) rather than just for the HLA antigens (from an unrelated individual) (254). MIC would certainly be a good candidate for histocompatibility, particularly in view of the widespread expression on epithelial cells. A recent study has demonstrated the presence of antibodies to MIC in rejected 'histocompatible' kidney transplants (139). Further analysis of the genetic and outcome data from patients who have undergone

transplantation should help to address what role MIC has to play in transplantation but it would certainly seem to be of some importance.

There is evidence that MICA may be aberrantly expressed and therefore possibly non-functional in certain individuals. The commonest allele of MICA, A5.1 results in a truncated molecule with no cytoplasmic tail. This has been shown to result in abnormal localisation of the molecule on the apical rather than the basolateral surface of intestinal epithelium. It is possible that this has resulted in some evolutionary advantage culminating in the high frequency of this particular allele in almost every population studied. Perhaps the native MICA molecule lies dormant and requires certain factors, either genetic or environmental, to become active. Certain polymorphisms may then alter the expression and function of the molecule which may then stimulate the immune response and produce disease.

It is also clear that some individuals can function without any MIC genes whatsoever. The so-called MIC 'null' haplotype (AH48.1) contains a complete deletion of both the MICA and MICB genes (130). This haplotype is extremely uncommon outside southeast Asia with the exception of some Amerindians. In southeast Asia, the AH48.1 is relatively common with a frequency of 3.2% and an expected homozygous carriage of 0.1%. So far, 8 individuals homozygous for the MIC null haplotype have been reported in the literature. Ostensibly, these individuals have no clinical symptoms and a limited exploration of their NK and T cell repertoires has proved unremarkable. This would suggest that the function of the MIC genes may be replaced in these individuals by functional homologues.

8.8 MICA and NK cells

Another factor in the functional role of MICA may be the repertoire of receptors on an individual's NK and T cells. Each NK cell bears many different types of both activatory and inhibitory receptors. There are two man types of NK cell receptors – the lectin-type receptors like NKG2D and the killer immunoglobulin-like receptors (KIRs). It has

recently emerged that there is a high degree of polymorphism and complexity in the genes that encode the KIRs on chromosome 19, resembling the MHC complex. A particular KIR haplotype will therefore encode a distinct set of receptors for an individual's NK cells. It is possible, therefore, that the presence of a particular MICA allele in the context of a particular KIR haplotype and HLA haplotype may influence the response of that individual to infection, cell damage or tumour cells.

There have been two recent publications examining the role of the KIR genes in the development of PsA and psoriasis. Both studies reported an association between the activating KIR2DS1 receptor and PsA (255) and psoriasis (256). Interestingly, the association between KIR2DS1 and PsA was only found if the HLA ligand for the corresponding inhibitory receptor (KIR2DL1) was absent. This may suggest one mechanism by which the presence of certain HLA Class I molecules, such as HLA-Cw6, can influence the NK cell response. It could be therefore that HLA-Cw6 and HLA-B27 are not simply markers for neighbouring disease-associated genes but are in fact directly involved in the immune response of an individual. Furthermore, which MICA allele an individual possesses may also influence NK cell activation via its interaction with the stimulatory NKG2D receptor.

8.9 HLA associations with PsA

The majority of the patients with PsA were typed for HLA-Cw6, HLA-B27 and HLA-DRB1 to examine any relationships to disease subset, clinical characteristics and disease severity. All the samples will be fully typed at all HLA Class I and II loci as part of the 13IHW but this work has yet to be completed.

The frequency of HLA-Cw6 was significantly increased in the PsA population compared with controls. HLA-Cw6 was associated with a higher nail score but not with a higher skin score. The reasons for this are not clear. As expected HLA-Cw6 was associated with a younger mean age of onset of psoriasis, confirming similar reports in

published studies. There were no other clinical or disease subgroup associations with HLA-Cw6.

In contrast, HLA-B27 was not found to be increased in the PsA population as a whole, nor was it increased in any of the disease subgroups. Some studies have reported associations between the spondyloarthritis subgroup and HLA-B27, but this has not been confirmed by others and was not confirmed here.

Overall, the only HLA Class II allele to be associated with PsA was HLA-DR7. This has been reported in previous studies (63) and is most likely to represent its presence on HLA-Cw6 containing ancestral haplotypes such as AH13.1 and AH57.1. There were no particular clinical characteristics associated with HLA-DR7. The prevalence of HLA-DR1 and HLA-DR4 was no greater than a control population, but a sub-analysis of the HLA-DRB1 SE alleles *0101, *0102, *0401, *0404, *0405, *0408 and *1001 has revealed some interesting associations within PsA.

8.10 The HLA-DRB1 shared epitope in PsA – what does it mean?

The HLA-DRB1 SE alleles have been consistently shown to be one of the strongest genetic associations with RA, particularly with the development of erosive disease. The frequency and relevance of these alleles in PsA has never been studied. It might be hypothesised that the SE alleles may be associated with a subset of PsA that resembles RA, perhaps with polyarthritis and erosive disease. The aims of this study were therefore to examine:

- The prevalence of the SE alleles in PsA
- Whether the presence of SE alleles was correlated with any particular clinical subgroup or phenotype of PsA
- Whether the SE alleles were associated with the presence of radiological erosions

The main findings of the study were:

- Overall the prevalence of the SE alleles was no different in PsA than in a control population
- There was no increase in the frequency of the SE in the polyarthritis subset
- There was a lower frequency of the SE in patients who remained in the oligoarthritis subset.
- A significantly greater number of patients with PsA who developed radiological erosions were SE positive (60% vs 43%, p=0.03, OR 2.11)

The findings add further evidence for the clear distinction between patients with PsA who have a polyarthritis and patients who have RA. It is possible that the SE influences the *severity* of RA as well as *susceptibility* to RA by contributing in some way to the development of erosions. It is interesting that the only significant association between the SE and PsA was in the development of erosions. This may therefore be a feature in common between the two arthritis populations. It is possible that the SE alleles are one of the genetic contributors to the *severity* of PsA.

8.11 Anti-CCP antibodies - are they a useful serological marker in PsA?

The development of an ELISA to detect the presence of anti-CCP antibodies in patients with early inflammatory arthritis has increased the specificity and sensitivity for diagnosing RA. Anti-CCP antibodies certainly seem to be superior to RF in their specificity for RA particularly with regards to other connective tissue diseases such as Sjogrens syndrome and SLE. However, there has been very little data on the prevalence of anti-CCP antibodies in PsA. It could be hypothesised that anti-CCP antibodies may be found in a subgroup of patients with PsA with more severe, erosive disease resembling RA. The aims of this study were therefore:

- To assess the prevalence of anti-CCP antibodies in PsA
- To elicit any associations with disease subset
- To elicit any associations with markers of disease severity
- To investigate any HLA associations including HLA-DRB1 and the SE

- To investigate whether the presence of RF had any impact on disease subset or severity

Overall the prevalence of anti-CCP antibodies was no higher in PsA than in a control population. However, there were some interesting features in the 7 patients (5.6%) who were positive for anti-CCP antibodies. Firstly, there was a significant association between anti-CCP positivity and DMARD use and number of swollen joints, indicating that it may be a marker for severity. In addition, there was a significant association with the presence of erosions similar to that seen in RA. The mechanism for the production of erosions is still poorly understood but it is possible that the anti-CCP antibodies play a role in the process possibly by the perpetuation of the immune response. There was no association between the presence of anti-CCP antibodies and clinical phenotype with an even distribution of polyathritis, oligoarthritis and spondyloarthritis in the positive patients. Thus, when present in PsA, anti-CCP antibodies would appear to be associated with indices of more severe disease.

There was a strong association between the presence of anti-CCP antibodies and the SE in all 7 positive patients. This is interesting and may relate to the pathology within the joint. It has recently been demonstrated that the SE alleles present peptides containing *citrulline* but not *arginine* to T cells. Thus, SE positive patients are able to produce anti-CCP antibodies in response to an injured cell converting its arginine residues to citrulline. The antibodies may then play a direct pathogenic role in the perpetuation of the immune response, leading to the development of a chronic inflammatory disease. Patients with PsA who are positive for anti-CCP antibodies also have a higher median number of swollen joints than anti-CCP negative patients. This would suggest that they may be a marker for or indeed a contributory factor in more severe disease.

One of the most important questions to resolve is whether the anti-CCP positive patients truly represent patients with more severe PsA or whether the patients really have RA. Some might see the associations with erosive disease, the SE, higher DMARD use and greater number of swollen joints as support for a diagnosis of RA. However, only 2/7

patients were also positive for RF. In addition, 3/7 patients had a true asymmetrical oligoarthritis and one of the polyarthritis patients had an associated spondyloarthritis with radiological sacroiliitis. Most patients (6/7) had radiological features of PsA such as new bone formation, DIP joint involvement and asymmetry. All patients had confirmed psoriasis of the skin and nails, 50% had a family history of psoriasis but none had a family history of RA and there was an equal sex incidence. However, more patient numbers would be required to improve the chances of establishing a definite conclusion. Even if the definitive diagnosis remained in doubt, any patient with PsA who has anti-CCP antibodies falls into a poor prognostic category and should be treated aggressively. However, the small proportion of patients who are positive for anti-CCP antibodies would not justify the routine testing of all patients with PsA. The test is most likely to be informative in an early arthritis setting as a predictor of persistence and severity of inflammatory arthritis.

Another interesting result of this study was that, in contrast to anti-CCP antibodies, there was no evidence that the presence of RF in PsA was associated with a more severe outlook. There was no association between RF and erosive disease, DMARD use, subgroup, the number of swollen joints or the presence of the shared epitope. Therefore, the presence of RF should not exclude patients from research studies on PsA. Neither should it be regarded as a barrier to making the correct diagnosis on the basis of classic clinical and radiological characteristics of PsA.

In summary, there are many challenges in the study of a complex heterogeneous disease such as PsA. Clear definition of clinical phenotype and prospective collection of data over extended periods of time should limit some of the variability. Another challenge is dissecting those factors that are predominantly involved in the development of psoriasis from those that predispose to a complicating inflammatory arthritis. Thus, the study of a control population with psoriasis who have been screened clinically and radiologically for the presence of arthritis is vital in the search for factors involved in the aetiology of PsA. This study has presented some significant immunogenetic associations with both PsA and psoriasis. Some of the associations are common to the two diseases whilst

others are independent and specific for the development of an inflammatory arthritis. Further work on these factors will hopefully establish why some patients with psoriasis develop arthritis whilst others never do.

8.12 Future work

8.12.1 Patient material

The significance of the genetic results from this study would be enhanced by higher patient numbers. There are considerably more patients with PsA who have been added to the database at the Royal National Hospital for Rheumatic diseases in Bath, many of whom have had DNA extracted to allow an extension of this study. In addition, it is hoped that ethical approval will be granted to allow the collection of DNA and demographic data from family members of the patients with PsA. There is a definite need to increase the numbers of patients with Type I psoriasis who have been screened for arthritis and recruitment from a further General Practice will hopefully commence soon.

8.12.2 Immunohistochemistry

The immunohistochemistry study presented in this thesis demonstrates some interesting preliminary results. Analysis of further samples is needed to establish clear expression patterns for MICA. The technique will need to be optimised for the monoclonal antibody to MICA so that some of the problems encountered with the polyclonal antibodies can be minimised. The use of further antibodies to MICB, other members of the extended MHC Class I family, NK cell receptors and T cell receptors will complement the results seen with MICA.

In addition, the collection of fresh skin and synovial samples from patients whose clinical details are known and whose DNA is available for analysis would be preferable to the use of archived material. Comparison of the expression patterns of MICA with the genotype of MICA will provide more information on whether there is any allele-specific expression.

8.12.3 Further genetic analysis

A major addition to the genetic analysis will be the availability of the results of the full HLA Class I and II typing on the patient and control samples. This will allow analysis of haplotypes of MICA and comparison of haplotype frequencies between patient and control populations. There is additional data available on polymorphisms of the TNF genes within the same population. This would allow the construction of extended haplotypes of MICA, TNF and the HLA Class I and II alleles. Further work is planned to elucidate the KIR haplotypes of the same patients. This could provide a fascinating insight into the interactions between MICA and NK cells and the roles they may play in the aetiology of PsA.

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Appendix 1

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PATIENT INFORMATION SHEET FOR PATIENTS WITH PSORIASIS AND ARTHRITIS

Markers of outcome in Psoriatic Arthritis Study

Introduction
This information sheet is for patients who have Psoriatic Arthritis. We would like you to take part in a research project. This is absolutely voluntary and if you would prefer not to take part your decision will be accepted without question. You would not have to give a reason and your future care would certainly not be affected in any way.

What is the purpose of the study ?
Arthritis associated with psoriasis is a very variable condition and affects patients in different ways. We are interested in finding out if there are any factors which influence why some patients develop more severe arthritis than others. In order to do this we would like to take a small sample of your blood to look for the presence of particular genes and a new antibody in the blood which may help us to understand why some people develop psoriatic arthritis and why some people have more problems from their joints than others.

Why have you been chosen ?
You have been asked to participate because you have arthritis associated with psoriasis.

Who is organising the study ?
Dr McHugh, Dr Korendowych and Dr Ravindran are organising the study which is funded by the Royal National Hospital for Rheumatic Diseases in Bath.

What will happen if you take part ?
We would ask you to give one small blood sample (approximately 15mls or 3 teaspoons). The blood will only be taken on one occasion. You will not need to make any special trips to the hospital to take part. If you agree to take part the blood sample will be taken at the end of your normal clinic appointment. We would also ask that you take a minute or two to fill in a short questionnaire. The doctor who sees you for your normal clinic appointment may take a little extra time examining your joints so that we have an accurate picture of how your arthritis affects you. Studies have also suggested that a proportion of patients with psoriasis and arthritis may have inflammation and arthritis in the spine and in some patients this may occur in the absence of any particular symptoms. We would also like to investigate this further and would also ask you to have an x-ray of your lower spine.

What are the possible benefits of taking part ?
The results of this study will not be of benefit directly to you but may eventually benefit others by increasing our understanding of the condition.

What are the possible risks of taking part ?
The insertion of a needle into a vein for collection of blood may produce minor pain, bruising or bleeding at the insertion site. There is a small risk of an infection at the site.

What will happen to my blood sample ?
Your sample will be kept in secure storage at the Bath Institute for Rheumatic diseases. The sample will be used to test for the genes and a new antibody we are interested in for this project. Some of this project involves working closely with a laboratory in Australia which has specialist facilities to look at certain genes. Dr Korendowych has been awarded a fellowship to travel to Australia to use their facilities. A small amount of your blood sample will also be transported to Australia where storage will be similarly rigorous to Bath and the sample will only be used by Dr Korendowych and then destroyed The sample will be kept for a further 10 years and then destroyed. The sample will only be used for the purposes of the tests described in this sheet. If any further tests are required in the future you will be informed and asked to give your permission before these could be carried out.

Confidentiality – who will know you are taking part ?
Data collected for this research will be treated as confidential information.Your GP will be informed that you have agreed to take part. Your blood sample will be allocated a study number and your name will not be on the sample. Only Dr McHugh, Dr Korendowych and Dr Ravindran will know that your study number applies to you. The results of the study on your blood will not be given to you. The overall results of the many patients who take part in the study will increase our understanding of psoriatic arthritis as a whole but will not be of benefit for individual patients. Summary results of this research may be published in the scientific literature but you will never be individually identified in any publication.

Will anybody benefit financially from the study ?
No

Ethics Approval
This study has been approved by the Bath Local Research Ethics Committee.

What happens now ?
You are free to choose whether you would like to take part. If you would like to take part you will be asked to complete and sign a consent form. If you do not it will not affect your usual care in any way. Thank you for reading this information sheet. If you have any questions about the study please ask the doctor who sees you in the clinic or Dr Ravindran/ Dr Korendowych who can be contacted in the clinic or at the Royal National Hospital for Rheumatic Diseases on telephone 01225 465941. Please keep this information sheet.

Thank you very much

Dr Ellie Korendowych and Dr Jaya Ravindran
Research Fellows to Dr McHugh

Appendix 2.

Royal National Hospital for Rheumatic Diseases
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INFORMED CONSENT FORM : Markers of outcome in psoriatic arthritis

Have you read the patient information sheet ? Yes / No

Have you had an opportunity to ask questions and discuss this study ? Yes / No

Have you received satisfactory answers to all your questions ? Yes / No

Have you received enough information about the study ? Yes / No

Who have you spoken to ?_____

Do you understand that you are free to withdraw from the study :

- At any time
- Without having to give a reason
- Without affecting your future care Yes / No

Do you agree to take part in this study ? Yes / No

Signed_____ Date _____

Name in block capitals_____

Signed (Researcher)_____ Date _____

linic date: __ / __ / ____ Study no: B P S A _ _ _

Appendix 3. Psoriatic Arthritis Follow-up form

orename (s)

urname

OB __ / __ / ____

Iospital No

Iew NHS No.

ddress

'elephone no

ection 1 – Joint Scores

	Right Tender	Right Swollen	Right Damaged	Left Tender	Left Swollen	Left Damaged
Temp. mand.						
Sterno. Clav.						
Acro. Clav.						
Shoulders						
Elbows						
Wrist						
CMC						
MCP1						
MCP2						
MCP3						
MCP4						
MCP5						
PIP1						
PIP2						
PIP3						
PIP4						
PIP5						
DIP1						
DIP2						
DIP3						
DIP4						
DIP5						

	Right Tender	Right Swollen	Right Damaged	Left Tender	Left Swollen	Left Damaged
Hips		■			■	
Knees						
Ankles						
Tarsi						
MTP1						
MTP2						
MTP3						
MTP4						
MTP5						
Toes(PIP)1						
Toes(PIP)2						
Toes(PIP)3						
Toes(PIP)4						
Toes(PIP)5						
Toes(DIP)2						
Toes(DIP)3						
Toes(DIP)4						
Toes(DIP)5						

Total Tender Joints

Total Swollen Joints

Total Damaged Joints

Total Involved Joints

Section 2– Clinical subgroup, Schobers, DAS and HAQ scores

a) Which subgroup best fits the current clinical picture? ☐ Mutilans ☐ Mono ☐ Oligo ☐ Poly ☐ Spond ☐ Dip

b) Schobers ☐☐☐☐☐

c) DAS score ☐☐☐☐☐

c) HAQ raw score ☐☐☐☐☐

Section 3 – Nail score

	R THUMB (1)	(2)	(3)	(4)	(5)	L THUMB (1)	(2)	(3)	(4)	(5)
Pitting	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Onycholysis	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hyperkeratosis	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Severe nail deformity	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Total Nail Score *(max 40)* ☐☐

Section 4 – PASI score

Extent:	<10%	10-29%	30-49%	50-69%	70-89%	90-100%	**Severity:**	Nil	Slight	Moderate	Severe	Very severe
Extent score:	1	2	3	4	5	6	**Severity score:**	0	1	2	3	4

Skin Area	Extent Score	Total Extent	{ SEVERITY: Erythema	Infiltration	Desquamation }	Total Severity	Total Extent x Total Severity
Head	☐	x 0.1= ☐·☐	☐ +	☐ +	☐	= ☐☐	☐☐·☐
Arms	☐	x 0.2= ☐·☐	☐ +	☐ +	☐	= ☐☐	☐☐·☐
Trunk	☐	x 0.3= ☐·☐	☐ +	☐ +	☐	= ☐☐	☐☐·☐
Legs	☐	x 0.4= ☐·☐	☐ +	☐ +	☐	= ☐☐	☐☐·☐

PASI (PsA Skin Index) = extent x severity *(max score 72)* ☐☐·☐

Section 5 - Investigations

a) Research bloods requested: ☐ Yes ☐ No ☐ N/A

b) Xrays requested: ☐ Hands ☐ Feet ☐ Heels ☐ C spine ☐ L spine ☐ Other

If other please specify:

Section 6 – Medication since last visit

DMARDS

Name	Start date	End date	Reason for stopping
a) Methotrexate	/ /	/ /	
b) Sulphasalazine	/ /	/ /	
c) Leflunomide	/ /	/ /	
d) Hydroxychoroquine	/ /	/ /	
e) Anti-TNF	/ /	/ /	
f) Azathioprine	/ /	/ /	
g) Cyclosporine	/ /	/ /	
h) IM Gold	/ /	/ /	
i) Prednisone	/ /	/ /	
j) Other	/ /	/ /	

If other please specify:

Reasons for stopping codes:

SR=Skin rash	H=Hepatic	R=Renal	N=Neurologic	P=Pulmonary
I=Ineffective	N= Not drug related	IR=In remission	U=Unknown	O=Other

Section 7 - Hospital Admissions

(related to arthritis since last clinic visit)

Section 8 – Additional comments

Clinic date: |__|__| / |__|__| / |__|__|__|__| Study no: |B|P|S|A|__|__|__|

Appendix 4. Psoriatic Arthritis Initial form

Forename (s) |__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|

Surname |__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|

DOB |__|__| / |__|__| / |__|__|__|__|

Hospital No |__|__|__|__|__|__|__|__|

New NHS No. |__|__|__|__|__|__|__|__|__|__|

Address |__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|

|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|

|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|__|

Telephone no |__|__|__|__|__|__|__|__|__|__|__|

Section 1 - Arthritis

a) What was the time course of onset of arthritis? ☐ After onset of psoriasis ☐ Before onset of psoriasis ☐ At same time as psoriasis

b) Mode of onset ☐ Mutilans ☐ Mono ☐ Oligo ☐ Poly ☐ Spond ☐ Dip

Section 2 - Psoriasis

a) Has Psoriasis ever been present? ☐ Yes ☐ No

b) Has there ever been any nail involvement? ☐ Yes ☐ No

Section 3 –Extra-articular features

	Present	Not Present	Ever
a) Dactylitis	☐	☐	☐
b) Any tender enthesis	☐	☐	☐
c) Diffuse enthesis pain	☐	☐	☐
d) Chest wall pain	☐	☐	☐
e) Inflammatory heel pain	☐	☐	☐
f) Clinical sacroliitis	☐	☐	☐
g) Inflammatory lower back pain	☐	☐	☐
h) Inflammatory thoracic back pain	☐	☐	☐
i) Inflammatory neck pain	☐	☐	☐
j) Iritis	☐	☐	☐
k) Clinical small joint	☐	☐	☐
l) Inflammatory bowel disease	☐	☐	☐

Section 4 – Medical History

DMARDS

Name	Start date	End date	Reason for stopping
a) Methotrexate	/ /	/ /	
b) Sulphasalazine	/ /	/ /	
c) Leflunomide	/ /	/ /	
d) Hydroxychoroquine	/ /	/ /	
e) Anti-TNF	/ /	/ /	
f) Azathioprine	/ /	/ /	
g) Cyclosporine	/ /	/ /	
h) IM Gold	/ /	/ /	
i) Prednisone	/ /	/ /	
j) Other	/ /	/ /	

If other please specify:

Reasons for stopping codes:

SR=Skin rash	H=Hepatic	R=Renal	N=Neurologic	P=Pulmonary
I=Ineffective	N= Not drug related	IR=In remission	U=Unknown	O=Other

Section 5 – Lab data

Name	Date taken	result
a) PV	/ /	
b) ESR	/ /	
c) RF	/ /	
d) ANTI CCP	/ /	

Section 6 – X-rays

Have any X-rays been taken?

	Yes	Result
a) C spine	☐	
b) L spine	☐	
c) Pelvis	☐	
d) Hands	☐	
e) Feet	☐	
f) Heels	☐	
g) Other	☐	

If other please specify:

Section 7 – Joint Scores

	Right			Left		
	Tender	Swollen	Damaged	Tender	Swollen	Damaged
Temp. mand.	☐	☐	☐	☐	☐	☐
Sterno. Clav.	☐	☐	☐	☐	☐	☐
Acro. Clav.	☐	☐	☐	☐	☐	☐
Shoulders	☐	☐	☐	☐	☐	☐
Elbows	☐	☐	☐	☐	☐	☐
Wrist	☐	☐	☐	☐	☐	☐
CMC	☐	☐	☐	☐	☐	☐
MCP1	☐	☐	☐	☐	☐	☐
MCP2	☐	☐	☐	☐	☐	☐
MCP3	☐	☐	☐	☐	☐	☐
MCP4	☐	☐	☐	☐	☐	☐
MCP5	☐	☐	☐	☐	☐	☐
PIP1	☐	☐	☐	☐	☐	☐
PIP2	☐	☐	☐	☐	☐	☐
PIP3	☐	☐	☐	☐	☐	☐
PIP4	☐	☐	☐	☐	☐	☐
PIP5	☐	☐	☐	☐	☐	☐
DIP1	☐	☐	☐	☐	☐	☐
DIP2	☐	☐	☐	☐	☐	☐
DIP3	☐	☐	☐	☐	☐	☐
DIP4	☐	☐	☐	☐	☐	☐
DIP5	☐	☐	☐	☐	☐	☐

	Right			Left		
	Tender	Swollen	Damaged	Tender	Swollen	Damaged
Hips	☐	■	☐	☐	■	☐
Knees	☐	☐	☐	☐	☐	☐
Ankles	☐	☐	☐	☐	☐	☐
Tarsi	☐	☐	☐	☐	☐	☐
MTP1	☐	☐	☐	☐	☐	☐
MTP2	☐	☐	☐	☐	☐	☐
MTP3	☐	☐	☐	☐	☐	☐
MTP4	☐	☐	☐	☐	☐	☐
MTP5	☐	☐	☐	☐	☐	☐
Toes(PIP)1	☐	☐	☐	☐	☐	☐
Toes(PIP)2	☐	☐	☐	☐	☐	☐
Toes(PIP)3	☐	☐	☐	☐	☐	☐
Toes(PIP)4	☐	☐	☐	☐	☐	☐
Toes(PIP)5	☐	☐	☐	☐	☐	☐
Toes(DIP)2	☐	☐	☐	☐	☐	☐
Toes(DIP)3	☐	☐	☐	☐	☐	☐
Toes(DIP)4	☐	☐	☐	☐	☐	☐
Toes(DIP)5	☐	☐	☐	☐	☐	☐

Total Tender Joints ☐☐

Total Swollen Joints ☐☐

Total Damaged Joints ☐☐

Total Involved Joints ☐☐

Section 8 – Nail score

	R THUMB (1)	(2)	(3)	(4)	(5)	L THUMB (1)	(2)	(3)	(4)	(5)
Pitting	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Onycholysis	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hyperkeratosis	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Severe nail deformity	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Total Nail Score *(max 40)* ☐☐

Section 9 – PASI score

Extent:	<10%	10-29%	30-49%	50-69%	70-89%	90-100%	Severity:	Nil	Slight	Moderate	Severe	Very severe
Extent score:	1	2	3	4	5	6	Severity score:	0	1	2	3	4

Skin Area	Extent Score	Total Extent	{ SEVERITY } Erythema	Infiltration	Desquamation	Total Severity	Total Extent x Total Severity
Head	☐	x 0.1= ☐·☐	☐	+ ☐	+ ☐	= ☐☐	☐☐·☐
Arms	☐	x 0.2= ☐·☐	☐	+ ☐	+ ☐	= ☐☐	☐☐·☐
Trunk	☐	x 0.3= ☐·☐	☐	+ ☐	+ ☐	= ☐☐	☐☐·☐
Legs	☐	x 0.4= ☐·☐	☐	+ ☐	+ ☐	= ☐☐	☐☐·☐

PASI (PsA Skin Index) = extent x severity *(max score 72)* ☐☐·☐

Section 10 – Clinical subgroup, Schobers and HAQ scores

a) Which subgroup best fits the current clinical picture? ☐ Mutilans ☐ Mono ☐ Oligo ☐ Poly ☐ Spond ☐ Dip

b) Schobers ☐☐☐☐

c) HAQ raw score ☐☐☐☐

Section 11 - Investigations

a) Research bloods requested: ☐ Yes ☐ No ☐ N/A

b) Xrays requested: ☐ Hands ☐ Feet ☐ Heels ☐ C spine ☐ L spine ☐ Other

If other please specify:

Section 12 – Additional comments

Study no: B P S A

Appendix 5. Psoriatic Arthritis Background information

(please check your contact details and if there are any incorrect or missing details then please amend)

Forename (s)

Surname

DOB / /

Hospital No

Address

Telephone no

Section 1 - Background data

1. Are you currently working? ☐ Yes ☐ No

   If yes what is your current occupation?

   If no what was you last occupation?

   when did you last work? / /

2. What is your marital status? ☐ Married ☐ Single ☐ Divorced ☐ Separated ☐ Widowed

3. What is your ethnic group?

White:	☐ British	☐ Irish	☐ Other	
Asian or Asian British:	☐ White & Black Caribbean	☐ White & Black African	☐ White & Asian	☐ Other
Black or black British:	☐ Indian	☐ Pakistani	☐ Bangladeshi	☐ Other
Chinese or other:	☐ Chinese	☐ Other		

(if other please give details)

4. Have you ever drunk alcohol? ☐ Yes ☐ No

   If yes then please provide details: ☐ I currently drink alcohol ☐ I used to drink alcohol

   Average number of units per week

5. Have you ever smoked? ☐ Yes ☐ No

   If yes then please provide details. ☐ I currently smoke ☐ I used to smoke

   No. Per day Years smoked Years since smoked

Section 2 - Arthritis

1. What year did the symptoms of arthritis start? |_|_|_|_|

2. What year was your arthritis diagnosed? |_|_|_|_|

3. Which joint was first affected by arthritis? |_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|
|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|_|

Section 3 - Psoriasis

1. What year did the symptoms of psoriasis start? |_|_|_|_|

2. What year was your psoriasis diagnosed? |_|_|_|_|

Section 4 – Past medical history

Do you have or is there a family history of any of the following:-

	Yes	Yes, family history	No
Cardiac problems	☐	☐	☐
Pulmonary problems	☐	☐	☐
Liver problems	☐	☐	☐

(If other medical problems then please provide details below)

Section 5 – Past joint surgery

If you have had any joint surgery then please provide details below:-

Section 6– Family history

	Yes	No
Have any of your family had any type of arthritis?	☐	☐

If YES then please describe which relation had the arthritis and what type of arthritis they had.

	Relation *E.g. Parental grandfather, mother, brother*	**Psoriasis**	**Psoriatic arthritis**	**Osteoarthritis**	**Rheumatic Arthritis**	**Unknown type of arthritis**
1.		☐	☐	☐	☐	☐
2.		☐	☐	☐	☐	☐
3.		☐	☐	☐	☐	☐
4.		☐	☐	☐	☐	☐
5.		☐	☐	☐	☐	☐
6.		☐	☐	☐	☐	☐
7.		☐	☐	☐	☐	☐
8.		☐	☐	☐	☐	☐
9.		☐	☐	☐	☐	☐
10.		☐	☐	☐	☐	☐

Section 7 - Previous Hospital Admissions

(Please provide details below of any hospital admissions you have had which were related to your arthritis)

Section 8 - Medications

Have you been prescribed any of the following drugs. If yes please provide details about when you started and finished them and why you stopped taking the medication.

Drug	Start date	End date	Reason for stopping
Methotrexate			
Sulphasalazine			
Leflunomide			
Hydroxychoroquine			
Anti – TNF drugs *(e.g. etanercept, infliximab, adalimumab)*			
Cyclosporin			
IM Gold			
Prednisone			

Appendix 6. HEALTH ASSESSMENT QUESTIONNAIRE

Forename: ______________________
Surname: ______________________
Date: __ __ / __ __ / __ __ __ __
Hospital Number: __________
Trial Number: ______________

We are interested in learning how your illness affects your ability to function in daily life. Please feel free to add any comments at the end of this form.

PLEASE TICK THE ONE RESPONSE WHICH BEST DESCRIBES YOUR USUAL ABILITIES OVER THE PAST WEEK.

	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	Unable to do
1) Dressing and Grooming				
Are you able to:				
- Dress yourself including shoelaces and doing buttons?	☐	☐	☐	☐
- Shampoo your hair?	☐	☐	☐	☐
2) Rising				
Are you able to:				
- Stand up from an armless straight chair?	☐	☐	☐	☐
- Get in and out of bed?	☐	☐	☐	☐
3) Eating				
Are you able to:				
- Cut your meat?	☐	☐	☐	☐
- Lift a full cup or glass to your mouth?	☐	☐	☐	☐
- Open a new carton of milk (or soap powder)?	☐	☐	☐	☐
4) Walking				
Are you able to:				
- Walk outdoors on flat ground?	☐	☐	☐	☐
- Climb up 5 steps?	☐	☐	☐	☐

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

- ☐ Walking stick
- ☐ Walking frame
- ☐ Crutches
- ☐ Wheelchair
- ☐ Devices used for dressing (button hook, zipper pull, long handled shoe horn etc)
- ☐ Built-up or special utensils
- ☐ Special or built-up chair
- ☐ Other (specify) ____________________

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

☐ Dressing and Grooming ☐ Rising ☐ Eating ☐ Walking

Please turn over

PLEASE TICK THE ONE RESPONSE WHICH BEST DESCRIBES YOUR USUAL ABILITIES OVER THE PAST WEEK.

	Without ANY difficulty	With SOME difficulty	With MUCH difficulty	Unable to do
1) Hygiene				
Are you able to:				
- Wash and dry your entire body?	☐	☐	☐	☐
- Take a bath?	☐	☐	☐	☐
- Get on and off the toilet?	☐	☐	☐	☐
2) Reach				
Are you able to:				
- Reach and get down a 5lb object (e.g. bag of potatoes) from just above your head?	☐	☐	☐	☐
- Bend down to pick clothing from the floor?	☐	☐	☐	☐
3) Grip				
Are you able to:				
- Open car doors?	☐	☐	☐	☐
- Open jars which have previously opened?	☐	☐	☐	☐
- Turn taps on and off?	☐	☐	☐	☐
4) Activities				
Are you able to:				
- Run errands and shop?	☐	☐	☐	☐
- Get in and out of a car?	☐	☐	☐	☐
- Do chores such as vacuuming, housework or light gardening?	☐	☐	☐	☐

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

☐ Raised toilet seat
☐ Bath seat
☐ Jar opener (for jars Previously opened)
☐ Bath Rail
☐ Long-handled appliances for reach
☐ Other (specify) ☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐☐

PLEASE TICK ANY ACTIVITIES OR AREAS THAT YOU USUALLY NEED HELP FROM ANOTHER PERSON

☐ Hygiene ☐ Reach ☐ Gripping and opening things ☐ Errands and housework

HOW STRONG HAS THE PAIN IN YOUR JOINTS BEEN IN THE PAST WEEK?

Please mark on the line to indicate the severity of the pain

No pain _____ Pain as bad as it could be

HOW ACTIVE HAS YOUR ARTHRITIS BEEN IN THE PAST WEEK?

Please mark on the line to indicate your level of activity

Not active at all _____ Extremely active

Appendix 7

Royal National Hospital for Rheumatic Diseases
Upper Borough Walls
Bath
BA1 1RL
Tel : 01225 465941
Fax: 01225 473435

INFORMATION SHEET FOR PATIENTS WITH PSORIASIS
Name of research project : Markers of outcome in psoriatic arthritis

Introduction
This information sheet is for people who have psoriasis. We would like you to help with a research project. This is absolutely voluntary and if you would prefer not to take part you would not have to give a reason and your future care would not be affected in any way.

What is the purpose of the study ?
Psoriasis is a very common skin condition which affects approximately one in fifty of the population. The majority of people with psoriasis never have problems with their joints but unfortunately about one in ten people with psoriasis develops arthritis. This can present problems with pain, stiffness and swelling in the joints or pain and stiffness in the spine.. Arthritis associated with psoriasis is a very variable condition and affects patients in different ways. We are interested in finding out what factors influence why some people with psoriasis develop arthritis and why some people get more severe arthritis than others. We have already studied a group of our patients who have psoriasis and arthritis and we would now like to study people who have psoriasis but not arthritis. In order to do this we would like to take a small sample of your blood to test for the presence particular genes and a new antibody in the blood which may help us to understand why some people with psoriasis develop arthritis and, if they do, how severely.

Why have you been chosen ?
You have been asked to participate because you have psoriasis.

Who is organising the study ?
Dr McHugh, Dr Korendowych and Dr Ravindran are Rheumatologists working at the Royal National Hospital for Rheumatic Diseases in Bath. They have a particular interest in arthritis associated with psoriasis. They are organising a study to try to find out why some people with psoriasis develop arthritis and why the majority do not.

What will happen if you take part ?
We would ask you to see either Dr Ravindran/ Dr Korendowych or our specialist therapist to discuss the study with you and give you an opportunity to ask any questions. This will be in the Dermatology unit at the Royal United Hospital after your normal appointment or if your GP looks after your psoriasis we could also see you at the Royal National Hospital for Rheumatic diseases in Bath or at the GP practice. We would ask you to give one small blood sample (approximately 15mls or 3 teaspoons) on one occasion only. We would also ask that you take a minute or two to fill in a short questionnaire and allow us to briefly examine your joints and skin. If there is any evidence of inflammation in your joints we may ask you to have

X-rays of those joints. Studies have also suggested that some patients with psoriasis may also have arthritis in the spine and in some this may occur in the absence of symptoms. We would also like to investigate this further and would ask you to have an x-ray of your lower spine.

What are the possible benefits of taking part ?
The results of this study will not be of benefit directly to you but may eventually benefit others by increasing our understanding of psoriasis and arthritis.

What are the possible risks of taking part ?
The insertion of a needle into a vein for collection of blood may produce minor pain, bruising or bleeding at the insertion site. There is a small risk of an infection at the site.

What will happen to my blood sample ?
Your sample will be kept in secure storage at the Bath Institute for Rheumatic diseases. The sample will be used to test for the genes we are interested in for this project. Some of this project involves working closely with a laboratory in Australia which has specialist facilities to look at these genes. Dr Korendowych has been awarded a fellowship to travel to Australia to use their facilities to complete this work. In order to do this a small amount of your blood sample will be transported to Australia where storage will be similarly rigorous to Bath and the sample will only be used by Dr Korendowych and then destroyed. The remainder of the sample will be kept in Bath for a further 10 years and then destroyed. The sample will only be used for the purposes of the tests described in this sheet. If any further tests are required within 10 years, you will be asked to give your permission before these could be carried out.

Confidentiality – who will know you are taking part ?
Data collected for this research will be treated as confidential information. Your blood sample will be allocated a study number and your name will not be on the sample. Only Dr McHugh, Dr Korendowych and Dr Ravindran will know that your study number applies to you. The results of the study on your blood will not be given to you. The overall results of the many patients who take part in the study will increase our understanding of psoriatic arthritis as a whole but will not be of benefit for individual patients. Summary results of this research may be published in the scientific literature but you will never be individually identified in any publication.

Will anybody benefit financially from the study ?
No

Ethics Approval
This study has been approved by the Bath Local Research Ethics Committee.

What happens now ?
You are free to choose whether you would like to take part. If you do not it will not affect your usual care. Thank you for reading this information sheet. If you have any questions about the study please ask the doctor or nurse who sees you in the clinic or Dr Ravindran/ Dr Korendowych who can be contacted in the clinic or at the Royal National Hospital for Rheumatic Diseases on telephone 01225 465941. Please keep this information sheet.

Thank you very much

Dr Ellie Korendowych and Dr Jaya Ravindran
Research Fellows to Dr McHugh

Appendix 8.

RECRUITMENT FOR PSORIASIS AND ARTHRITIS STUDY

Dear Patient

You are invited to take part in a research project involving patients with psoriasis taking place at the Royal National Hospital for Rheumatic Diseases (RNHRD). About one in ten patients with psoriasis develop arthritis, although it is not clear why some do and others do not. The Research Team at the RNHRD think that genes may play an important role. Therefore, we are looking for people with psoriasis to assist us in investigating this area. This is purely voluntary and if you would prefer not to take part you do not have to give a reason and your future care will not be affected in any way.

The study takes place at the RNHRD, in Bath. In a one off appointment, at a time to suit you, a Doctor or Clinical Research Therapist will ask some questions about your psoriasis, examine your joints, nails and skin, and ask you to provide a blood sample and an X-Ray of your lower spine. The appointment is likely to take about an hour and reasonable transport costs can be reimbursed.
Further details are given in the information sheet provided.

If you are interested in taking part and would be happy for us to contact you, please fill in the detachable section at the bottom of this letter and return it in the stamped envelope provided. Alternatively, please contact Dr Ravindran or Jenny Lewis, members of the research team at the RNHRD on 01225 465941 extension 432 or extension 267.
Thank-you for taking the time to read this letter

Yours sincerely

Dr Linda McHugh
General Practioner

.....

I am / am not interested in taking part in the psoriasis and arthritis study
(please delete as appropriate)

Signature.....Name.....Date.....

Address .....
.....
..... Telephone number.....

Appendix 9.

Royal National Hospital for Rheumatic Diseases
Upper Borough Walls
Bath
BA1 1RL
Tel: 01225 465941
Fax: 01225 473435

INFORMED CONSENT FORM FOR PATIENTS WITH PSORIASIS

Name of research project : Markers of outcome in psoriatic arthritis

Have you read the patient information sheet ? Yes / No

Have you had an opportunity to ask questions and discuss this study ? Yes / No

Have you received satisfactory answers to all your questions ? Yes / No

Have you received enough information about the study ? Yes / No

Who have you spoken to ?_____

Do you understand that you are free to withdraw from the study :

- At any time
- Without having to give a reason
- Without affecting your future care Yes / No

Do you agree to take part in this study ? Yes / No

Signed_____ Date _____

Name in block capitals_____

Signed (Researcher)_____ Date _____

Appendix 10

SCREENING QUESTIONNAIRE FOR PATIENTS WITH PSORIASIS

1. How old were you when you first developed psoriasis ? .....

2. Are you under the care of a dermatologist ? Yes / No
   If yes, who? .....

3. Does anyone else in your family have psoriasis ? Yes / No

   If yes, who ? (please include brothers, sisters, parents, grandparents and children)

   .....

4. Does anyone in your family have arthritis ? Yes / No

   If yes, who ? .....

   What type of arthritis do they have and do they have psoriasis ?.....
   .....

5. Have you ever had psoriasis affecting your nails (eg thickened yellow ridged nails, pitting of nails) Yes / No

6. Have you had any of the following problems with your joints that have lasted for more than a week? :

Swollen Joints	Yes / No
Painful Joints	Yes / No
Stiff Joints	Yes / No
Back problems	Yes / No
Neck problems	Yes / No

   If yes, tell us more .....
   .....

7. Have you ever seen your GP due to problems with your joints?

   Yes / No

   If yes, please tell us more .....

   .....

8. Have you ever needed to take medication for your joints for longer than a week ?

   Yes / No

   If yes, please tell us more .....

   .....