Genetic Associations in Ankylosing Spondylitis

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Summary

Ankylosing spondylitis is a common inflammatory arthritis that is characterised by inflammation of the spine and sacroiliac joints. The genetic association between AS and the MHC Class I gene B27 is one of the strongest disease associations known, however the evidence points to AS being an oligogenic disease. The aim of this study was to investigate several candidate genes that may contribute to the susceptibility to AS.

The MHC Class II region is a highly polymorphic area where associations with particular alleles and other autoimmune diseases have been previously demonstrated. This study investigated the distribution of the MHC Class II DRβ1 alleles and the DRβ1*04 subtypes in a large well defined group of AS patients in comparison to both normal healthy controls and HLA B27 positive healthy controls all from the West of Scotland. A significant increase in the distribution of the DRβ1*01 alleles was seen in the AS patients in comparison to both control populations. No significant differences were seen in the distribution of the DRβ1*04 subtypes between the AS patients and the B27 positive controls.

The TNF genes lie within the MHC Class III region on chromosome six in close proximity to the B27 gene. There are several polymorphic sites within the TNF genes, some of which have been linked to differences in production of the TNF proteins. TNF is an important pro-inflammatory cytokine and it has been suggested that increased levels of TNF could contribute to disease pathogenesis. Six different polymorphic sites were

investigated in total. A significant increase in the 1* allele at the –308 site within the TNF promoter was seen in the AS patients compared to the B27 positive controls. Other associations were seen at other sites between the patients and the normal population but they would appear to be linked to the presence of B27 and not to the disease. None of the associations demonstrated would appear to be with any of the alleles linked to increased production of TNF.

Recent whole genome screens have highlighted areas other than the MHC that may have a role to play in susceptibility to disease. One in particular is on chromosome two where the IL-1 genes lie. The IL-1 genes are also important pro-inflammatory cytokines where particular alleles have been associated with other autoimmune diseases. Three sites were investigated, one within the IL-1 alpha gene, one within the IL-1 beta gene and the third within the natural antagonist IL-1 receptor antagonist. No associations were seen in the IL-1 alpha or IL-1 beta, however a significant increase in the carriage of the 2* allele of the IL-1 receptor antagonist 86 base pair variable number tandem repeat was demonstrated in the AS patients compared to B27 positive controls. This allele has previously been shown to predispose to increased levels of the anti-inflammatory IL-1 receptor antagonist.

Overall this study has demonstrated three different associations at three different loci, one within the MHC class II region, one within the MHC class III and one within the IL-1 region. This study has also demonstrated the possible existence of extended haplotypes

that could cross the TNF locus into the MHC Class II region that would incorporate the HLA B27 gene. Large family studies would be required to confirm this finding.

In conclusion none of the associations demonstrated in this study are with any of the alleles that have previously been linked to increased pro-inflammatory cytokines. In fact it could be hypothesised that the associations demonstrated in this study could in theory lead to a reduced pro-inflammatory response.

Acknowledgements

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For his vision, direction, enthusiasm, invaluable advice, humour and never ending patience with me I would like to say a very big thank you to Dr Max Field. What are you going to find to nag me about now?

All my love to my family who have supported me for years and continue to do so, they mean the world to me. And finally to Joe for being there, all my love.

In the field of observation, chance favours only the mind that is prepared.

-Louis Pasteur

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CHAPTER ONE:

INTRODUCTION

1.1 Ankylosing spondylitis

Ankylosing Spondylitis [AS] is an inflammatory condition primarily affecting the axial spine although other systems may be involved. The name is derived from the greek words *ankylos* meaning bent or crooked and *spondylos* meaning vertebra. In severe conditions the spine can become rigid due to the vertebra joints fusing together but the condition tends to start with inflammation in the sacroiliac joints. Patients will therefore have varying degrees of restricted movement in their spine.

AS has been described as far back as 2900 B.C. where illustrations in mummies and graves of ancient Egypt provide convincing evidence of its existence (Bourke, 1967). The existence of AS was firmly established at the end of the nineteenth century by individual descriptions by Bechterew, Strumpell and the description now generally agreed as the most detailed by Marie on two autopsies in 1898. With the advent of X-rays by 1896 the first spines showing AS were X-rayed by Beneke in 1897 and in 1906 Schlayer was the first to show the spine in an X-ray of a living patient. This aided diagnosis but it was not until 1930 that sacroiliac disease was fully recognised.

During the first and second world wars a large number of young men underwent general screening including X-rays giving rise to an abundance of information. The insidious nature of this disease that often went unrecognised even by the patient and the realisation that this was a familial disease came to light. A National Insurance Service formed in 1912 and a National Health Service formed in 1948 meant patients could be referred to hospitals with X-ray facilities. This meant patients who were less severely affected and a wider spectrum of clinical disease was recognised.

The term Ankylosing Spondylitis describes a condition that belongs to a group of inter-related disorders termed the Spondyloarthropathies [SpA]. Other members of this

group such as Reiter's syndrome, reactive arthropathies, psoriatic arthropathy, enteropathic spondylitis and other rare disorders share a number of clinical, epidemiologic and genetic features. The spondyloarthropathies share several characteristics in common such as the involvement of the sacroiliac joints, peripheral inflammatory arthropathy, the absence of rheumatoid factor in serum and a strong association with the major histocompatibility complex [MHC] class I gene product the human leukocyte antigen [HLA] B27. The various members of the spondyloarthropathies can be distinguished from one another on the basis of the particular peripheral joints involved, the time course of the disease (i.e., remission or relapse) and the associated clinical features (i.e., urethritis, conjunctivitis, skin involvement). However there is clinical evidence of overlap between different members in that a patient with inflammatory bowel disease may go on to develop AS or an individual with reactive arthropathy may develop uveitis or sacroiliitis.

1.2 Clinical Features

The majority of AS patients present with lower lumbar pain approximately three months after onset of symptoms. They find it difficult to locate accurately the site of pain. They complain of early morning stiffness that eases with exercise. The onset is insidious and normally occurs in patients below the age of 40. Patients will have various constitutional features such as fatigue, weight loss, low-grade fever, hypochromic anaemia and increased erythrocyte sedimentation rate. Some patients complain of 'pleuritic' chest pain that may affect sleep patterns. The pain is worse on inspiration and is due to insertional tendinitis of the many costosternal and costovertebral muscle insertions.

The disease progresses with a series of excacerbations and remissions with spinal limitation and deformity increasing with time, although few patients seem to progress relentlessly to the classic stage of bamboo spine (fig 1). The course of the disease can be very different from patient to patient and no two cases are exactly the same. In the classic case the spine becomes very stiff and movement is severely restricted but in many cases the patient can play a significant role in influencing how serious the condition becomes by paying particular attention to posture and maintaining a regular exercise regime under the supervision of a physiotherapist.

It is now recognised that AS is not the 'immunologically silent' disease that it was once thought to be and it is evident that other body systems may be involved. Approximately 20-40% of patients will also present with extraspinal joint disease at some point in the course of their disease. This peripheral joint disease [PJD] frequently affects the lower limbs and although is similar to rheumatoid in appearance it is normally less severe. Hip and shoulder involvement may cause major disability although any joint may be involved. The initial changes seen in peripheral joints are similar to those seen in rheumatoid arthritis [RA] with tissue swelling and periarticular demineralisation followed by narrowing of the joint space and bony erosion. Bony ankylosis is the final change and occurs more often in AS than in RA. Bony erosions can be seen in the region of the Achilles bursa and plantar spurs.

In 1850 Sir Benjamin Brodie (Brodie, 1850) gave a detailed clinical description of a 31-year-old man, with onset three years before, becoming completely rigid but without pain. This was described as a 'long continued chronic rheumatism of the spine with a hoop like deformity' and 'occasionally suffering severe inflammation of the eyes'. This was one of the first descriptions of an association between AS and Uveitis,

(inflammation of the eye). Uveitis has been shown to occur in approximately 25% of AS patients. The iris itself or the iris and the ciliary body can become inflamed. Frequently both eyes can be involved although it is uncommon for both eyes to be affected at the same time.

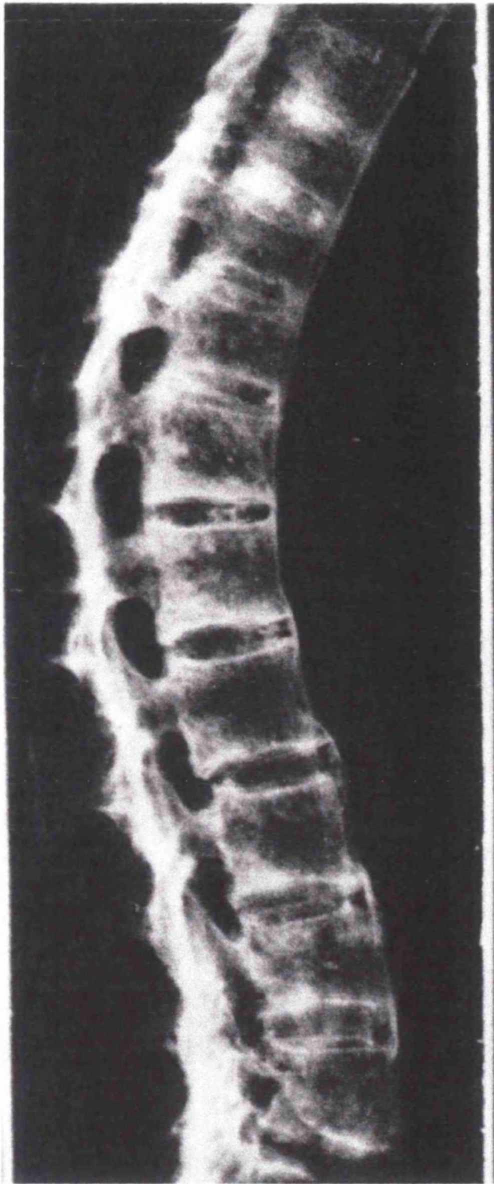
The inflammation can occur as often as every two to three weeks and it has been suggested that attacks may be seasonal (Ebringer, 1985). The presence of uveitis has been suggested as indicating a more severe form of the disease. (Nussenblatt, 1996).

AS patients may also have pulmonary involvement where chronic infiltration and fibrotic changes in the upper lobe of the lungs may occur. This slow progressive fibrosis tends to appear on average 18 years after onset and radiographs depict bilateral, upper lobe pulmonary fibrosis with occasionally cyst formation and parenchymal destruction (Court-Brown, 1965).

Approximately 3.5% of AS patients with a disease duration of less than 15 years and 10% of those with a disease duration of up to 30 years have cardiovascular involvement. In those patients with more severe disease, heart disease is more common especially in those patients with peripheral joint disease or extra articular features. The most common findings are aortic incompetence, cardiomegaly and persistent defects in cardiac conduction.

Ileocolonoscopic studies have demonstrated the presence of gut inflammation in as many as 60% of AS patients as well as being present in other spondyloarthropathies suggesting a common pathogenic mechanism in both diseases. The successful treatment of inflammatory bowel disease by sulphasalazine has also been shown to be effective in the treatment of SpA. A prospective study carried out by Mielants demonstrated a link between the presence of gut inflammation and active joint

inflammation and correspondingly the absence of gut inflammation with disease remission in patients with a variety of SpA's including AS (Mielants, 1995).



Legend for Figure 1

Post mortem X-ray of spine from AS patient showing classical Kyphosis (Picture courtesy of Dr. Max Field, Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow).

1.3 Diagnosis

The first internationally defined and agreed criteria for the diagnosis of AS was in Rome in 1961, which was revised in New York in 1966 (Bennett, 1968). The definition of the New York criteria for AS is described in Table 1. Diagnosis is based on clinical features and radiographic evidence of grade III or IV bilateral sacroiliitis, together with a history of pain. There are five grades of sacroiliitis according to the New York criteria ranging from 0 where the joint is normal to grade IV where there is definite ankylosis. The condition is considered to be primary if no other rheumatological symptoms are present and secondary if conditions such as psoriatic arthropathy, inflammatory bowel disease or Reiter's syndrome accompany the sacroiliitis.

A limitation of the Rome criteria was that spinal movement was not defined. The New York criteria does define a limitation of chest expansion as 2.5cm or less and although chest expansion is normally reduced in AS its measurement does have drawbacks in that some patients may be reluctant to take a deep breath. Also reduced chest expansion is normally seen late on in the course of the disease when the diagnosis has been well established. Other measurements such as spinal movement especially lumbar and cervical spines, wall-tragus distance and in early cases tenderness of the sacro-iliac joints all aid diagnosis. There are several features that help distinguish AS from mechanical back pain such as morning stiffness, which is nearly always a symptom of spondyliitis. The AS patient's pain improves with activity and will worsen with inactivity and pain relief can be achieved with non-steroidal anti-inflammatory drugs.

AS has in the past often gone undiagnosed since low grade back pain may be mistaken for mechanical back pain. An individual may have pain free sacroiliitis which unless they were being X-rayed for another reason would go undiagnosed.

In the past women have gone undiagnosed due possibly to a reluctance to carry out pelvic X-rays and a tendency to blame female pelvic organs for chronic low back pain (Calin, 1975). However the treatment of AS and mechanical back pain differ greatly and therefore an early diagnosis is of great importance in order to establish the correct therapy.

There are obviously other criteria used in the diagnosis of AS such as the European Spondylarthropathy study group (ESSG) criteria however the patients in this study were diagnosed on the basis of the New York criteria. The purpose of this study was not to evaluate the different diagnostic criteria and therefore they were not discussed here.

Table 1: New York Criteria for AS

Clinical criteria

1. Limited movement of the lumbar spine in all three planes (anterior flexion, lateral flexion and extension).
2. History or pain in lumbar spine or at dorsolumbar junction.
3. Chest expansion limited to less than 2.5cm

Radiological Grading

Definite AS

1. Grade 3-4 bilateral sacroilitis with at least one clinical criterion
2. Unilateral grade 3-4 sacroilitis, or grade 2 bilateral sacroilitis with clinical criterion 1 or both clinical criterion 2 and 3.

Probable AS

1. Grade 3-4 bilateral sacroilitis with no clinical criterion.

1.4 Epidemiology

With increased awareness of the condition, better diagnostic criteria and the link with B27 well established it has been possible to carry out population studies to give a clearer picture of the epidemiology of AS. AS was originally thought to be a male orientated disease with often a ratio of 10:1 being quoted (West, 1949). However this picture has changed and several large studies have put the ratio at nearer 3:1 in favour of males. There have been several reports suggesting that the progression and disease severity tends to develop more rapidly in males than in females. This leads to a delay in diagnosis in females to an average of 10 years after onset compared with 3 years in males (Hill, 1976). One major difference between males and females is that females are reported to have more peripheral joint involvement, which has sometimes led to a misdiagnosis of seronegative rheumatoid arthritis.

In comparison to rheumatoid arthritis AS has an early onset with sacroiliac and spinal disease developing in males with primary AS around the age of 20. An early onset was considered to indicate a more severe form of the disease as is the presence of extra spinal joint disease (Wilkinson, 1958). The pattern of onset also appears to differ in different parts of the world with an early onset seen in developing countries compared to the later onset seen in developed countries. A study from 1992 in patients from the United Kingdom has also suggested that the age of onset is increasing in this country (Will, 1992).

The prevalence of AS throughout the world has been a subject of much debate. The under diagnosis in females as described earlier and the lack of facilities in developing countries to confirm diagnosis by X-ray have all lead to conflicting rates of incidence. The prevalence of AS has been easier to establish since the discovery of the link with

B27. The true prevalence of AS in developed countries ranges from 0.25-1%, with a peak of around 2.5% being found in Norway. The apparent low prevalence of AS in black Africans appears to follow the distribution of the B27 gene, which has a lower incidence in this population than the West were B27 is seen in approximately 8% of the general population. A recent study has put the incidence of B27 in a population of black West Africans at 6% (Brown, 1997). This is confirmed in a population of Haida Indians were the incidence of B27 is found to be approximately 18-50% and correspondingly the prevalence of AS is increased. AS is seen less frequently in Japanese populations where the incidence of B27 is less than 1%.

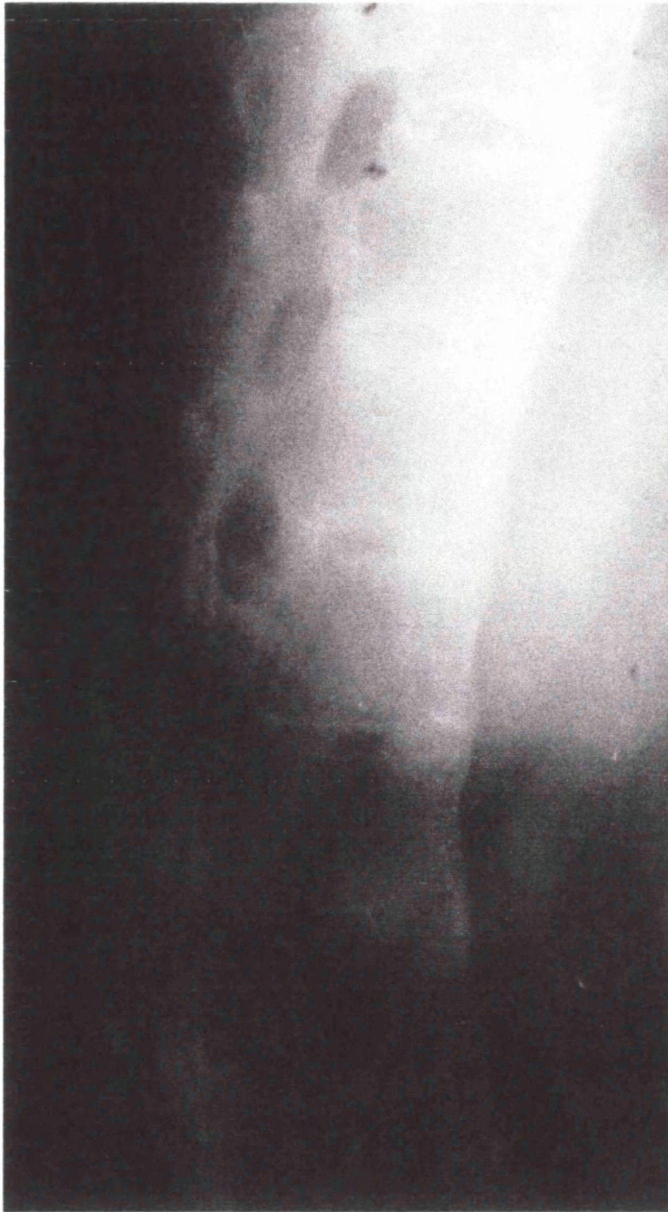
1.5 Pathology

The non infective inflammatory nature of AS has been well established with the primary pathologic site being the enthesis (insertion of ligaments into bone) in comparison to rheumatoid arthritis where the main pathologic site is the synovium (Bywaters, 1968). The initial lesion appears to be non-specific inflammation that tends to be brief but recurrent. Rheumatoid arthritis is characterised by joint destruction and instability whereas AS is characterised by fibrosis and ossification. In the spine this process leads to squaring of the vertebral bodies as can be seen in Figure 2. Periods of inflammation followed by new bone growth leads to syndesmophyte formation, which are vertical outgrowths of bone from a vertebra. Fusion of these syndesmophytes across joints between vertebrae contributes to the rigidity of the spine. The process normally begins in the sacroiliac joints and Figure 3 demonstrates sclerosis (hardening of the tissue due to fibrosis following inflammation) in the sacroiliac joint of an AS patient. The disease tends to progress from this joint in an ascending pattern although

progression may be sporadic and slow. Not all patients progress to end stage disease where the spine takes on the classical bamboo shape seen in Figure 1. The fused spine has the classic kyphosis shape that gives rise to the posture seen in Figure 4 of a patient with severe end stage disease.

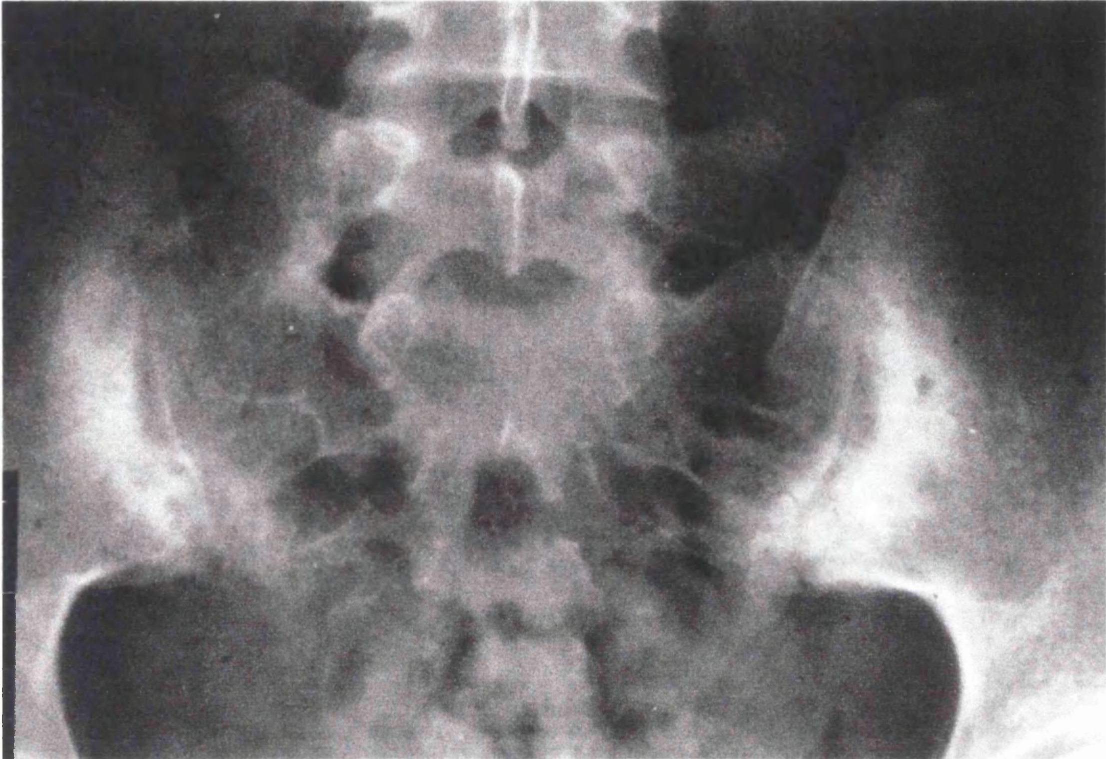
The pathology of the inflammatory enthesopathy has been difficult to determine as biopsy material is not readily available and most work has been established using post mortem tissue. The histopathology at this stage may differ from the initial stages of the disease. It has been shown that there is an inflammatory cell infiltrate that tends to spread along small vessels that lie within or close to the ligament causing lesions, which are directly repaired by the deposition of reactive bone. It is this new bone that leads to the formation of the bony spurs. It is this cycle of inflammation followed by ossification and the localisation of the disease to the spine and sacroiliac joints, which has made the pathogenesis of AS difficult to understand. A recent study examined the histology of computed tomography assisted biopsies from the sacroiliac joint of AS patients (Braun, 1995). They confirmed the presence of T cells and macrophages in the cellular infiltrates of the joints of five AS patients with active disease. Messenger RNA for the cytokine Tumour Necrosis Factor [TNF] was detected in abundance along with message for Transforming Growth Factor β2 [TGFβ2] at the site of new bone formation. The presence of TNF may have important implications for a possible therapeutic intervention in AS as has been seen in rheumatoid arthritis (Elliot, 1995). A further study has used this technique to make a quantitative analyses of sacroiliac biopsies confirming the predominate role of T cells and macrophages in early and active sacroiliitis (Bollow, 2000).

Approximately 30% of AS patients also develop a peripheral arthritis at some point in their disease whereby joints out with the axial skeleton become inflammed. Biopsy material from diarthrodial joints of AS patients appears to be very similar in the type and density of inflammatory cell infiltrate to that of rheumatoid patients (Julkunen, 1966). The synovial fluid of AS patients was reported to contain less polymorphonuclear cells and a higher percentage of lymphocytes than fluid from rheumatoid patients (Kendall, 1973).



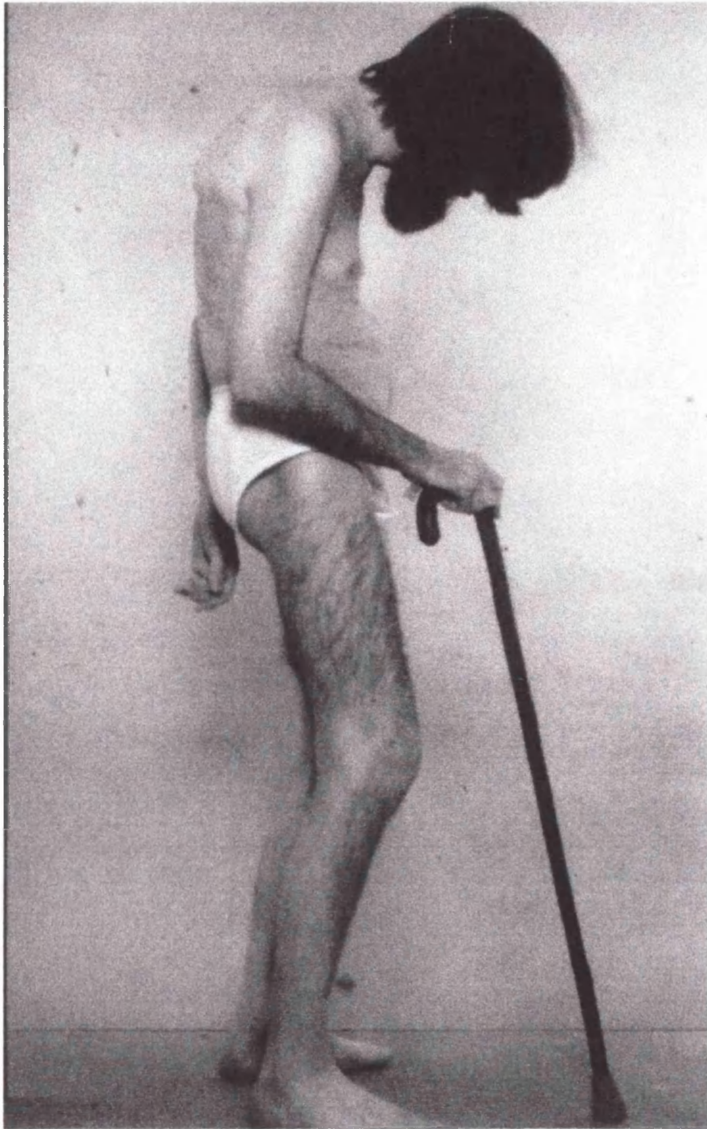
Legend for Figure 2

X-ray of spine from AS patient showing fused lumbar vertebrae with syndesmophytes linking vertebrae. (Picture courtesy of Dr. Max Field, Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow)



Legend for Figure 3

Pelvic radiograph of AS patient showing sclerosis and loss of joint space in sacroiliac joints. (Picture courtesy of Dr.Max Field, Centre for Rheumatic Diseases, Glasgow Royal Infirmary, Glasgow).



Legend for Figure 4

Figure 4 shows a male AS patient attending the Centre for Rheumatic Diseases showing severe end stage of disease. (Picture Courtesy of Professor Sturrock, Centre for Rheumatic Disease, Glasgow Royal Infirmary, Glasgow).

1.6 Genetics of AS

Since the 1950's it has been recognised that, AS has a strong hereditary component with twin and family studies suggesting a strong genetic link long before the association of AS with HLA B27 was established. For example the disease was said to be 30 times more prevalent in relatives of AS patients compared to controls (Strecher, 1957). The discovery in 1973 by two independent groups of the strong link between AS and the MHC class I gene HLA B27 has helped in the explanation of this family association (Brewerton, 1973). HLA B27 has been shown by several groups to be present in approximately 95% of AS patients whereas the prevalence of B27 in the normal West of Scotland population is approximately 9%. The prevalence of AS in the West of Scotland is between 0.5 and 1% of the population. The fact therefore that there are individuals who carry the B27 gene who do not have the disease and vice versa suggests that there may be other genetic or environmental factors involved. A recent study has been undertaken by a group in Germany where they have investigated a large group of blood donors for the prevalence of spondylarthropathy in conjunction with the presence of B27 and found that 6.4% of the B27 positive subjects investigated had definite SI changes indicative of AS (Braun, 1998). This may have consequences for any study where normal B27 positive blood donors are used as the control population as they may have asymptomatic sacroilitis thereby under-estimating the risk of AS in B27 positive individuals. However the contamination of the blood donors with 6.4% having AS is not likely to have a major effect, as the genetic risks we are looking at are relatively weak.

Since the discovery of the link between AS and B27 estimates of the risk apportioned to B27 have varied from between 35-50% (Rubin, 1994, Brown, 1995). However a

recent study on a large cohort of monozygotic and dizygotic twins has claimed the overall disease risk attributable to B27 to be approximately 16% (Brown, 1997). Due to the different statistical techniques used in these studies however it is unlikely that the risk apportioned to B27 is probably not significant. Although there may be controversy as to the true risk defined by the gene for B27 and also to the role of B27 in the pathogenesis of AS, it is well recognised that there is increased prevalence among relatives of AS patients. Several family studies have shown that for first degree relatives of B27 positive AS patients the risk of developing the disease is approximately 20% (van der Linden, 1984). Pooled data from all published studies (ascertainment bias minimised) on family recurrence however puts the risk in first degree relatives at considerably less than 20% (Brown, 2000). If only a single gene was involved the concordance rate amongst monozygotic twins would be 100%. This is not the case in AS. Also most B27 positive relatives of B27 positive AS probands show no evidence of disease and these two facts point to the involvement of additional genetic and environmental factors. This raises the question as to what other genes might be involved?

1.7 MHC Class I Associations

Since the discovery of the association of AS with the MHC class I molecule HLA B27 its role in the disease pathogenesis has been investigated. However even though it is one of the strongest known associations between an autoimmune disorder and an MHC gene its actual role has yet to be established and several theories have yet to be confirmed or denied. One suggestion is that a bacterial agent is involved in the disease pathogenesis of AS and that the B27 molecule through its antigen presentation role is

crucial to this process. This has been demonstrated by the use of transgenic animals expressing human B27 genes whereby the animals develop a spondyloarthropathy similar to the disease seen in humans when the animals are maintained in conventional facilities (Hammer, 1990). However the disease does not develop if the animals are maintained in a germ free environment highlighting the need for both genetic and environmental susceptibility factors in this disease.

Molecules of the MHC class II and class I regions are vital to the initiation of the immune response in that they are necessary for the presentation of antigen to T cells. Only antigens bound to membrane associated MHC molecules can be recognised by T lymphocytes. The class II and class I loci differ in the type of antigen they present and in the population of T cells they present to. The class I MHC molecules tend to present endogenously synthesised proteins to $CD8^+$ T cells whereas the MHC class II molecules present antigens from extracellular proteins to $CD4^+$ T cells.

All class I molecules B27 included consist of two separate polypeptide chains, the polymorphic α chain which noncovalently pairs with a non-polymorphic molecule β_2 microglobulin. The 44 kilodalton [kD] α chain is encoded by the MHC and the 12kD β_2 microglobulin is encoded on chromosome 15. Three quarters of the α chain protudes into the extracellular matrix with a short hydrophobic segment crossing the membrane and a carboxy terminal with 30 amino acids located in the cytoplasmic region. The β chain interacts non-covalently with the extracellular portion of the α chain known as $\alpha 3$ and has no connection to the cell. Peptides bind to the class I molecules through approximately 180 amino acid residues of the amino terminus of the class I α chain. This region consists of two homologous segments of about 90

amino acids each termed $\alpha1$ and $\alpha2$ which are linked by a single N-linked oligosaccharide at the junction between $\alpha1$ and $\alpha2$. The $\alpha1$ and $\alpha2$ segments interact to form a platform of an eight-stranded β sheet supporting two parallel strands of α-helix (see figure 5).

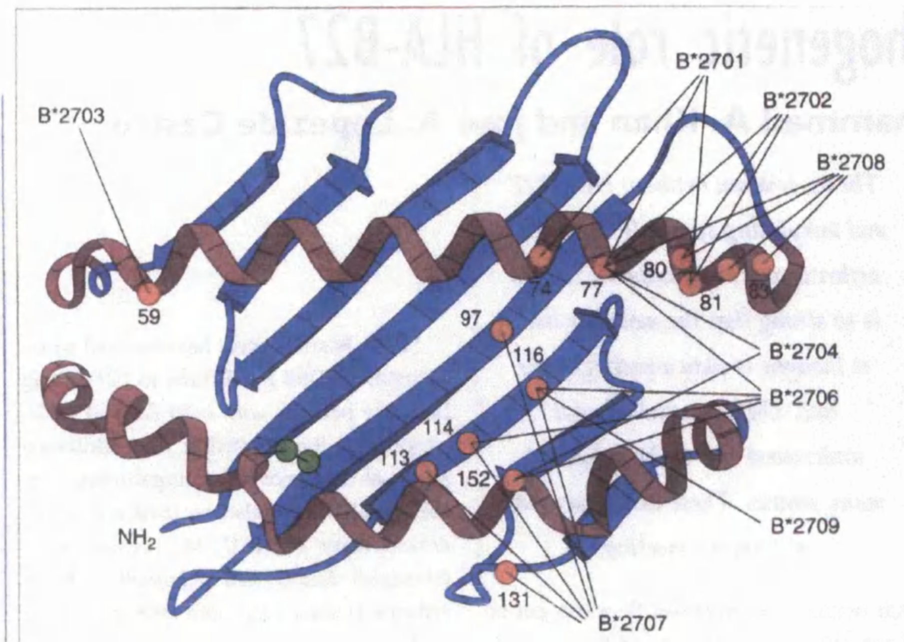
The two α-helices form the sides of a cleft whose floor is formed by the strands of the β-pleated sheet. This cleft, which is capable of binding 10-20 amino acid protein fragments is the presumed site of peptide binding. The small size of the cleft means proteins have to be processed to smaller fragments that can be bound and presented to T cells. It is differences in the amino acids in the α-helical sides of the cleft or the β strands that form the floor of the cleft that give rise to the ability to bind different peptides. As was mentioned before MHC class I molecules present peptides to CD8+ T cells and it has been shown that it is the nonpolymorphic $\alpha3$ segment that contain the binding sites for the T cells.

The gene for the B27 molecule lies within the MHC class I region on the short arm of chromosome six. The MHC itself can be divided into three regions; the class II, which includes the DR, DP and DQ loci, the class III which contains genes some of which are involved in the inflammatory process such as complement genes and the gene for tumour necrosis factor and the class I which contains the HLA A, B and C loci. A diagrammatic representation of the MHC is shown on figure 6.

The MHC genes are highly polymorphic and many different alleles exist within the population and these alleles differ in their ability to bind and present many different antigens thereby allowing the MHC to control the immune response to protein antigens. The region consists of several tightly linked genes that are co-dominantly

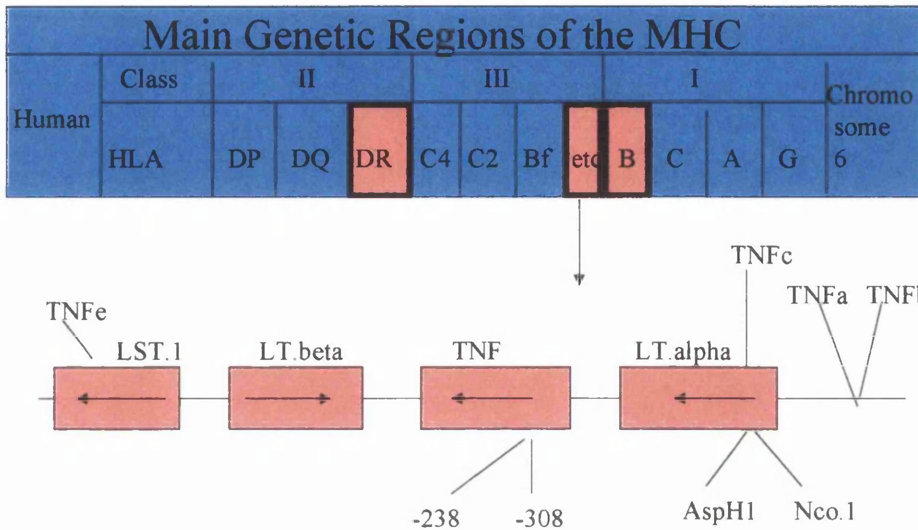
expressed. The total set of alleles for all the MHC genes gives rise to the MHC haplotype. In humans certain HLA alleles at different loci are inherited together in linkage disequilibrium. There is evidence that such haplotypes may be associated with certain autoimmune diseases (Dawkins, 1993).

There are a large number of alleles at the HLA-A, B and C loci each having 59, 118 and 36 alleles respectively. At present there are 22 different B27 subtypes with B*2705 being the most common in European Caucasians followed by B*2702. Several groups have investigated the distribution of these subtypes among different populations along with possible associations of a particular subtype with the presence of AS. This has been disappointing as most studies have shown an equal distribution of the subtypes in normal and disease populations (Breur-Vriensendorp, 1987), with B*2702, B*2704 and B*2705 all equally linked to disease. The subtypes B*2703, B*2706 and B*2709 have not been shown to be associated with disease in population studies with B*2706 and B*2709 being suggested as having a possible protective role for AS (Ren, 1997). Larger patient and control populations are required to confirm the distribution of susceptible and protective subtypes along with a better understanding of how the disease pathogenesis can be altered by the presence of just a few different amino acids which is all that distinguishes these different subtypes. Studies involving animals that are transgenic for these different subtypes are under way and will hopefully shed light on the role of B27 in AS.



Legend for Figure 5

Diagrammatic representation of an HLA B*2705 molecule highlighting the sites (shown in red) at which it differs from other subtypes of HLA B27 (taken from Immunology Today, 1996;17:6).



Legend for Figure 6

Figure 6 is a diagrammatic representation of the main regions of the MHC. Highlighted below are some of the polymorphic sites that are known within the TNF locus.

1.8 MHC Class II Associations

Along with the clear association of AS with HLA B27 there have been several reports of associations with MHC Class II genes. A recent study of dizygotic twins found those carrying the B27-DR1 haplotype were more concordant for disease than B27 positive DR1 negative twins (Brown, 1997). Another association between a small group of Sardinian AS patients and a B27-DR2 haplotype was observed (La, 1993) which is consistent with the finding of similarities in peptide presentation by DR2 and B27 (Lopez, 1993), although this observation has not been confirmed in other populations. Other observations have linked the presence of particular alleles with the presence of extra spinal features such as the carriage of DRβ1*0803 being a risk factor for the development of Acute Anterior Uveitis [AAU] in a group of Japanese AS patients (Monowarul Islam, 1995). This finding has been subsequently attributed to DR8 being associated with a younger age of onset, not AAU primarily (Ploski, 1996). The presence of DR4 has also been reported as a risk factor for the presence of peripheral joint disease (Miehle, 1985).

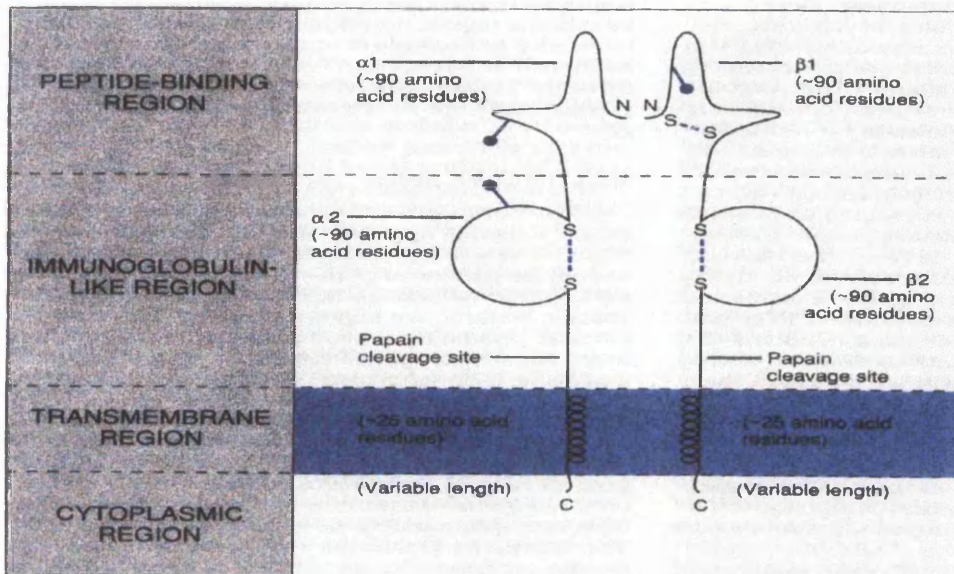
The MHC class II molecules consist of two polypeptide chains of similar size and structure. The α chain is 32-34KDa and is non-covalently associated with the β chain which is approximately 29-32KDa. Each chain consist of an external region linked by a connecting peptide to a transmembrane region followed by a cytoplasmic tail with two thirds of the chains lying in the extracellular space (see figure 7). The two chains are synthesized separately but associate shortly afterwards in the cytoplasm along with a third chain γ. The three chains only remain associated as long as they remain inside the cell once they reach the plasma membrane the γ chain dissociates itself leaving just

the α and β chain expressed on the cell surface. The class II molecules domain structure is similar to the class I domains (figure 5) with the α1-β1 domains forming the groove which is believed to serve as the binding site for the processed antigen. This antigen binding groove is formed by a floor of β-pleated sheet conformation with the sides formed by the α helices. The ends of the floor are open allowing access for a larger peptide, which is clipped to a smaller peptide of about 25 residues before the complex is transported to the cell surface. As with the class I molecules it is genetic polymorphisms that determine the amino acids that lie within the α1, β1 residues that give rise to the ability to recognise different antigens.

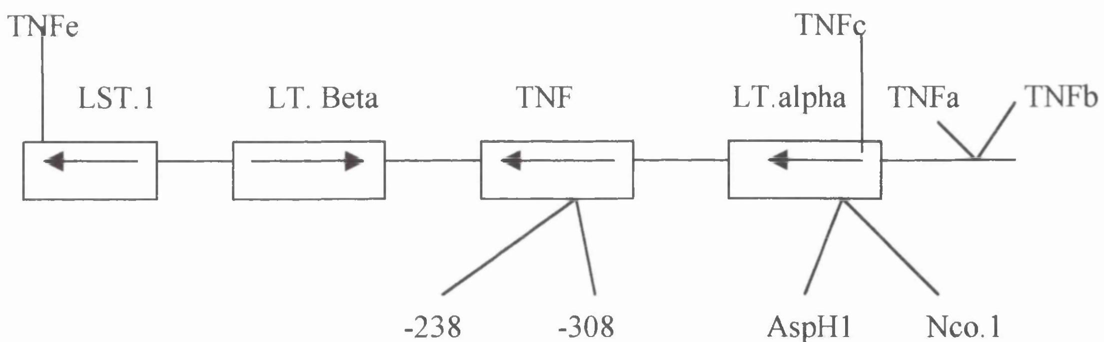
The MHC Class II genes lie closest to the centromere on the short arm of chromosome six (as shown in figure 6). This region consist of several genes the majority of which are associated with immunity such as the DR, DQ and DP loci which are the major products and are involved in antigen presentation to $CD4^+$ T cells. It has been shown that there is variation in the number of functional α and β chains with for example the DR loci having a single α gene and up to nine β genes. This along with the large number of potentially different alleles at each loci due to genetic polymorphism made the standardisation for the nomenclature of this region very important. The class II genes are designated by three capital letters followed by a series of numbers of which the first letter identifies the gene. The second letter specifies the family and the third letter indicates the gene type i.e. A for the α chain and B for the β chain. The first number specifies the locus and the remaining numbers the allele, which are usually separated by an asterisk. The first two digits indicate major alleles and the following digits the minor variants e.g. DRB1*0401.

There are a large number of possible alleles at each locus for example HLA-DRB1 has 168. These nucleotide variations lead to discrete changes in the gene product, which in turn may have functional consequences in that they influence the way the HLA molecule binds and presents the antigenic peptide. This large number of possible alleles means a large number of possible peptides may be recognised.

The evolutionary mechanisms that appear to have brought about this extensive genetic polymorphism seems to utilise a form of segmented exchange in which genes are interspersed and interchanged with other HLA genes. This means specific sequence patterns at particular gene loci are often repeated in other alleles. These regions are often called " hypervariable regions". This mechanism sheds light on the "shared epitope" theory that has been implicated in RA. Originally RA was associated with DR4 but with the advent of molecular typing techniques and the clearer understanding of the genetic organisation of this region it became apparent that RA was associated with not all of the DR4 alleles and was also associated with other DRB1 genes. It would appear that the acquisition and or severity of RA depend on the presence of a particular sequence in the third hypervariable region of the DRB1 chain. This region termed the "shared epitope" has particular amino acids at residues 67-74 which are thought to be important in the way the HLA-DR antigen complex is presented to T cells.



Legend for Figure 7
Schematic diagram of a class II molecule (Abbas AK. Lichtman AH. Pober JS. Cellular and Molecular Immunology, 1991, pg 108).



Legend for Figure 8
A diagrammatic representation of the TNF gene cluster highlighting some of the polymorphic sites investigated in this study, along with some other sites that were not studied.

If the class II loci have an important role to play in RA may it also be involved in any other autoimmune disorders such as AS? The class II is involved in presenting antigen to $CD4^+$ T cells and the class I presents to $CD8^+$ T cells as mentioned before, therefore in AS which is linked to class I the number of circulating $CD8^+$ T cells would be expected to be more than the number of $CD4^+$ but this is not the case there appears to be more $CD4^+$ T cells. The previous literature suggests also that the class II loci may be additional susceptibility loci for AS with links with particular alleles and disease itself or with markers of disease severity.

1.9 Tumour Necrosis Factor

Tumour necrosis factor [TNF] is an important proinflammatory cytokine that plays a key role in the pathogenesis of many infections and inflammatory diseases. It is a principal mediator of the host response to gram-negative bacteria contributes to the regulation of normal homeostasis and has both beneficial and adverse effects in infectious disease. TNF was originally identified through its ability to lyse tumour cells (Carswell, 1975), although its ability to do so was actually noted nearly 100 years ago when a tumour necrosing effect coincident with infection was seen by Coley in a patient with a sarcoma of the neck. Subsequently Coley's toxins (sterilised gram-negative bacteria) were used to treat tumours in the early 1900's (Coley, 1906). By the mid 1980's the protein had been purified and sequenced following the cloning of the gene.

TNF belongs to a family of proteins that, includes TNFα, lymphotoxin α (previously known as TNFβ) and lymphotoxin β. Activated monocytes (macrophages) are the

major source of TNFα, which is synthesised as a 20kDa pro-protein that is cleaved by TNFα converting enzyme [TACE] to a 17kDa monomeric form. Under physiological conditions TNFα circulates as a stable cone-shaped homotrimer (Jones, 1989). This soluble trimer mediates its effects by binding to two receptor molecules TNF RI (p55) and TNF RII (p75). TNF induces a number of effects including cytokine production, expression of adhesion molecules, activation of neutrophils, costimulator for T cell activation and stimulates antibody production by B cells. TNFα contributes to the regulation of normal homeostasis as well as playing an important role in inflammation. In autoimmune conditions such as rheumatoid arthritis TNFα contributes to the pathogenesis of synovitis and joint destruction through its ability to stimulate fibroblast growth, induction of prostaglandin E2 and collagenase release from synovial cells. It is able to induce the resorption of bone and cartilage and inhibit the synthesis of proteoglycan in cartilage, increases adherence of granulocytes and macrophages and regulates the production of Interleukin 1 [IL1]. TNFα has been detected in synovial fluid from RA patients and mRNA has been detected in synovial cells confirming the local production of TNFα in inflammed tissues (Chu, 1991).

Lymphotoxin β is a transmembrane protein that belongs to the TNF related ligand family along with lymphotoxin α previously known as TNFβ which was originally identified as a soluble cytotoxic factor involved in type I delayed hypersensitivity reactions. The activities of both cytokines overlap due to their binding and signaling through the same two receptors. The primary pathophysiological role of the soluble forms of these cytokines is to promote defence against infection. Lymphotoxin α can be secreted or retained at the surface of the cell by binding to LTβ in a 1:2 ratio. This

surface heterotrimer called lymphotoxin recognises a different receptor than TNFα and LTα alone and is made specifically by lymphocytes. The role of lymphotoxin in autoimmune conditions is not fully understood.

The genes for TNFα, LTα and LTβ have all been cloned, sequenced and mapped to the MHC Class III loci on the short arm of chromosome six. This places the TNF genes in close proximity to the HLA B27 locus. The TNF genes are tandemly arranged within a 7Kb region 250Kb centromeric to the HLA B locus and 340Kb telomeric to the C2/BF locus. The location of the TNF genes, its well established proinflammatory activity and its importance in joint destruction in rheumatoid arthritis as confirmed by the therapeutic use of monoclonal antibodies to TNF in clinical trials have suggested that the TNF loci may be important in HLA associated diseases. A recent study has shown increased messenger RNA levels for TNF in biopsies taken from the sacroiliac joint of AS patients suggesting that this cytokine has a role to play in AS (Braun, 1995). The search for a possible involvement of TNF in autoimmune and infectious diseases led to the discovery of polymorphisms within the TNF genes. There have been several polymorphic sites described some of which are highlighted on figure 8.

Previously it has been reported that there are significant inter-individual variations in the level of TNFα induction in normal individuals suggesting production may be genetically controlled (Jacob, 1990). In autoimmune animal models it has been demonstrated by several groups that there is abnormal TNFα production especially in models of genetically determined IDDM in BB rats and NOD mice (Jacob, 1988) suggesting even minor stable differences in TNF production may have a role to play in the resulting immune response. Previous work has shown associations with particular

alleles at individual polymorphic sites to be associated with differences in TNF production *in vitro* (Pociot, 1995). Several groups have also demonstrated associations of individual alleles with susceptibility to different autoimmune diseases such as IDDM (Pociot, 1993), SLE (Wilson, 1994) and RA (Brinkman, 1997).

This raises the question are there particular alleles that predispose individuals to higher levels of TNF making them more susceptible to HLA associated disease? The individual polymorphic sites examined in this study will be discussed separately in the methods section.

1.10 Interleukin 1

A recent genome screen of AS patients and their families for non-HLA susceptibility markers highlighted several other regions has having a significant contribution to AS (Brown, 1998). They suggested the contribution by non- MHC genes was approximately 60% and that AS is a model of a polygenic disease. One of the areas highlighted was on chromosome 2 close to the loci coding for the Interleukin 1 genes. There are two proteins that are designated as Interleukin 1(IL-1), IL-1α and IL-1β which are products of distinct genes but both proteins recognise the same cell surface receptors. IL-1 is another cytokine that has an important role to play in the inflammatory response and its production is closely linked with that of TNF in the rheumatoid synovium. Like TNF stable variations in production rates have been observed, which may be genetically controlled (Molvig J, 1988). Several polymorphisms have been described that lie within the promoter regions of both IL-1 α and IL-1 β genes that have been suggested may alter transcription rates of these

cytokines. These facts suggested that differences in frequencies of these alleles may have a role to play in the pathogenesis of AS.

IL-1 was initially described in the late 1960's as a protein produced by macrophages in response to infection or inflammation that induced fever hence its previous name of endogenous pyrogen. It is known to induce acute phase reactions, aid in lymphocyte proliferation, induce release of collagenase and prostagladins from synovial cells. It is also involved in proliferation of fibroblasts, helps promote bone resorption and cartilage destruction and has been implicated in the destruction of the rheumatoid joint. Normal production of IL-1 is crucial for the host response to injury or infection but inappropriate or prolonged exposure has been implicated in a number of different pathological conditions such as sepsis, RA, inflammatory bowel disease, IDDM and atherosclerosis. IL-1 is produced by a number of cell types including monocytes, macrophages, fibroblasts, keratinocytes and osteoblasts.

IL-1α and IL-1 β are both synthesized as 31kDa precursors, which are subsequently cleaved to proteins of 17 kDA, which show approximately 25% homology at the amino acid level and have similar biological properties. The mature 17kDa protein is the physiologically significant form, which is secreted from the cells. There is also a membrane bound from of IL-1α but its biological and physiological role is unclear. IL-1 mediates its response through two specific receptors termed IL-1R type I and IL-1R type II. Type I is a 80kDA protein found mainly on T cells, fibroblasts and keratinocytes and as a receptor binds IL-1α better than IL-1β. Type II is a 68kDa protein found mainly on B cells and PMN's and binds IL-1β more strongly than IL-1α. The two receptors show approximately 28% homology in their extracellular

domains but differ in their cytoplasmic domains. Only ligand binding to the Type I receptor induces signal transduction and all biological effects are attributed to IL-1 binding to this receptor. Both receptors are members of the Ig superfamily. The Type II receptor is membrane bound and acts as a precursor for a soluble IL-1 binding factor that can be shed under appropriate conditions which antagonises and modulates the activity of IL-1 (Sims, 1993). Glucocorticoids such as Dexamethasome are able to upregulate this receptor explaining some of the anti-inflammatory properties of these compounds.

Another naturally occurring IL-1 inhibitor is a protein termed IL-1 receptor antagonist (IL-1Ra), which is produced by monocytes, neutrophils, macrophages and fibroblasts. This secreted molecule of 25 kDa shows 26% homology to IL-1β and 19% homology to IL-1α. By binding to the IL-1 Type I receptor, IL-1Ra competitively inhibits the action of IL-1 since its binding does not result in signal transduction. IL-6, INFγ, IL-4, GM-CSF and TGFβ are able to upregulate the production of secretory IL-1Ra. Pre-treatment with IL-1Ra has been shown *in vitro* to prevent death due to septic shock produced by LPS injection in rabbits (Ohlsson, 1990). Both IL-1 and IL-1Ra are present in the joints of Rheumatoid patients but it has been suggested that the concentration of IL-1Ra is too low to effectively inhibit the action of IL-1 (Firestein, 1994). Although IL-1Ra binds to the receptor with high affinity a 10-100 fold excess is required to inhibit the biological response of IL-1 by 50%. Pre-clinical and clinical trials are underway to study the use of IL-1Ra in RA.

As was mentioned before stable inter individual differences in cytokine production by monocytes stimulated by LPS suggest that production may be genetically controlled to

produce higher or lower amounts of IL-1 (Danis, 1995). The three genes coding for IL-1α, IL-1β and IL-1Ra all lie in close proximity on chromosome 2. Recently a restriction map of this region has been constructed showing the three genes mapped to a common restriction fragment of approximately 430Kb (Nicklin, 1994). The three genes were mapped relative to one another at the following intervals: IL-1α was between +0 and +35Kb, IL-1β between +70 and +110Kb, and IL-1Ra between +330 and +430Kb. Polymorphisms have been recently described which lie within the promoter region of the IL-1α and IL-1β gene and within intron 2 of the IL-1Ra gene. It is proposed that variations within these polymorphic sites may be involved in the susceptibility or severity of AS. The individual sites examined will be described more fully in the methods section.

1.11 Aims

Although the association between B27 and AS is very strong it is not thought to be the only gene involved and other candidate genes have been proposed as contributing to the susceptibility of AS.

I. The MHC class II alleles have been proposed as further candidate genes due to their location on chromosome six, the strong association with other autoimmune disorders such as RA, their role in antigen presentation and work by previous studies suggesting associations with extra spinal features such as peripheral joint disease and uveitis. The occurrence of ancestral haplotypes spanning the Class I, II and III loci which appear to have relationships with TNF levels also suggest that this loci may have a role to play in the pathogenesis of AS. The aim was to type the AS patients and controls at the DRβ1 loci and to subtype the DRβ1*04 positive individuals to examine for the presence of associations with disease as a whole or with the presence of extra spinal features.

II. The TNF genes have been implicated because of their location within the Class III loci in close proximity to HLA B27, the demonstration of increased mRNA levels in biopsies from the sacroiliac joints of AS patients and its well established proinflammatory properties. The existence of polymorphisms some of which have been shown to be associated with variations in TNF production may be a possible contributing factor to the pathogenesis. The aim was to investigate several of these polymorphic sites spanning the TNF locus in a large group of clinically well defined AS patients and controls to determine if there were any significant differences in the frequency of the alleles at each site. The distributions of the

alleles were also investigated for associations with any extra spinal features that may indicate a more severe pattern of disease.

III. Polymorphisms within the Interleukin 1 genes were examined because of the results of a whole genome screen, which highlighted a significant relationship with an area on chromosome 2 where the IL-1 genes lie. The pattern of AS in patients and family members suggest other genes are involved. The pro-inflammatory nature of this cytokine and the association of particular alleles with other autoimmune diseases and the functional differences seen with particular alleles have suggested that this area may be important in susceptibility to AS. The aim was to examine three polymorphic sites in AS patients and controls in order to determine any association with particular alleles and the presence of AS or the presence of extra spinal features.

It is strongly suggested that AS is a polygenic disease and it is hoped that this study may highlight the possible role of other genes involved in the pathogenesis of AS.

CHAPTER TWO:

MATERIALS

AND METHODS

2.1 Patients and controls

The patients enrolled in this study were all attending the outpatients department of the Centre for Rheumatic Diseases, University Department of Medicine, Glasgow Royal Infirmary, Glasgow. All patients had previously been examined by a clinician (RDS) and fulfilled the New York criteria for the diagnosis of AS (Bennett,P.H, 1968). At the time of sample collection a detailed history was recorded (see appendix A for copy of form). This included screening the patients for the presence of extra-spinal features such as peripheral joint disease and uveitis. The diagnosis of peripheral joint disease was based on the presence of inflammed joints outwith the axial skeleton. 10mls of blood in EDTA tubes was collected from each patient. All patients in the study had previously been tissue typed and were known to be HLA B27 positive.

Purified DNA was supplied from a panel of 93 healthy unrelated normal volunteers who were HLA B27 negative by the Department of Tissue Typing, Glasgow Royal Infirmary, Glasgow. A second control group consisted of purified peripheral blood mononuclear cells of 88 individuals who had been previously tissue typed as HLA B27 positive by Law Blood Transfusion Services, Carluke, Scotland. At the time of sample collection a general health questionaire was completed (see appendix B for copy of questionnaire) and although no specific questions relating to ankylosing spondylitis were asked this group of individuals they were taken as healthy controls.

2.2 DNA Extraction

Traditionally the extraction of mammalian DNA has been based on the digestion of cells by proteinase K in the presence of EDTA and a detergent such as SDS, followed by extraction with phenol/chloroform and finally precipitation with ethanol. However the use of phenol is particularly hazardous and over the past few years there have been a number of DNA extraction kits that have come onto the market that avoid the use of phenol are less time consuming and increase reproducibility. In this study three methods were compared as to their safety, efficiency, reproducibility, yield and quality of DNA. The first was based on modifications of the method of (Blin,N., 1976) which uses the traditional method of lysing the red cells, purifying the white cells which are then lysed followed by extraction with phenol chloroform and finally precipitation with ethanol. The second method investigated was the Genomix kit (Talent srl, Trieste, Italy) which following initial lysis of the whole blood via a cationic detergent uses a simplified purification step involving another cationic detergent that forms a micellar complex with DNA . The detergent is removed via ionic exchange during resuspension of the pellet followed by precipitation of the DNA with ethanol. The third method was the Nucleon II genomic DNA extraction kit (Scotlab Limited, Lanarkshire, Scotland), which uses the proprietory Nucleon resin to remove all unwanted cell impurities without binding the DNA. To investigate the advantages and disadvantages of each method 30mls of whole blood was collected from five normal volunteers in EDTA tubes and divided into 10ml aliquots from which DNA could be extracted by each method. From the results (data not shown) the Nucleon method was best suited for the purposes of the study and therefore was the method used.

For the Nucleon DNA extraction kit 10mls of whole blood was collected in EDTA tubes, 4x the volume of reagent A (10mM Tris-HCl; 320mM sucrose; 5mM $MgCl_2$; 1% Triton X-100; adjusted to pH 8.0 using 40% NaOH and autoclaved) was added in a 50ml polypropylene tube which was rotary mixed for 4 minutes at room temperature. This was centrifuged at 1300g for 4 minutes (Heareus megafuge 1.0R, Essex, UK) and the supernatant discarded without disturbing the pellet into bleach. The process was repeated with the pellet being resuspended in 500ul of reagent A before being made up to 40mls. This second wash was not recommended in the protocol book but it appeared to help in removal of more of the red cell debris than just one wash. Contaminating red cell debris may inhibit the PCR process due to the presence of heme from the red cells, which has the ability to bind to the polymerase enzyme. The resultant pellet was resuspended in 2ml of Reagent B (400mM Tris-HCL; 60mM EDTA; 150mM NaCL; 1% SDS [added after autoclaving]; adjusted to pH8.0 with 40% NaOH) and vortexed briefly to ensure the pellet was fully resuspended. The cell suspension was then transferred to a 14ml polypropylene tube, 500ul of sodium perchlorate added and mixed by inverting at least 7 times. After the addition of 2ml of chloroform and mixing by inversion at least 7 times 300ul of the Nucleon resin was added. No further mixing was required before centrifuging at 1300g for 5 minutes. Without disturbing the interface the upper layer was removed to a fresh tube. Two volumes of ice-cold absolute ethanol were added and the tube inverted several times until the DNA was seen to precipitate out of solution. The DNA was pelleted by centrifuging at 4000g for 5 minutes and the supernatant discarded before washing the pellet with 70% ethanol. The pellet was air dried for 15 minutes and

then resuspended in 500ul of sterile distilled water. The pellet was left overnight at 37°C to ensure it had fully resuspended before reading the absorbance.

2.3 DNA Quantitation

In order to estimate the quantity of DNA in each sample an absorbance reading was taken at both 260nm and 280nm on a Cecil Bioquest CE2501 spectrophotometer. An aliquot of each sample was diluted in sterile distilled water (normally a 1:500 dilution) and placed in a quartz cuvette. The spectrophotometer was first blanked with water alone before the samples were measured. The reading at 260nm gives an indication of the amount of nucleic acid in this case DNA in each sample and the reading at 280nm an estimate of the amount of protein. The ratio of the 260/280 readings give an indication of the purity of the sample, for DNA a ratio of approximately 1.8 is optimum. The 260nm reading was used to calculate the yield, as 1OD unit at 260nm is equivalent to 50ug/ml of double stranded DNA.

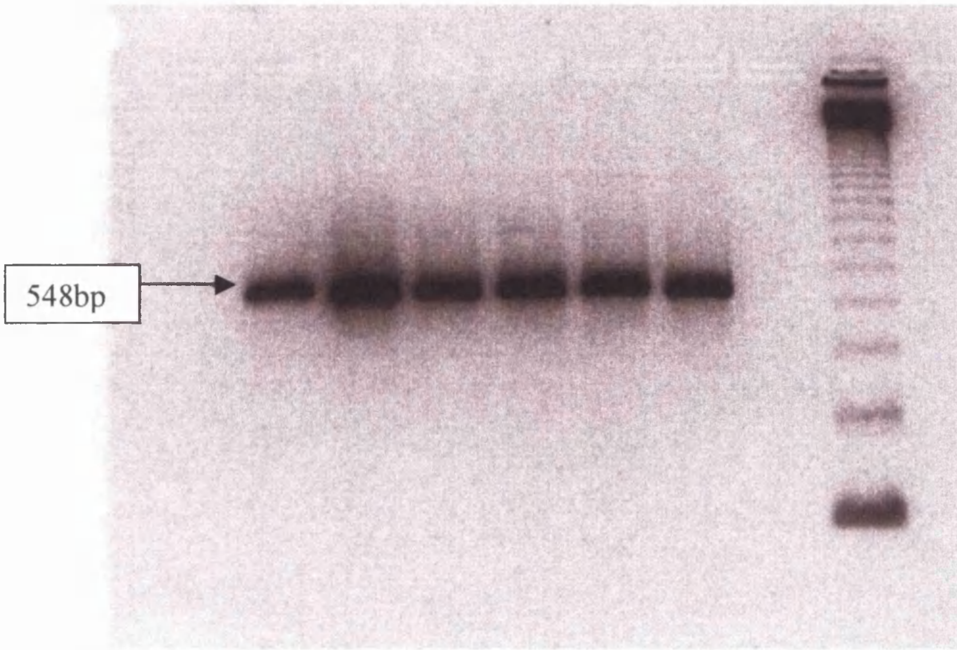
The DNA was standardised in order to obtain a final concentration of between 100 to 500ng in each PCR reaction.

2.4 β Actin Polymerase Chain Reaction

The polymerase chain reaction (PCR) was first described in 1985 by Saiki R.K., and is a method of amplifying a segment of DNA that lies between two regions of known sequence.

β Actin is known as a housekeeping gene in that it is found in multiple copies in most cells and acts as a control gene for the PCR since if this gene cannot be amplified it is unlikely that the gene of interest can be amplified. The DNA samples were amplified by PCR for the βActin gene first and only those samples that gave a positive result were included in the study. The two primers specific for the β Actin gene (primer 1 5' GTG GGG CGC CCC AGG CAC CA 3' and primer 2 5' CTC CTT AAT GTC ACG CAC GAT TTC 3') give rise to a PCR product of 584 base pairs. All PCR's were carried out on a Techne Genius PCR machine (Scotlab,Lanarkshire). Briefly 100ng of template DNA was amplified using 0.5 units of Taq (Advanced Biotechnologies) in a final volume of 1X Buffer IV [200mM $(NH_4)_2SO_4$, 750mM Tris-HCL, pH8.8, 0.1%(v/v) Tween 20] (Advanced Biotechnologies, Surrey, UK);1.5mM $MgCl_2$; 200mM dNTP's (dATP, dGTP, dTTP,dCTP); 0.12uM of each primer made up to a final volume of 25ul. This was subjected to 5 minutes at 95°C followed by 30 cycles of 95°C for 1 minute, 60°C for 1minute and 72°C for 2minutes with a final extension of 72°C for 10minutes. An aliquot of the PCR product was mixed with loading buffer (50% glycerol, 50% 10X TBE with orange G) and run out on a 2% agarose (Gibco Life Technologies, Paisley, Scotland) gel in a horizontal gel tank (Anachem, Bedfordshire, UK) in 1X TBE (Tris base, boric acid,

EDTA) with a constant current of approximately 50mAmps. The gel was stained with ethidium bromide before photographing the gel on a UV transilluminator (see figure 9).



Legend for Figure 9
In Figure 9 lanes 1-6 represent the amplified products from six individuals amplified with primers specific for the β actin gene. The product of 548bp can be sized in reference to the molecular weight ladder seen in lane 8 (123bp Ladder). Lane 7 is the negative control (i.e. no template added).

2.5 MHC Class II Associations

2.5.1 DRβ1* Typing by SSP

HLA-DR typing in clinical practice has until recently been carried out by serological methods but the new advances in molecular techniques has greatly improved the reproducibility, resolution and accuracy of the typing. By using PCR to identify the allelic polymorphism of the DRβ1 series the typing can be carried out in a fraction of the time needed for serology testing. The typing method relies on the specificity of sequence specific primers amplifying the template DNA in a PCR reaction where completely matched primer will be amplified more efficiently than a primer with one or several mismatches. The presence or absence of an amplified product can be detected by agarose gel electrophoresis. This sequence specific PCR was first used to distinguish between the wild type and mutant alleles for the diagnosis of sickle-cell anemia (Wu DY, 1989). The method is now becoming more widespread for the typing of MHC class I and class II alleles and a number of companies now supply kits containing all the relevant primers.

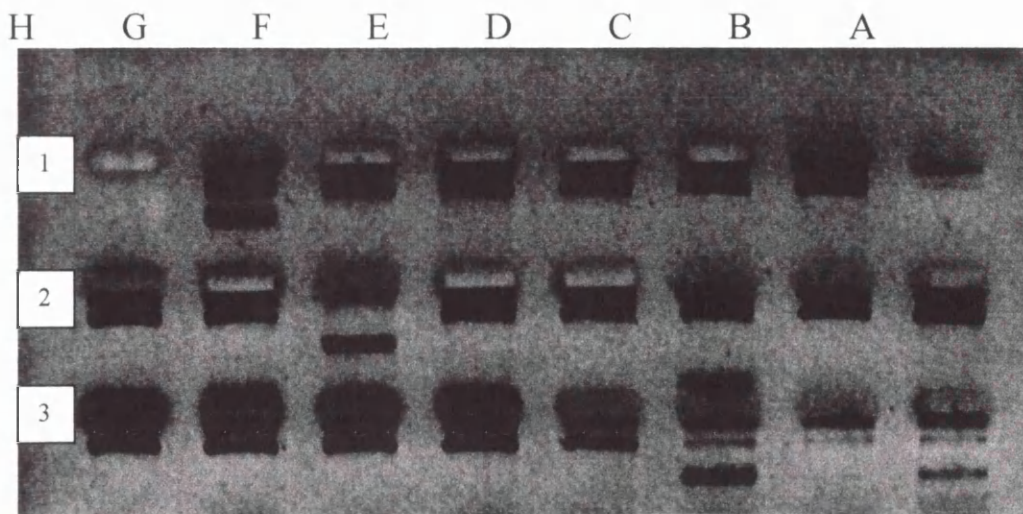
The Micro SSP Generic HLA DRB kit used in this study was from One Lambda, Inc. (Canoga Park, CA) and includes sequence specific primers for amplification of DRβ1, 3, 4, 5 alleles of HLA Class II gene locus and the Human β-globin gene. The amplification of the Human β-globin gene, which is present in all DNA samples acts as an internal control to verify the integrity of the PCR reaction.

The primers used in this kit have been pre-optimised and dried onto a 96 well 0.2ml thin-walled tube tray ready for the addition of the template, specially formulated dNTP-buffer mix and enzyme (Taq polymerase, Advanced Biotechnologies, Surrey, UK). The tray is

then placed in the PCR Express thermocycler (Hybaid, Middlesex, UK) for the required number of cycles.

The actual cycling conditions and full procedure can be found in the manufacturers instruction booklet. An example of a worksheet, which was filled out for each sample tested can be seen in Appendix C. The results were then recorded against the reaction pattern sheet supplied (appendix D) and the alleles assigned. A copy of the primer sequences is also in appendix E.

The PCR products were loaded directly from the typing tray onto a 2.5% agarose gel using the Micro SSP gel system (supplied by VH BIO, Newcastle, UK). This was run for five minutes before being photographed. An example of a gel of a patient typed at the DRβ locus is given in Figure 10.



Legend for Figure 10

Figure 10 is a representative gel picture of an individual typed at the generic HLA DRB locus. The 24 wells each represent unique primer sets that distinguish which alleles are present. The upper band in each well is of the control primers for β-globin gene. The lower band indicates which alleles are present. Well H/1 is the negative control (no template added).

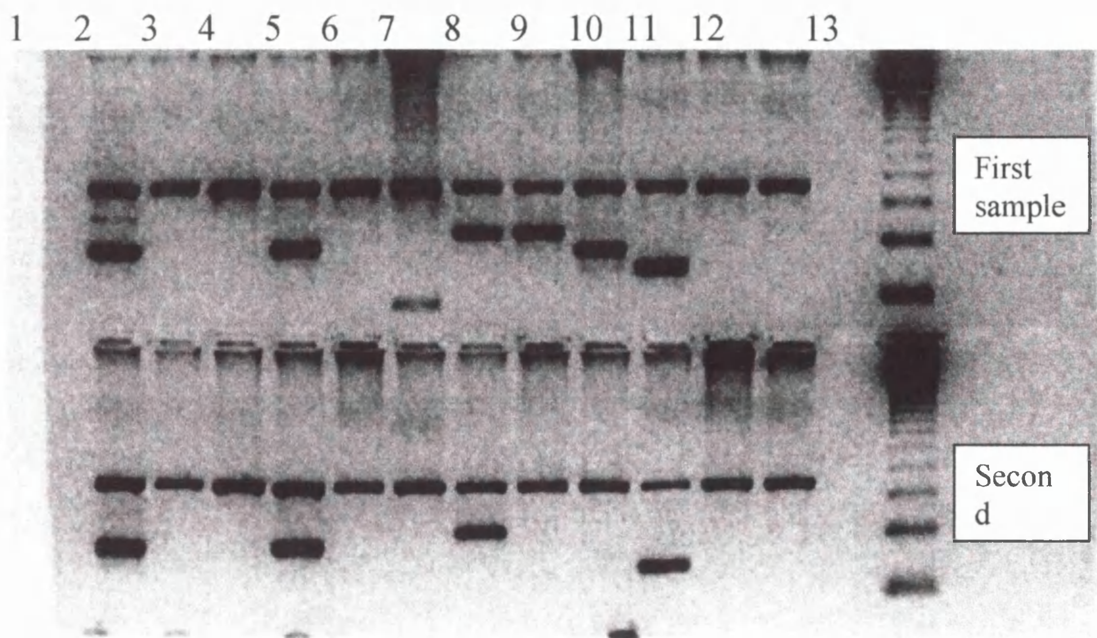
By recording which bands are present, for example in the gel pictured above wells G/1, F/2, C3 and A3 all have the lower band, and by referring to the table supplied by the manufacturers the individual alleles present can be determined. In this example the individual is heterozygous for alleles HLA DRβ* 01 and 13.

2.5.2 DRβ1*04 Subtyping by SSP

A single HLA-DR type represents a serologically defined specificity, which can be present on many different molecules encoded by distinct genes for example HLA-DR4 has at present 22 different alleles. These alleles known as DRβ1*0401-0422 are closely related to each other and differ by only a few amino acids. Interest in the DRβ1*04 alleles has arisen through the association with Rheumatoid arthritis.

The kit used in this study to identify the DRβ1*04 subtypes was from Dynal (Oslo, Norway) and incorporated primer sets for identifying DRβ1*0401 to DRβ1*0422 recognised by the HLA Nomenclature Committee in February 1996 (Tissue Antigens 1995: 46: 1-18) as well as DRβ1*1122 and DRβ1*1410 (see appendix G for the DRβ1*04 specificities). All primers were supplied in a diluted form in sufficient quantities for 50 reactions. Each primer solution consists of specific primer pair as well as control primers. A reaction mix suitable for 30 samples was prepared containing 500ul of 10x reaction buffer IV (Advanced Biotechnologies), 10ul of each dNTP (stock concentration 100mM), 300ul of 1.5mM $MgCl_2$, 250ul of glycerol, 25ul of 10mg/ml of cresol red and 385ul of sterile distilled water. This was vortexed to make sure the glycerol was well mixed and was stored frozen until required. For each individual sample 5ul of each primer mix was added to 12 0.2ml thin walled PCR tubes (Advanced Biotechnologies, Surrey, UK). To 42ul of the reaction mix was added 1.1ul of Taq (Advanced Biotechnologies, Surrey, UK) and 1.4ug of template DNA in 27ul of sterile water and 5ul of this was added to each of the 12 tubes. These were then subjected to 10 cycles of 94°C for 10sec, 65°C for 1min followed by 20 cycles of 94°C for 10sec, 61°C

for 50sec and 72°C for 30 sec on a Techne Genius PCR machine (Techne, Anachem, UK). The samples were then loaded directly onto a 2% agarose gel run in 1x TBE at 50mAmps. The gel was stained with ethidium bromide and viewed on a UV transilluminator and photograhed. A representative gel picture of AS patients typed for DRβ1*04 is shown in Figure 11. By comparing the pattern of bands with the manufacturers table of specificities (appendix G) the individual can be assigned their particular alleles.



Legend for Figure 11

The results of two individuals subtyped at the HLA DRβ1*04 locus are shown in Figure 11. The 12 lanes on the 2% agarose gel represent 12 different primer sets that distinguish between the different alleles. Lane 13 is the 123bp molecular weight marker. The top band in each lane is of the PCR product for the Human growth hormone gene, which is the internal control. The lower band identifies the specific alleles present. By comparing the allele specific amplifications with the interpretation table supplied by the manufacturer the individual alleles present can be identified. For example the individual in the first sample is heterozygous for alleles HLA DRβ1*0401 and 0404. The individual in the second sample is homozygous for allele HLA DRβ1*0401.

2.6 TNF Polymorphisms

2.6.1 HLA Defined Cell Lines

In order to correctly identify the individual alleles at each polymorphic site and to act as positive controls for each PCR reaction DNA was prepared by the Nucleon method described previously from HLA defined cell lines from the European Collection of Animal Cell Cultures (Porton Down, Salisbury). The cells were maintained in RPMI 1640 (Sigma, Poole, UK) supplemented with 10% foetal calf serum (Sigma, Poole, UK), penicillin, streptomycin and L-glutamine (Sigma, Poole, UK) in a 5% CO_2 humidified incubator at 37°C. The cells were pelleted at 600g for 5minutes at 4°C before DNA was extracted. The cell lines used along with their typing results at each site are given in Table 2.

Name	Ecacc Number	DR	TNFa	TNFc	TNFe
HOM-2	88052005	1	6	1	3
SAVC	88052034	4	11	1	3
DKB	88052074	9	2	2	1
WT47	88052063	13	4	1	3
MOU	88052050	7	8	1	3

Legend for Table 2
The names of the individual cell lines used as internal controls along with their typing results are given in Table 2.

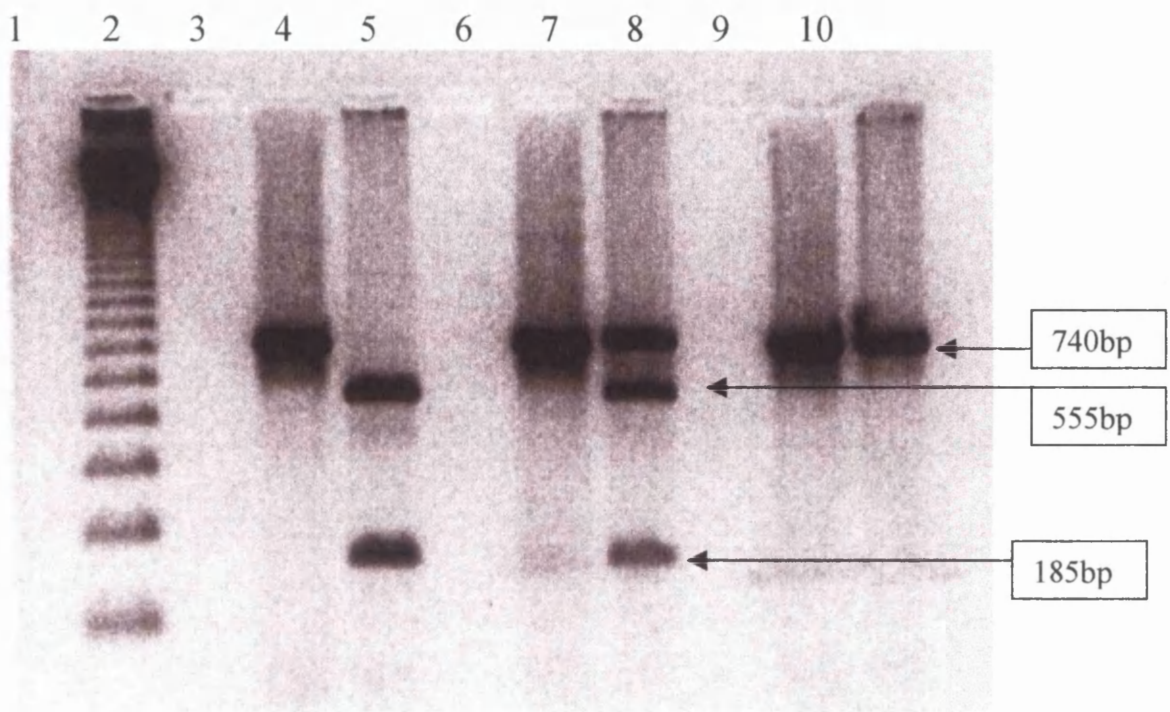
2.6.2 Nco.1 Polymorphism

The first site investigated was the bi-allelic restriction fragment length polymorphism in the first intron of the lymphotoxin alpha gene (Messer G., 1991). This RFLP designated Nco.1 is due to a single base substitution of a cytosine for an adenosine which corresponds to an amino acid change of a threonine for asparagine at position 26.

This gives rise to two alleles, which can be distinguished by the restriction enzyme Nco.1. The Nco.1*1 allele (TNF B*1 as described in Messer's paper) contains the restriction enzyme recognition site and the Nco.1*2 allele (TNF B*2) does not have the recognition site (see figure 12). Primers have been designed that flank this recognition site and therefore by amplifying this segment of DNA with these primers followed by digestion of the PCR product with the Nco.1 enzyme the two different alleles can be identified.

In order to investigate the distribution of the Nco.1 alleles in the AS patients and controls the following method was carried out. The PCR was carried out using 0.5units of Taq (Advanced Biotechnologies) in 1x Buffer IV; 1.5mM $MgCl_2$ (Advanced Biotechnologies); 200mM of each deoxyribonucleotide; 1uM of sense primer (5' CCG TGC TTC GTG CTT TGG ACT A 3') and 1uM of antisense primer (5' AGA GCT GGT GGG GAC ATG TCT G 3') and 500ng of template DNA in a total volume of 25ul. Cycles were 95°C for 6 minutes followed by 35 cycles of 95°C for 1 minute; 64°C for 1minute; 72°C for 1minute and a final elongation step of 72°C for 5minutes on a Techne Genius PCR machine. A 10ul aliquot of the PCR reaction was then digested with Nco.1 restriction enzyme (New England Biolabs, Herts, UK) for two hours at 37°C. The alleles were determined by running an aliquot of the undigested PCR product adjacent to the

digested product on a 2% agarose gel run in 1xTBE, stained with ethidium bromide and visualised on a UV transilluminator. The Nco.1 RFLP gives rise to two alleles and therefore three genotypes, which can be distinguished by the band pattern on the gel as can be seen in figure 12.



Legend for Figure 12

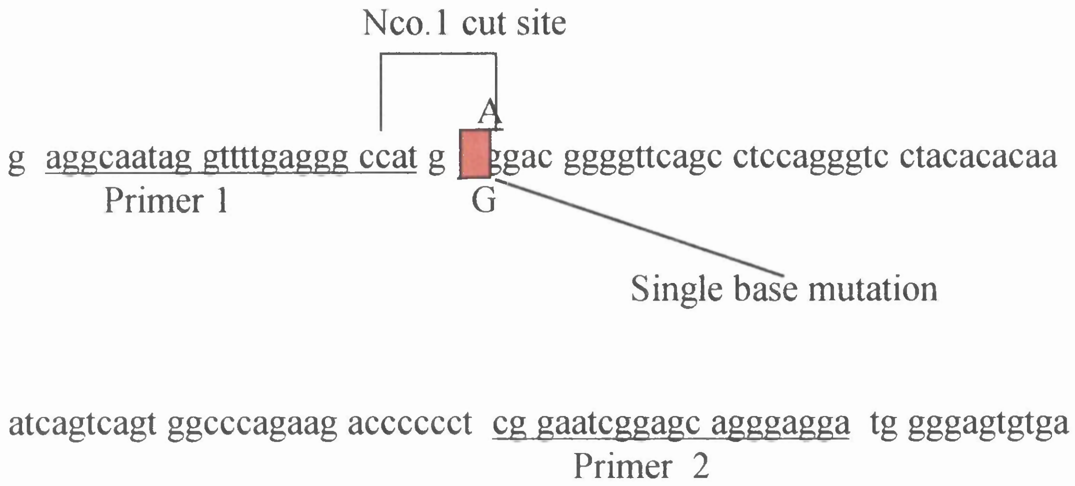
There are two possible alleles at the Nco.1 RFLP corresponding to three possible genotypes. Examples of these are depicted in Figure 12. Lane 1 represents the 123bp molecular weight marker. Lanes 3, 6 and 9 represent undigested PCR products of 740bp. Lane 4 is an example of an individual homozygous for allele 1 where the restriction enzyme has cut the PCR product into two bands of 555 and 185bp. Lane 10 is an individual homozygous for allele 2 where the enzyme has not cut the PCR product and Lane 7 is an individual heterozygous for alleles 1and 2.

2.6.3 -308 polymorphism

The -308 RFLP first described by Wilson lies in the promoter region of the TNF alpha gene (Wilson A.G, 1992). This RFLP is due to a substitution of guanine by adenosine and gives rise to two alleles distinguished by a Nco.1 restriction site (see figure 13).

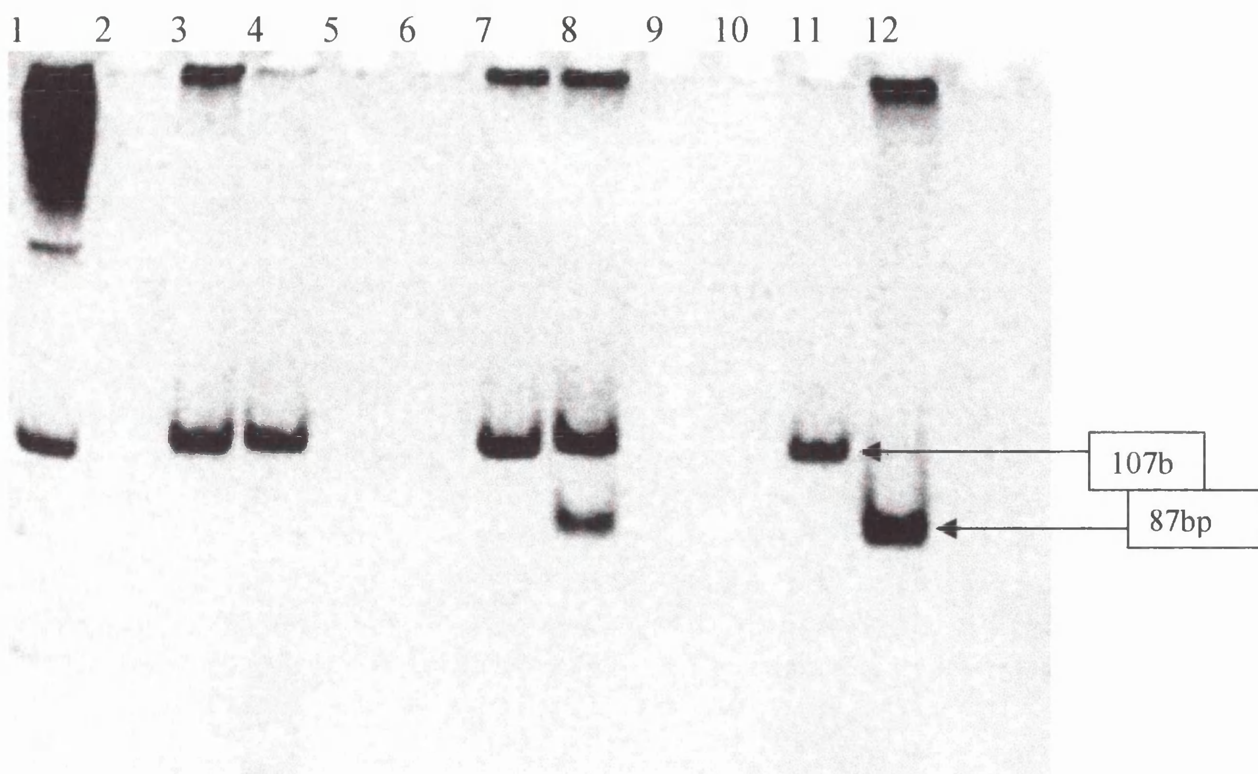
Analysis of the -308 RFLP was determined using the following primers; sense primer 5' AGG CAA TAG GTT TTG AGG GCC AT 3' and anti sense primer 5' TCC TCC CTG CTC CGA TTC CG 3'. The reaction was carried out in a volume of 25ul containing 1x Buffer IV, 1.5mM MgCL2 (Advanced Biotechnologies, Surrey, UK), 200mM dNTP's, 1uM of each primer, 0.5 units of Taq (Advanced Biotechnologies, Surrey, UK) and 500ng of template DNA. The reaction mix was subjected to one cycle of 94°C for three minutes, 60°C for one minute, 72°C for one minute, then 35 cycles of 94°C for one minute, 60°C for one minute, 72°C for one minute with a final elongation step of 72°C for five minutes. A 10ul aliquot of the PCR product was then digested with Nco.1 enzyme at 37°C for two hours. An aliquot of the undigested and digested PCR products were run out on an 8% polyacrylamide gel on a Sturdier vertical gel (Hoeffer, UK) in 1x TBE at 150volts, approximately 20mAmps. The gel was removed from the gel apparatus, stained with ethidium bromide and visualised on an UV transilluminator. Allele 1 (-308.1) which has guanine at position -308 contains the Nco.1 recognition site and therefore is cut by the enzyme giving rise to two bands (as can be seen in Figure 14) and allele 2 (-308.2) with adenosine at this position is uncut. A polyacrylamide gel was used to give better separation of the PCR fragments.

-308 RFLP



PCR product using primers 1 and 2	107 base pairs
Digest with Nco.1; cut site present (G)	87 and 20 base pairs
cut site absent (A)	107 base pairs

Legend for Figure 13
A diagrammatic representation of the –308 RFLP is shown in Figure 13. The single point mutation is highlighted along with the PCR product sizes after digestion with the restriction enzyme.



Legend for Figure 14

At the –308 locus there are two possible alleles and figure 12 represents the pattern of bands seen when undigested and digested PCR products are run out on an 8% polyacrylamide gel (as described in the methods).

Lane 1 is of the 123bp molecular weight marker. Lanes 3, 7 and 11 are undigested PCR products of 107bp. Lane 4 is an individual homozygous for allele 2 (i.e no cut site present). Lane 8 is an individual heterozygous for alleles 1 and 2 and lane 12 is an individual homozygous for allele 1 (cut site present).

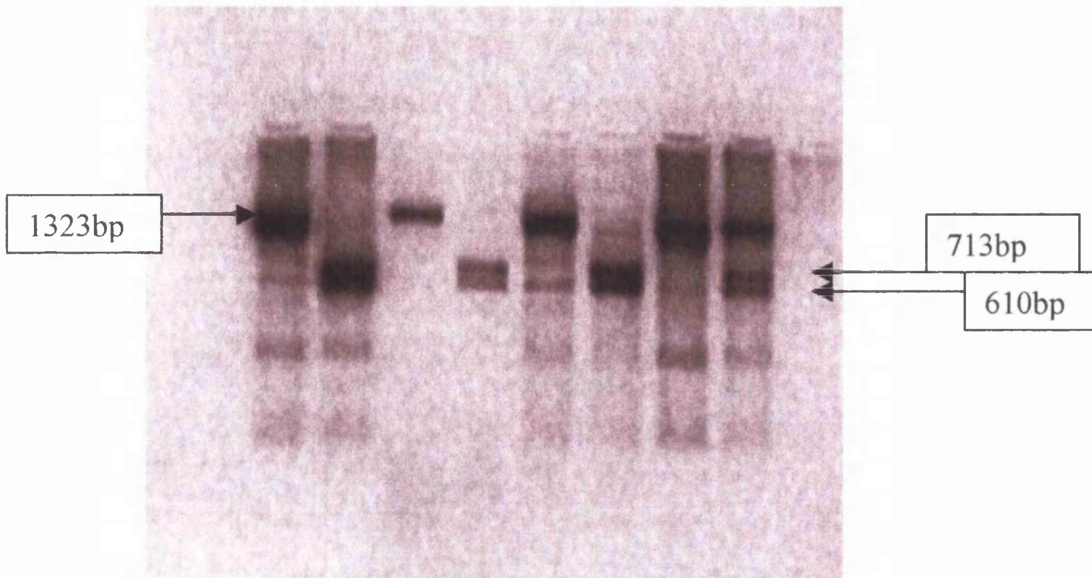
2.6.4 LST 1 polymorphism

The Leucocyte-specific transcript (LST-1) the human homologue of the mouse B144 gene lies between the Lymphotoxin β gene and the BAT1 loci within the MHC class III region. Although no known function has been found so far its close proximity to the TNF locus and pattern of expression point to a possible role in the immune response (Holzinger I., 1995). The gene is known to be transcribed in B cells, T cells, macrophages and haemtopoietic tissues. Analysis of the nucleotide sequence of LST-1 implies a possible signal function (Abraham L.J., 1992). Clones for full-length cDNA of LST-1 have been isolated and show that LST-1 codes for INF-γ inducible transcripts found in lymphoid tissues, T cells and macrophages. The LST-1 RFLP lies at position 2794 and can be identified by Pvu II restriction enzyme digestion. A previous study has investigated this polymorphic site in patients with type I diabetes and patients with Graves disease but no association was demonstrated (Rau H., Usadel K.H., Ommert S. and Badenhoop K. European Journal of Immunogenetics, 22 277-282, 1995). However the position of the LST-1 site close to the TNF genes and its pattern of expression suggest it may have a possible role in autoimmune diseases.

Using primers LST-1 A (5' AGC AGG AAC TCC ACT ATG C 3') and LST-1 B (5' GGC ATC TAC GTG TGC AG 3') spanning the polymorphic site a 1323bp product was amplified under the following conditions: 1x Buffer IV, 200uM dNTP's, 1.5mM $MgCL_2$, 1uM of each primer, 0.4ul of Taq and 500ng of template DNA. The reaction mix was subjected to one cycle of 95°C for 5minutes followed by 30 cycles of 95°C for 30 seconds; 56°C for 1 minute; 70°C for 1.5 minutes and a final elongation step of 70°C for 5minutes. An aliquot of the PCR product was then digested with Pvu II (N.E.B, Herts,

UK) at 37°C for two hours before being run out on a 2% agarose gel, stained with ethidium bromide and visualised on an UV transilluminator. An example of the LST-1 RFLP is shown in Figure 15.

This PCR gave multiple bands on the PCR gel picture but since it was apparent early on that there was no significant differences between the groups it was decided not to spend precious time optimising the PCR.



Legend for Figure 15

The LST.1 RFLP gives rise to possible alleles which are depicted on Figure 15. Lanes 1,3,5 and 7 are examples of undigested PCR products of 1323bp in length from four different AS patients. Lanes 2,4 and 6 are examples of the digested PCR product where the cut site is present giving rise to two bands of 713 and 610bp in length. These patients are therefore homozygous for allele 2. The patient in lane 8 has both the uncut and the cut site and is therefore heterozygous for alleles 1 and 2.

2.6.5 TNFa Microsatellite

Microsatellites are tracts of DNA in which a single base or a small number of bases are repeated. Expansions of such tracts have previously been associated with several human disorders such as the fragile X syndrome (Caskey C.T, 1992). Within the TNF locus are several microsatellites as described by Udalova (Udalova I.A., 1993) which have been associated with an apparent genetic predisposition to higher or lower levels of production of TNF *in vitro* (Pociot F., 1993).

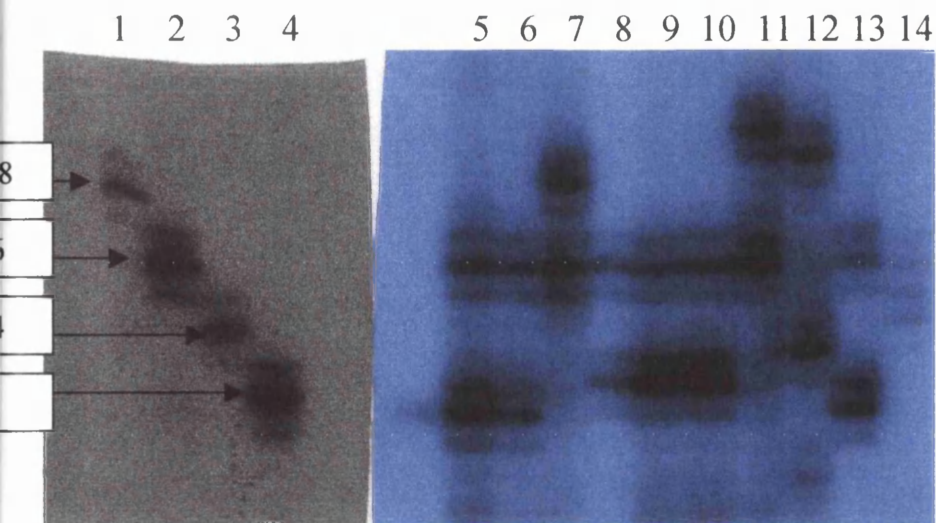
The TNFa microsatellite is highly polymorphic in that there are a possible 13 different alleles that have been found in a number of different ethnic populations. The TNFa microsatellite is an AC/GT repeat that is found in the upstream region of the TNF-β (lymphotoxin) gene. Previous work has shown an association with alleles 6 and 2 with differences in production levels of TNFα protein. These alleles differ in size due to the different number of repeats they contain that can be detected by amplifying the region containing the microsatellite by PCR and then separating the products out by electrophoresis. A diagrammatic representation of the TNFa microsatellite is shown in Figure 16.

The method for identifying these 13 different alleles is based on the method of Udalova with the following primers being used; primer 1 5'GCC TCT AGA TTT CAT CCA GCC ACA 3', primer 2 5' CCT CTC TCC CCT GCA ACA CAC A 3'. An initial first round reaction contained 1x Buffer IV (Advanced Biotechnologies), 200uM dNTP,s, 1.25mM $MgCL_2$, 1uM of each primer, 0.02 units of Taq and 500ng of template DNA in a final volume of 10ul. This was subjected to 96°C for 3 minutes, followed by 30 cycles of 94°C for 30s, 65°C for 30s and 72°C for 1min with a final extension of 74°C for 10min. A

second round PCR was carried out on 0.5ul of the first round product incorporating a ^{32}P labelled dCTP. The reaction mix contained 1x buffer IV, 1.25mM $MgCl_2$, 1uM of each primer, 0.02units of Taq, 200uM dATP,dGTP,dTTP and 20uM dCTP along with 0.05ul of α^{32}PdCTP (3000Ci/mol) in a final volume of 10ul. This was subjected to 5 cycles of the same parameters as before.

The PCR reaction was stopped by the addition of 5ul of formamide buffer. The samples were heated at 80°C for three minutes after which they were kept on ice before being loaded onto a 6% denaturing polyacrylamide gel (7.6m urea). The gel was run in 1x TBE at 75W for approximately 90minutes. The gel was transferred onto 3mm filter paper (Whatman, Kent, UK) before being dried at 80°C for 90minutes. The dried gel was then transferred to a film cassette with intensity screens and exposed to BioMax film (Kodak, Herts, UK) overnight.

This method of two rounds of PCR was established after several protocols were examined to try and clean up the PCR products. This was important when the gels were examined as there is an inherent problem in this type of microsatellite analysis. This type of analysis suffers from the problem of shadow bands that occur when there is incomplete copying of the PCR product. This makes it very difficult to read the autorads due to the large number of erroneous bands. The difficulty in assigning these alleles is overcome by the addition of HLA defined cell lines that have previously been characterised at the TNFa locus. A representative gel of the TNFa alleles in AS patients is shown in Figure 17.



Legend for Figure 17

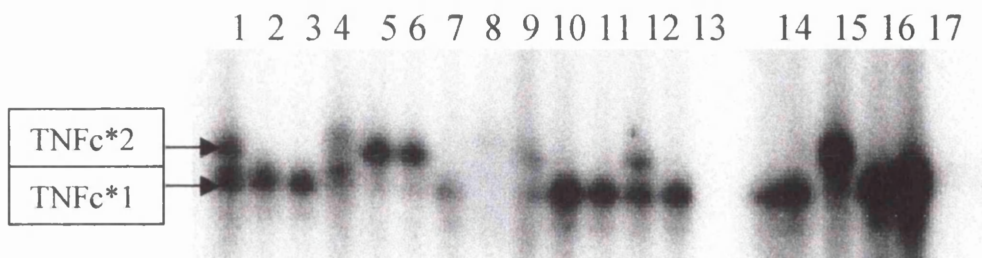
A typical autorad of the TNFa microsatellite is depicted in Figure 17.

The left hand panel is of the cell lines, which have previously been characterised (as described in Table 2). These were used as markers to assign the alleles to the patients samples 10 of which are represented on the right hand panel. For example the individual in lane 5 is heterozygous for alleles 2 and 6. Lane 7 is an example of an individual heterozygous for alleles 6 and 8.

2.6.6 TNFc Microsatellite

The TNF c microsatellite lies within the intron 1 of the LTα alpha gene and consists of a TC/GA biallelic repeat that gives rise to two alleles depending on the number of repeats i.e. allele 1 contains 9 repeats and allele 2 contains 10 repeats (Nedospasov SA, 1991). It is thought that variation in the length of these microsatellites does not itself affect the regulation or structure of the gene but that they act as markers for another gene that may have direct effects.

The two alleles can be distinguished from each other on the basis of size by first amplify the region by PCR followed by separation of the PCR products by electrophoresis. Primers designed to flank this microsatellite; primer 1 TNFc (1) 5' GGG AAG TCT GTC TTC CGC CG 3' and primer 2 TNF c (2) 5' CGT TCA GGT GGT GTC ATG GG 3' were used in a PCR reaction containing 1x Buffer IV, 200mM dATP, dGTP, dTTP, 20mM dCTP, 1.5 mM $MgCL_2$, 1uM of each primer, 0.02 units of Taq, $\alpha^{32}P$ dCTP (3000Ci/mol) and 500ng of template DNA in a total volume of 10ul. This was subjected to one cycle of 94°C for five minutes followed by 35 cycles of 94°C for 25 seconds, 60°C for 1minute, 74°C for 1 minute with a final extension of 74°C for 10 minutes. The reaction was stopped by the addition of formamide/EDTA gel loading buffer. The samples were heat denatured for 3 minutes at 80°C before loading onto a 6% denaturing (7.6M urea) polyacrylamide gel run at 75 watts in 1X TBE. The gel was vacuum dried and exposed overnight to BioMax film (Kodak). Characterised cell lines as described were run on each gel in order to size the PCR products as can be seen in figure 18.

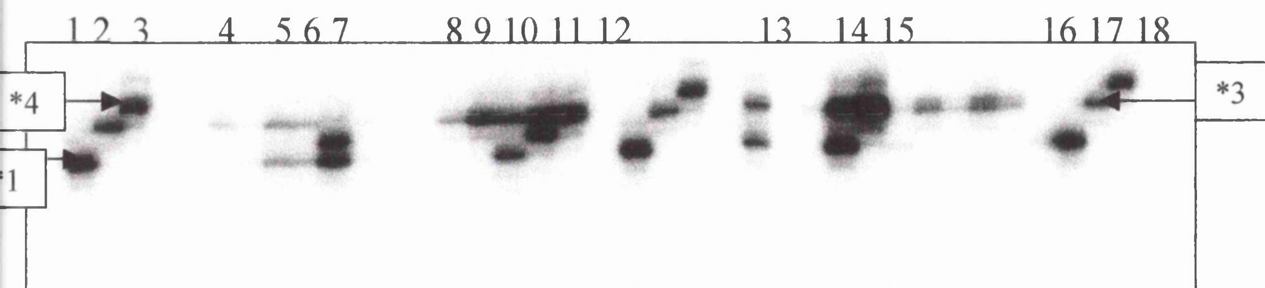


Legend for Figure 18

A representative autorad of a TNFc polyacrylamide gel is shown in Figure 18. Lanes 15,16 and 17 samples of the characterised cell lines were used as markers for the two possible alleles. Lane 2 is a representative of an individual homozygous for allele 1 (9 repeats) and Lane 5 is an individual homozygous for allele 2 (10 repeats). Lane 1 is an example of an individual heterozygous for alleles 1 and 2.

2.6.7 TNFe Microsatellite

The TNFe microsatellite, which lies at the 3' region of the Lst-1 gene, 8-10 Kb downstream of the TNF alpha gene, has been shown to have at least three alleles depending on the number of TC/GA repeats. The repeats were amplified using the following primers based on the method of Udalova (ref). Primer 1 5' GTG CCT GGT TCT GGA GCC TCT C 3' and primer 2 5' TGA GAC AGA GGA TAG GAG AGA CAG 3' were used at a final concentration of 1uM in a total reaction mix of 10ul containing 1X Buffer IV, 1.5mM $MgCL_2$, 200uM dATP, dGTP, dTTP, dCTP, 0.02 units of Taq and 100ng of template DNA. The reaction was subjected to 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 1minute and a final extension of 72°C for 5 minute. A 0.5ul aliquot of the PCR product was removed and added to a fresh tube containing the following reaction mix; 1X Buffer IV, 1.5mM $MgCl_2$, 200mM dATP, dGTP, dTTP, 20mM dCTP, 0.04ul α^{32}PdCTP (3000Ci/mol) and 0.02 units of Taq in a final volume of 10ul. This was subjected to the five of the cycles described above. The reaction was stopped by the addition of formamide/EDTA gel loading buffer. The samples were heat denatured at 80°C for 3 minutes before loading onto a 6% denaturing (7.6M Urea) polyacrylamide gel run in 1X TBE at 75 watts in a Stratagene squencing gel rig (company address). The film was vacuum dried before being exposed overnight to BioMax film (Kodak). An example of a TNFe autorad is shown in Figure 19.



Legend for Figure 19

A representative autorad of the TNFe microsatellite is shown in Figure 19. Lanes 1,2 and 3 are results from the characterised cell lines where the alleles have been assigned as alleles 1, 3 and 4. These are used as markers in order to assign alleles to the patient samples. For example the patient in lane 7 is heterozygous for alleles 1 and 2. Lane 11 is an example of an individual homozygous for allele 3 and lane 14 is a patient heterozygous for alleles 1 and 3.

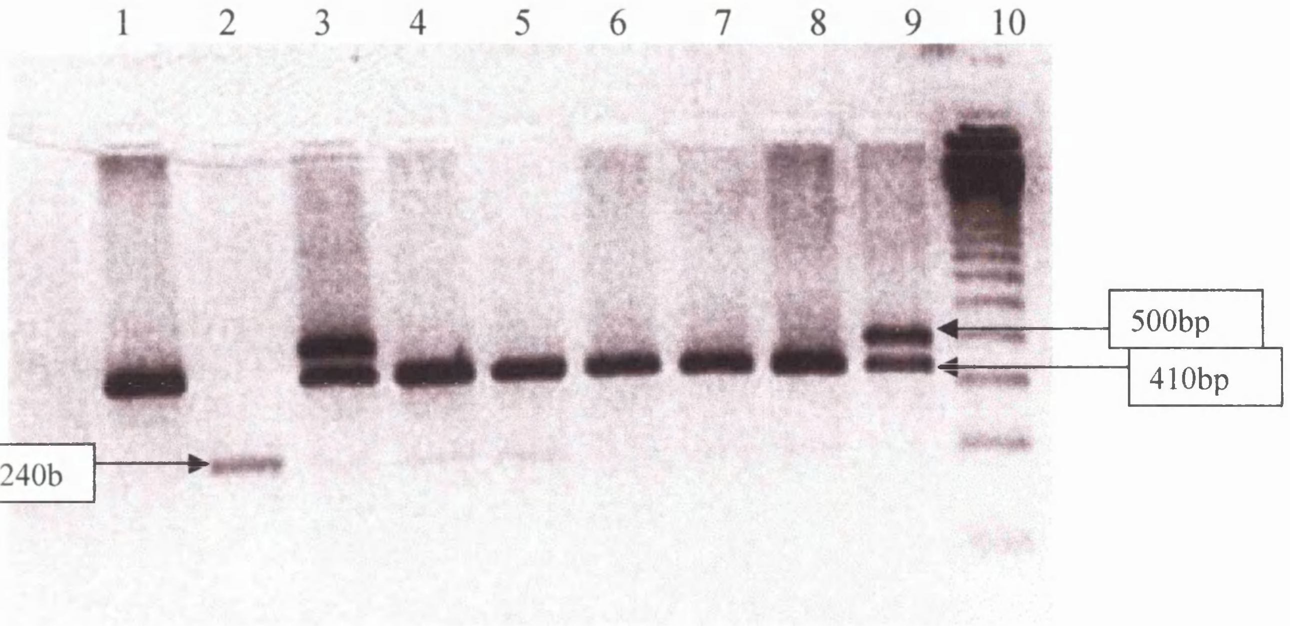
2.7 IL-1 Polymorphisms

2.7.1 IL-1Ra VNTR

Within the intron 2 of the IL-1Ra gene is a variable number tandem repeat (VNTR) of 86bp length that has previously been shown to be polymorphic (Tarlow JK, 1993). This VNTR gives rise to 4 different alleles depending on the number of times the 86bp fragment is repeated. A previous report has shown allele 2 at this site to be over represented in a population of patients with Ulcerative Colitis (UC) (Mansfield JC, 1994). This increase of allele 2 was not seen in patients with Crohns disease. The development of immune-mediated colitis has been shown to be prevented by treatment with IL-1Ra in animal models and has led to clinical trials using human recombinant IL-1Ra in UC patients (Dinarello CA 1993). Previous work has shown allele 2 to be associated with increased production of IL-1Ra and decreased production of IL-1α *in vitro* (Danis VA, 1995).

Primers flanking this region were used to amplify this polymorphic site, which could then be analysed by gel electrophoresis to distinguish the different alleles by size. Allele 1 has been shown to contain 4 repeats and therefore gives a band size of 410bp, allele 2 has 2 repeats and has a PCR product of 240bp and allele 3 has 5 repeats and gives rise to a product of 500 base pairs. Only three alleles were found in this study although a fourth allele has been seen in other populations (ref). Figure 20 shows a representative gel of AS patients amplified for this site.

Primer 1 5' CTC AGC AAC ACT CCT AT 3' and primer 2 5' TCC TGG TCT GCA GGT AA 3' were used at a final concentration of 1uM in a total reaction volume of 25ul



Legend for Figure 20

The PCR products of nine individuals amplified for the IL-1Ra polymorphism analysed on a 2% agarose gel are shown in Figure 20. Lane 10 is of the 123bp molecular weight marker. Lanes 1,4,5,6,7, and 8 represent individuals homozygous for allele 1 (410bp). Lane 2 is an individual homozygous for allele 2 (240bp) and lanes 3 and 9 represent individuals heterozygous for alleles 1 and 3 (410 and 500bp).

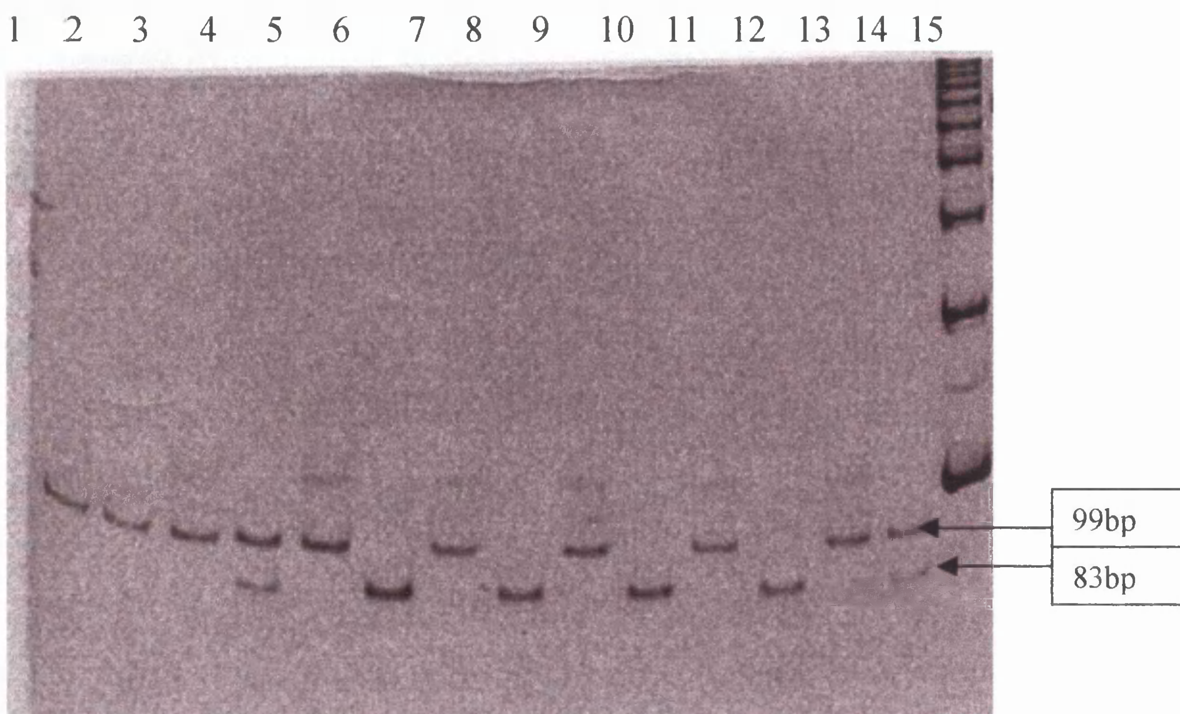
containing 100ng of template DNA, 200uM dNTP's, 1.5uM $MgCL_2$, 1X Buffer IV and 0.1ul of Taq. The reaction mix was subjected to an initial denaturation at 96°C for 1 minute followed by 30 cycles at 94°C for 1 minute, 60°C for 1 minute and 70°C for 1 minute with a final extension of 74°C for 10 minutes. An aliquot of the PCR product was mixed with orange G loading buffer (50% glycerol, 50% 1X TBE, orange G) and run out on a 2% agarose gel in 1XTBE at 50mAmps and stained with ethidium bromide. The gel was viewed on a UV transilluminator and photographed (Genetic Research Instrumentation, UK).

2.7.2 IL-1 alpha RFLP

The 5' flanking region of the IL-1α gene contains a single base pair polymorphism at position -889 (cytosine for thymine substitution). This polymorphism lies within the promoter region upstream of a sequence involved in transcriptional silencing of the Il-1α gene. It is known that single base changes can have an effect on transcriptional rates either by affecting the binding of trans-activating factors or by alterations in secondary structure. To date no known functional differences have been associated with any particular alleles. There are other polymorphic sites within the IL-1α gene such as the 46bp VNTR in intron 6, which do however have functional significance (Bailly S, 1995). Only the polymorphism at position -889 was investigated in this study as it has been shown previously to be associated with other inflammatory conditions. An increased carriage of the IL-1α 2 allele was found in patients with early onset pauciarticular

juvenile rheumatoid arthritis (EOPA-JRA) and in particular was strongly linked to those patients who developed chronic iridocyclitis.

Primers were designed to create a recognition site for the restriction enzyme Nco.1 in one allele but no restriction site in the other. The PCR was carried out on 100ng of template DNA with the following primers; primer1 5' AAG CTT GTT CTA CCA CCT GAA CTA AGG C 3', primer 2 5' TTA CAT ATG AGC CTT CCA TG 3', in a total volume of 25 ul. The reaction mix also contained 1X Buffer IV, 1.5mM $MgCL_2$, 200mM dNTP's and 0.1ul of Taq and underwent the following cycles of an initial denaturation at 96°C for 5 minutes, 35 cycles of 94°C for 1 minute, 46°C for 1 minute and 74°C for 2 minutes with a final extension of 74°C for 10 minutes. A 10ul aliquot of the PCR product was removed and added to a fresh tube containing 1X restriction digest buffer (N.E.B. buffer 4), 0.5ul of Nco.1 restriction enzyme (N.E.B) in a total volume of 5ul. This was incubated at 37°C for 2 hours. The digested PCR products were loaded onto an 8% polyacryamide gel in formamide/EDTA gel loading buffer, run in 1X TBE at 150 volts for approximately 2 hours. The gel was stained with ethidium bromide and photographed on a UV transilluminator. As can be seen on Figure 21 allele 1 gives rise to two bands one of 83bp and one of 16bp and allele 2 gives one band of 99bp.



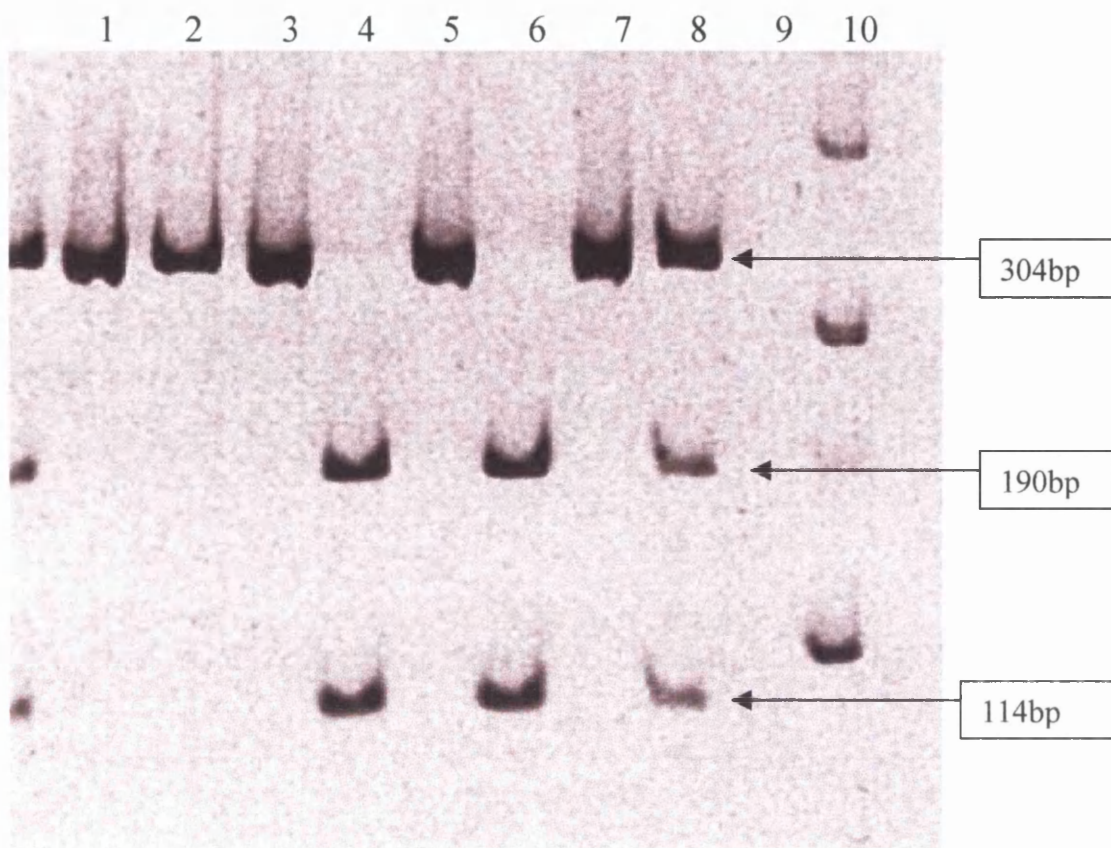
Legend for Figure 21

A representative polyacrylamide gel of AS patients amplified for the IL-1α single base pair polymorphism is shown in Figure 21. Lane 15 is the 123bp molecular weight marker. Lanes 1,3,5,7,9,11 and 13 are undigested PCR products. Lanes 6,8 10 and 12 represent individuals homozygous for allele 1 where the restriction cut site is present giving bands of 83 and 16bp's (the smaller fragment is not visible on the gel). Lane 2 represents an individual homozygous for allele 2 (no cut site present) and lanes 4 and 14 represent individuals heterozygous for alleles 1 and 2.

2.7.3 IL-1 beta RFLP

Within the promoter region at position -511 of the IL-1 beta gene is a cytosine to thymine single base pair substitution that gives rise to a restriction enzyme site recognised by the enzyme Ava I. No previous functional difference of any of the alleles has been demonstrated and no disease associations have been found so far. However the three genes do all lie in close proximity on chromosome 2 and it has been suggested that there may be some linkage between the genes as the common alleles of IL-1Ra have been shown to be associated with the common alleles of IL-1 β.

Using primers that flank this region the DNA can be amplified, followed by digestion with the products being detected by electrophoresis in order to distinguish the individual alleles. The primers, 1 5' TGG CAT TGA TCT GGT TCA TC 3' and 2 5' GTT TAG GAA TCT TTC CCA CTT 3' were used at a concentration of 0.1uM in a reaction mix containing 2.5ul of 10X reaction buffer IV, 2.5ul of dNTP's (2mM), 1.5ul $MgCL_2$ (1.5mM), 0.1ul of Taq (Advanced Biotechnologies) in a total volume of 15ul. Template DNA was added at 100ng in a volume of 10ul which was then added to the reaction mix. The PCR conditions were an initial denaturation at 95°C for 2min followed by 35 cycles of 95°C for 1 minute, 53°C for 1 minute, 74°C for 1 minute with a final extension at 74°C for 10 minutes. A 10ul aliquot of the PCR product was digested with 0.5ul of Ava I restriction enzyme (New Eng Biolabs) in a digestion mix containing 3ul of water and 1.5ul of buffer. This was incubated at 37°C for 2 hours. The digested and undigested PCR products were run out on an 8% acrylamide gel in 1x TBE at 150 volts for 2-2.5 hours before being stained with ethidium bromide and photographed on a UV transilluminator. An example of an IL-1β gel can be seen in Figure 22.



Legend for Figure 22

Figure 22 is a representative picture of an 8% polyacrylamide gel of the undigested and digested PCR products of the IL-1β polymorphism. Lane 10 is of the 123bp molecular weight marker. Lanes 1, 3,5 and 7 are the undigested PCR products of four AS patients. Lane 2 is an individual homozygous for allele 2 (uncut PCR product, 304bp). Lanes 4 and 6 are individuals homozygous for allele 1 (cut PCR product, 190 and 114bp). Lane 8 is an individual heterozygous for alleles 1 and 2.

2.8 Statistics

Population genetics is the study of the distribution of genotypes in a population and over the past few years has become very much the field of mathematicians. There has been much debate as to the most appropriate statistical test to use when comparing frequencies of different genotypes and alleles. In case-control analysis the aim is to detect whether certain marker alleles have different frequencies among cases and controls, suggesting that the marker may lie close to a disease gene or may itself affect susceptibility. Normally this would be assessed by applying a chi-squared test to a table of the allele counts observed in cases and controls. However microsatellite markers may have many alleles, some of which are relatively rare. If expected counts are less than 5-10 in a contingency table then the standard chi-squared statistic may be inaccurate. Standard approaches to avoid this problem include pooling rare alleles together, or testing each allele against the rest and then applying a Bonferroni correction for the number of alleles tested. For this study it was decided beforehand that for multiple allelic sites such as the HLA DRβ1*, TNFa microsatellite and the IL-1Ra VNTR the best approach was to use the clump software programme which assesses significance using a Monte Carlo approach. The clump programme is designed to assess the significance of the departure of observed values in a contingency table from the expected values conditional on the marginal totals. This works on the basis of a 2x N contingency table especially when N is large and the table is sparse. The significance is assessed using a Monte Carlo approach, by performing repeated simulations to generate tables having the same marginal totals as the one under consideration and counting the number of times that a chi-squared value

associated with the real table is achieved by the randomly simulated data. This means that the empirical significance levels assigned should be accurate (with precision dependent on the number of simulations performed) and that no special account needs to be taken of continuity corrections or small expected values.

All statistics were carried out using Minitab version 10 for personal computers. For comparison of genotype and allelic frequencies of bi-allelic sites p-values were calculated by the chi square statistics. Odds Ratio and 95% confidence intervals were calculated using the cross-products ratio. In order to take into account the number of analyses undertaken in the primary calculation, $p<0.017$ was taken as significant.

Polymorphic sites with more than two alleles were assessed by the Monte-Carlo simulation index following the method of Sham and Curtis (Sham, 1995). With the result of the T_4 calculation being quoted.

In the IL-1Ra polymorphic site the carriage rates (number of individuals with at least one copy of the test allele) were also calculated.

CHAPTER THREE:

CLINICAL AND MHC ASSOCIATIONS

3.1 Clinical Data Results

A total of 203 patients diagnosed with AS were enrolled in the study. A clinician saw all patients and a detailed clinical history was recorded. The overall clinical picture can be seen in Table 3. The ratio of males to females in this patient population was 3.2 :1. AS differs from RA in that the average age of onset is much younger (in this case median age of onset 21). Disease onset may also occur much later though, the oldest patient presenting in this study was 54 years old. A number of previous studies have suggested that a significant proportion of AS patients develop the disease before the age of 16 (Burges-Vargas R., 1989). In this study information on the age of onset was available in 192 out of 203 patients. The age of onset was defined by the clinician based on radiographic evidence. The overall number of individuals with an age of onset below or equal to 16 was 42 (22%). The distribution of the age of onset in the AS patients are depicted in Figure 23. This early onset means that patients will be seen that have had the disease for a long time. The median disease duration in this population was 24 years with a range of 1 to 59 years. The distribution of disease duration is shown in Figure 24.

Increased family prevalence is well documented in AS with 21% of this patient group reporting a family history. Information regarding a family history was available in 186 out of 203 patients but this information did not distinguish which family members were involved. It may be of interest to determine which other family member had the disease i.e mother, father, brother, sister as there has been recent evidence regarding increased incidence of AS in families where the mother is the proband.

It is also known that other systems may be involved in particular the eyes with 39% of these patients having a history of uveitis. Peripheral joint disease was recorded as not including hip and shoulder involvement and was seen in 33% of this group. Other clinical features recorded are described in Table 3. There were 19 patients who had undergone joint replacement surgery: only one female had joint surgery. It is worth remembering that the patients enrolled in this study were all attending the outpatients department at the Centre for Rheumatic diseases, which is a tertiary referral centre and therefore all the patients are more likely to have a more severe disease than individuals in the community. It has been suggested that the under diagnosis in females was due to females having a less severe disease. A comparison of the female and male AS patients in this group was carried out to determine if their clinical picture differed in particular the presence of extra spinal features which are taken as indicating a more severe form of the disease. The results are shown in tables 4, 5 and 6. It can be seen from table 4 that the median age, age of onset and disease duration of both the female and male AS patients were very similar. The presence of extra spinal features such as peripheral joint disease, uveitis and a family history in both males and females is examined in table 5. The percentage of both males and females who had peripheral joint disease did not differ with 36% of females compared to 32% of males having peripheral joint disease. Neither was there a difference in the presence of a family history (22% of both males and females). In females there was a slight increase in the number of individuals having a history of uveitis (47% compared to 37%) but this was not statistically significant.

Individuals who develop the disease early are thought to have a more severe disease. This was examined in the male and female AS patients with the results shown in table 6. The

two groups of patients were subdivided into those with an age of onset below 21 years and those with an onset equal to or above 21 years. The additional presence of either peripheral joint disease or uveitis separately or together was included. As can be seen from table 6 the number of females who had both an early onset and peripheral joint disease was increased when compared to those with a later onset (43% compared to 31%) although this failed to reach significance. In the male AS patients the number of individuals with a younger onset of disease having peripheral joint disease was significantly increased ($p=0.029$) when compared to those with an onset greater than 21 years. The number of females with an early onset along with a history of uveitis was decreased (38% compared to 52%) although this was not statistically different. There was no difference in the males.

Of 191 AS patients on whom information was available 19 required joint replacement, 18 males and 1 female therefore males were significantly more likely to require joint replacement surgery than females ($p=0.044$). The percentage of patients having joint replacement in comparison to their length of disease duration is shown on Figure 25. It would be predicted that the number of patients requiring joint surgery would increase with increasing disease duration however as can be seen from Figure 25 the majority of patients who required surgery actually had it carried out between 20 and 30 years after onset. The distribution of the age at which the patients required joint surgery is given in Figure 26. The most common age of the patients receiving joint surgery is between 41 and 50 years.

Overall the AS patients in this study are comparable to other groups of patients found in the UK.

Total number of AS patients	203
Male/Female Ratio	3.2:1
Mean Age (years)	47 Range [18-77]
Median Age of Onset (years)	21 Range [3-54]
Median Disease Duration (years)	24 Range [1-59]
Family History	21%
Uveitis	39%
Peripheral Joint Disease	33%
Inflammatory Bowel Disease	4%
Psoriasis	7%
Cardiac Defects	2%
Amyloid	0.5%
Lung Disease	1%
Joint replacement surgery	10%

Legend for Table 3

Table 3 describes the demographic and clinical details of the 203 AS patients enrolled in the study.

Years	Females			Males		
	Number	Median	Range	Number	Median	Range
Age of Onset	46	21	11-52	146	20	3-54
Disease Duration	45	23	5-58	146	25	1-59
Age	46	43	26-74	145	48	18-77

Legend for Table 4
No differences were seen in the male and female AS patients with regards to their age, age of onset and disease duration as described in Table 4.

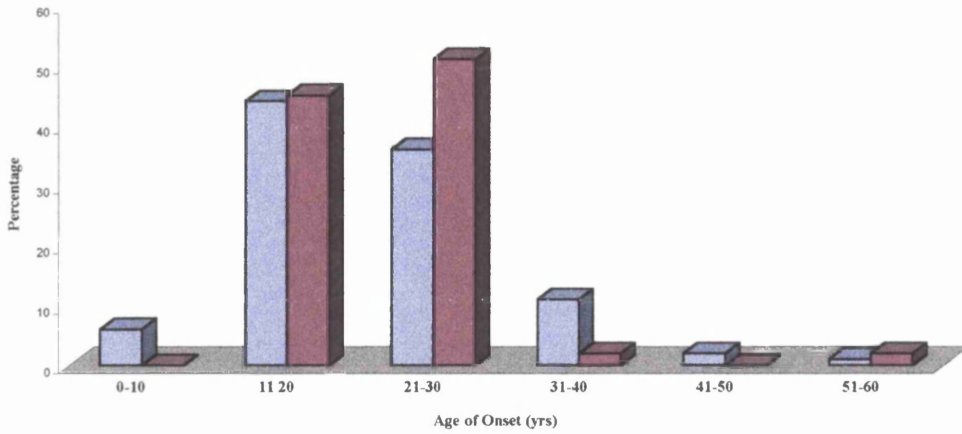
Extra spinal features	Total AS patients (%)	Female AS patients (%)	Male AS patients (%)
Peripheral Joint Disease Present	63(33)	17(36)	46(32)
Uveitis Present	74(39)	21(47)	53(37)
Family History Present	36(21)	9(22)	27(22)
Joint surgery	19(10)	1(2)*	18(12)*

Legend for Table 5
Table 5 describes the distribution of different extra spinal features in the AS patients. A slight increase in the number of female AS patients with uveitis was seen in comparison to the male AS patients (47% cf 37%). The number of males requiring joint replacement surgery was significantly increased compared to the females (12% cf 2%, *p=0.044).

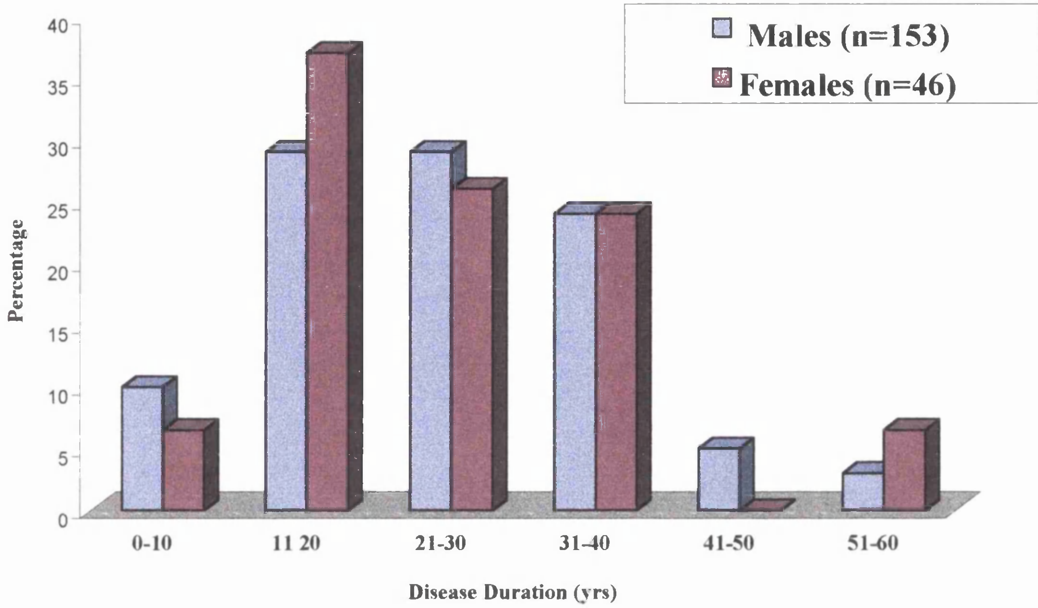
Extra spinal features	Females Onset <21 (%)	Females Onset >21 (%)	Males Onset <21 (%)	Males Onset >21 (%)
Peripheral joint disease present	9 (43)	8 (31)	28 (39)*	16 (22)*
Uveitis present	8 (38)	13 (52)	28 (39)	24 (33)
P.J.D + Uveitis Present	3 (14)	3 (12)	14 (20)	7 (10)

Legend for Table 6
A comparison between male and female AS patients with P.J.D., uveitis or both is shown in Table 6. The number of male AS patients who had an early onset of disease and peripheral joint disease was significantly increased compared to those with a later onset (39% cf 22%, *p=0.029).

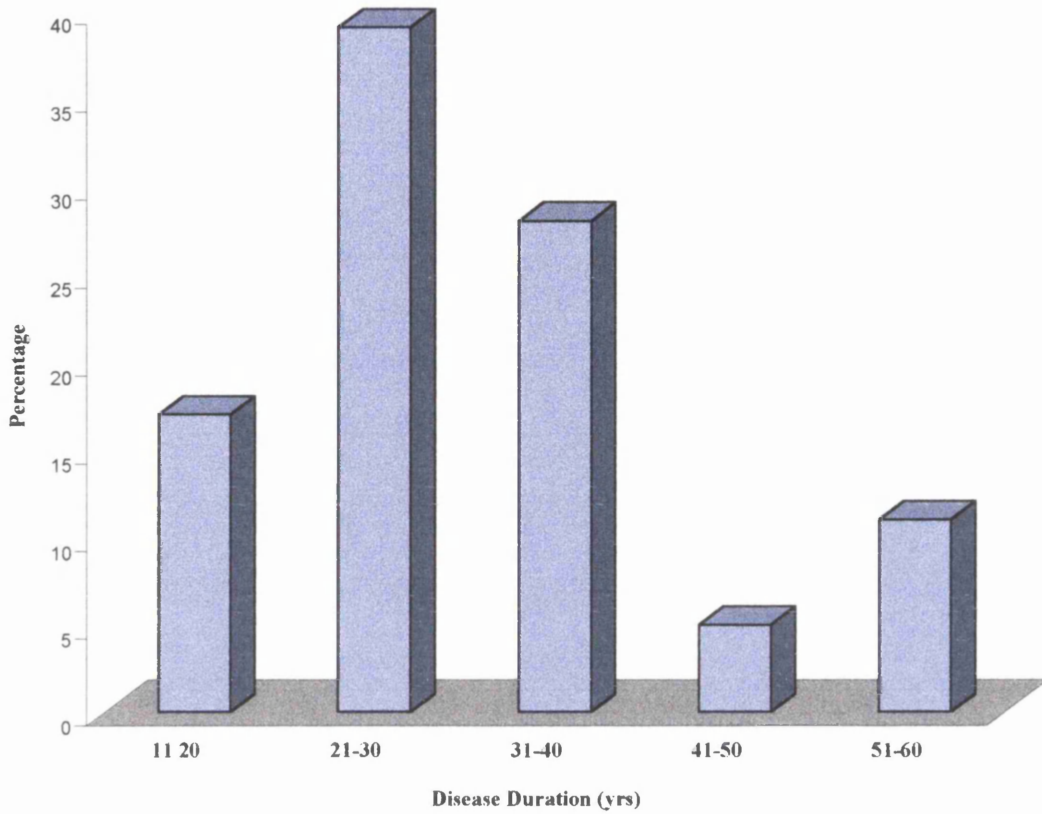
Distribution of Age Onset in AS patients



Legend for Figure 23
The distribution of the age of onset in the AS patients is shown in Figure 23. No differences between the male and female AS patients were seen.



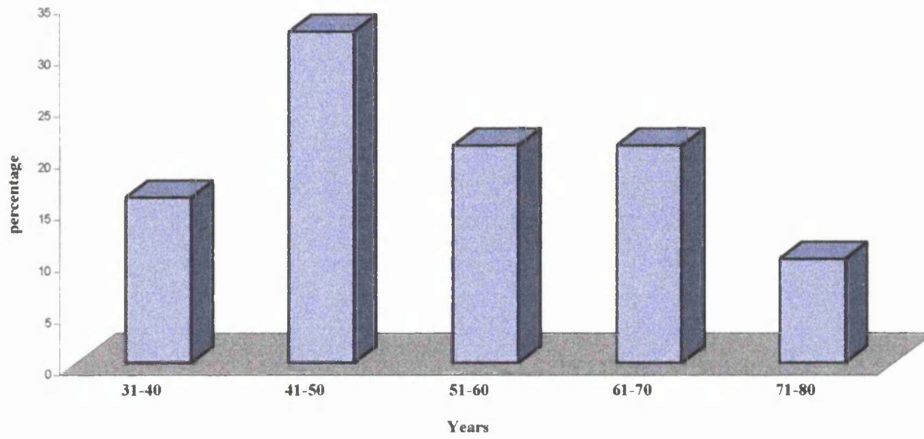
Legend for Figure 24
The distribution of the disease duration in the AS patients are shown in Figure 24. No differences between the male and female AS patients were seen.



Legend for Figure 25

Figure 25 describes the distribution of the percentage of AS patients having joint replacement surgery in comparison to the length of their disease duration.

Distribution of age of AS patients who have had Joint replacement surgery



Legend for Figure 26
The distribution of AS patients requiring joint replacement surgery in comparison to their ages is described in Figure 26.
35% of patients required surgery between the ages of 41-50.

3.2 Clinical Data Discussion

In the United Kingdom at present approximately 0.5-1% of the population are diagnosed with AS (Gran, 1998). The use of a uniform diagnostic criterion such as the New York criteria allows comparisons to be drawn between groups studied in different centres. Overall the 203 patients in this study who were all diagnosed using the New York criteria are very comparable in their features with other groups of patients. All patients in this study had been typed positive for the HLA B27 gene using the polymerase chain reaction by the Tissue Typing Department (Glasgow Royal Infirmary, Glasgow). Previous studies using the standard serology testing may underestimate the number of B27 positive individuals due to false negative results, however there are patients who have been diagnosed with AS who are negative for B27 but it was decided to only look at B27 positive patients in this study.

Original estimations put the ratio of males to females at nearly ten to one in favour of males however nowadays the ratio is normally put at three to one in favour of males. This study has confirmed that finding with a ratio of 3.2:1 in favour of males. In this study the male and female patients had very similar clinical patterns in that the average age of onset, disease duration and age of the two groups where very similar. The group of patients as a whole had similar median age of onset, disease duration and occurrence of extra-articular features as that described in the literature for other groups. However the incidence of inflammatory bowel disease is rather low at 4% since some groups have reported the incidence as being higher (Meilants,1996). This depends on how diagnosis is made and several papers report the incidence of inflammatory bowel lesions, which are much more common than full blown inflammatory bowel disease. For instance

inflammatory bowel lesions may be recorded in as many as 60% of AS patients (Mielants H, 1996). In this study the low incidence may be due to patients not undergoing specific endoscopic examination for the confirmation of gut lesions. Many patients may be asymptomatic for bowel disease but will have definite lesions visible upon investigation. The incidence of gut inflammation has been shown to be high (58%) in a group of Korean patients and not just in Western populations (Lee YH, 1997), confirming the strong link between the bowel and AS.

The strong family association has long been established with 21% of the patients in this study confirming a family history. However no information on which members of the family were affected was available. This information may have been interesting as it has been suggested that there is increased risk of developing the disease for first-degree relatives of female AS probands (Kennedy LG, 1993). It also has been suggested that where patients have a family history they may have a milder course of disease compared to those with no family history (Falkenbach A, 1998). This did not appear to be the case in this patient population in that those individuals with a family history had similar patterns of disease to those with no family history.

It is often postulated that disease outcome is milder in females compared to males with more peripheral joint involvement, less dramatic spinal changes and overall less rapid progression. The process of ankylosis has been said to proceed much faster and be more complete in males compared to females (Feldtkeller E, 1998). The presence of extra spinal features in males and females were compared and it was noted that slightly more females had a history of uveitis compared to the males (47% cf 37%) although this failed to reach statistical significance. The percentage of patients with peripheral joint disease

was the same in both sexes. There were no differences in the sexes with regard to the presence of a family history. However it was noted that out of the nineteen patients requiring joint surgery all but one of them were males. This lends weight to the argument that males have a more severe outcome in their disease in terms of their peripheral joint disease.

It has also been suggested that patients whose disease develops early are also more likely to have a more severe outcome. The incidence of peripheral joint disease and or uveitis was investigated in those patients whose disease onset was below the age of 21 years compared to those with a disease onset greater than or equal to 21 years. In the male patients the incidence of peripheral joint disease was significantly increased in the patients with a younger onset compared to those with a later onset $p=0.029$. Overall the male patients did appear to have a more severe outcome if their disease developed early on but this was not so convincing in the female patients again highlighting the milder outcome predicted for female patients. Of course the increased incidence of peripheral joint disease in those with earlier joint disease may simply be due to longer disease duration.

Two control populations were investigated in this study. The first group consisted of normal, healthy, unrelated blood donors who were all HLA B27 negative (kindly supplied by the Tissue Typing Department, Glasgow Royal Infirmary). The second group were normal, healthy, unrelated blood donors who were all HLA B27 positive (kindly supplied by Dr R. Green, Blood Transfusion, Law Hospital). All patients and controls were from the West of Scotland as it is important to use locally recruited controls that match the patient population. The B27 positive control group completed a general health

questionnaire that does not directly ask about back pain. None of these individuals have undergone a radiological examination to rule out any sacroiliac damage. A recent study has shown a high percentage of B27 healthy blood donors have signs of asymptomatic sacroiliac damage upon radiological examination. (Braun, 1998). It could be possible therefore that some of the B27 positive control population may have undiagnosed AS.

3.3 MHC Class I: HLA B27 Results

All the AS patients in this study were HLA B27 positive as confirmed by the Department of Tissue Typing in the Glasgow Royal Infirmary and information regarding the presence of other B genes in these patients was available in 120 cases. Previous work has reported the risk of AS to be increased three fold in patients with HLA B60 in addition to HLA B27, (Rubin, 1994). Although the association with HLA B60 has also been demonstrated in HLA B27 negative AS patients, suggesting HLA B60 may act independently as a disease susceptibility gene (Brown, 1996). In B27 negative patients it has been suggested that HLA B39 may be increased (Yamaguchi, 1995). Peptide binding studies predict that particular peptides may be capable of binding to both B27 and B39.

Epidemiology studies have concentrated more on the actual subtypes of B27 itself to determine if particular subtypes are disease associated. HLA B27 represents a family of 22 subtypes that differ only in a few amino acids. The most common subtype found in European Caucasians is B2705. Some subtypes appear to be disease associated whereas others such as B2706 and B2709 appear to have a protective effect (Lopez-Larrea, 1995). 100 AS patients in this study were subtyped all were B2705 (Alan Walkinshaw, Department of Tissue Typing, Glasgow Royal Infirmary). The subtypes of the B27 positive healthy controls were unknown.

The distribution of the other B antigen in the AS patients in comparison to the normal distribution of B antigens in the West of Scotland population are described in Table 7.

A significant difference (T_4=0.01) was seen using the Clump statistical test (Sham, 1995) in the AS patients compared to the West of Scotland controls. In the AS patients the

incidence of B27 was increased compared to the control population (10% compared to 3%, T_4=0.01).

B60 and B61 are splits of B40 with the majority being B60 in the West of Scotland. In the Table the results are given for B40. The distributions were very similar between the two groups however the incidence of B40 (B60 and B61) is relatively small in these West of Scotland populations. In this study all patients were B27 positive and therefore no comment on the distribution of B antigens in B27 negative patients can be made.

The distributions of the B antigens were investigated in the AS patients in comparison to their additional disease features. Table 9 describes the results for the AS patients with and without peripheral joint disease and those patients with an early onset of disease. No significant differences were noted. Table 8 describes the results for the AS patients with and without a family history and those with and without uveitis. In those patients with and without a family history there were no significant differences. However when the 50 AS patients who had a history of Uveitis were compared to the 64 patients who had no history of Uveitis there was a significant difference in the distribution of the other B antigen by the Clump test (T_4=0.01). The frequency of the B7 gene was significantly increased in the AS patients with Uveitis (T_4=0.036) whereas the B12 gene was significantly decreased in these patients (T_4=0.0069), when analysed by Monte Carlo simulations.

Unfortunately the number of the AS patients on whom the information about the other B gene was available was relatively small (n=120) and therefore it is difficult to draw any conclusions. However it is of interest to note that the distribution of the B40 gene was not increased in this population although in those patients with a family history there was a

slight increase in its frequency. The different distribution of the other B gene in those patients with a history of Uveitis is also of note although again numbers are small.

HLA B Antigens	AS Patients (n=120) [%]	West of Scotland B27 HealthyControls (n=890) [%]
B5	6 (5)	24 (3)
B7	23 (19)	163 (18)
B8	19 (16)	155 (17)
B12	19 (16)	155 (18)
B13	5 (4)	12 (1)
B14	6 (5)	49 (6)
B15	11 (9)	46 (5)
B17	2 (2)	38 (4)
B18	2 (2)	31 (3)
B22	1 (1)	20 (2)
B27	12 (10)*	25 (3)*
B35	6 (5)	50 (6)
B40	5 (4)	46 (5)
OTHERS	2 (2)	76 (9)

Legend to Table 7

Table 7 describes the distribution of the HLA B antigens encoded by the non-B27 chromosome in 120 AS patients compared to 890 West of Scotland B27 healthy controls (kindly supplied by the Tissue Typing Department, Glasgow Royal Infirmary). The AS patients differed significantly from the West of Scotland controls by Clump (T_4=0.01). The frequency of AS patients homozygous for the B27 gene was significantly increased (*T_4=0.01) when compared to the West of Scotland controls.

	Family History		**Uveitis**	
HLA Antigens	**Present n=27 (%)**	**Absent n=76 (%)**	**Present n=50 (%)**	**Absent n=64 (%)**
B5	2 (7.4)	4 (5.3)	3 (6)	3 (4.7)
B7	9 (33.3)	12 (15.8)	13 (26)*	7 (10.9)*
B8	0 (0)	15 (19.7)	9 (18)	9 (14)
B12	3 (11.1)	14 (18.4)	3 (6)**	16 (25)**
B13	1 (3.7)	4 (5.3)	0 (0)	5 (7.8)
B14	1 (3.7)	3 (3.9)	5 (10)	1 (1.6)
B15	2 (7.4)	6 (7.8)	4 (8)	6 (9.3)
B17	0 (0)	1 (1.3)	0 (0)	2 (3.1)
B18	1 (3.7)	0 (0)	1 (2)	0 (0)
B22	0 (0)	1 (1.3)	1 (2)	0 (0)
B27	4 (14.9)	6 (7.8)	6 (12)	6 (9.3)
B35	2 (7.4)	4 (5.3)	3 (6)	3 (4.7)
B37	0 (0)	1 (1.3)	1 (2)	0 (0)
B39	0 (0)	1 (1.3)	0 (0)	1 (1.6)
B40	2 (7.4)	3 (3.9)	1 (2)	4 (6.3)
B50	0 (0)	1 (1.3)	0 (0)	1 (1.6)

Legend for Table 8

The distribution of the HLA B antigens encoded by the non-B27 chromosome in AS patients are shown in Table 8. The distribution is compared in 27 AS patients with and 76 patients without a family history and in 50 patients with and 64 without uveitis. No significant differences were seen in the AS patients with a family history compared to those without. In the AS patients with uveitis the incidence of B7 was significantly increased (*T_4=0.036) compared to the patients with no history of uveitis. The frequency of B12 was significantly decreased in this group of patients (**T_4=0.0069) when analysed by Monte Carlo Simulations.

	PJD		Age of onset	
HLA B antigens	**Present [n=34] (%)**	**Absent [n=82]**	**<21 [n=61]**	**>21 [n=59]**
B5	2 (5.9)	4 (4.8)	3 (4.9)	3 (5.1)
B7	8 (23.5)	14 (17)	13 (21.3)	10 (16.9)
B8	7 (20.6)	11 (13.4)	10 (16.4)	9 (15.3)
B12	7 (20.6)	12 (14.6)	10 (16.4)	9 (15.3)
B13	2 (5.9)	3 (3.6)	1 (1.6)	4 (6.8)
B14	2 (5.9)	4 (4.8)	4 (6.5)	2 (3.4)
B15	1 (2.9)	10 (12.2)	5 (8.2)	6 (10.2)
B17	0 (0)	2 (2.4)	2 (3.3)	0 (0)
B18	1 (2.9)	0 (0)	0 (0)	2 (3.4)
B22	0 (0)	1 (1.2)	1 (1.6)	0 (0)
B27	1 (2.9)	10 (12.2)	7 (11.5)	5 (8.5)
B35	2 (5.9)	4 (4.8)	3 (4.9)	3 (5.1)
B37	1 (2.9)	0 (0)	1 (1.6)	0 (0)
B39	0 (0)	1 (1.2)	0 (0)	1 (1.7)
B40	0 (0)	5 (6.1)	1 (1.6)	4 (6.8)
B50	0 (0)	1 (1.2)	0 (0)	1 (1.7)

Legend for Table 9
The distribution of the HLA B antigens encoded by the non-B27 chromosome in AS patients are shown in Table 9. The distribution of the B antigens are compared in the 34 AS patients with peripheral joint disease to the 82 patients without and in the 61 patients with an age of onset below 21 years to the 59 with an onset greater than or equal to 21.

3.4 MHC Class I: HLA B27 Discussion

It is now firmly established that AS is a genetic disorder that shows familial aggregation but with a variable pattern of segregation. From family and twin studies where concordance rates for monozygotic twins is approximately 67% (Brown, 1997) it can be deduced that there more than one gene is involved. The strong association with HLA B27 and its direct effect on disease pathogenesis as demonstrated in B27 transgenic animals (Khare, 1995) is not in doubt. However approximately 9% of the population carry the B27 gene but only a small fraction (0.5-1%) go on to develop AS.

It is of interest that of the 22 B27 subtypes known there appears to be no difference between the subtypes found in the patients compared to the subtypes found in healthy controls. Of the subtypes that have been shown to be present in AS patients, such as B2702, 2704, 2705 these are also found at the same frequency in the healthy community. Several reports have postulated that there are some subtypes that may be protective such as B2706 in a Thais population (Lopez-Larrea, 1995) and B2709 in a Sardinian population (D'Amato, 1995). These subtypes differ by as little as one amino acid residue that can alter the peptide binding specificity. However the very low frequency of these subtypes in the population make it difficult to confirm their protective role and further studies are required.

120 of the AS patients in this study were typed to determine the B27 subtypes present. All the patients in this study were B2705 (Alan Walkinshaw, personal communication). The subtypes of the B27 positive healthy controls were unknown.

It has been suggested that individuals heterozygous for both HLA B27 and B60 have a three fold increased risk of developing the disease (Robinson, 1989). Some studies have

suggested that B60 may also act independently as a disease susceptibility gene (Brown, 19'6). Information regarding which other HLA B genes were present was available in 12(of the AS patients. When the distribution of these B genes was compared between the AS patients and the normal West of Scotland population by the Clump test a sigiificant difference was seen in the two populations (T_4=0.01). The overall distribution of he HLA B27 gene in the 890 West of Scotland normal individuals was 3%. This is slightly less than the normally accepted distribution which ranges from 5% to 14% in Caicasians and which has been previously reported at 9.5% for UK populations (Dipartment of Tissue Typing, Glasgow Royal Infirmary, personal communication).

In his population 10% of the AS patients were homozygous for B27 with B7 being the most common gene to be carried with B27. HLA B60 and B61 are splits of B40 with B60 most commonly found in the West of Scotland. The information regarding the disribution of the B60 and B61 genes in the West of Scotland normal population was conbined and presented as B40 (supplied by the Tissue Typing department, Glasgow Roial Infirmary). No increase in the patients heterozygous for B27 and B40 (B60+B61) wa seen in this group although numbers are small. With advances in molecular typing tecmiques an HLA B27-B40 hybrid allele has been observed in some German patients whch could be mistyped by some assays as a B27/B40 heterozygote this could artifically infate the risk associated with this genotype (Weissensteiner, 1998). In this group of AS patents therefore it cannot be confirmed that the additional presence of B60 increases therisk of developing the disease.

Th HLA B39 gene has previously been reported to have an association with B27 negative AS patients (Yamaguchi, 1995). In this study all the patients were B27 positive

and none of the patients were heterozygous for B27 and B39. This gene would have to be investigated in a population of B27 negative AS patients to see if an association could be made. Conversely another study has shown that there is no significant association between B39 and B27 negative disease (Brown, 1996).

The distribution of the other B gene was also investigated in the AS patients in comparison to several disease features. Of the features studied no differences were seen those patients with and without peripheral joint disease or those with a younger onset of disease. Neither were there any differences in the patients with a family history compared to those without apart from a slight increase in B40 in those patients with a family history. However in those patients with a history of uveitis there was a significant increase in the number of individuals heterozygous for B27 and B7 compared to those without uveitis. This was in comparison to the significant decrease in the number heterozygous for B27 and B12 (T_4=0.0069). The presence of B7 in conjunction with B27 would appear to increase the risk of developing uveitis in these AS patients but greater numbers would have to be investigated to confirm this finding.

3.5 MHC Class II: DRβ1* Alleles Results

The MHC is a highly polymorphic region that has several genes associated with the inflammatory response. Examples include the class I genes which are involved in antigen presentation to CD8 positive T cells, the complement and TNF genes in the class III region, and the Class II genes which are also involved in antigen presentation to CD4 positive T cells. Recent genome scans have confirmed the strong linkage disequilibrium with the MHC that has been shown to extend across the MHC from the marker D6S276 to the HLA DRβ1 locus and beyond (Brown, 1998). Only a few studies have investigated the role of the MCH class II alleles in AS with some studies suggesting as association with the DRβ1*01 alleles. In this study the distribution of the MHC Class II DRβ1 alleles were investigated in 139 AS patients compared to the two control populations.

The distribution of the DRβ1 alleles in 139 AS patients compared to 216 normal West of Scotland healthy controls is shown in Table 10 and Figure 27. The overall distribution between the two groups was statistically different when examined by the Clump test ($T_4 < 0.0005$). On closer examination this was due in particular to the increased frequency of the DRβ1*01 alleles in the AS patients (27% versus 8.8%, $p < 0.0005$) and the increased frequency of the DRβ1*04 alleles (31% versus 20.8%, $p = 0.038$). This was partly compensated for by the decrease in frequency of the DRβ1*03 alleles (9% versus 19%) although this failed to reach statistical significance.

The distribution of the DRβ1*alleles were compared between the 139 AS patients and 61 B27 positive controls as can be seen in Table 11 and Figure 28. The overall distribution between the two groups was significantly different as determined by the Monte Carlo

Simulation test (T_4=0.03). As seen with the normal controls this is mainly due to a significant difference in the distribution of the DRβ1*01 alleles. The frequency of the DRβ1*01 is significantly increased in the AS patients compared to the controls (27% versus 16%, p=0.013). Unlike the HLA B27 negative normals the distribution of the DRβ1*04 alleles is not different in the two groups. The distribution of the DRβ1*03 alleles is decreased in the AS patients compared to the B27 positive controls, but this was not statistically significant.

Several disease features have been suggested as indicators of a more severe outcome in AS such as an early onset, the presence of uveitis and peripheral joint disease. The distribution of the DRβ1* alleles was examined in the AS patients in relation to the presence or absence of these particular features. The results are described in Tables 12 to 15. In the 64 patients with an early onset compared to the 71 with a later onset there were no statistical significant differences. A slight increase in the frequency of DRβ1*04 was seen in the patients with a younger onset (35% versus 27%). This was in contrast to the 52 AS patients with a history of uveitis where a slight decrease of the DRβ1*04 alleles was seen in comparison to the 82 without uveitis (25% versus 36%). In the 20 AS patients with a family history the frequency of both the DRβ1*04 and 06 alleles were increased compared to the 104 patients with no family history. This was compensated for by a decrease in the incidence of the DRβ1*03 and 07 alleles. Previous studies have suggested an increased frequency of DRβ1*04 alleles in patients with peripheral joint disease (PJD) (Miehle, 1985). In the 44 AS patients with PJD there was no association

with DRβ1*04 with the frequency the same in those patients without (36% and 30% respectively).

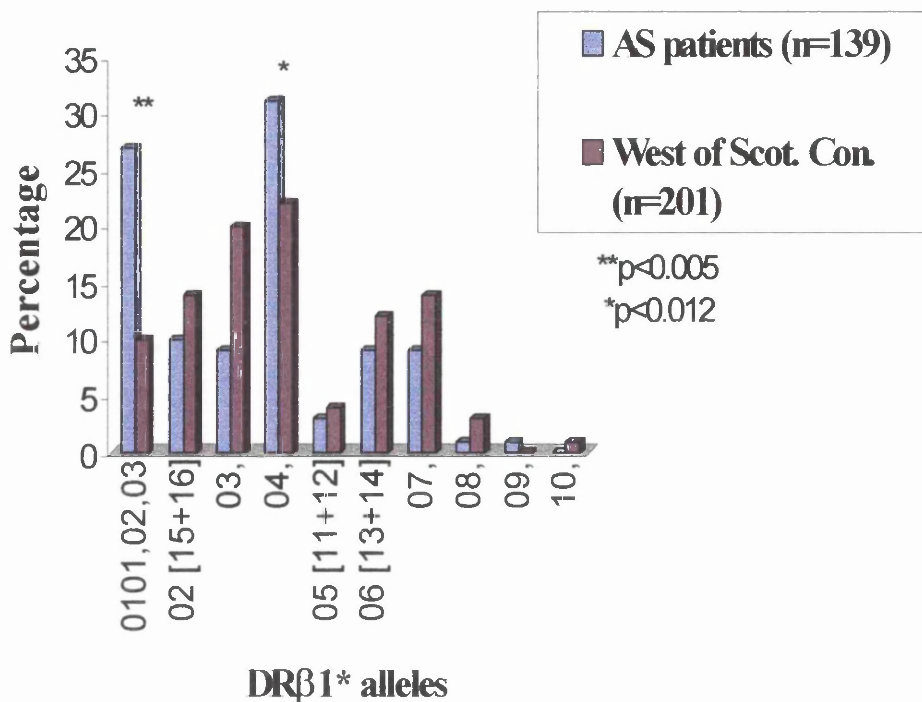
The DRβ1*04 subtypes were determined in 68 AS patients, 24 of which were homozygous. The results of the distribution of the DRβ1*04 subtypes in the AS patients compared to the two control groups are described in Table 16. Overall the distribution was significantly different in the AS patients compared to the West of Scotland controls as determined by the Clump test ($T_4<0.0005$). This was due to the increased frequency of the DRβ1*0401 and 0404 subtypes in the patients ($p<0.0005$). In contrast the DRβ1*0405 alleles were significantly decreased in the patients compared to the normal controls.

The distribution was compared in the AS patients to the B27 positive controls but no significant differences were seen. There was an increase in the frequency of the DRβ1*0404 in the AS patients compared to the B27 positive controls (24% versus 13%) but this failed to reach significance. However numbers of control subjects was small.

In conclusion there appears to be a significant association of the DRβ1*04 subtypes in the AS patients compared to the West of Scotland healthy controls that looks to be linked with the presence of B27 however numbers of control individuals would have to be increased to confirm this.

DRβ1* Alleles	AS Patients [n=139] (%)	West of Scotland Controls [n=201] (%)
0101,02 +0103	75 (27)**	19 (10)**
02 [15+16]	28 (10)	28 (14)
03	26 (9)	41 (20)
04	86 (31)*	45 (22)*
05 [11+12]	9 (3)	8 (4)
06 [13+14]	24 (9)	25 (12)
07	25 (9)	28 (14)
08	2 (1)	6 (3)
09	3 (1)	0 (0)
10	0	1(1)

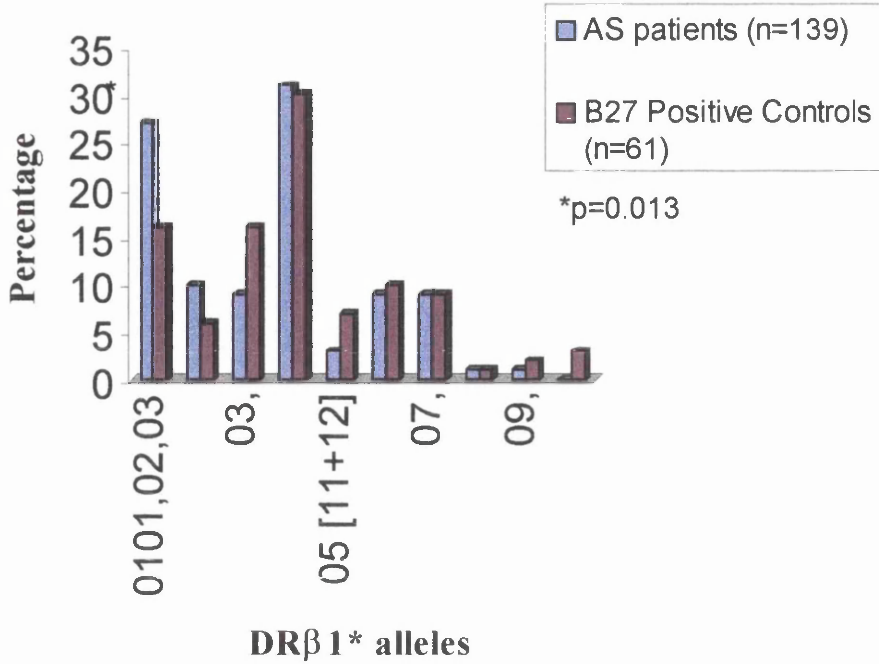
Legend for Table 10
Distribution of MHC Class II DRβ1* alleles in AS patients compared to normal unrelated blood donors from the West of Scotland are shown in Table 10. The overall distribution of DRβ1* alleles differ in the AS population when compared to the West of Scotland controls when analysed by Clump ($T_4<0.0005$). In particular the frequency of the DRβ1*01 alleles are significantly increased in the AS patients compared to the controls (**$p<0.0005$). The frequency of the DRβ1*04 alleles are also increased (*$p=0.038$)



Legend for Figure 27
The distribution of the DRb1* alleles in AS patients compared to the West of Scotland controls are described in Figure 27. Overall the distribution differed significantly between the two groups (T_4<0.0005)

DRβ1* Alleles	AS Patients [n=139] (%)	B27 Positive Controls [n=61] (%)
0101,02 +0103	75 (27)*	19 (16)*
02 [15+16]	28 (10)	7 (6)
03	26 (9)	19 (16)
04	86 (31)	37 (30)
05 [11+12]	9 (3)	9 (7)
06 [13+14]	24 (9)	12 (10)
07	25 (9)	11 (9)
08	2 (1)	1 (1)
09	3 (1)	3 (2)
10	0	4 (3)

Legend for Table 11
The distributions of the DRβ1* alleles in the 139 AS patients are compared to the 61 B27 positive controls in Table 11. Overall the distributions between these two groups are different when analysed by Clump (T_4=0.03). The frequency of the DRβ1*01 alleles are increased in the AS patients compared to the B27 positive controls (*p=0.013).



Legend for Figure 28
The distribution of the DRβ1*alleles in the AS patients compared to the B27 positive controls are described in Figure 28. Overall the distribution differs between the two groups (T_4=0.03).

DRβ1* Alleles	Age of Onset <21yrs n=64 (%)	Age of Onset >21yrs n=71 (%)
0101,02+0103	32 (25)	39 (27)
02[15+16]	10 (8)	18 (12)
03	12 (9)	15 (10)
04	45 (35)	39 (27)
05[11+12]	7 (5)	2 (2)
06[13+14]	10 (8)	12 (9)
07	11 (8)	13 (9)
08	0 (0)	2 (1)
09	1 (1)	2 (1)
10	0 (0)	0 (0)

Legend for Table 12

Table 12 describes the distributions of the DRβ1* alleles in the 64 AS patients with a disease onset of less than 21 years compared to the 71 with an onset of greater than or equal to 21 years. A slight increase in the number of patients with a younger onset carried the DRβ1*04 allele than those with a later onset (35% cf 27%) but this was not statistically significant.

DRβ1* Alleles	Uveitis Present n=52 (%)	Uveitis Absent n=82 (%)
0101,02+0103	30 (29)	39 (24)
02[15+16]	10 (9)	16 (10)
03	11 (10)	16 (10)
04	26 (25)	60 (36)
05[11+12]	6 (6)	3 (2)
06[13+14]	10 (10)	11 (7)
07	8 (8)	17 (10)
08	2 (2)	0 (0)
09	1 (1)	2 (1)
10	0 (0)	0 (0)

Legend for Table 13

The distribution of the DRβ1* alleles were compared in the 52 AS patients with uveitis with the 82 AS patients without, the results are described in Table 13. A slight non-significant decrease in the frequency of the DRβ1*04 alleles was seen in those patients with uveitis compared to those without.

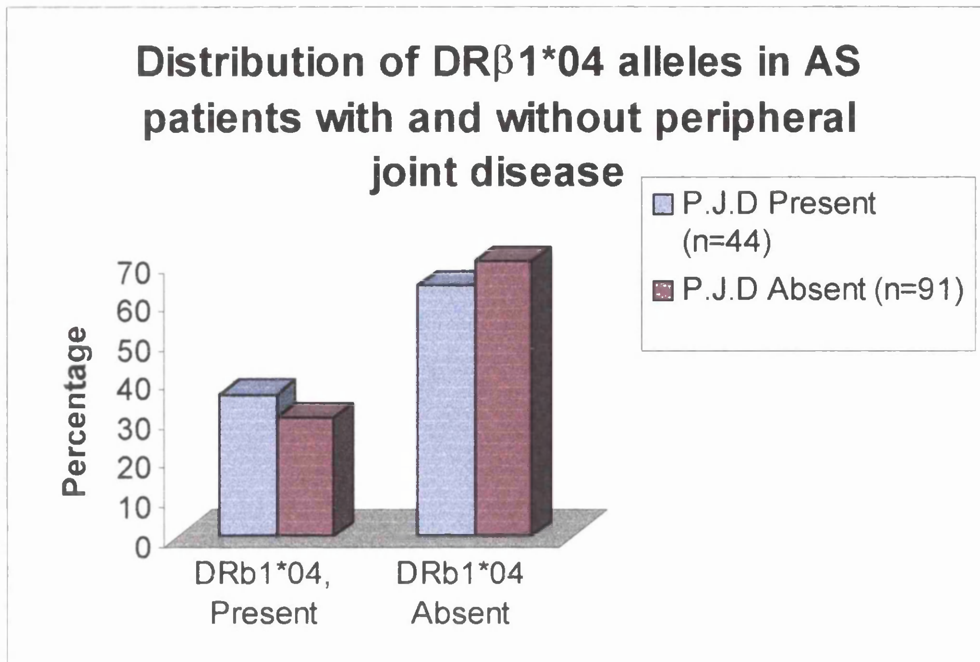
DRβ1* Alleles	Family History Present n=20 (%)	Family History Absent n=104 (%)
0101,02+0103	9 (23)	57 (28)
02[15+16]	5 (12)	21 (10)
03	1 (2)	21 (10)
04	15 (38)	59 (29)
05[11+12]	0 (0)	8 (3)
06[13+14]	7 (18)	15 (7)
07	2 (5)	23 (11)
08	0 (0)	2 (1)
09	1 (2)	2 (1)
10	0 (0)	0 (0)

Legend for Table 14
The distribution of the DRβ1* alleles in the 20 AS patients with a family history were compared to the 104 without and the results are shown in Table 14. In those patients with a family history the incidence of the DRβ1*04 and 06 alleles was increased with a decrease in the incidence of DRβ1*03 and 07. However this was not statistically significant.

DRβ1* Alleles	**P.J.D Present n=44 (%)**	**P.J.D Absent n=91 (%)**
0101,02+0103	25 (28)	49 (27)
02[15+16]	6 (7)	18 (10)
03	3 (3)	24 (13)
04	32 (36)	54 (30)
05[11+12]	4 (4)	4 (2)
06[13+14]	8 (9)	13 (7)
07	8 (9)	17 (9)
08	1 (1)	1 (1)
09	1 (1)	2 (1)
10	0 (0)	0 (0)

Legend for Table 15

The distribution of the DRβ1* alleles in the 44 AS patients with peripheral joint disease is compared to the 91 patients without in Table 15. There were no significant differences between the two groups.

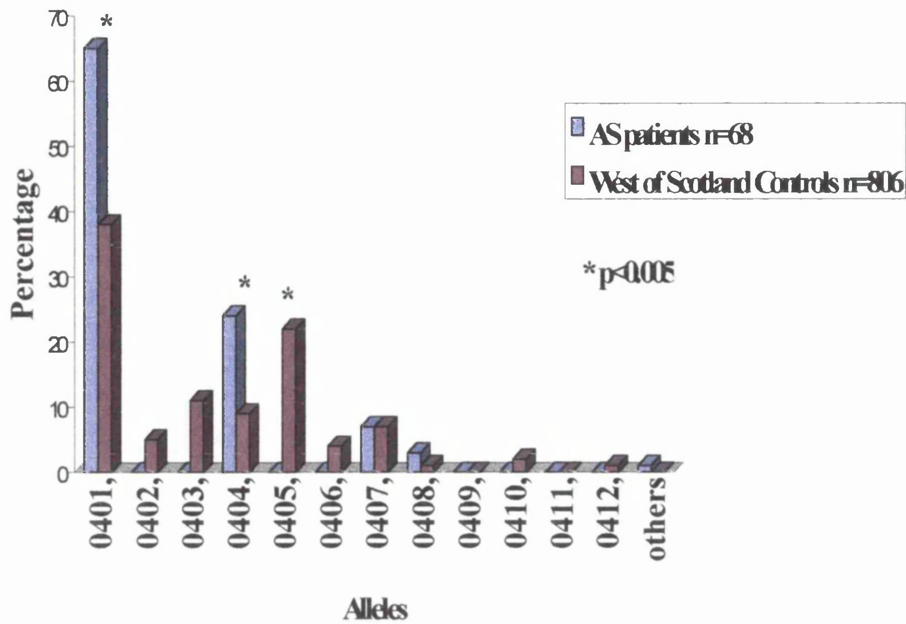


Legend for Figure 29
No significant differences were seen in the distribution of DRb1*04 alleles in AS patients with peripheral joint disease compared to those without.

DRβ1*04 Subtypes	AS patients n=68 (%)	West of Scotland Controls n=806 (%)	B27 Positive Controls n=24 (%)
0401	44 (65)*	306 (38)*	14 (58)
0402	0 (0)	41 (5)	2 (8)
0403	0 (0)	89 (11)	0 (0)
0404	16 (24)*	73 (9)*	3 (13)
0405	0 (0)*	177 (22)*	0 (0)
0406	0 (0)	32 (4)	0 (0)
0407	5 (7)	56 (7)	2 (8)
0408	2 (3)	8 (1)	1 (4)
0409	0 (0)	0 (0)	0 (0)
0410	0 (0)	16 (2)	0 (0)
0411	0 (0)	0 (0)	0 (0)
0412	0 (0)	8 (1)	0 (0)
others	1 (1)	0 (0)	2 (8)

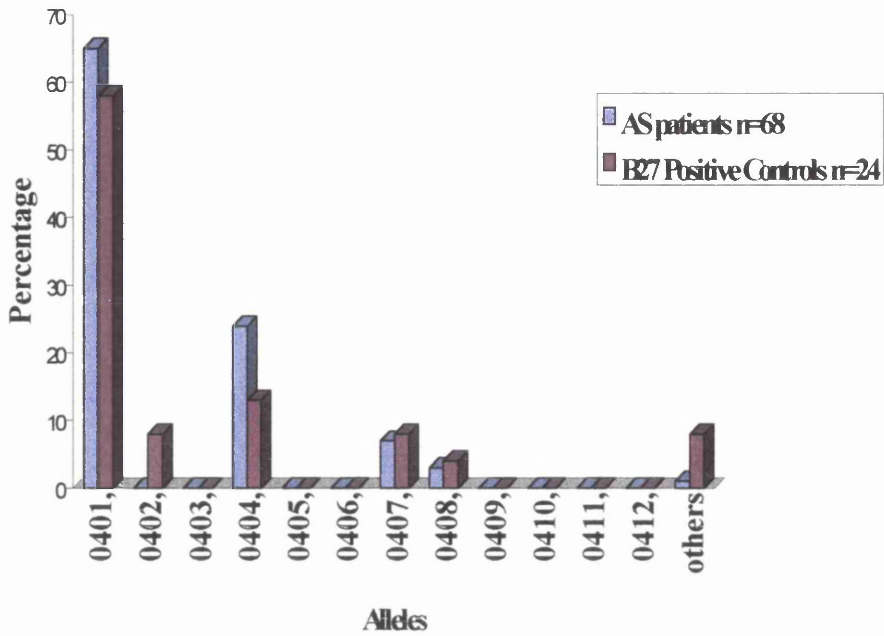
Legend for Table 16

The results of the distribution of the DRβ1*04 subtypes in the AS patients compared to the West of Scotland controls and the B27 positive controls are given in Table 16. The overall distribution of the subtypes in the AS patients is significantly different to the West of Scotland controls ($T_4 < 0.0005$). This is due to the significant increase in the DRβ1*0401 and 0404 alleles in the AS patients ($p<0.0005$) compared to the controls. This is balanced by the significant decrease of the DRβ1*0405 alleles in the AS patients ($p<0.005$). No significant differences were seen in the AS patients compared to the B27 positive controls although the distribution of the DRβ1*0404 was increased in the patients but failed to reach statistical significance.



Legend for Figure 30

The distribution of the DRβ1*04 subtypes in the AS patients compared to the West of scotland controls are described in Figure 30. In the AS patients the frequency of the DRβ1*0401 and 0404 are significantly increased ($p<0.005$), with a significant decrease in the frequency of the DRβ1*0405.



Legend for Figure 31

Figure 31 demonstrates the distribution of the DRβ1*04 subtypes in the AS patients compared to the B27 positive controls. No significant differences between the two groups were seen.

3.6 MHC Class II: DRβ1* Alleles Discussion

Several studies have investigated the role of other MHC genes in AS since it is well established that B27 is not the only gene involved. Some of these studies have looked at genes involved in the antigen-processing pathway such as the TAP genes (Maksymowych, 1995) or genes involved in the inflammatory response such as the TNF genes. Other groups have suggested that the MHC Class II genes may also have a role to play.

The MHC Class II region contains 17 HLA Class II genes and pseudogenes but of greatest interest is the HLA DRβ genes which encode the β1 chain of the DR molecule since this is the most polymorphic region with approximately 180 alleles described (Beck, 1999). There are five distinct human HLA-DR haplotypes that differ in their genomic organization and number of HLA DRβ Class II genes (Anderson G. Evolution of Human HLA DR region). The recent genome wide scan has confirmed the strong linkage disequilibrium with the MHC region and AS. This linkage has been shown to extend at least 8cM across the region between markers D6S276 and DRβ1 (Brown, 1998). It has been proposed that this may simply be due to linkage disequilibrium with HLA B27 or may be due to extended haplotypes that may contain several genes relevant to susceptibility to AS (Martinez-Borra, 2000). However the broad linkage reported by Brown is due to the weakness of linkage as a method of fine mapping because of the limited numbers of recombination events in families over short distances, not due to linkage disequilibium which refers to population effects.

In this study the distribution of the HLA DRβ1 alleles was investigated in 139 AS patients and compared to the control populations. Overall the distribution of the DRβ1 alleles was significantly different in the AS patients compared to the West of Scotland controls as determined by the Clump test. This appeared to be due to the significant increase in the distribution of the DRβ1*01 alleles in the AS patients as compared to the controls ($p<0.0005$), along with a significant increase in the DRβ1*04 alleles ($p=0.012$). A corresponding decrease in the DRβ1*03 alleles was seen but this was not statistically significant. Similarly when the AS patients were compared to the B27 positive controls the overall distribution was significantly different as determined by the Clump test ($T4=0.03$). However although the DRβ1*01 alleles were still significantly increased in the AS patients ($p=0.013$) the DRβ1*04 distribution was the same between the two groups. It could be that the DRβ1*04 alleles are in linkage disequilibrium with the B27 gene but family studies would be required to confirm this. Linkage disequilibrium between B27 and DRβ1*04 has been demonstrated in Scandinavian countries (Westman, 1996) and in British Caucasians (Brown, 1998). Again the distribution of the DRβ1*03 alleles is reduced in the AS patients compared to the controls although this is not statistically different.

Not many studies have looked at the distribution of the HLA DRβ1 alleles in AS but one important study by Brown and collegues has also shown a significant increase in the distribution of the DRβ1*01 alleles (Brown, 1998). They suggest that at most the effect of the DRβ1*01 alleles are minor on disease susceptibility. Dizygotic twins carrying both B27 and DRβ1*01 are said to be more concordant for spondylarthritis than those without

DRβ1*01(Brown, 1997). Haplotypes containing DR1;DQ5 have been shown to be transmitted preferentially within families to children with AS (Nahal, 1996). An early British study has also suggested an association with DRβ1 alleles and AS susceptibility but this study did not investigate B27 positive controls (Armstrong, 1983).

It has been demonstrated previously that TNFα production varies between individuals according to their HLA DR alleles (Jacob, 1990). They can be divided into two groups, high producers that carry DRβ1*03 and 04 alleles and low producers that carry DRβ1*02 [15+16] alleles. In this study the incidence of DRβ1*01 alleles is increased in the AS patients but there is also a decrease in the high producer phenotype of the DRβ1*03 alleles. This significant increase would appear to be disease associated since the increase is seen in comparison to both the normal and the B27 positive population.

So DRβ1 appears to have an effect on disease susceptibility in AS patients but does it have an effect on disease phenotype. It has been suggested in polygenic disorders that genes may determine clinical phenotype without conferring overall disease susceptibility. Previous studies have suggested particular DRβ1* alleles may have increased risk with particular clinical manifestations. Studies in Japanese patients have reported associations of DRβ1*08 with increased risk of acute anterior uveitis [AAU], (Islam, 1995). This has also been confirmed in another study of Norwegian AS patients but was not replicated in a study of Mexican patients (Maksymowych, 1997) and in a UK population (Brown, 1998). Associations with DRβ1*0103 have also been described for AS patients with the complication of inflammatory bowel disease (Orchard, 2000). Both DRβ1*04 and more commonly DRβ1*07 have previously been associated with peripheral arthritis (Sanmarti,

1987). Although, other groups have not confirmed the link with DRβ1*04 and peripheral arthritis.

In the present study the distribution of the DRβ1* alleles was investigated in the AS patients in relation to different clinical features. No significant differences were seen at all in any of the features studied. In those patients with an early onset of disease compared to those with a later onset only a slight increase in the 04 alleles was seen although previously an association with DRβ1*08 and an early onset has been suggested (Ploski, 1996). Interestingly a slight decrease in the DRβ1*04 alleles was seen in the AS patients with uveitis compared to those without but no associations with DRβ1*08 were seen. However this is not surprising as the incidence of DRβ1*08 in the West of Scotland overall is very low. Of the only two patients in the study that had DRβ1*08 alleles however both did have uveitis. Of the patients that had a family history the DRβ1*04 and 06 alleles were slightly increased with a corresponding decrease of the DRβ1*03 and 07 alleles but again these were not statistically significant. No differences were seen in the patients with or without a peripheral joint disease. It has been said that DR12, which is a split of DRβ1*05 may have a protective role in AS. The distribution of DRβ1*05 was very similar between the AS patients and the West of Scotland controls.

The overall conclusion therefore is that DRβ1*01 may be an additional susceptibility gene but that it appears to not play a role in determining disease phenotype.

CHAPTER FOUR:

TUMOUR NECROSIS FACTOR POLYMORPHISMS

4. Polymorphisms within the Tumour Necrosis Factor region

AS is understood to be a polygenic disease and one candidate gene that has been proposed as having a role to play is that of the gene for the proinflammatory cytokine Tumour necrosis factor (TNF). TNF is an important proinflammatory cytokine and as has been discussed in the introduction this cytokine may have a role to play in the disease pathogenesis of AS. It has been suggested that particular polymorphisms within the TNF region may predispose individuals to higher levels of TNF protein leading to joint destruction. Certain TNF polymorphic sites previously associated with high or low levels of the actual proteins have been associated with other inflammatory conditions. This raised the question was the distribution of any of these polymorphisms associated with increased production different in the AS patients when compared to controls.

Six individual polymorphic sites spanning the TNF region from the Class I region through towards the Class II region were investigated. The actual sites examined are highlighted in figure 8. The six polymorphisms were investigated in a large population of well characterised AS patients and two control populations as discussed in the materials and methods section.

4.1 Nco.1 Polymorphism results

The first site to be examined was the Nco.1 RFLP, which lies within the first intron of the lymphotoxin alpha gene. This single base substitution gives rise to two possible alleles Nco.1.1 and Nco.1.2 and therefore three possible genotypes. The Nco.1.2 allele has previously been shown to be associated with increased levels of TNFα *in vitro*.

148 AS patients were examined at the Nco.1 site and the results are shown in Table 17. Comparison of the alleles and genotypes of the 148 AS patients with the 82 HLA B27 negative controls are shown in Figure 32a and b. This comparison revealed a significant increase (p=0.0014) in the Nco.1 2*2 genotype in the HLA B27 negative controls compared to the AS patients (42% in the controls compared to 22% in the patients). This was also confirmed in the distribution of the alleles with a significant increase (p=0.0007) of the Nco.1.2 allele in the HLA B27 negative controls compared to the AS patients (64% compared with 48%) as seen in Figure 32b. The Nco.1.2 allele previously associated with increased production of TNFα *in vitro* is significantly decreased in this group of AS patients compared to the normal control population.

However when the distribution of the alleles and genotypes in the 148 AS patients were compared to 54 HLA B27 positive controls no significant differences were found (see Table 18). The distribution of the Nco.1 2*2 genotype was 22% in the AS patients compared to 24% of the B27 positive controls and the Nco.1.2 allele was found in 48% of the patients compared to 51% of the HLA B27 positive controls. This suggests that the differences seen in the Nco.1 distribution between AS patients and HLA B27 negative controls could therefore be related to the presence of HLA B27 itself and not to the disease.

During the clinical examination of the AS patients various other clinical findings were recorded such as whether the patient had a history of the disease in the family, the age of onset of the disease, the presence of extra-spinal manifestations such as uveitis, peripheral joint disease etc. All the clinical findings are discussed in chapter 3.1. Previous work in other inflammatory conditions has suggested that the presence of certain TNF alleles is associated with disease severity and not just the

presence of disease. The distribution of the Nco.1 alleles and genotypes were examined in the AS patients along with the presence of extra features of disease which may be regarded as indicators of disease severity. The results are summarised in Table 19.

Information regarding the presence of uveitis was recorded in a total of 145 out of the 148 AS patients who had been investigated at the Nco.1 site. The distribution of the Nco.1 alleles and genotypes were examined in 56 AS patients who had a previous history of uveitis and compared to 89 AS patients who had never had an episode of uveitis. There were no significant differences between the two groups of patients.

The presence or absence of a family history was recorded in 131 of the 148 AS patients who had been investigated at the Nco.1 site. There were no significant differences between the 26 AS patients who had a history of disease in the family and the 105 AS patients were no family history had been noted.

Another indicator of a more severe disease is an early onset of disease. In this case an onset of disease before the age of 21 was regarded as early and was compared to those patients who had an onset equal to or above the age of 21. Information regarding the onset of disease was recorded in 144 of the AS patients. The results of the 69 AS patients with an onset below the age of 21 compared to the 75 AS patients with an onset of 21 years or above are shown in table 19. There was a slight increase in the distribution of the Nco.1 2*2 genotype in the AS patients with an early onset compared to those with a later onset (29% compared to 16%) but this was not statistically significant.

At some point throughout the course of their disease approximately 30-40% of AS patients will suffer from peripheral joint disease. Joints other than the axial spine

but not including hips and shoulders may become inflamed. In this study 33% of the AS patients have recorded a history of peripheral joint disease (P.J.D). Out of the 148 AS patients examined at the Nco.1 site 143 have a record of the presence or absence of P.J.D. The results of the distribution of the Nco.1 alleles and genotypes in the 48 AS patients with a history of P.J.D compared to the 95 AS patients with no history of P.J.D are shown in table 19. No significant differences where seen in the two groups of patients.

Overall the differences seen at the Nco.1 site appear to be associated with the presence of HLA B27 and not with the disease itself.

Nco.1	AS Patients n=148 (%)	B27 -ve Controls n=82 (%)
1*1	39 (26)	11 (13)
1*2	77 (52)	37 (45)
2*2	32 (22)*	34 (42)*
1*	155 (52)*	59 (36)*
2*	141 (48)	105 (64)

Legend for Table 17
The frequency of the distribution of the Nco.1 genotypes and alleles in the AS patients compared to the B27 negative control group are shown in Table 17. The frequency of the Nco.1 2*2 genotype was significantly decreased (*p=0.0014) in the AS patients compared to the B27 negative controls (22% versus 42%). This was reflected in a significant increase in the frequency of the Nco.1.1* allele in the AS patients (*p=0.007, 52% compared to 36%) compared to the B27 negative controls

Nco.1	AS Patients n=148 (%)	B27 +ve Controls n=54 (%)
1*1	39 (26)	12 (22)
1*2	77 (52)	29 (54)
2*2	32 (22)	13 (24)
1*	155 (52)	53 (49)
2*	141 (48)	55 (51)

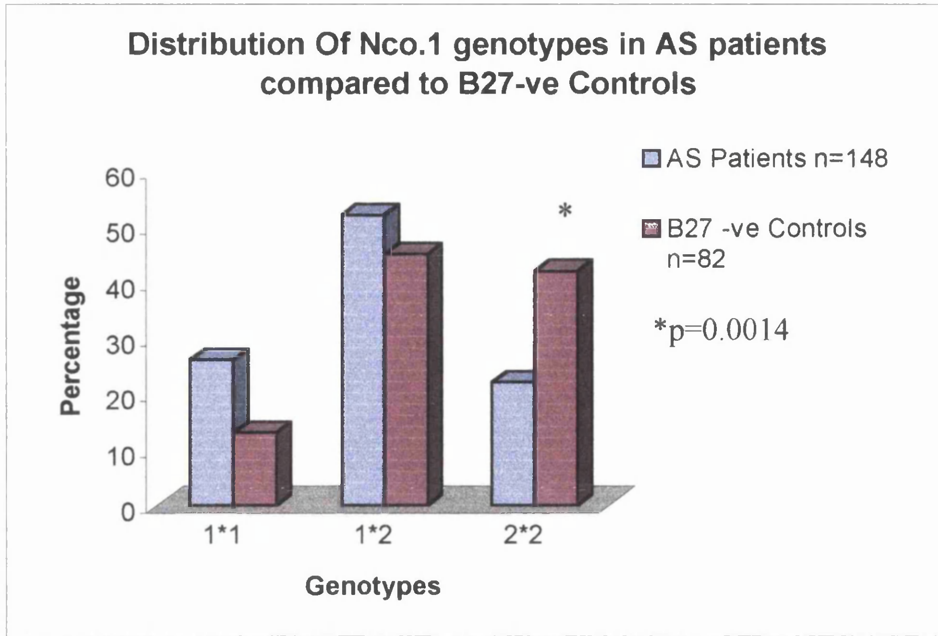
Legend for Table 18
The results of the distribution of the Nco.1 genotypes and alleles in the AS patients compared to the HLA B27 +ve controls are depicted in table 18. There were no significant differences between the two groups.

Nco.1 Genotypes and alleles

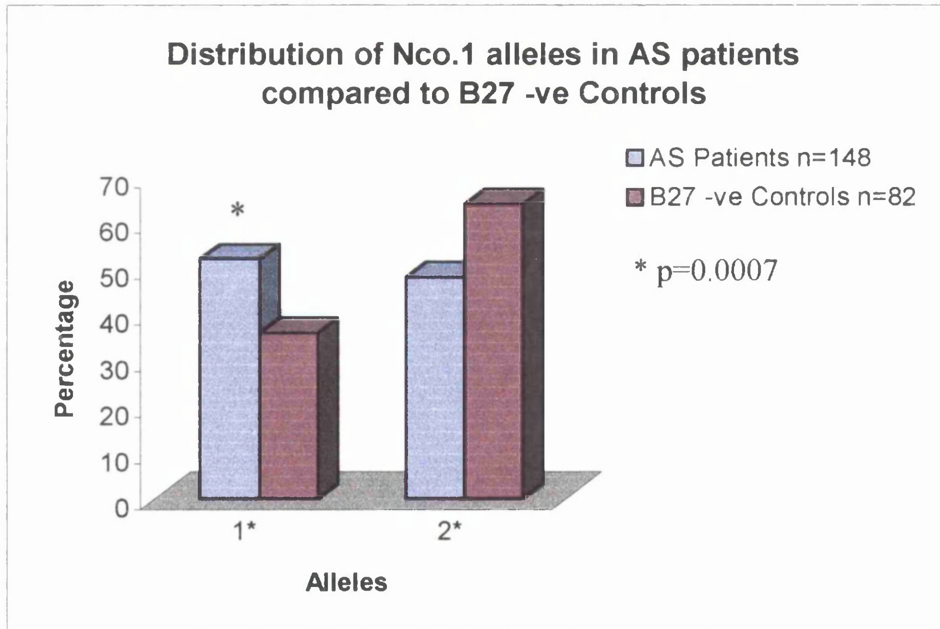
Extra Features of Disease	1*1	1*2	2*2	1*	2*
Age of Onset<21 n=69 (%)	17 (25)	32 (46)	20 (29)	66 (48)	72 (52)
Age of Onset>21 n=75 (%)	20 (27)	43 (57)	12 (16)	83 (55)	67 (45)
Uveitis Present n=56 (%)	15 (26)	30 (54)	11 (20)	60 (54)	52 (46)
Uveitis Absent n=89 (%)	24 (27)	45 (51)	20 (22)	93 (52)	85 (48)
Family History Present n=26 (%)	8 (31)	16 (62)	2 (7)	32 (62)	20 (38)
Family History Absent n=105 (%)	25 (24)	52 (49)	28 (27)	102 (49)	108 (51)
Peripheral joint disease Present n=48 (%)	16 (33)	23 (48)	9 (19)	55 (57)	41 (43)
Peripheral joint disease Absent n=95 (%)	22 (23)	52 (55)	21 (22)	96 (51)	94 (49)

Legend for Table 19

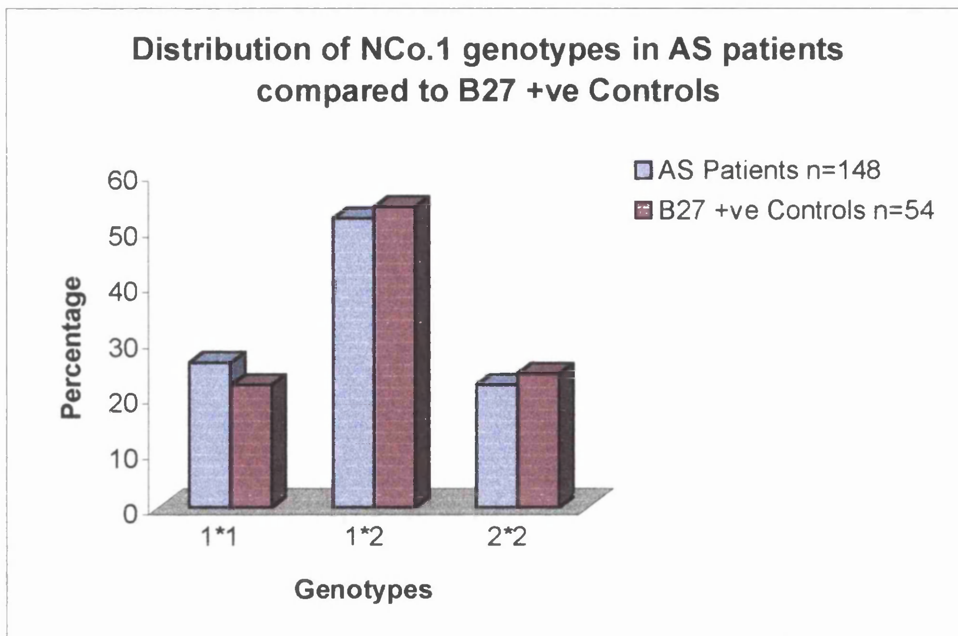
A comparison of the distribution of the Nco.1 alleles and genotypes in the AS patients with and without extra disease features is shown in Table 19. In the 69 AS patients with an age of onset below the age of 21 years a slight increase in the 2*2 genotype was seen compared to those with an onset greater than 21 years (29% versus 16%), although this was not statistically significant. The Nco.1 1*allele was increased in the 26 AS patients with a family history compared to those without (62% versus 49%) but this failed to reach statistical significance. No significant differences where seen in those patients with or without uveitis or those with or without peripheral joint disease.



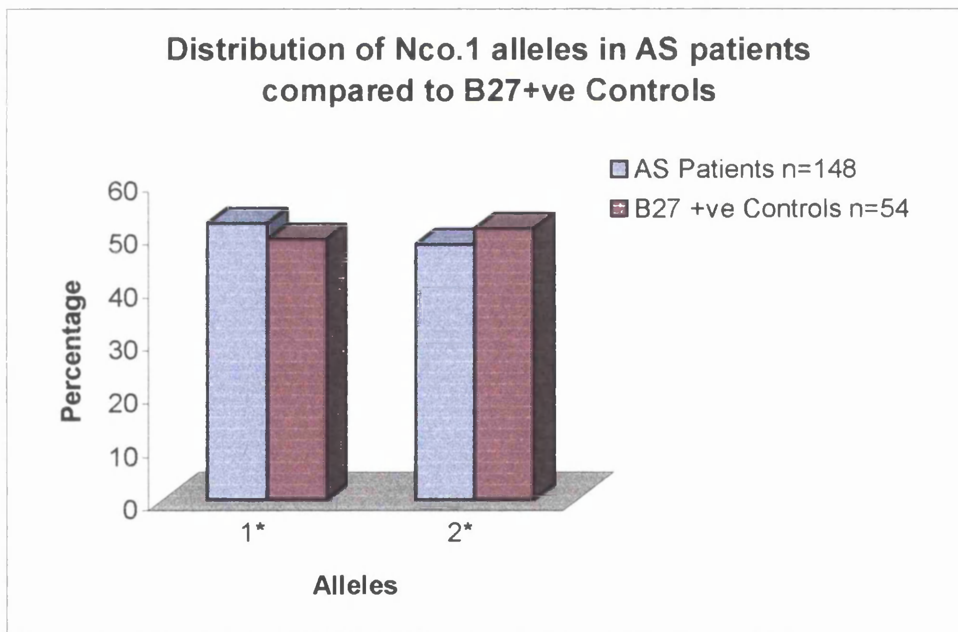
Legend for Figure 32a
The distributions of the Nco.1 genotypes in 148 AS patients compared to 82 B27 negative controls are given in Figure 32a. The 2*2 genotype was significantly decreased in the AS patients compared to controls (p=0.0014).



Legend for Figure 32b
The distributions of the Nco.1 alleles in the AS patients compared to the controls are described in figure 32b. The frequency of the 1* allele was significantly increased in the AS patients compared to the controls (p=0.0007).



Legend for Figure 33a
The distributions of the Nco.1 genotypes in the AS patients compared to the 54 B27 positive controls are described in figure 33a. There were no significant differences between the two groups.



Legend for Figure 33b
The distributions of the Nco.1 alleles in AS patients compared to the B27 positive controls are described in figure 33b. There were no significant differences.

4.2 –308 Polymorphism Results

The second polymorphism to be investigated was the –308 RFLP, which lies within the promoter region of the TNFα gene. This single base substitution gives rise to two alleles. The –308.2 allele has previously been suggested to be involved with increased risk of developing cerebral malaria and mucocutaneous Leishmaniasis. A decreased frequency of this allele has also been linked to patients with ulcerative colitis. There have also been some reports that allele 2 may be able to induce increased transcription of TNF. This link with other diseases and the ability to affect transcription of the protein suggest that this polymorphic site may be involved in the pathogenesis of AS.

A total of 203 AS patients who are all HLA B27 positive were examined at this site and compared to 91 normal controls who were known to be HLA B27 negative. The distribution of the two alleles and the three genotypes in these two groups are shown in Table 20 and Figure 34a and b. It can be seen that there is a significant increase in the frequency of the -308.1 allele in the AS patients compared to the B27 negative controls (p=0.0028), OR 2.0, 95%CI 1.3-3.1, which is reflected in the decreased frequency of the -308.2 allele. The distribution of the genotypes also differ with a significant increase (p=0.011), OR 2.0, 95%CI 1.2-3.4 in the 1*1 genotype in the AS patients compared to the B27 negative controls (78% versus 64%).

When the 203 AS patients where compared to the second control group of 81 HLA B27 positive individuals (shown in Table 21 and Figure 35a and b) the -308. 1*1 genotype was significantly increased (p=0.0015), OR 2.4, 95%CI 1.4-4.0, [78% versus 59%] with an expected concomitant decreased in the -308. 1*2 genotype [22% versus 41%]. The rarer -308.2*2 genotype was not seen in either the AS

patients nor the B27 positive controls but was seen in 4% of the B27 negative controls. The allele distribution showed a significant increase in the -308.1 allele in the AS patients compared to the B27 positive controls (p=0.0037), OR 2.1, 95%CI 1.3-3.4, with the expected decrease in the frequency of the -308.2 allele.

The results for the distribution of the –308 alleles and genotypes in the AS patients with and without extra features of disease are summarised in Table 22. Of the 203 AS patients examined at the -308 site information regarding their age of onset was available in 199 of which 100 had an age of onset below the age of 21 and 99 had an age of onset equal to or greater than 21. The distribution of the -308 alleles and genotypes did not significantly differ between the two groups. A slight increase in the -308.1 allele was seen in the 35 AS patients with a history of disease in the family compared to the 141 AS patient where there was no family history (86% compared to 76%). This was not statistically significant.

The distribution of the -308 alleles and genotypes in the 199 AS patients where information was available regarding the presence of peripheral joint disease are shown in Table 22. Out of the 199 patients 66 had a history of peripheral joint disease compared to 133 with no history. There were no statistically differences between the two groups of patients.

Information was recorded in 196 AS patients with respect to the presence of uveitis and the results are shown in Table 22. The distribution of the alleles and genotypes did not differ between the 74 AS patients with uveitis compared to the 122 without.

The results clearly show an association with AS and the presence of the -308.1 allele. This difference appears to be disease associated and not with the presence of HLA B27 as it was confirmed in the HLA B27 positive population. However this allele has not previously been associated with increased production of TNF. In fact

the -308. 2 allele that has been associated with increased production was significantly decreased with the -308. 2*2 genotype being completely absent. No apparent associations were seen between the presence of the -308 alleles and any of the extra disease features.

-308	AS patients n=203 (%)	B27 Negative Controls n=91 (%)
1*1	158 (78)*	58 (64)*
1*2	45 (22)	29 (32)
2*2	0 (0)	4 (4)
1*	361 (89)**	145 (80)**
2*	45 (11)	37 (20)

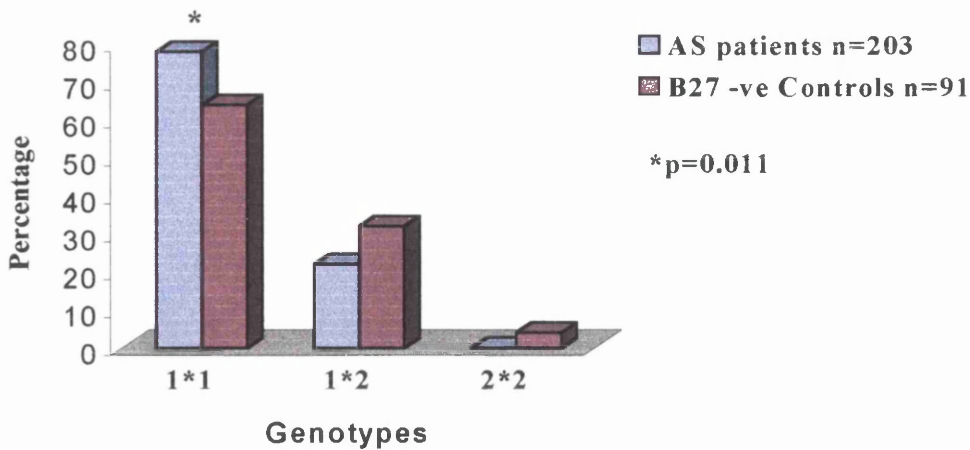
Legend for Table 20
The results of the distribution of the -308 alleles and genotypes in 203 AS patients compared to 91 B27 negative controls are shown in Table 18. A significant increase (*p=0.011) in the frequency of the 1*1 genotype was seen in the AS patients (78% versus 64%) compared to the controls. The frequency of the 1* allele was significant increased (**p=0.0028) in the AS patients compared to the B27 negative controls (89% versus 80%).

-308	AS patients n=203 (%)	B27 Positive Controls n=81 (%)
1*1	158 (78)*	48 (59)*
1*2	45 (22)*	33 (41)*
2*2	0 (0)	0 (0)
1*	361 (89)*	129 (79)*
2*	45 (11)*	33 (21)*

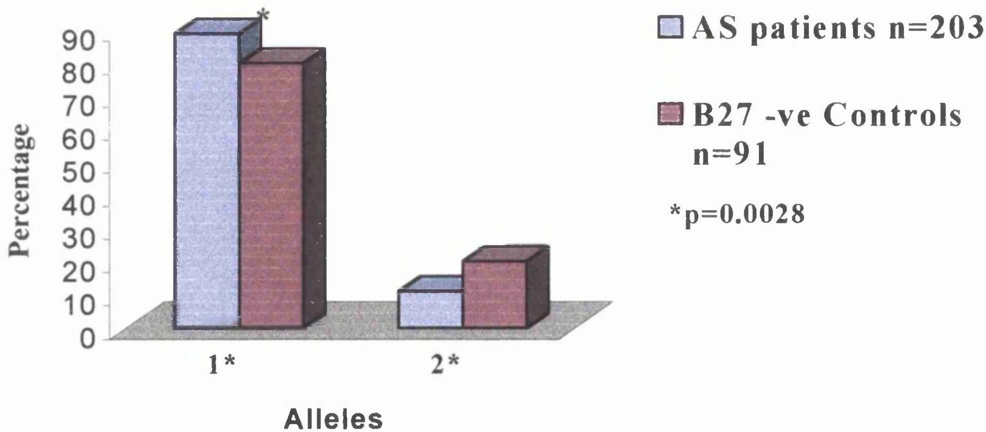
Legend for Table 21
The distribution of the -308 genotypes and alleles in 203 AS patients compared to 81 B27 positive controls is given in Table 19. The 1*1 genotype was significantly increased (*p=0.0015) in the AS patients compared to the controls. Correspondingly the 1*2 genotype was significantly decreased in the patients. The -308 1* allele was significantly increased (*p=0.0037) in the patients compared to the B27 positive controls (89% versus 79%).

-308 Genotypes and Alleles					
Extra Features of Disease	**1*1**	**1*2**	**2*2**	**1***	**2***
Age of Onset <21n=100 (%)	75 (75)	25 (25)	0 (0)	175 (88)	25 (12)
Age of Onset >21 n=99 (%)	80 (81)	19 (19)	0 (0)	179 (90)	19 (10)
Uveitis Present n=74 (%)	58 (78)	16 (22)	0 (0)	132 (89)	16 (11)
Uveitis Absent n=122 (%)	94 (77)	28 (23)	0 (0)	216 (88)	28 (12)
Family History Present n=35 (%)	30 (86)	5 (14)	0 (0)	65 (93)	5 (7)
Family History Absent n=141 (%)	107 (76)	34 (24)	0 (0)	248 (88)	34 (12)
Peripheral Joint Disease Present n=66 (%)	53 (80)	13 (20)	0 (0)	119 (90)	13 (10)
Peripheral Joint Disease Absent n=133 (%)	101 (76)	32 (24)	0 (0)	234 (88)	32 (12)

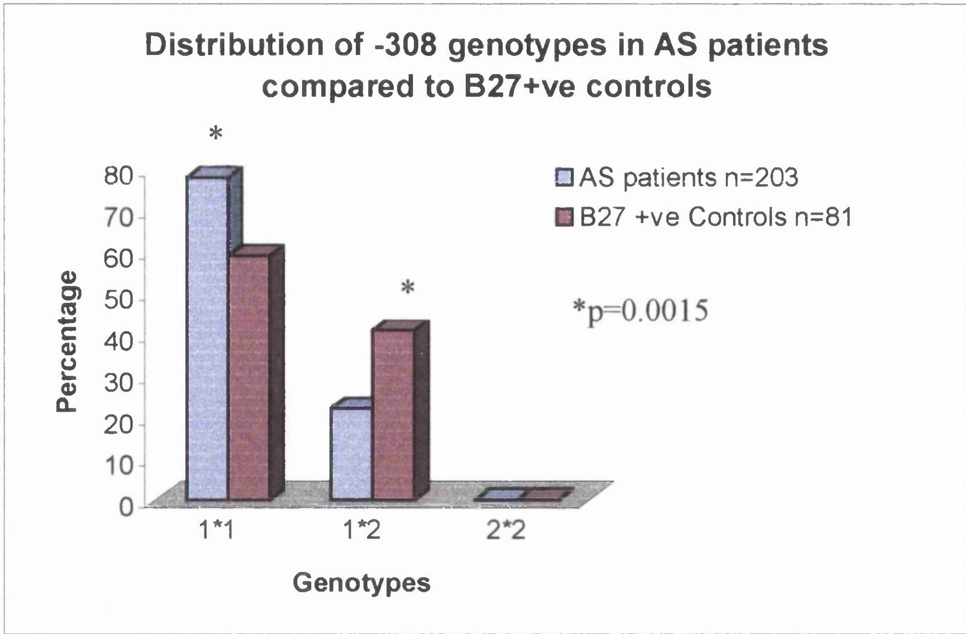
Legend for Table 22
The distribution of the –308 alleles and genotypes in the AS patients with and without extra features of disease are shown in Table 22. No significant differences were seen in the patients with or without any of the other features examined.



Legend for Figure 34a
The distributions of the –308 genotypes in the AS patients compared to the B27 negative controls are shown in Figure 34a. A significant increase (p=0.0011) in the frequency of the 1*1 genotype was seen in the AS patients compared to the controls.

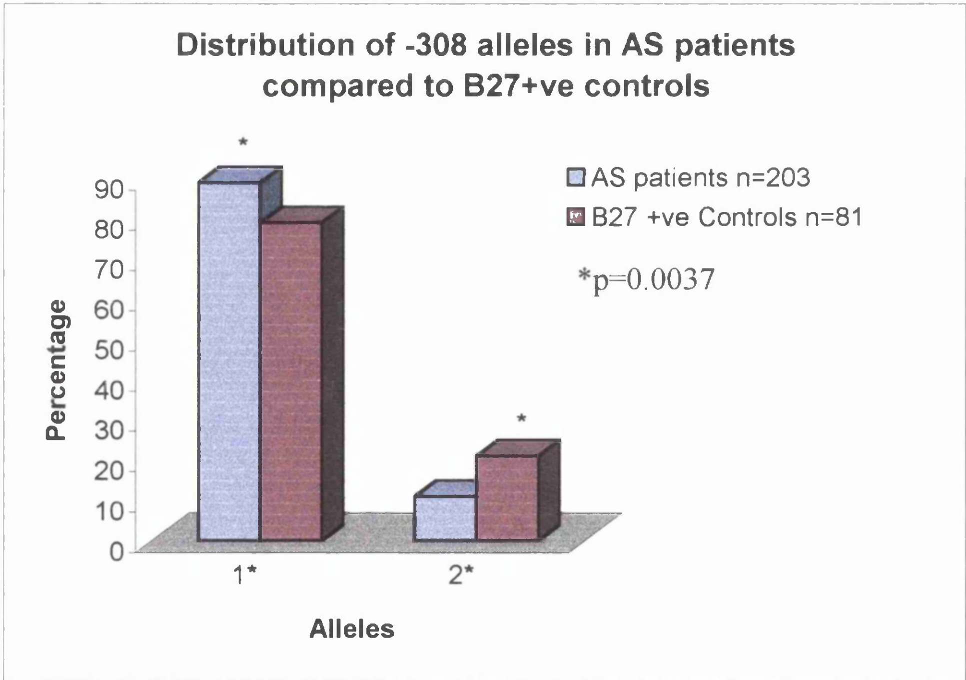


Legend for Figure 34b
The distributions of the –308 alleles in the AS patients compared to the B27 negative controls are shown in Figure 34b. The frequency of the –308*1 allele was significantly increased (p=0.0028) in the AS patients compared to the controls.



Legend for Figure 35a

The distributions of the –308 genotypes in the AS patients compared to the B27 positive controls are described in figure 35a. The frequency of the 1*1 genotype was significantly increased in the AS patients compared to the controls (p=0.0015)



Legend for Figure 35b

The distributions of the –308 alleles in the AS patients compared to the B27 positive controls are shown in figure 35b. The frequency of the 1* allele was significantly increased in the AS patients compared to the controls (p=0.0037).

4.3 LST.1 Polymorphism

The LST.1 gene located 4 kilobases upstream of the lymphotoxin b gene contains a polymorphic Pvu II restriction site 260 base pairs downstream of the polyadenylation signal. The LST.1 gene codes for an IFN gamma inducible 800 nt transcript which has been shown to be present on macrophages and T cells. The position of this polymorphic site and its pattern of expression have implied it may have a role in autoimmune diseases.

A total of 142 AS patients were investigated at this site and the results compared to 48 normal controls as can be seen in Table 23. The majority of individuals studied carried the wild type allele. No significant differences were seen in either the distribution of the alleles or genotypes. Similarly in the AS patients compared to 52 B27 positive controls (results in Table 24) no significant differences were seen.

The distribution pattern was examined in the AS patients with regard to the presence or absence of extra features of disease, there were no significant differences (data not shown).

In conclusion there appears to be no significant associations with this polymorphic site and AS.

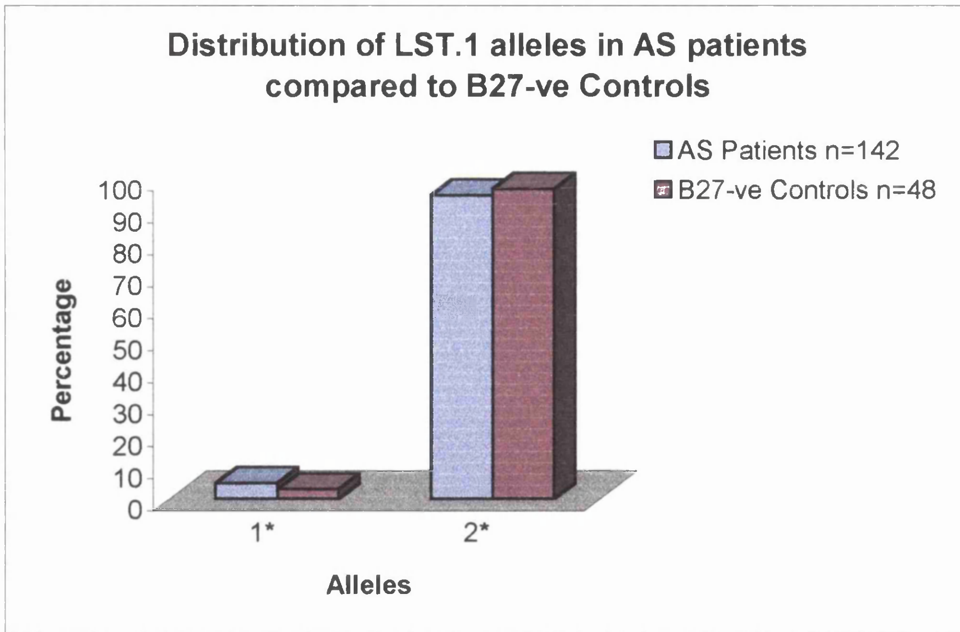
LST.1	AS Patients n=142 (%)	B27 –ve Controls n=48 (%)
1*1	1 (1)	0 (0)
1*2	13 (9)	3 (6)
2*2	128 (90)	45 (94)
1*	15 (5)	3 (3)
2*	269 (95)	93 (97)

Legend for Table 23

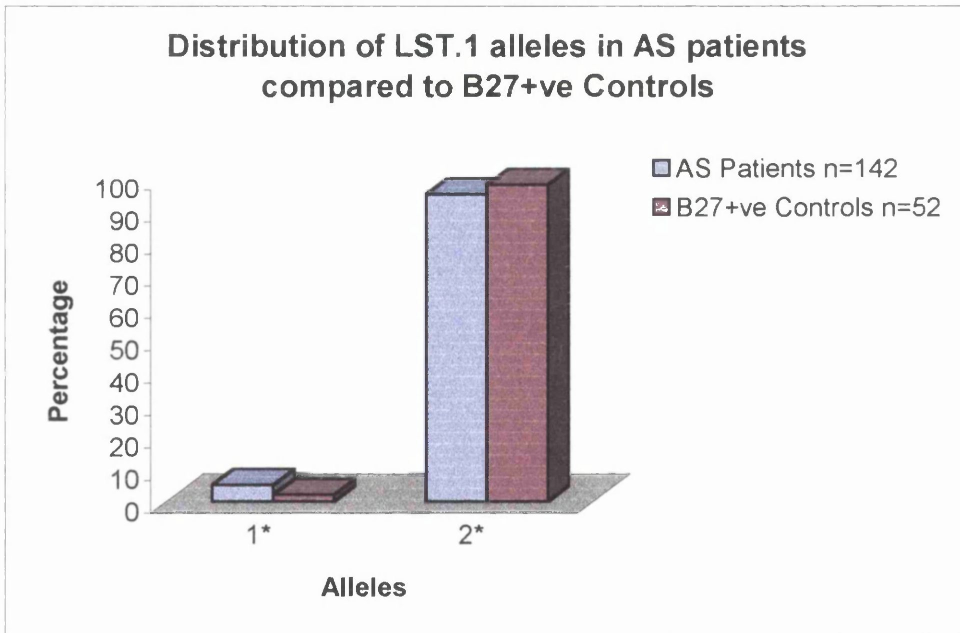
The distribution of the LST.1 alleles and genotypes in the 142 AS patients compared to 48 B27-ve controls are shown in Table 23. There were no significant differences between the two groups.

LST.1	AS Patients n=142 (%)	B27+ve Controls n=52 (%)
1*1	1 (1)	0 (0)
1*2	13 (9)	2 (4)
2*2	128 (90)	50 (96)
1*	14 (5)	2 (2)
2*	269 (95)	102 (98)

Legend for Table 24
The distribution of the LST.1 genotypes and alleles in the AS patients compared to the B27 positive controls are shown in Table 24. There were no significant differences between the two groups.



Legend for Figure 36a
The distributions of the LST.1 alleles in the AS patients compared to the B27-ve controls are shown in figure 36a. There were no significant differences between the two groups.



Legend for Figure 36b
The distributions of the LST.1 alleles in the AS patients compared to the B27+ve controls are shown in figure 36b. There were no significant differences between the two groups.

4.4 Tumour Necrosis Factor Polymorphisms Discussion

The position of the TNF genes in the MHC class III region and their role in the inflammatory process has suggested they may have a role to play in MHC associated diseases such as AS. As mentioned previously there are numerous polymorphic sites in the TNF locus some of which have been associated with alterations in the actual levels of protein produced. High levels of mRNA for TNFα have been detected in biopsies from the sacroiliac joints of AS patients (Braun, 1995). In transgenic mice the dysregulation of TNFα expression has been shown to induce arthritis (Kontoyiannis, 1999). Serum levels for TNFα in AS patients have been shown to be increased (Gratacos, 1994). This study was therefore carried out to investigate a number of these polymorphic sites in the AS patients in comparison to the controls in order to determine if there was an increased frequency of any of the alleles associated with increased production.

A total of six polymorphic sites were investigated, three biallelic RFLP's and three microsatellites. These polymorphic sites were chosen as they span the TNF locus from close to the B27 locus towards the DR locus. Each site was investigated in the AS patients compared to the two control populations.

4.5 Nco.1 Discussion

The first site to be investigated was the Nco.1 biallelic RFLP, which lies within the first intron of the LTα gene and gives rise to two alleles. Previous work has associated the Nco.1*2 allele with increased levels of TNFα in some populations (Abraham, 1993). In this study the distribution of the Nco.1 alleles were significantly different in the AS patients compared to the normal control group who

were all healthy unrelated B27 negative individuals. The Nco.1*1 allele was significantly increased in the AS patients (p=0.0007). This appeared to be due to a significant decrease in individuals homozygous for the Nco.1*2 allele (p=0.0014). In a similar study the distribution of the Nco.1 alleles have also been shown to be significantly different in a group of AS patients when compared to the normal population (Fraile, 1998). A significant decrease in the frequency of the 2*2 genotype was also seen in the AS patients with a similar distribution as was seen in this study. However this is in contrast to another study of AS patients where no differences where seen (Verjans, 1991).

The Nco.1*1 allele has previously been shown to be associated with increased production of LTα and it would appear that this allele is significantly increased in the AS patients compared to the normal population. However it could be that this difference in distribution is due to linkage disequilibrium with the B27 gene since this significant difference disappeared when the AS patients where compared to the B27 positive control population. The frequency of each of the genotypes and alleles were very similar between these two groups. This is comparable to the Spanish study where they also saw no difference between the AS patients and B27 positive controls. They go on to suggest that the Nco.1*1 allele is in strong linkage disequilibrium with B27. It has been demonstrated that B27 positive cells have increased production of TNFα compared to B27 negative cells (Repo, 1988). This could be due to the increased frequency of the Nco.1*1 allele, associated with increased production, in B27 positive individuals.

The role of TNFα in other autoimmune diseases such as Rheumatoid arthritis is much more established and several studies have suggested that particular TNF alleles may not only be disease associated but may also be a marker for disease

severity. In order to investigate this in relation to disease severity in the AS patients several features were examined. There were no significant differences found in any of the extra disease features investigated. Although in other studies the Nco.1*1 allele has been shown to be associated with an early onset of disease in patients with myasthenia gravis (Hjelmstrom, 1998).

In conclusion at this site a significant increase of the Nco.1*1 allele was seen in the AS patients compared to the normal population but that this difference disappeared when the patients where compared to the appropriate control population. This would predict that the Nco.1 polymorphic site does not play a primary role in the disease pathogenesis of AS. But that it points to possible haplotypes that may exist across the MHC.

4.6 –308 Discussion

The –308 polymorphic site within the TNF promoter region has been the subject of much interest in a variety of autoimmune diseases including SLE, IDDM, Crohns disease as well as parasitic diseases such as cerebral malaria and Leishmaniasis. Increased frequency of the –308*2 allele has been demonstrated in other autoimmune conditions such as SLE (Wilson, 1994).

Previous studies have led to conflicting results as to the functional consequences of this polymorphism. Some *in vitro* studies have shown increased levels of TNFα associated with the –308*2 allele (Louis, 1998). It has been suggested that this difference in TNFα levels is due to ability of the allele*2 to bind nuclear factors involved in transcription differently to allele*1 (Abraham, 1997). This gives rise to a two-fold increase in the levels of transcription in PMA-stimulated Jurkat and U937 cells. This has been attributed to a 10bp sequence around the –308 site that is

homologous to the binding site of an activator protein-2 (AP-2) element (Kroeger, 1996). However other studies have failed to confirm these findings in a transient transfection system where both alleles were said to contribute equally to the total amount of mRNA (Brinkman, 1996). Associations with TNFα production and particular –308 genotypes have been described in peridontal disease where the 1*2 genotype was correlated with increased production in patients with advanced disease (Galbraith, 1998). This raised the question was the distribution of the –308 alleles in AS patients different from the control populations and was the incidence of the –308*2 allele, which is associated with higher levels of TNFα, increased in this patient group.

The results clearly show a significant increase in the distribution of the –308*1 allele in the AS patients not only when compared to the normal control group but also when compared to the B27 positive controls. This implies a link between this allele in the TNF promoter region in these patients that is not present in the HLA-B27 positive controls. It would be of interest to know if the increased expression of this allele is also found in B27 negative AS patients, but as they number less than 10% of the AS population, multicenter studies would be required to achieve sufficient numbers for statistical analysis.

A number of other groups have investigated this site in AS and found conflicting results. In the study by Verjans no significant differences where seen, however only a small number of patients and controls where examined (66 patients and 19 controls). It may also be that there are regional differences as seen in the distribution of the –308*2 allele which in the Verjans study had a very low frequency in the control group compared to other Western European control groups (Verjans, 1994). A similar study carried out on British Caucasians has also failed to

demonstrate this association with allele*1 and AS and in fact found a weak association with the rare -308*2 allele being increased in AS patients. (Brown, 2000). Brown and colleagues have since carried out a further study on a second cohort of patients and have established an association with the –308*1 allele in a cohort of German AS patients (Milicic, 2000).

One alternative interpretation of the data seen in this study could be that there is a reduction in the frequency of the –308*2 allele as was implied in a recent study where it was suggested that the –308*2 allele had a protective role against the development of AS in B27 positive individuals (Hohler, 1998). The association of the –308*2 allele with the HLA DRβ1*03 alleles as seen in SLE patients also suggest a protective role against the development of nephritis (Jacob, 1990). The –308*2 allele in conjunction with the IL-1Ra*2 allele may also be protective against infection with cytomegalovirus (Hurme, 1998). Cytomegalovirus has been implicated in patients with uveitis (De Boer, 1996) So could it be in diseases such as AS where infections have been implicated in the pathogenesis, B27 positive individuals carrying the –308*2 allele are better equipped to combat these infections.

Previous work has shown the –308*2 allele to be in linkage with the HLA DRβ1*03 allele in SLE patients, although it has also been suggested that the –308*2 allele may have an independent effect as well (Sullivan, 1997). This is part of the HLA A1-B8-DR3-DQ2 extended haplotype, which is known to confer susceptibility to celiac disease (McManus. 1996). TNFα levels are also known to vary according to particular HLA DR alleles (Jacob, 1990, Bendtzen, 1988). Individuals carrying the HLA-DR2 allele are low producers compared to those with HLA-DR3 and HLA-DR4 alleles that are high producers. It is of interest to note

that in this study the incidence of HLA-DRβ1*03 alleles was reduced which is what would be expected if the –308*2 allele is part of an extended haplotype.

As with the other polymorphic sites investigated the distribution of the –308 alleles were compared in the AS patients with and without several different disease features. In a study of primary biliary cirrhosis patients the –308 1*1 genotype was associated with more advanced disease suggesting that this allele may be linked to disease progression rather than disease susceptibility (Jones, 1999). No significant differences were seen in the distribution of the –308 alleles in any of the features examined in our AS patients. In conclusion this study has shown a significant increase of the –308*1 allele in the AS patients when compared to both control groups implying that this region of the genome is associated with AS, independent of HLA B27. However as it is the –308*2 allele that is associated with higher mRNA production then the increase in –308*1 allele would imply that if TNF is important in the pathogenesis of sacroilitis in AS, any genetic influence over the differential production is unlikely to be important.

4.7 T NFa Microsatellite Results

The TNFa microsatellite is highly polymorphic and contains at least 13 alleles that differ according to the number of AC/GT repeats. The TNFa microsatellite is located upstream of the LTα gene and in vitro stimulation studies have indicated that TNFa levels vary dependant on different TNFa alleles (Pociot, 1993). Previous studies have shown associations with TNFa6 allele in RA patients and TNFa2 with IDDM. The TNFa6 allele has been linked with decreased secretion of TNFa in IDDM patients whereas TNFa2 has been associated with higher production. The TNFa microsatellite was investigated in 138 AS patients, 91 B27 negative controls and 72 B27 positive controls, the results are depicted in Table 25 and 26.

The distribution of the TNFa alleles in the AS patients compared to the B27 negative controls are shown in Figure 37. Overall the distribution of the TNFa alleles are significantly different between the two groups when analysed by Clump ($T_4<0.0005$). A significant increase in TNFa6 allele was seen in the AS patients compared to the B27 negative controls (40% versus 18 %, $p<0.005$). This was balanced by a significant decrease in the TNFa11 allele in the patients compared to the controls (6% versus 20.9%, $p<0.005$). None of the other alleles differed significantly.

As was seen in the Nco.1 site, however this significant difference did not hold true when examining the AS patients to the B27 positive controls. As can be seen in Figure 38 the distribution of all the alleles were very similar between these two groups suggesting that the differences seen may be part of an extended haplotype with B27 and not predisposing to disease.

Previous studies have suggested that particular alleles may predispose not only too higher levels of TNFa but may be associated with more severe forms of disease. The increased frequency of TNFa6 allele in a study of RA patients was even more pronounced in those patients with a more severe disease (Hajeer, 1996).

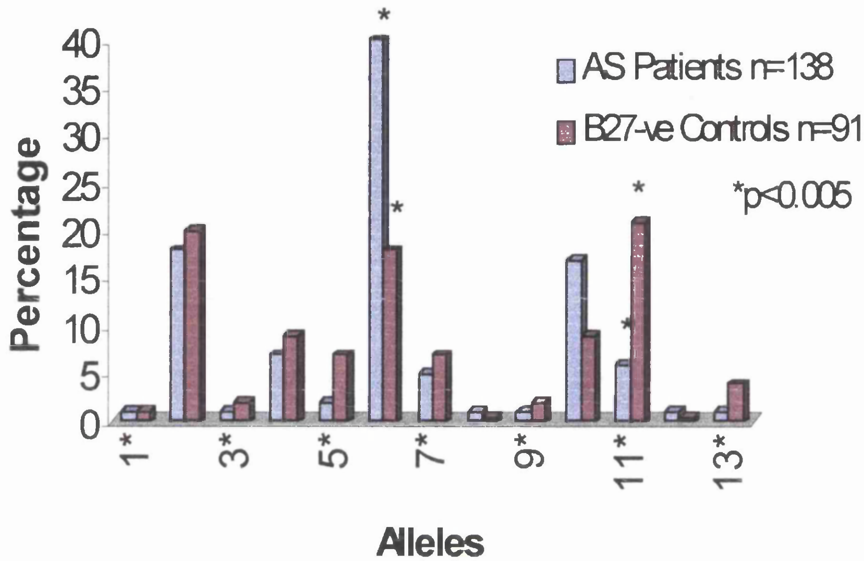
The distribution of the 13 alleles in the 67 AS patients with an onset of disease below the age of 21 years compared to the 68 AS with an onset greater than 21 years is described in Table 27. No significant differences between the two groups were seen. .

In the 48 AS patients with uveitis the TNFa4 allele was represented only once compared to the 86 AS patients without a history of uveitis where the TNFa4 allele was seen in 19 patients (p=0.04). The results can be seen in Table 28. The distributions of the 13 TNFa alleles in the 23 AS patients with a family history compared to the 98 patients with no family history are recorded in Table 29. Some differences were noted although none reached statistical significance. For instance the frequency of the TNFa2 allele was reduced in the patients with a family history compared to those without (7% versus 21%). Also an increase in the frequency of the TNFa6, 7 and 10 alleles was seen in the patients with a family history. A reduction of the frequency of the TNFa2 allele was also seen in the 41 AS patients with peripheral joint disease compared to the 94 without as can be seen in Table 30 (12% versus 20%).

Although some differences were seen in the distribution of the TNFa alleles in the AS patients with and without extra features of disease they failed to reach statistical significance.

TNFa Alleles	AS Patients n=138 (%)	B27 Negative Controls n=91 (%)
1	3 (1)	2 (1)
2	49 (18)	37 (20)
3	2 (1)	3 (2)
4	18 (7)	17 (9)
5	5 (2)	12 (7)
6	111 (40)*	33 (18)*
7	14 (5)	12 (7)
8	2 (1)	1 (0.5)
9	2 (1)	3 (2)
10	47 (17)	16 (9)
11	16 (6)*	38 (21)*
12	3 (1)	1 (0.5)
13	4 (1)	7 (4)

Legend for Table 25
The distribution of the 13 different alleles at the TNFa locus in 138 AS patients compared to 91 B27 negative controls is described in Table 25. The TNFa6 allele was significantly increased in the AS patients compared to the controls ($p<0.005$). The TNFa11 allele was significantly reduced in the AS patients compared to the controls ($p<0.005$).

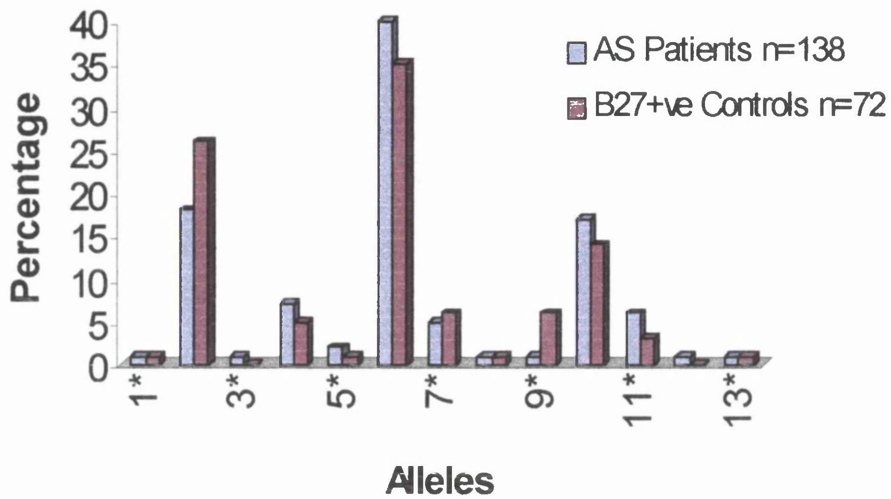


Legend for Figure 37
The distribution of the 13 different TNFa alleles in the 138 AS patients compared to the 91 B27-ve controls are shown in Figure 37. The frequency of the TNFa6 allele was significantly increased in the AS patients ($p<0.005$) compared to the controls. Correspondingly the TNFa11 allele was significantly decreased ($p<0.005$).

TNFa Alleles	AS Patients n=138 (%)	B27 positive controls n=72 (%)
1	3 (1)	1 (1)
2	49 (18)	38 (26)
3	2 (1)	0 (0)
4	18 (7)	7 (5)
5	5 (2)	2 (1)
6	111 (40)	51 (35)
7	14 (5)	9 (6)
8	2 (1)	2 (1)
9	2 (1)	9 (6)
10	47 (17)	20 (14)
11	16 (6)	4 (3)
12	3 (1)	0 (0)
13	4 (1)	1 (1)

Legend for Table 26

Table 26 depicts the distribution of the 13 TNFa alleles in the 138 AS patients compared to the 72 B27 positive. There were no significant differences between the two groups.



Legend for Figure 38

The distribution of the 13 TNFa alleles in the AS patients compared to the B27 positive controls are shown in Figure 38. There were no significant differences between the two groups.

TNFa Alleles	AS Patients with Age of Onset <21yrs n=67 (%)	AS Patients with Age of Onset >21yrs n=68 (%)
1	1 (1)	2 (1)
2	25 (19)	23 (17)
3	0 (0)	2 (1)
4	7 (5)	10 (7)
5	2 (1)	3 (2)
6	52 (39)	55 (40)
7	9 (7)	5 (4)
8	1 (1)	1 (1)
9	2 (1)	0 (0)
10	23 (17)	24 (18)
11	9 (7)	7 (5)
12	1 (1)	2 (1)
13	2 (1)	2 (1)

Legend for Table 27

Distributions of the 13 TNFa alleles in 67 AS patients with an onset of disease <21 years of age compared to 68 AS patients with an onset > than 21 years are shown in Table 27. No significant differences in the frequency of the alleles were seen between the two groups.

TNFa Alleles	Uveitis Present n=48 (%)	Uveitis Absent n=86 (%)
1	0 (0)	3 (2)
2	23 (24)	25 (15)
3	1 (1)	1 (1)
4	1 (1)*	19 (11)*
5	4 (4)	2 (1)
6	40 (42)	64 (37)
7	2 (2)	11 (6)
8	0 (0)	2 (1)
9	1 (1)	1 (1)
10	16 (17)	31 (18)
11	5 (5)	9 (5)
12	2 (2)	1 (1)
13	1 (1)	3 (2)

Legend for Table 28

The distribution of the TNFa alleles in 48 AS patients with uveitis compared to 86 AS patients without are shown in Table 28. The TNFa4 allele was represented only once in the 48 AS patients with uveitis compared to 19 out of 86 patients ($p=0.04$).

TNFa alleles	Family History Present n=23 (%)	Family History Absent n=98 (%)
1	0 (0)	2 (1)
2	3 (7)	42 (21)
3	0 (0)	2 (1)
4	2 (4)	12 (6)
5	0 (0)	5 (2.5)
6	22 (48)	73 (37)
7	6 (13)	7 (4)
8	0 (0)	1 (0.5)
9	0 (0)	1 (0.5)
10	10 (22)	34 (17)
11	2 (4)	12 (6)
12	1 (2)	1 (0.5)
13	0 (0)	4 (2)

Legend for Table 29
Distributions of the 13 different TNFa alleles in AS patients with and without a family history are depicted in Table 29. The frequency of the TNFa2 allele was reduced in the 23 AS patients with a family history compared to the 98 AS patients without (7% versus 21%). In the patients with a family history the frequency of the TNF a6, a7 and a10 alleles were increased compared to the patients without a family history.

TNFa Alleles	PJD Present n=41 (%)	PJD Absent n=94 (%)
1	0 (0)	3 (2)
2	10 (12)	38 (20)
3	0 (0)	2 (1)
4	3 (4)	15 (8)
5	2 (2)	3 (2)
6	40 (49)	68 (36)
7	7 (9)	7 (4)
8	0 (0)	2 (1)
9	1 (1)	1 (1)
10	12 (15)	35 (19)
11	5 (6)	9 (5)
12	1 (1)	2 (1)
13	1 (1)	3 (2)

Legend for Table 30
The distribution of the 13 TNFa alleles in 41 AS patients with PJD compared to 94 AS patients without PJD are shown in Table 30. The frequency of the TNFa2 allele was reduced in the AS patients with PJD compared to those without (12% versus 20%).

4.8 TNFc Microsatellite results

The TNFc microsatellite is a biallelic polymorphism that lies within intron 1 of the LTα gene. Of the two possible alleles allele *2 has been previously associated with increased secretion of TNFα . In the same publication the TNFc *1 allele was shown to be associated with low TNFα production in IDDM patients (Pociot, 1993). The TNFc *1 allele has also shown an association with RA in a multiplex family study (Mulcahy, 1996). Several reports have suggested that some of the TNF polymorphisms play a role in extended haplotpyes that extend across the MHC Class I, II and III regions. The TNFc *2 allele has been shown to be linked to the presence of DR4 (Jongeneel, 1991). However in a study of Reactive arthritis patients in which B27 plays a role the TNFc *1 allele was proposed to have an independent role on disease susceptibility (Tuokko, 1998).

A total of 159 AS patients were investigated at the TNFc site and the results were compared to 98 B27 negative controls. The results of the distribution of alleles and genotypes are described in Table 31 and depicted in figures 39a and b. The distributions were very similar between the two groups.

However when the 159 AS patients were compared to the 50 B27 positive controls the TNFc 1*2 genotype was significantly decreased in the patients (30% versus 50%, $p=0.01$). The results are described in Table 32 and shown in Figure 40a and b. No statistical differences in the distribution of alleles were seen in the AS patients compared to the B27 positive controls.

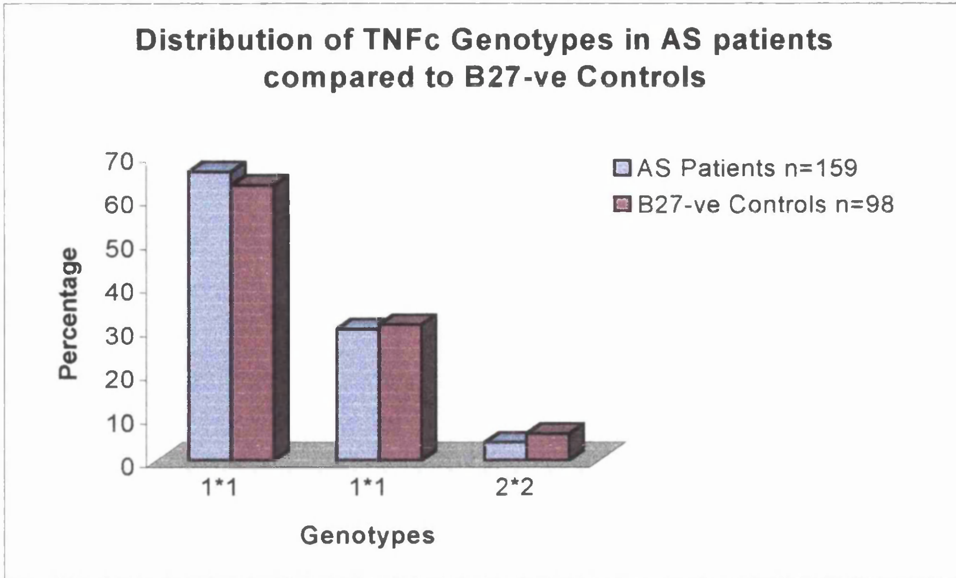
The distribution of the TNFc alleles and genotypes were investigated in the AS patients with and without the extra disease features as described in Table 33. There were no significant differences in any of the features investigated.

TNFc	AS Patients n=159 (%)	B27-ve Controls n=98 (%)
1*1	105 (66)	62 (63)
1*2	48 (30)	30 (31)
2*2	6 (4)	6 (6)
1*	258 (81)	154 (79)
2*	60 (19)	42 (21)

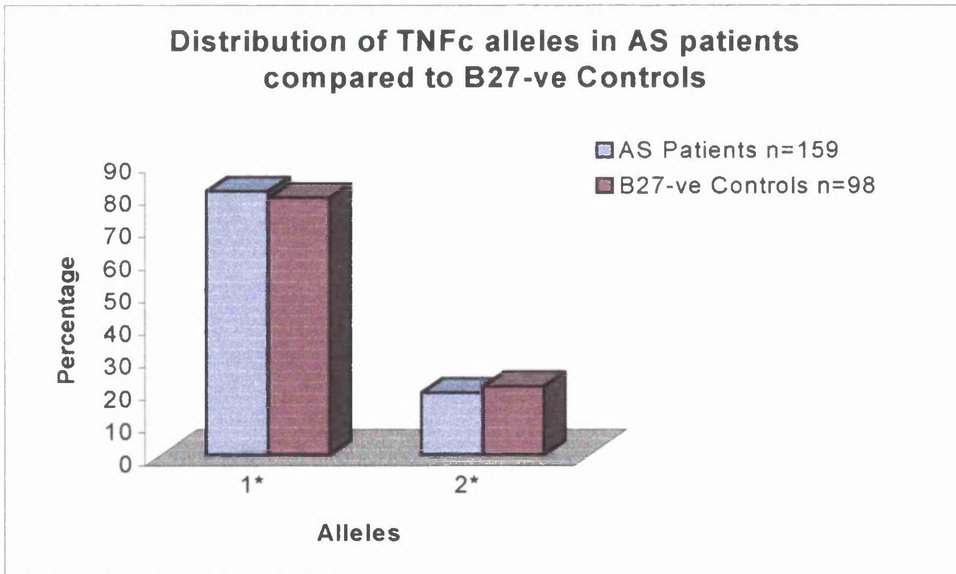
Legend for Table 31
The distribution of the TNFc genotypes and alleles in the AS patients compared to the B27 negative controls are displayed in Table 31. There were no significant differences between these two groups

TNFc	AS Patients n=159 (%)	B27 +ve Controls n=50 (%)
1*1	105 (66)	25 (50)
1*2	48 (30)*	25 (50)*
2*2	6 (4)	0 (0)
1*	258 (81)	75 (75)
2*	60 (19)	25 (25)

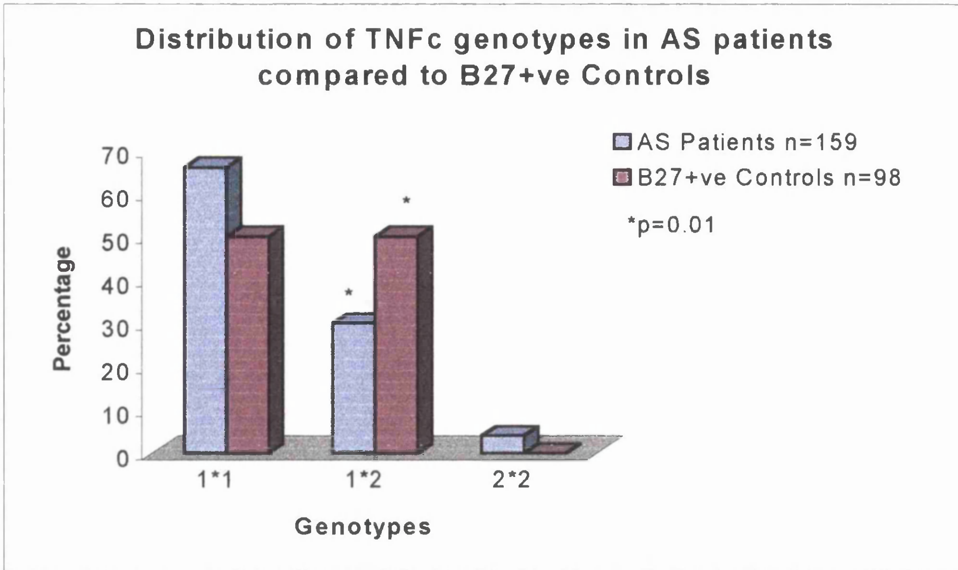
Legend for Table 32
The results of the distribution of the TNFc genotypes and alleles in the AS patients compared to the B27 positive controls are shown in Table 32. The TNFc 1*2 genotype was significantly increased ($p=0.01$) in the B27 positive controls compared to the AS patients (50% versus 30%). There were no significant differences in the distribution of the alleles.



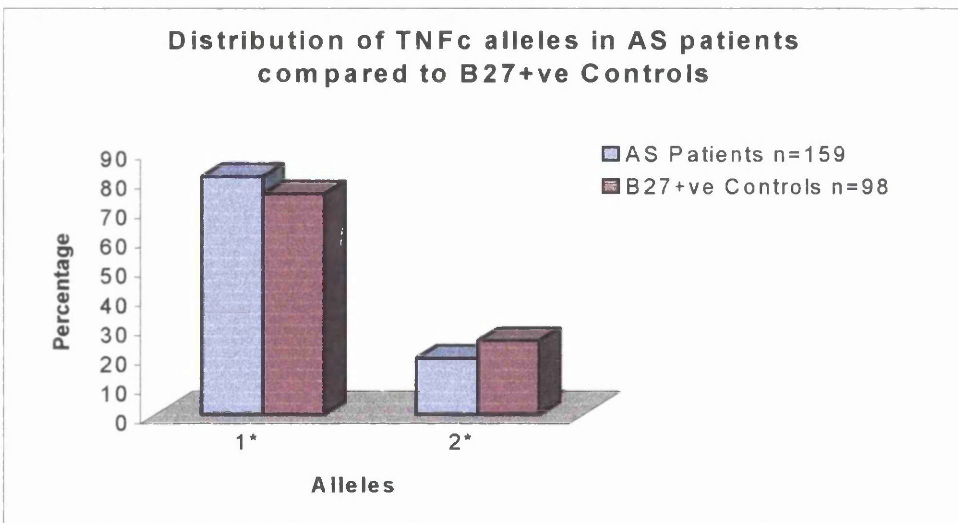
Legend for Figure 39a
The distributions of the TNFc genotypes in the AS patients compared to the B27-ve controls are shown in Figure 39a. There were no significant differences between the two groups.



Legend for Figure 39b
The distributions of the TNFc alleles in the AS patients compared to the B27-ve controls are shown in Figure 39b. There were no significant differences between the two groups.



Legend for Figure 40a
The distributions of the TNFc genotypes in the AS patients compared to the B27+ve controls are shown in Figure 40a. The frequency of the TNFc 1*2 genotype was significantly decreased in the AS patients compared to the controls (p=0.033).



Legend for Figure 40b
The distributions of the TNFc alleles in the AS patients compared to the B27+ve controls are shown in Figure 40b. There are no significant differences between the two groups.

	TNFc Genotypes and Alleles				
Extra Features of Disease	**1*1**	**1*2**	**2*2**	**1***	**2***
Age of Onset <21 n=78 (%)	50 (60)	24 (31)	4 (5)	124 (79)	32 (21)
Age of Onset >21 n=78 (%)	52 (67)	24 (31)	2 (2)	128 (82)	28 (18)
Uveitis Present n=59 (%)	35 (59)	20 (34)	4 (7)	90 (76)	28 (24)
Uveitis Absent n=95 (%)	66 (70)	27 (28)	2 (2)	159 (84)	31 (16)
Family History Present n=25 (%)	19 (76)	5 (20)	1 (4)	43 (86)	7 (14)
Family History Absent n=116 (%)	72 (62)	39 (34)	5 (4)	183 (79)	49 (21)
Peripheral Joint Disease Present n=47 (%)	35 (74)	10 (21)	2 (5)	80 (85)	14 (15)
Peripheral Joint Disease Absent n=109 (%)	68 (62)	37 (34)	4 (4)	173 (79)	45 (21)

Legend for Table 33
The distribution of the TNFc alleles and genotypes in the AS patients with and without extra features of disease are shown in Table 33. The TNFc 1 allele was reduced in the AS patients with uveitis (76% versus 84%) with a corresponding increase in the TNFc 2 allele but this was not statistically significant. Similarly the TNFc 1*1 genotype was increased in those patients with a family history (76% versus 62%). Overall there were no statistical differences.

4.9 TNFe Microsatellite Results

At the centromeric end of the TNF locus, which still remains largely undefined, lies the TNFe microsatellite. This site contains at least three alleles with some studies detecting four. No known associations with any of the TNFe alleles and alterations in protein levels have been established. However the TNFe *3 allele has been demonstrated to be over-represented in a group of RA patients compared to controls (Field, 1997). The TNFe locus may also be linked with possible extended haplotypes spanning the MHC as in the same study the increase of the TNFe *3 allele was accounted for by the presence of DR4 in these RA patients.

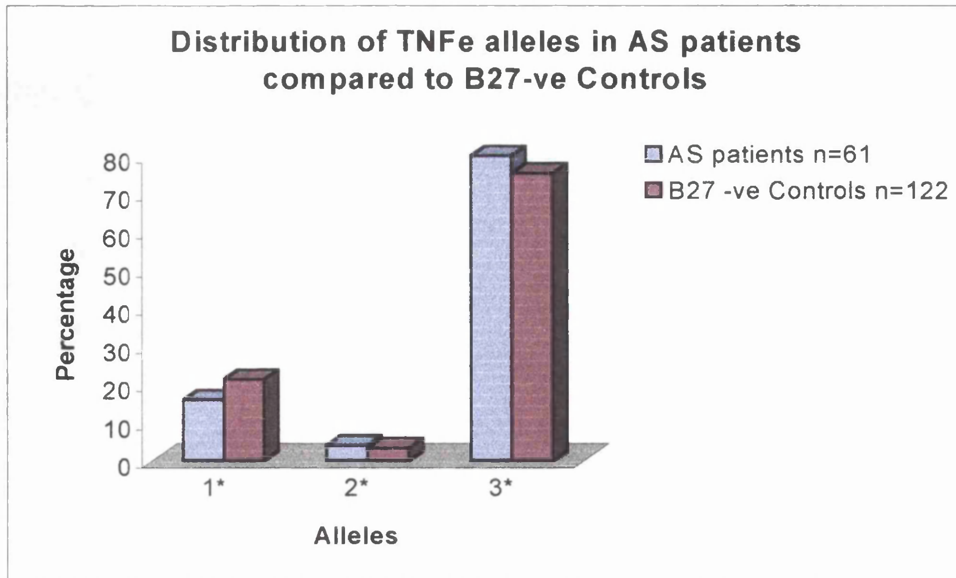
Since this site was not associated with any functional difference only a small number of the AS patients and controls were investigated. The results of the distribution of the TNFe alleles in the AS patients are described in Table 34. There were no significant differences between the two groups. Similarly the distribution of the TNFe alleles in the AS patients compared to the B27 positive controls are shown in Table 35. A slight decrease in the distribution of the TNFe *3 allele was seen in the AS patients (80% versus 90%) but this was not statistically significant. No significant differences in the distribution of the genotypes were seen (data not shown) in the AS patients compared to both control groups.

TNFe	AS Patients n=61 (%)	B27-ve Controls n=122 (%)
1*	19 (16)	26 (21)
2*	5 (4)	4 (3)
3*	98 (80)	92 (76)

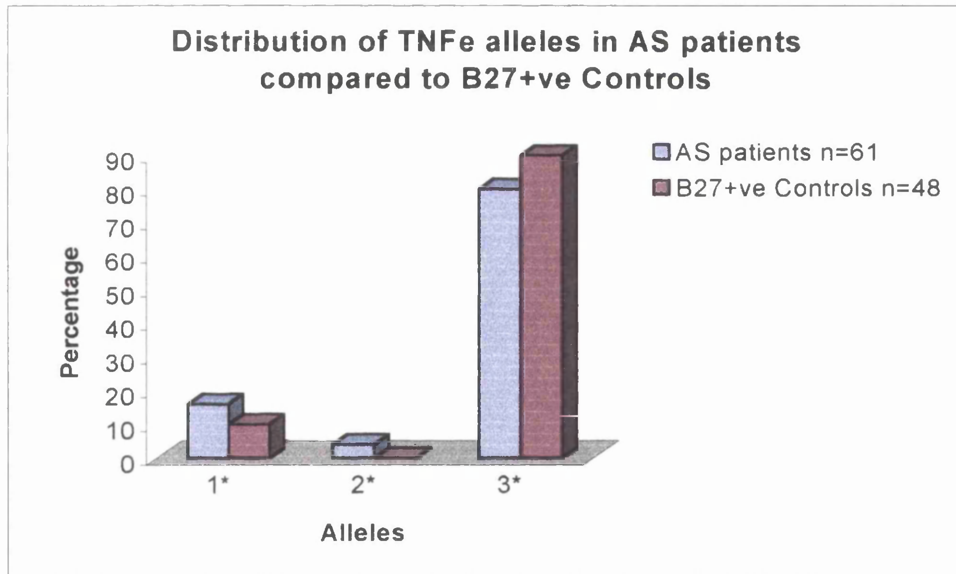
Legend for Table 34
The distribution of the TNFe alleles in the AS patients compared to the B27 negative controls are described in Table 34. There were no significant differences between the two groups either in the distribution of the alleles or the genotypes (data not shown).

TNFe	AS Patients n=61 (%)	B27 +ve Controls n=48 (%)
1*	19 (16)	5 (10)
2*	5 (4)	0 (0)
3*	98 (80)	43 (90)

Legend for Table 35
No significant differences were seen in the distribution of the TNFe alleles in the AS patients compared to the B27 positive controls either in the frequency of alleles or the genotype frequencies (data not shown).



Legend for Figure 41
The distribution of the TNFe alleles in the AS patients compared to the B27-ve controls are described in Figure 41. There were no significant differences between the two groups.



Legend for Figure 42
The distribution of the TNFe alleles in the AS patients compared to the B27 +ve controls are shown in Figure 42. There were no significant differences between the two groups.

4.10 TNF Microsatellite Discussion

Three microsatellite loci were investigated in this study, with the TNFa being one of the more informative regions. This is a highly polymorphic site with at least 13 possible alleles being described. Previous work has attributed differences in production of TNFα to particular alleles at this site. The TNFa2 allele linked with DRβ1*03 in an extended haplotype correlates with increased production whereas TNFa6 is associated with decreased production (Pociot, 1993).

In the present study a significant increase in the frequency of the TNFa6 allele was seen in the AS patients compared to the normal control population. This was in comparison to the significant decrease of the TNFa11 allele in the patients. However this association appears to be secondary to the presence of B27 since the distributions did not differ between the patients and the B27 positive controls. This would suggest that any association with the TNFa microsatellite alleles is part of an extended haplotype and not predisposing to disease. Large multi-case family studies would be required to confirm this finding.

Similar results have been described for Reactive arthritis patients where an increase in the TNFa6 allele and decrease in TNFa11 was also seen. Reactive arthritis also has an association with B27 and it would appear in this study that the differences seen are also secondary to B27 (Tuokko, 1998). In RA patients an independent association with TNFa6 has also been described (Mulcahy, 1996) along with studies showing the allele to be in linkage with DRβ1*0401 (Hajeer, 1996). The TNFa6 has been associated with decreased secretion of TNFα in IDDM patients but to date information on levels in AS patients in relation to these alleles have not been studied.

It would appear that these TNFa alleles are part of an extended haplotype in our population involving both MHC Class I (B27) and MHC Class II (DRβ1) genes and therefore do not have an effect on disease susceptibility in AS patients. It has been suggested that these alleles may have a role in disease severity with TNFa11 linked to severity in RA patients (Mu, 1999). Therefore the distributions of these alleles were examined in relation to different features of disease. Only in the AS patients with and without uveitis was a difference seen in that AS patients with uveitis had a decreased incidence of TNFa4 allele. However numbers where small and no functional relevance of the TNFa4 allele has been described. It would be interesting to investigate this further in AS patients with uveitis who where B27 negative to determine if this was independent of B27.

Of the other microsatellite regions investigated no significant differences were seen at the TNFe locus. At the TNFc locus the AS patients differed significantly in the distribution of the TNFc 1*2 genotype which was decreased in the AS patients. Associations with TNFc*1 allele with decreased production and TNFc2 allele with increased production of TNFα have been demonstrated in IDDM patients. The TNFc*1 allele has also shown an association with RA in a family study (Mulcahy, 1996). The TNFc alleles have also been demonstrated to be part of an extended haplotype with particular TNFc alleles being linked with particular Nco.1 alleles (Pociot, 1993). The significance of the decreased frequency of the TNFc1*2 genotype in the patients is unclear as it is not seen in the normal population and does not hold for the distribution of the alleles themselves. With the small numbers studied it may just reflect linkage patterns across the MHC.

CHAPTER FIVE

IL-1 POLYMORPHISMS

5.1 Interleukin 1 Polymorphism

A recent genome screen carried out on a large number of AS patients highlighted areas other than the MHC as candidate sites, which could play a role in the pathogenesis of AS. One area lies within six centimorgans of the genes for the three interleukin 1 proteins (IL-1α, IL-1β, and IL-1Ra). Il-1α and IL-1β are important proinflammatory cytokines whose prolonged production has been implicated in autoimmune diseases.

IL-1 is a powerful stimulant of osteoclastic bone resorption, has potent catabolic effects on cartilage, induces the acute phase response and increases the release of other inflammatory mediators. In particular IL-1 has been shown to be important in animal models such as the rabbit model of immune mediated colitis and in cartilage destruction in the collagen induced model (Cominelli,1992). Although there is no direct evidence of the involvement of IL-1 proteins in AS the presence of TNF in sacroiliac biopsies suggest IL-1 may be important since it is well recognised that TNF can induce IL-1. IL-1Ra blocks the effects of IL-1α and β and it is thought to be the balance between the three proteins that is important in limiting the activity of IL-1.

The genes for the three proteins along with the genes for the IL-1 receptors are all found on chromosome two. The IL-1 α,β and Ra genes all map to a common restriction site where known linkage between the genes has been recognised (Cox, 1998). Stable variations in protein levels between individuals have been reported previously (Danis, 1995), implying genetic factors may be important. Several polymorphic sites have been identified in all three genes some of which are known to affect cytokine production. Particular alleles at some of these sites have also been associated with other autoimmune conditions.

It is therefore feasible that in a condition such as AS where fibrous joints are destroyed that IL-1 may be implicated. It could be that AS patients have an increased frequency of particular IL-1 alleles linked to high IL-1 production that may contribute to disease pathogenesis. In order to investigate this the frequency of alleles at three polymorphic sites, the –889 RFLP in IL-1α, the –511 RFLP in IL-1β and the 86 base pair repeat in the IL-1Ra gene were examined in the AS patients and compared to controls.

5.2 Interleukin 1 Receptor Antagonist Results

The role of the IL-1Ra is as a naturally occurring inhibitor, which is capable of binding to the IL-1 receptor without inducing any intracellular response. Although there are two structural variants of IL-1Ra, a secreted form and a form that remains in the cytoplasm, both are transcribed from the same gene through the use of alternative first exons (Arend, 1998). The human IL-1Ra gene has been mapped to the band q14-q21 on the long arm of chromosome 2 and contains within intron 2 an 86 base pair variable number tandem repeat (VNTR). This VNTR has been reported to have five alleles depending on the number of repeats of the 86bp fragment. There are three potential protein binding sites in this region suggesting a possible functional role although none is known at present. Allele 2 which has two repeats has previously been associated with a variety of diseases including, ulcerative colitis, Graves disease, SLE, severe peridontitis and reduced bone loss in women with an early menopause (Keen, 1998).

A total of 182 AS patients where examined by PCR for the IL-1Ra 86bp VNTR as described in the materials and methods section. The distribution of the alleles and genotypes where compared to two control populations, the first group consisted of 115 unrelated healthy blood donors who were all known to HLA B27 negative and the second group consisted of 76 HLA B27 positive healthy individuals. The results of the AS patients compared to the normal HLA B27 negative controls are described in Table 36. Only three of the possible five alleles and only four genotypes were seen in this study. Alleles 4 and 5 are uncommon and where not seen in either the patients or both control groups in this study.

In the AS patients the frequency of the IL-1Ra 1*1 genotype was decreased compared to the control population (80% Vs 90%). The frequency of the 1*2 genotype was 8% in the patients and was only seen in one of the controls (1%) and the 2*2 genotype was seen in 9% of the patients compared to 7% of the controls (Fig 43a). The overall frequency of the genotypes in the AS patients as seen in Figure 43a differed significantly from the B27 negative controls when examined using the Monte-Carlo simulation index (T_4=0.02). Carriage of at least one copy of allele 2 in the AS patients was 16% compared to 8% in the B27 negative controls giving an odds ratio of 2.3 with 95% confidence limits of 1.1 to 4.9, p=0.03. An increased carriage of allele 2 has been shown previously for other autoimmune diseases such as ulcerative colitis (Mansfield,1994).

The frequency and distribution of the IL-1Ra alleles in the AS patients were compared to a second group of healthy individuals who were known to HLA B27 positive. The results are described in Table 37 and shown on Figure 44a and b. The 1*1 genotype was decreased in the patients compared to controls (80% Vs 91%). None of the B27 positive controls carried the 1*2 genotype. The overall distribution of the genotypes in the AS patients was significantly different to the B27 positive controls when analysed by the Monte-Carlo simulation index (T_4=0.04). This accounted for the slight increase in the frequency of allele 2 seen in the patient group (13% Vs 8%). The carriage rate therefore of allele 2 was higher in the patients compared to the B27 positive controls (16% Vs 8%) giving an odds ratio of 2.3 with 95% confidence levels of 0.91 to 5.8, p=0.06.

Although the IL-1 genes lie on chromosome two and therefore the possibility of any association with genes on chromosome six is unlikely the distributions were still examined in the AS patients compared to the two individual control populations. If the distributions were examined in the AS patients in comparison to the combined control groups a significant difference was seen. The carriage rate of the allele 2 was higher in the patients when compared to the combined control population (16% cf 8%, OR 2.3, 95% CI 1.2-4.4, p=0.01).

A comparison between the distribution of the IL-1Ra alleles and genotypes in the normal control population in this study and two other control populations from other studies was carried out and the results are shown in Table 38. It can be seen that the frequency of the 1*1 genotype is increased in the West of Scotland controls compared to the control group from Sheffield and the second control group from Germany (89% cf 54% and 46%). Similarly the 1*2 genotype is decreased (0% cf 35% and 41%). A corresponding increase of allele 1 and decrease of allele 2 was seen in the three groups (90% cf 73% and 69% for allele 1) and 6% cf 24% and 27% for allele 2. The carriage rate of allele 2 in the West of Scotland controls is 9% compared with 42% for the controls from Sheffield and 49% for the controls from Germany. Although all three control groups are European Caucasian healthy blood donors different ethnic differences have previously been shown to affect the distribution of alleles at this site (Tountas, 1996). The number of control individuals in the West of Scotland group is small. Other studies carried out by different investigators in the same laboratory on different patient groups (e.g. Rheumatoid Arthritis patients and patients with Septic arthritis) have shown very similar distributions of alleles.

In summary an increased carriage of allele 2 was seen in the AS patients compared to both the B27 negative healthy controls and the B27 positive healthy controls. The controls were pooled in order to increase the power of the study as for these sites they did not require to be separated. The results were no different in the AS patients when compared to the pooled control populations (McGarry, 2001).

IL-1Ra	AS patients n=182 (%)	B27 Negative Controls n=115 (%)
1*1	147(80)*	104(90)*
1*2	14(8)	1(1)
2*2	16(9)	8(7)
1*3	5(3)	2(2)
1*	313(86)	211(92)
2*	46(13)	17(7)
3*	5(1)	2(1)

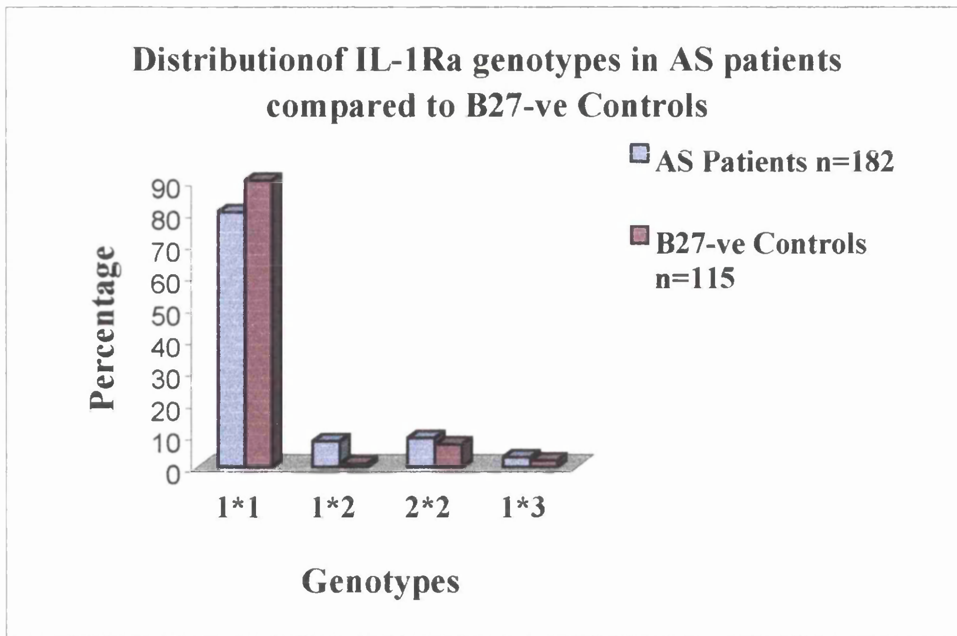
Legend for Table 36
Only three alleles at the IL-1Ra polymorphic site were seen in these populations the distribution of which are shown in Table 36. The distribution of the 1*1 genotype was significantly decreased in the AS patients compared to the B27 negative controls. Overall the distribution of the genotypes were significantly different (T_4=0.02)

IL-1Ra	AS patients n=182 (%)	B27 positive controls n=76 (%)
1*1	147(80)*	69(91)*
1*2	14(8)	0(0)
2*2	16(9)	6(8)
1*3	5(3)	1(1)
1*	313(86)	139(91)
2*	46(13)	12(8)
3*	5(1)	1(1)

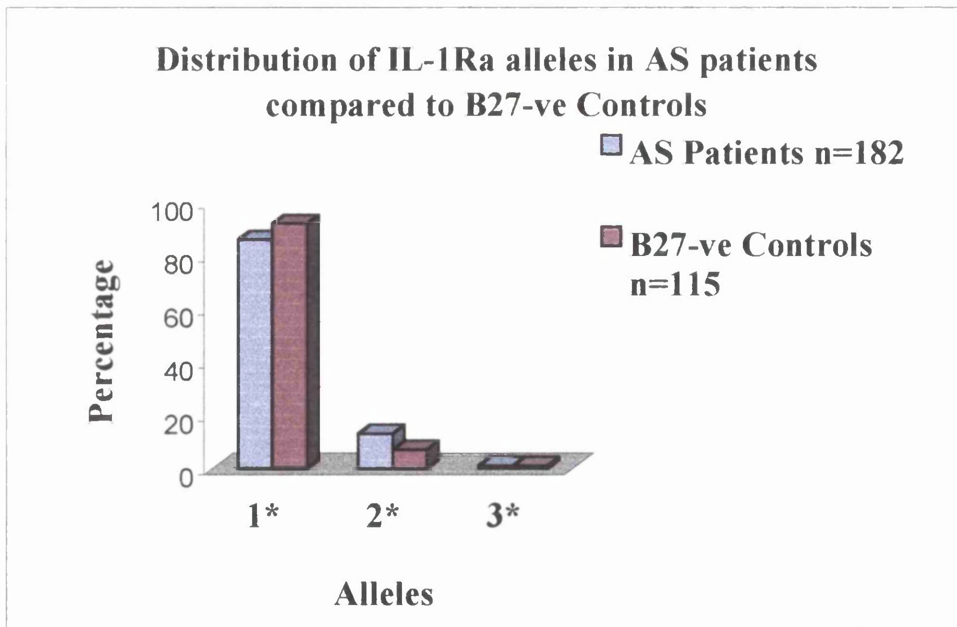
Legend for Table 37
The results of the distribution of the IL-1Ra alleles and genotypes in 182 AS patients compared to 76 B27 positive controls are recorded in Table 37. Overall the distribution of the genotypes differed significantly between the two groups (T_4=0.04).

IL-1RA	West of Scotland Controls n=115 [%]	Healthy Controls (Sheffield)* n=261 [%]	Healthy Controls (Germany)* * n=234 [%]
1*1	104 [90]	141 [54]	107 [46]
1*2	1 [1]	90 [35]	97 [41]
2*2	8 [7]	17 [6]	13 [5]
1*3	2 [2]	9 [3]	10 [4]
2*3	0 [0]	2 [1]	4 [2]
1*5	0 [0]	0 [0]	2 [1]
1*4	0 [0]	2 [1]	1 [1]
1*	211 [92]	383 [73]	324 [69]
2*	17 [7]	126 [24]	127 [27]
3*,4*,5*	2 [1]	13 [3]	17 [4]

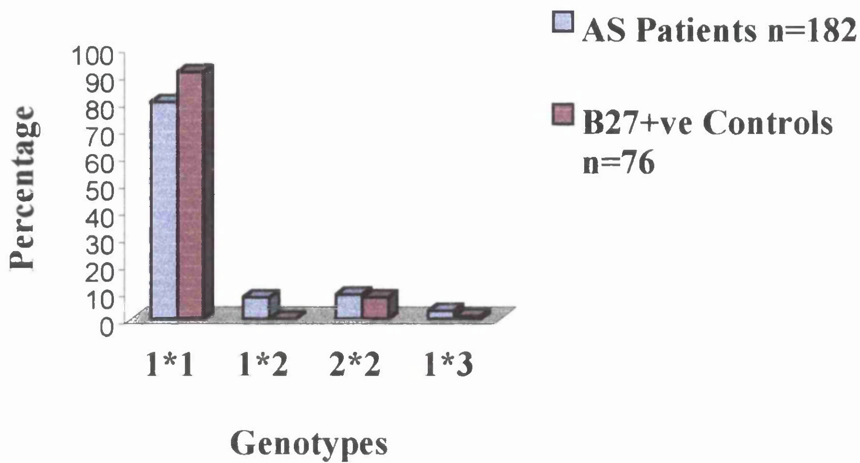
Legend for Table 38
A comparison between the distribution of the alleles and genotypes of the IL-1Ra VNTR between three different healthy populations are depicted in Table 38. The frequency of allele 2* and 1*2 genotype are decreased in the West of Scotland healthy controls compared to the Sheffield group (Mansfield, 1994) and the German healthy control group (Hacker, 1997).



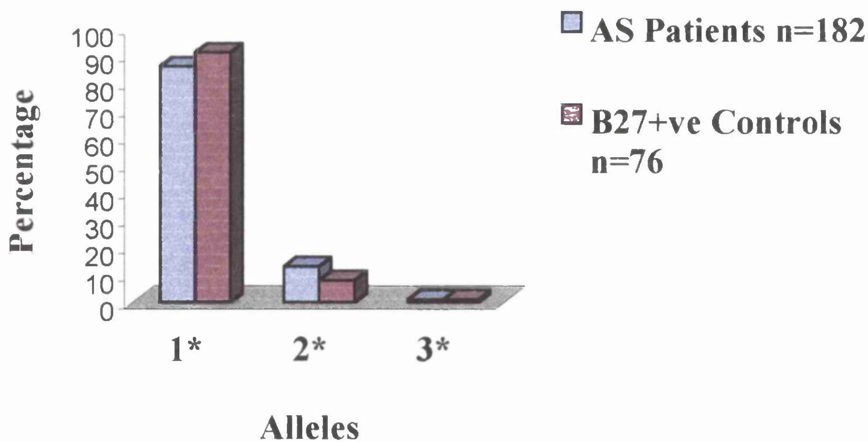
Legend for Figure 43a
The distributions of the IL-1Ra genotypes in the AS patients compared to the B27-ve controls are shown in Figure 43a. Overall the distributions are significantly different when analysed by the Monte-Carlo simulation index (T_4=0.02).



Legend for Figure 43b
The distribution of the IL-1Ra alleles in the AS patients compared to the B27-ve controls as is shown in Figure 43b.



Legend for Figure 44a
The distributions of the IL-1Ra genotypes in the AS patients compared to the B27+ve Controls are shown in Figure 44a. Overall the distributions differed significantly when analysed by the Monte-Carlo simulation index (T_4=0.04).



Legend for Figure 44b
The distributions of the IL-1Ra alleles in the AS patients compared to the B27+ve controls are given in Figure 44b.

5.3 Interleukin 1 Alpha Results

Interleukin 1 alpha (IL-1α) is an important proinflammatory cytokine that is known to contribute to inflammatory joint disease. It is thought to be the balance between the agonists (IL-1α and β) and the antagonist IL-1Ra that is important in controlling the effects of IL-1. In patients with inflammatory bowel disease an imbalance at the mRNA level between IL-1 and IL-1Ra has been seen in biopsy specimens (Isaacs,1992). A number of polymorphic sites in the IL-1α gene have been investigated in autoimmune diseases but it was decided to examine the single base pair change at position –889 in this study. This particular site has previously been linked with early onset pauciarticular juvenile rheumatoid arthritis with increased carriage of allele 2 (McDowell, 1995).

A total of 182 AS patients where examined for the frequency of the IL-1α alleles and genotypes in comparison to 113 healthy controls. The results are described in Table 39 and shown in Figure 45a and b. The frequency of the IL-1α alleles 1 and 2 in the AS group was 66% and 34% respectively compared to 72% and 28% in the controls. This slight increase in allele 2 was reflected in a carriage rate of 58% in the As patients compared to 47% in the controls. There were no significant statistical differences between the two groups.

The frequency of the IL-1α alleles and genotypes were compared between the AS patients and the HLA B27 positive controls, the results are given in Table 40 and Figure 46a and b. There were no statistical significant differences in the distribution of the genotypes in the AS patients compared to the controls although the 1*2 genotype was increased slightly in the AS patients (43% cf 38%).

The distribution of the IL-1α alleles and genotypes in the control populations were very similar to distributions seen in other control groups such as the Norwegian randomly selected controls from the study by McDowell etal and the healthy blood donors from Sheffield in the Study by Mansfield etal. The frequencies of these alleles would appear to be stable throughout European populations which differs from the distributions of the IL-1Ra alleles.

At this polymorphic site no significant differences were seen in the distribution of alleles or genotypes in the AS patients compared to either control group. Although a small increase in the carriage of at least one copy of allele 2 giving an odds ratio of 1.7 was seen in the patients compared to the B27 negative controls. It would appear that the IL-1α –889 polymorphic site is not a marker for disease susceptibility to AS.

IL-1 alpha	AS patients n=188 (%)	B27 Negative Controls n=113 (%)
1*1	82(45)	62(55)
1*2	80(43)	39(35)
2*2	22(12)	12(10)
1*	244(66)	163(72)
2*	124(34)	63(28)

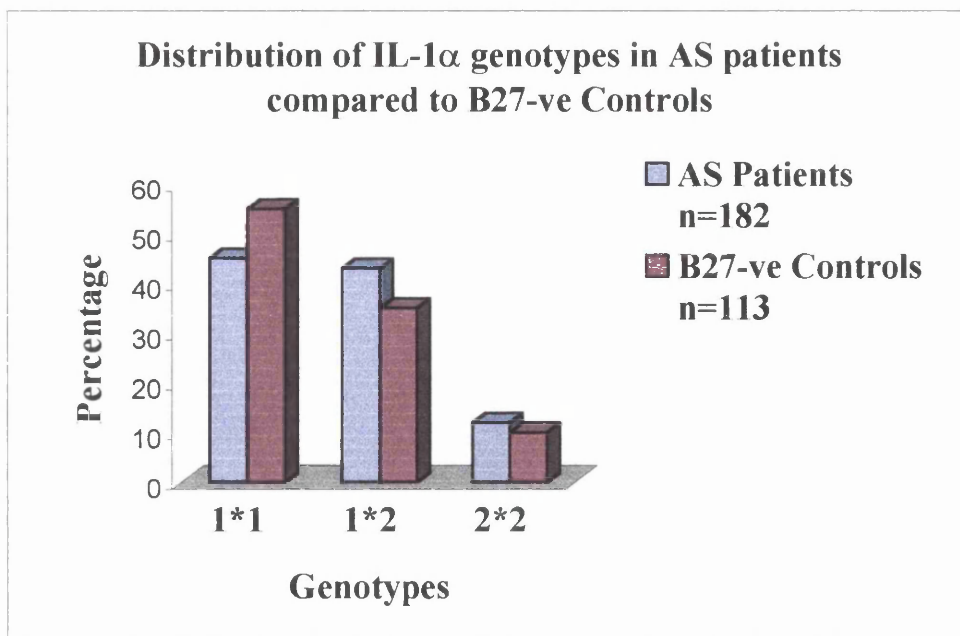
Legend for Table 39

The distribution of the IL-1 α genotypes and alleles are depicted in Table 39. A slight decrease in the 1*1 genotype was seen in the 188 AS patients compared to the 113 B27 negative controls (45% Vs 55%). Correspondingly a slight decrease in the 1* allele (66% Vs 72%) was seen in the patients but this did not reach statistical significance.

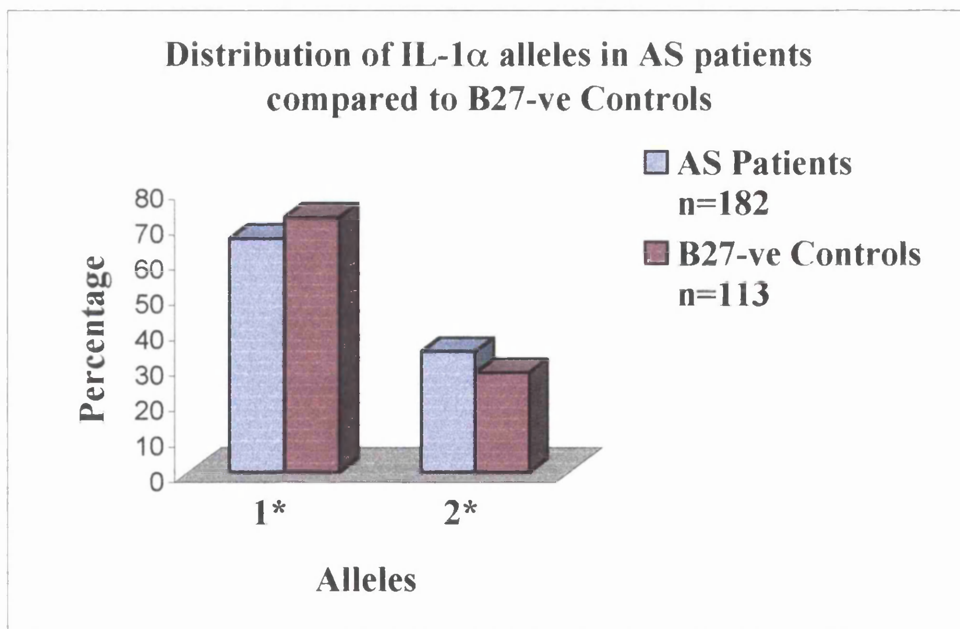
IL-1 alpha	AS patients n=188 (%)	B27 Positive Controls n=81 (%)
1*1	82(45)	41(51)
1*2	80(43)	31(38)
2*2	22(12)	9(11)
1*	244(66)	113(70)
2*	124(34)	49(30)

Legend for Table 40

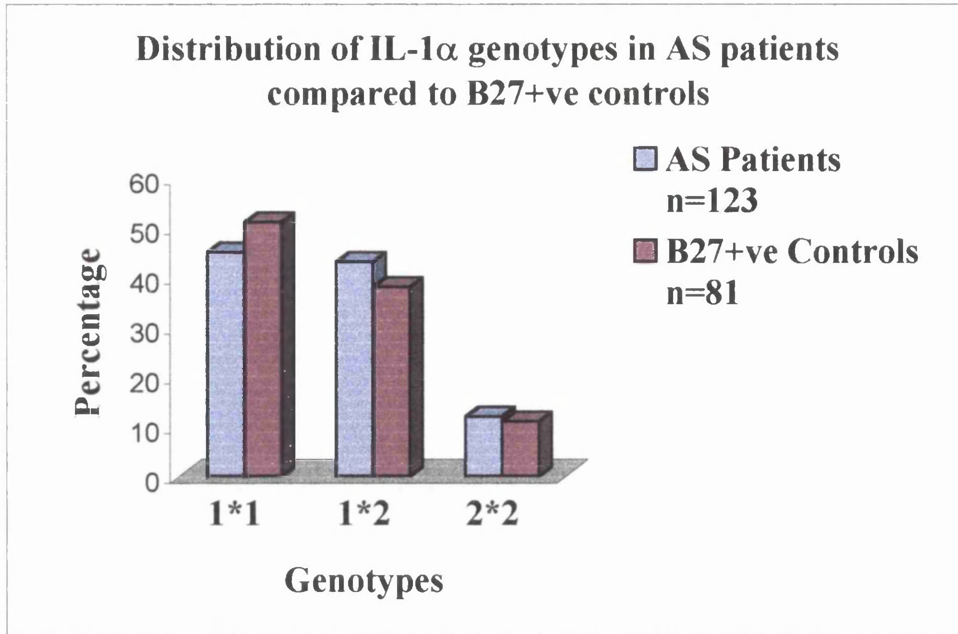
Table 40 represents the results of the distribution of the IL-1 α alleles and genotypes in 188 AS patients compared to 81 HLA B27 positive controls. There were no significant differences between these two groups.



Legend for Figure 45a
The distributions of the IL-1α genotypes in the AS patients compared to the B27-ve controls are shown in Figure 45a. No significant differences were seen between the two groups.

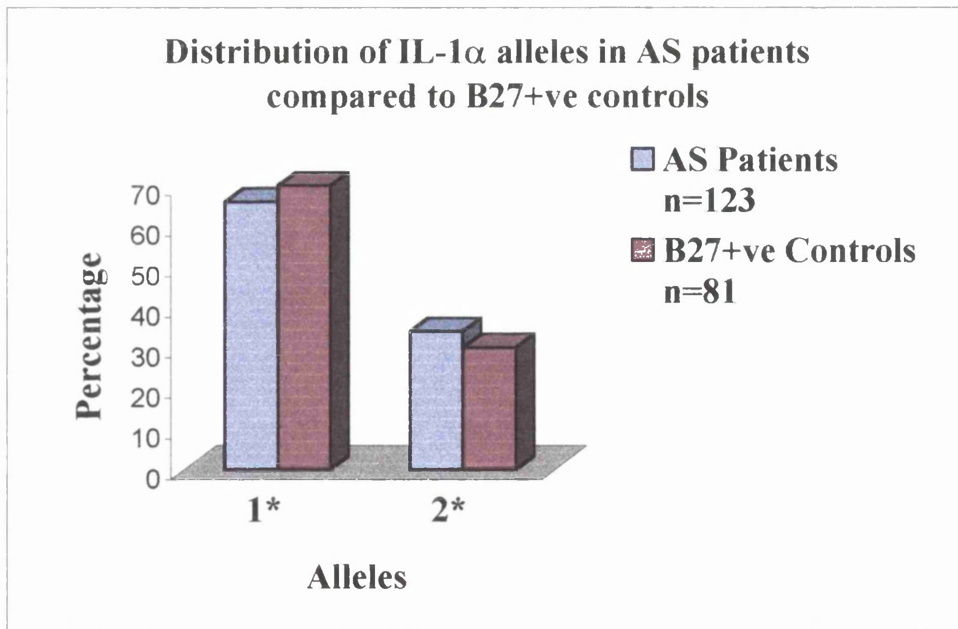


Legend for Figure 45b
The distribution of the IL-1a alleles in the AS patients compared to the B27 –ve controls is described in Figure 45b. There were no significant differences between the two groups.



Legend for Figure 46a

The distributions of the IL-1α genotypes in the AS patients compared to the B27+ve controls are shown in Figure 46a. No significant differences between the two groups were seen.



Legend for Figure 46b

The distributions of the IL-1α alleles in the AS patients compared to the B27+ve controls are shown in Figure 46b. There were no significant differences between the two groups.

5.4 Interleukin 1 Beta Results

The normal production of IL-1 is critical for the host response to injury and infection with prolonged or inappropriate production implicated in a variety of pathological conditions. The major extracellular from of IL-1 found in humans is IL-1β since IL-1α appears to remain membrane bound. IL-1β has been shown to be present at the cartilage-pannus interface in RA patients (Chu, 1992).

Within the IL-1β gene lie several polymorphic sites but the site investigated in this study was the bi-allelic site at –511 within the promoter region. To date no functional relevance has been assigned to this site. No significant associations with the site and any autoimmune diseases have been established but it has been proposed that the IL-1β and IL-1Ra genes are in linkage disequilibrium. Although IL-1β -511 has been implicated in inflammatory bowel disease (Nemetz, 1999).

A total of 188 AS patients and 111 B27 negative controls were investigated. The results of the distribution of the alleles and genotypes are described in Table 41 and Figure 47a and b. No significant differences were seen between the two groups. The patients were compared to 81 B27 positive controls with the results described in Table 42 and Figure 48a and b. Again the distribution of both the alleles and genotypes were very similar between the two groups. No significant differences were seen.

Several studies have investigated the distribution of alleles not in relation to disease susceptibility but to disease severity markers. For example an association with IL-1α and rheumatoid arthritis suggest the presence of IL-1α allele 2 could constitute a risk factor for a more destructive form of the disease (Jouvenne, 1999). A statistically significant increase of allele 2 of the IL-1β gene has been correlated with a high

secretor phenotype in patients with diabetic nephropathy (Loughrey, 1998). Allele 2 of the IL-1Ra gene has been suggested as being a marker of more severe forms of Sjogren's syndrome (Perriere, 1998).

The distribution of alleles and genotypes of each of the IL-1 polymorphic sites were investigated in relation to different disease features. The results are described in Tables 43,44 and 45. There were no significant differences in the patients with or without any of the features examined. In the 52 patients with an early onset the IL-1Ra 1*allele was increased compared to the 55 patients with a later onset (70% versus 59%) but this was not statistically significant.

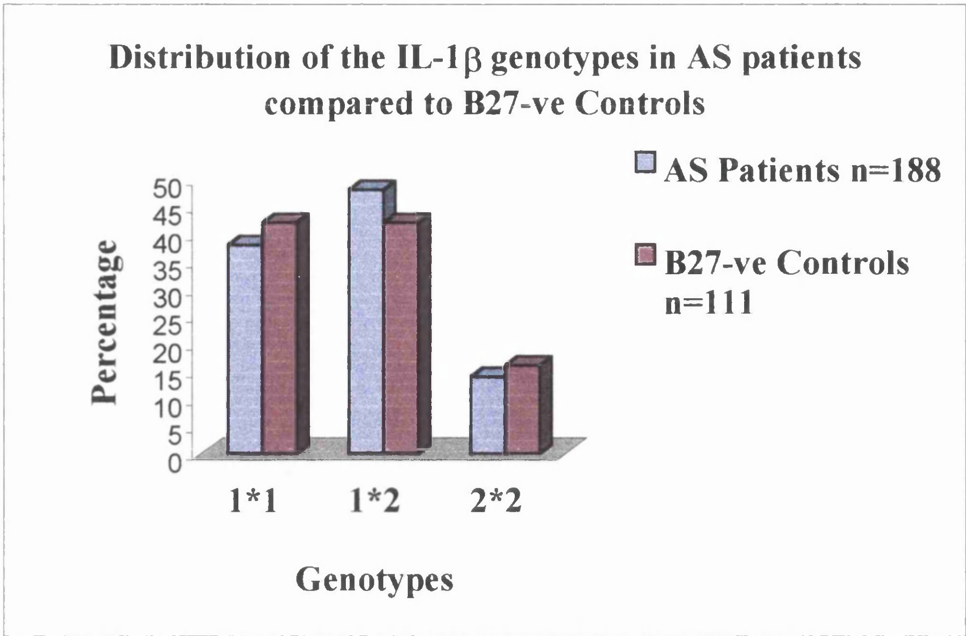
In conclusion of the three IL-1 sites investigated a significant association was seen in the carriage of the IL-1Ra 2*allele in the AS patients as compared to controls. This appeared to be associated with disease susceptibility and not with disease phenotype. The correlation with the increased levels of IL-1Ra and allele 2 warrant further investigation.

IL-1 beta	**AS patients n=188 (%)**	**B27 Negative Controls n=111 (%)**
1*1	71(38)	46 (42)
1*2	91(48)	47(42)
2*2	26(14)	18(16)
1*	233(62)	139(63)
2*	143(38)	83(37)

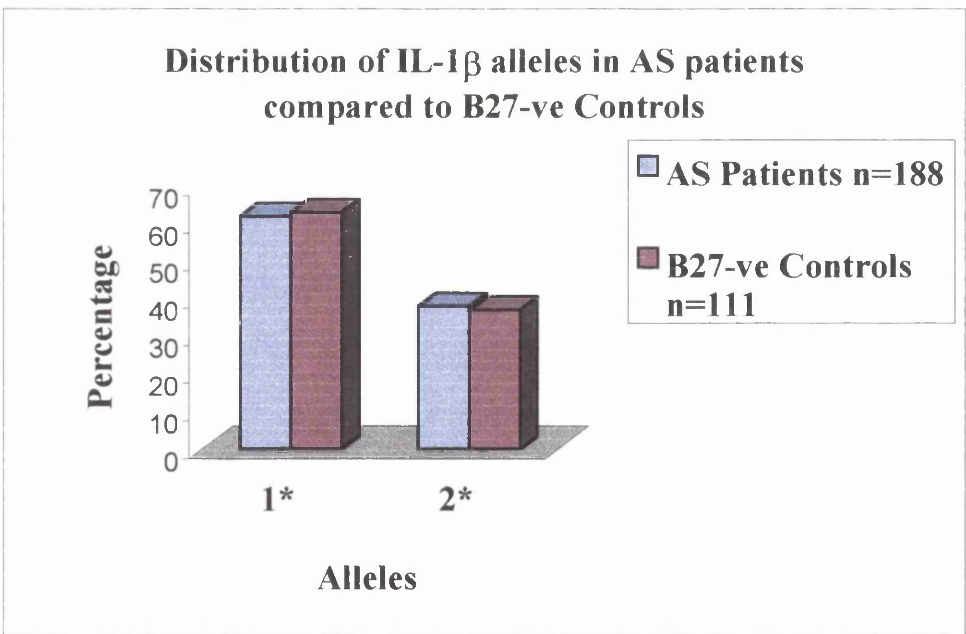
Legend for Table 41
The distributions of the IL-1 β alleles and genotypes in 188 AS patients compared to 111 B27 negative controls are shown in Table 41. No significant differences were seen between the two groups.

IL-1 beta	**AS patients n=188 (%)**	**B27 Positive Controls n=81 (%)**
1*1	71(38)	32(40)
1*2	91(48)	39(48)
2*2	26(14)	10(12)
1*	233(62)	103(64)
2*	143(38)	59(36)

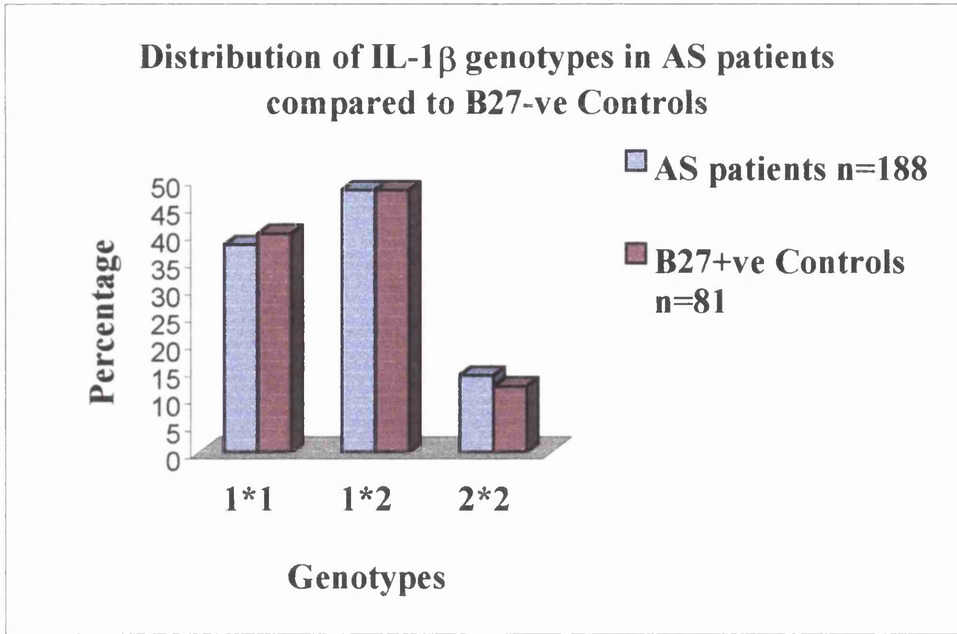
Legend for Table 42
No significant differences were seen in the distribution of the IL-1 β alleles and genotypes between the 188 AS patients and the 81 B27 positive controls.



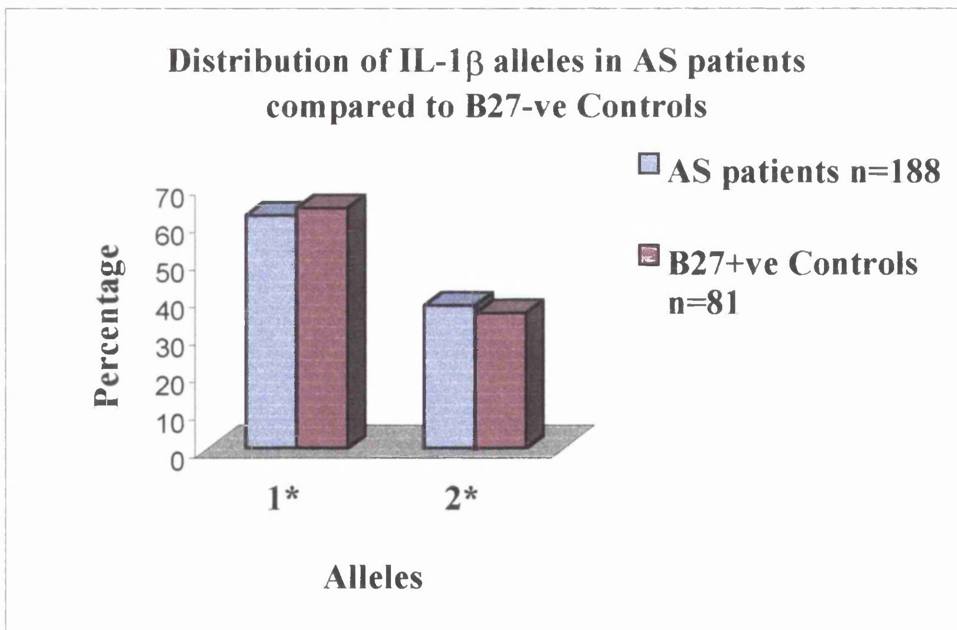
Legend for Figure 47a
The distributions of the IL-1b genotypes in AS patients compared to B27-ve Controls are shown in Figure 47a. No significant differences were seen between the two groups.



Legend for Figure 47b
The distributions of the IL-1b alleles in the AS patients compared to the B27-ve controls are shown in Figure47b. There were no significant differences.



Legend for Figure 48a
The distributions of the IL-1b genotypes in the AS patients compared to the B27+ve controls are shown in Figure 48a. No significant differences were seen between the two groups.



Legend for Figure 48b
The distributions of the IL-1β alleles in the AS patients compared to the B27+ve controls are shown in Figure 48b. There were no significant differences.

Polymorphic site	**Alleles**	**Age of Onset <21 yrs (%)**	**Age of Onset >21 yrs (%)**
IL-1 α	1*	73 (70)	65 (59)
	2*	31 (30)	45 (41)
IL-1 β	1*	68 (65)	63 (58)
	2*	36 (35)	45 (42)
IL-1Ra	1*	79 (84)	88 (82)
	2*	14 (15)	20 (18)
	3*	1 (1)	0 (0)

Legend for Table 43
The distribution of the IL1 alleles in those patients with an onset of disease below the age of 21 years compared to those with an onset equal to or greater than 21 years is shown in Table 43. In the 52 AS patients with an onset below the age of 21 the IL-1 α 1* allele was increased compared to the 55 patients with a later onset (70% Vs 59%) but this was not statistically significant.

Polymorphic site	Alleles	Uveitis Present (%)	Uveitis Absent (%)
IL-1 α	1*	48 (63)	90 (65)
	2*	28 (37)	48 (35)
IL-1 β	1*	46 (59)	83 (63)
	2*	32 (41)	49 (37)
IL-1Ra	1*	66 (89)	100 (78)
	2*	8 (11)	26 (20)
	3*	0 (0)	2 (2)

Legend for Table 44
Table 44 describes the distribution of alleles at each of the IL1 sites investigated in AS patients with uveitis compared to those without. No significant differences were seen between the two groups.

Polymorphic site	Alleles	Peripheral joint disease present (%)	Peripheral joint disease absent (%)
IL-1α	1*	54 (68)	85 (62)
	2*	26 (32)	51 (38)
IL-1β	1*	48 (60)	82 (62)
	2*	32 (40)	50 (38)
IL-1Ra	1*	60 (83)	108 (82)
	2*	11 (15)	23 (17)
	3*	1 (2)	1 (1)

Legend for Table 45

The results of the distribution of each of the alleles at the three polymorphic sites investigated in AS patients with and without peripheral joint disease are given in Table 45. There were no significant differences between the AS patients who had a history of peripheral joint disease with those that did not at any of the sites investigated.

5.5 Interleukin 1 Receptor Antagonist Discussion

Several studies have been carried out to investigate associations with the IL-1Ra VNTR and inflammatory conditions in particular inflammatory bowel disease. Like AS inflammatory bowel disease has a genetic predisposition and has been associated with genes in the MHC Class II region such as HLA-DR2 with ulcerative colitis and HLA DR1 in Crohn's disease. Again like AS these are not thought to be the only genes involved (Toyoda., 1993).

The role of IL-1Ra in inflammatory bowel disease has suggested that the IL-1 genes may be suitable candidate genes for this disease, evidence for this comes from both animal models and studies on biopsy material from diseased tissue. Neutralisation of endogenous IL-1Ra with specific antibodies in an animal model of rabbit immune colitis failed to resolve the colitis or the chronic inflammation (Ferretti, 1994). Similarly the administration of recombinant IL-1Ra in these animals prevented the development of colitis (Cominelli, 1992). Several studies have been carried out to investigate the role of polymorphisms within the IL-1 genes in relation to susceptibility and severity of inflammatory bowel disease. In a study carried out by Mansfield etal an association between allele 2 of the IL-1Ra and patients with ulcerative colitis was seen although other studies have failed to confirm this (Hacker, 1997). Increased carriage of allele 2 has also been associated with other conditions such as reduced bone loss at the spine in early postmenopausal women (Keen, 1998). Inflammatory bowel disease is frequently seen in patients with AS and consequently it is feasible that IL-1Ra could have a role to play in disease susceptibility to AS.

The distribution of the IL-1Ra alleles of the 86bp VNTR in intron 2 of the IL-1Ra gene was investigated in 182 AS patients and compared to the control populations. A significant increase in the carriage of allele 2 was found in the AS patients compared to the controls.

Association studies and twin analyses have not previously examined the IL-1 locus in AS patients. However, the recent study of AS sibling pairs showed that one area on chromosome 2 marked by microsatellite D2S160 is strongly associated with AS (Brown, 1998). This marker is within 6 centimorgans of the genes in the IL-1 family (Genebank:http://www.ncbi.nlm.nih.gov/genemap/map.cgi?CHR=2),so the presence of a polymorphism in the IL-1Ra gene could represent a candidate gene in AS associated with the D2S160 marker. However, other important genes close to D2S160 could be relevant in which case the IL-1Ra association would be due to linkage disequilibrium.

An association with allele 2 of this polymorphic site and increased production of IL-1Ra protein has been established in mononuclear cells from healthy individuals when stimulated with GM-CSF (Danis, 1995). In a second study of 200 healthy blood donors allele 2 has been associated with higher levels of IL-1Ra in plasma (Hurme, 1998). Although in one study this allele was associated with reduced expression of mRNA for IL-1Ra in tissues from ulcerative colitis (Carter, 1999). These studies suggest that the presence of the IL-1Ra allele 2 could be linked with high steady state production of IL-1Ra, and increased levels of IL-Ra production in response to gram negative antigenic stimulation. By implication it would imply that in these AS patients a poorer response to the standard levels of IL-1 would ensue.

The apparent discrepancies between the distributions of the IL-1Ra alleles in the groups seen in this study in comparison to other groups could reflect differences in gene inheritance dependent on the local genetic pool. The results were similar between the local unselected normal group and the HLA B27 normals indicating this frequency is likely to be a true representation of gene frequencies in the West of Scotland. This is confirmed by the observation that in 100 rheumatoid arthritis patients, also local to the West of Scotland, the IL-1Ra 2 allele frequency was similar to those examined in this study (Crilly A Submitted to Ann Rheum Dis). In a study of Czech RA patients and controls the frequency of the IL-1Ra alleles were similar to those seen in this study (Vencovsky, 2000). This comparison between populations highlights the importance of using locally recruited controls in comparative studies.

In conclusion the increased carriage of the IL-1Ra allele 2, which has been suggested to be associated with increased production of IL-1Ra, may have a role to play in the disease pathogenesis in AS patients.

5.6 Interleukin 1 Alpha Discussion

IL-1α is known to contribute to inflammatory joint disease through its catabolic effects on cartilage and its ability to stimulate other inflammatory mediators. It also stimulates fever and directly or indirectly through IL-6 induces the acute phase response. High levels of IL-1α and β have been detected in synovial fluid from RA patients and serum levels of circulating IL-1 have been related to disease severity. Direct roles for IL-1 have come from animal studies where anti-IL-1 antibodies are able to reduce cartilage destruction in murine arthritis models (Van de Loo, 1990). In contrast the injection of IL-1 directly into the joints of animals causes leukocyte accumulation and cartilage degradation (Pettipher, 1986).

There are several polymorphic sites within the IL-1α gene which have been investigated in a variety of autoimmune diseases including inflammatory bowel disease, early onset pauciarticular juvenile rheumatoid arthritis (EOPA-JRA), Graves disease and rheumatoid arthritis. Within intron 6 of the gene is a 46bp VNTR with a total of seven alleles being demonstrated in Caucasian populations (Bailly, 1993). This VNTR may have some functional relevance to the actual protein levels produced. However in a study of RA patients no significant differences were seen compared to controls (Bailly, 1995). No associations for the AC dinucleotide repeat within intron 5 have been established as yet. However the Cytosine to Thymine substitution at position –889 relative to the transcription start site has been associated with EOPA-JRA. An increase in the carriage of allele 2 was seen in the patients compared to the controls (McDowell, 1995). However this was not confirmed in a second study where no association was seen in a group of British oligoarticular juvenile chronic arthritis

individuals (Donn, 1999). Several studies have established that single base pair changes can alter the transcription of proteins either by affecting the binding of trans-activating factors or by alterations of the secondary structure of the DNA leading to interference of the protein-protein interactions within the transcriptional assembly (Smith, 1992).

In this study the distribution of the IL-1α alleles were investigated in a large group of AS patients compared to controls. No significant differences were seen either in the distribution of alleles, genotypes or in comparison to extra disease features. Therefore it is unlikely that the IL-1α site has a role to play in the disease pathogenesis of AS.

5.7 Interleukin 1 Beta Discussion

IL-1β is structurally related to IL-1α with both exerting their effects through binding to two specific receptors IL-1R type I and type II. IL-1α binds preferentially to the IL-1R type I with IL-1β binding better to the type II receptor. It has been suggested that it is only binding to the type I receptor that is capable of transducing a signal (Sims, 1993). The binding to the type II receptor is thought to act as a decoy and therefore aids the regulation of IL-1 under appropriate conditions.

Within the IL-1β gene are several polymorphic sites the +3953, which is bi-allelic and the site investigated in this study the cytosine to thymine single base change at position –511 within the promoter region. To date no functional association has been assigned to this site but since it lies within the promoter region it cannot be ruled out. No significant associations with the site and any autoimmune diseases have been established but it has been proposed that the IL-1β and the IL-1Ra genes are in linkage

disequilibrium. From physical mapping studies the IL-1β gene has been shown to lie closer to IL-1Ra (Nicklin, 1994). Allelic associations between IL-1β and IL-1Ra have been investigated since it has been suggested that it is the ratio between the two proteins that is crucial in the regulation of inflammation. It could be that there is co-ordinate regulation between the two genes.

Several studies have shown associations between alleles at the –511 site and the VNTR in the IL-1Ra site. Allele 2 of the IL-1Ra site has been associated with allele 2 of the IL-1β gene and also with the absence of allele 2 at the +3953 site (Santtila, 1998). Mononuclear cells from carriers of allele 2 at –511 and non-carriers of allele 2 at +3953 had a slight increase in their ability to produce IL-1β *in vitro*. It has also been shown that the increase in the IL-1Ra protein plasma levels seen in association with the carriage of IL-1Ra allele 2 also requires the presence of the –511 allele 2. The regulation of the IL-1Ra production appears to be under the influence of both the IL-Ra and IL-1β genes (Hurme, 1998). Although there were no significant differences in the distribution of the IL-1β alleles or genotypes in this AS patient group compared to controls the association with allele 2 with the IL-1Ra allele 2 was demonstrated.

A statistically significant increase of allele 2 of the IL-1β gene has been correlated with a high secretor phenotype in patients with diabetic nephropathy (Loughrey, 1998). However no significant differences were seen in the distribution of the IL-1β alleles and genotypes in the AS patients with and without extra features of disease.

In conclusion a significant increase in the carriage of IL-1Ra allele 2 was seen in the AS patients compared to the controls with no differences seen at the other two sites investigated. If this IL-1Ra allele 2 is linked with increased IL-1Ra protein production,

then it could be suggested that since IL-1Ra prevents IL-1 signalling through the IL-1 receptor, the presence of this allele will exert some limitation of IL-1 mediated signals.

Chapter Six:

Conclusion

For over forty years the strong family association with Ankylosing Spondylitis has been known and for over twenty-five years the link with the MHC class I allele HLA B27 has been demonstrated. However the fact that only a small proportion of B27 positive individuals go on to develop the disease and the pattern of disease in monozygotic and dizygotic twins has suggested other factors must be involved. While environmental factors such as Klebsiella have been implicated, various studies confirm that other genes must be important. In fact it has been proposed that there are a further five to ten other genes that may interact together to cause the disease (Brown, 1998). This study was carried out to investigate several candidate genes that could possibly play a role in susceptibility to AS.

From the clinical information gathered this particular group of AS patients would appear to be very similar in their clinical features to those of other studies. The fact that all the patients and both sets of control patients all come from the West of Scotland is important to remember as it is well established that not only are there ethnical differences but as has been shown before there are regional differences in the distribution of particular alleles.

Although the association with HLA B27 is one of the strongest known associations between an autoimmune disease and a MHC molecule the pathogenesis of the disease is still poorly understood. The lack of association with any one particular subtype of the HLA B27 molecules has been disappointing. Of the twelve possible B27 subtypes only two have been suggested as being non-disease associated (B*2706 and B*2709), although these results need to be confirmed in other studies (Fiorilo, 1995). It has been suggested that it is the lack of a C-terminal Tyr motif in these two subtypes that do not allow arthritogenic peptides to be presented by HLA B27 molecules (Garcia, 1997). In

this study all the patients that had been subtyped were B*2705 which is the most common subtype found in nearly all populations and has been reported to be the ancestral haplotype from which all other subtypes have arisen. The HLA B27 subtype present on the contra-lateral chromosome may be of importance in the susceptibility to AS in that individuals homozygous for B27 may have increased risk of disease. The presence of HLA B60 has also been shown to increase the risk of developing AS (Brown, 1996). In this study the number of patients homozygous for HLA B27 was significantly increased in comparison to the control population. This confirms previous studies were the apparent frequency of homozygotes is greater than the expected frequency (Khan, 1978). However no increase in the incidence of HLA B60 was seen in our study.

Whilst the importance of B27 is not in question, the fact that 75% of HLA B27 positive identical twins are concordant for the disease compared to only 27% of HLA B27 positive non-identical twins confirm the significance of B27 to the development of the disease but highlight the requirement for other genes to be involved (Salvetti, 2000). Animal studies also confirm these findings along with the importance of an environmental trigger as can be seen when the animals are maintained in a germ free environment in that they do not develop disease. Rats transgenic for HLA-B*2705 and human β2-microglobulin are susceptible to developing an inflammatory arthritis similar to the B27-associated human disease (Hammer, 1990). However backcross studies in these animal models (Taurog, 1999) also confirm the importance of the particular genetic background of the animals in disease susceptibility confirming again that AS is an oligo-genetic disease.

To investigate other possible candidate genes, three regions were investigated, two within the MHC on chromosome six and one on chromosome 2. Within the MHC class II region lie the DRβ1* alleles where a previous association with DRβ1*01 alleles has been demonstrated in AS patients (Brown, 1998). This has been confirmed in our study where a significant increase in the distribution of the DRβ1* alleles was seen in the AS patients when compared to the control groups. This association with DRβ1* 01 alleles would appear to be independent of B27 confirming the work of Brown and colleagues. No associations where seen between any of the DRβ1* alleles and the presence of any extra disease features that may be indicators of disease severity.

Previous studies have shown inter-individual differences in the production of TNFα in relation to particular alleles of the class II locus. The presence of HLA-DR2 has been linked with decreased TNFα production following stimulation of PBMC's by LPS (Jacob, 1990), implying the MHC can exert some genetic control over TNFα production.

The close proximity between the MHC class II locus and the TNF locus has raised the possibility that any variations within the TNF locus could have arisen simply because of linkage disequilibrium with the MHC. Studies on cell lines have shown haplotypes that cross both the Class I and Class II loci are inherited as a group (Deglie-Esposit, 1992). The HLA A1-B8-DR3-DQ2 haplotype is frequently seen in autoimmune diseases in particular SLE and has been linked with increased TNFα production (Jacob, 1988). Further studies have confirmed the presence of certain TNF alleles in this haplotype, including the TNFb3 and TNFa2 microsatellite alleles and the rare TNF –308*2 allele (Gallagher, 1997). In this particular study the frequency of the DRβ1*03 alleles which are associated with increased production of the pro-inflammatory cytokine TNFα was

reduced in AS patients. This was in conjunction with the decreased frequency of the –308*2 allele. This implies that there is a haplotype in our AS patients covering DRβ1*03, and -308*2 as is seen in other normal and patient populations. However the lack of family studies in our population makes it difficult to confirm at this time. Therefore although the DRβ1* alleles appear to have a role to play in susceptibility to the disease it does not appear to be linked to increased production of TNF.

The second region investigated lies within the MHC class III region and involved the TNF locus itself. Six different polymorphic sites were examined; three single base pair substitutions that were examined by restriction enzymes and three microsatellites that where examined by PCR and electrophoresis. The results clearly showed a significant increase in the distribution of the –308*1 allele in the AS patients which implied a link between this allele in the TNF promoter that is present in the patients but not in the HLA B27 positive controls. It should be noted however that the HLA B27 positive normal individuals were not examined by a clinician, nor where they X-rayed they only completed a general health questionnaire. As has been reported the incidence of asymptomatic AS in the general population may be higher than expected and therefore some of the controls included in this study may have underlying disease or may go on to develop the disease (Braun, 1998). It would be of interest to the know the frequency of the –308*1 allele in HLA B27 negative AS patients but as this consists of less than 10% of patients multi-center studies would be required to achieve sufficient numbers. However at the –308 site the significant increase of the –308.1* allele in the patients was different in both control populations suggesting that this is disease associated. This finding has been confirmed by some groups and not by others however in the studies

were this increase has not been seen the number of subjects and controls were small (Verjans, 1994). It has been proposed that the –308*2 allele actually has a protective effect (Hohler, 1998). The functional consequences of this polymorphism have been the subject of much debate. Some studies have associated the –308*2 allele with increased production of TNFα, the increased frequency of the –308*1 allele in the AS patients suggest a less pro-inflammatory response in these patients. In fact T cell stimulation with PHA produces a response more characteristic of a Th_2 profile. T cells from AS patients produce less TNFα and IFN γ than controls when analysed by ELISA of supernatants and Facs analysis of stained PBMCs (Rudwaleit, 2001). This is also seen in other spondyloarthropathies such as Reactive arthritis where it has been proposed that the decreased TNFα production might lead to a less active pro-inflammatory response which contributes to the bacterial persistence seen in ReA (Braun, 1999).

The third region to be investigated was on chromosome two where the genes for the pro-inflammatory cytokine IL-1 lie. The genes for IL-1α, IL-1β and IL-1Ra all lie close together on this region that have been highlighted in a large genome screen as a possible candidate region. Single base pair substitutions where examined in the IL-1α and IL-1β genes along with an 86bp repeat in the IL-1Ra gene. No associations were seen in the IL-1α or IL-1β sites. However a significant increase in the carriage of the IL-1Ra 2*allele was seen in the AS patients compared to the controls. This allele which has been shown to predispose to increased levels of the anti-inflammatory cytokine IL-1Ra (Hurme, 1998) would appear to be disease associated. A recent study investigating the IL-1 gene cluster in multiplex families with spondylarthropathies did not find any associations in particular with the IL-1Ra 86bp VNTR. However numbers in this study where small and

not all the subjects studied where HLA B27 positive (Djouadi, 2001). Although no obvious link exists between the IL-1 cluster and the MHC, this does suggest that these patients are far from genetically homogeneous. Therefore it could be suggested that the carriage of an allele associated with increased production of IL-1Ra could lead to decreased effectiveness of the pro-inflammatory cytokines IL-1α and β. This in itself could lead to decreased TNFα production because IL-1 can initiate production of other pro-inflammatory cytokines.

The results in this study suggest associations with the IL-1Ra 2*allele on chromosome two, the –308 1* allele in the MHC class III region and the HLA DRβ1*01 alleles with predisposition to AS. However, we have also been able to show that in patients and controls that carry HLA-B27 other haplotypes could exist across the TNF locus into the MHC Class II region. However to confirm the presence of these haplotypes large family studies would be required.

Of the associations established in this study none were associated with any of the alleles that have previously been linked to increased production of TNFα. Although increased transcription and serum levels have been shown previously there is little evidence to suggest that there are increased levels of TNFα protein in the joint. In the AS patients mRNA for TNFα has been demonstrated in a small number of sacroiliac biospies, but there is no direct evidence that the actual TNF protein is increased in these patients. Increased serum levels of TNFα have been previously demonstrated levels are not as high as other autoimmune diseases such as rheumatoid arthritis and they have been shown not to correlate with clinical markers of disease activity (Gratacos, 1994). In fact in AS patients without additional peripherial joint disease, ESR and CRP levels would

appear to be normal (R.D.Sturrock, personal communication) suggesting that in patients with sacro-ileitis alone, these individuals are not undergoing a systemic pro-inflammatory response. In contrast the use of anti-TNF therapy has shown some promising results in the treatment of AS patients (Braun, 2000), but only those with peripheral joint disease. There is no evidence of change to the clinical or pathological features of sacro-ileitis. Efficacy, placebo-controlled trials and long term safety need additional evaluation.

It could be hypothesised that the genetic associations seen in this study could lead to a reduced pro-inflammatory response from monocytes; firstly reduced TNF production and secondly a less effective IL-1 response. Studies in systemic lupus erythematosus have shown an association between multiple polymorphic sites predicting an increased predisposition to apoptosis. It could be hypothesised that in AS a similar prediction could be built up for multiple polymorphisms linked with a less effective pro-inflammatory cytokine response. Since AS has the appearance of a fibrotic disease it could be that genes involved in the fibrosis process such as transforming growth factors (TGFβ) may be important. TGFβ has been implicated in the process of scarring and matrix synthesis and in AS skeletal lesions it is the process of fibrosis and ossification that predominate (Edwards, 2000). TGFβ is known to stimulate IgA production and in patients with AS increased levels of IgA have been detected in serum. In sacroiliac biopsies mRNA for TGFβ has been detected in particular in areas of fibroblast infiltration (Braun, 1998).

Future work is required to confirm this hypothesis and preliminary work investigating polymoprhisms within the TGFβ1 gene has shown promising results in that a particular allele associated with increased TGFβ production is increased in the AS patients (McGarry, 2001). Other studies have demonstrated associations with this allele and other

fibrotic diseases. Other genes involved in the fibrotic process may be of interest. For example the collagen genes may be likely candidates since levels of IgM and IgG antibodies to four of the collagens have been shown to be increased in AS patients (Tiwana, 2001).

This study would be strengthened if the results shown here could be demonstrated in a second population of AS patients. A second group of Hungarian patients and controls are currently under investigation.

References

Abraham LJ, Grimsley G and Zang WJ, Degli-Esposti MA, Dawkins RL. Polymorphism in the human B144 gene in different MHC haplotypes. European Journal of Immunogenetics.19(3):165-8,1992

Abraham LJ, French MA, Dawkins RL. Polymorphic MHC ancestral haplotypes affect the activity of tumour necrosis factor-alpha. Clin Exp Immunol. 92(1): 14-18, 1993

Kroger KM, Carville KS, Abraham LJ. The –308 tumour necrosis factor-alpha promoter polymorphism effects transcription. Molecular Immunology. 34(5): 391-399,1997

Anderson G, Evolution of Human HLA DR region, Frontiers in Bioscience. 3: 739-745,1998

Arend WP, Malyak M, Guthridge CJ, Gabay C. Interleukin-1 receptor antagonist: Role in biology. Annul Rev. Imunol. 16:27-55, 1998

Armstrong RD, Panayi GS, Welsh KI. Histocompatibility antigens in psoriasis, psoriatic arthropathy, and Ankylosing spondylitis. Ann Rheum Dis. 42: 142-6, 1983

Bailly S, Di Giovine FS, Duff GW, Polymorphic tandem repeat region in interleukin-1 alpha intron 6. Human Genetics. 9(1): 2327-33, 1993

Bailly S,Hayem G, Fay M, Kahn MF, Gougerot-Pocidalo MA. Absence of correlation between IL-1 alpha intron 6 polymorphism and rheumatoid arthritis. Br J Rheumatol. 34(12): 1123-1126, 1995

Beck S, Trowsdale J. Sequence organisation of the class II region of the human MHC. Immunol Rev, 167:201-210, 1999

Bendtzen K, Morling H, Fomsgaard A, Svenson M, Jakobsen B, Odum N, Svejgaard A. Association between HLA-Dr2 and production of tumour necrosis factor alpha and interleukin 1 by mononuclear cells activated by lipopolysaccharide. Scand J Immunol. 28(5):599-606, 1988

Bennett PH, and Burch TA, Population studies of Rheumatic Diseases. Excerpta Medica International Congress series, Amsterdam, no. 148: 456-457,1968

Blin N, Stafford DW. A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res 3(9):2303-2308, 1976

Bollow M, Fischer T, Rei hauer H, Backhaus M, Sieper J, Hamm B, Braun J. Quantitative analyses of sacroiliac biopsies in Spondyloarthropathies: T cells and macrophages predominate in early and active sacroiliitis cellularity correlates with the

degree of enhancement detected by magnetic resonance imaging. Ann Rheum Dis 59:135-140, 2000

Bourke JB, A review of the palaeopathology of Arthritic Diseases in Antiquity, Edited by D. Drothwell and A.T. Sardison p352. 1967

Braun J, Bollow M, Neure L, Seipelt E, Seyrekbasan F, Herbst H, Eggens U, Distler A. and Seiper J. Use of immunohistologic and in situ hybridization techniques in the examination of sacroiliac joint biopsy specimens from patients with ankylosing spondylitis. Arthritis and Rheumatism 38(4): 499-505, 1995

Braun J, Bollow M, Remlinger G, Eggens U, Rudwaleit M, Distler A. and Sieper J. Prevalence of Spondylarthropathies in HLA B27 positive and Negative Blood Donors, Arthritis and Rheumatism 41: 58-67, 1998

Braun J, Yin Z, Spiller I, Siegert S, Rudwaleit M, Distler A. and Sieper J. Low secretion of tumour necrosis factor alpha, but no other TH1 or Th2 cytokines, by peripheral blood mononuclear cells correlates with chronicity in reactive arthritis. Arthritis and Rheumatism. 42(10)2039-44, 1999

Breur-Vriensendorp BS, Dekker-Saeys AJ, Ivanyi P. Distribution of HLA-B27 subtypes in patients with ankylosing spondylitis: The disease is associated with a common determinant of the various B27 molecules. Annals of the Rheumatic Diseases. 46(6): 353-6, 1987

Brewerton DA, Hart FD, Nichols A, Caffery James DCO. And Sturrock RD. Ankylosing spondylitis and HL-A27, Lancet, I, 904-907, 1973

Brinkman BMN,Zuijdgeest D, Kaijzel EL, Breedveld FC, Verweij CL. Relevance of the tumour necrosis factor alpha (TNFalpha) –308 promoter polymorphism in TNFalpha gene regulation. Journal of Inflammation. 46(1): 32-41, 1996

Brodie BC, Pathological and surgical observations on the diseases of the joints. 5th Edition. London: Longman, Brown, Green and Longmans, 1850

Brown MA, Wordsworth BP. Predisposing factors to Spondyloarthropathies. Curr Opin Rheumatol. 9: 308-14,1997

Brown MA, Kennedy LG, McGregor AJ, Darke C, Duncan E, Shatford JL, Taylor A, Calin A. and Wordsworth BP. Susceptibility to Ankylosing spondylitis in Twins: The role of genes, HLA and the environment, Arthritis and Rheumatism, 40(10): 1823-1828, 1997

Brown MA, Pile KD, Kennedy LG, Calin A, Darke C, Bell J, Wordsworth BP. And Cornelis F. HLA class I associations of ankylosing spondylitis in the white population in the United Kingdom, Annals of the Rheumatic Diseases, 55(4): 268-270, 1996

Brown MA, Pile KD, Kennedy LG, Campbell D, Andrew L, March R, Shatford JL, Weeks DE, Calin A, Wordsworth BP. A genome-wide screen for susceptibility loci in ankylosing spondylitis. Arthritis and Rheumatism 41(4): 588-95, 1998

Brown MA, Kennedy LG, Darke C, Gibson K, Pile KD, Shatford JL, Taylor A, Calin A, Wordsworth BP. The effect of HLA-DR genes on susceptibility to and severity of ankylosing spondylitis. Arthritis and Rheumatism. 41(3): 460-465, 1998

Brown MA, Laval SH, Brophy S, Calin A. Recurrence risk modelling of the genetic susceptibility to Ankylosing spondylitis. Ann Rheum Dis 59:883-886

Burges-Vargas R, Naranjo A, Castillo J, etal AS in the Mexican Mestizo. Patterns of disease according to age at onset. J. Rheumatology, 16: 186-191, 1989

Bywaters EGL, Pathology of the Spondylarthropathies. In Spondylarthropathies [ed. A.Calin] 1984 pp 43-68. Grune and Stratton, Orlando, FA.

Calin A, Fries JF. Striking prevalence of ankylosing spondylitis in "healthy" w27 positive males and females. New England Journal of Medicine, 293(17): 835-839, 1975

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumours, Proc. Natl. Acd. Sci. USA 72: 3666-3670, 1975

Carter MJ, Duff GW, Lobo AJ, DiGiovine FS. Allele specific mRNA assessment in colonic mucosum ulcerative colitis: Association of IL-1Ra gene allele with reduced steady state mRNA. Gastroenterology 116: G2952, 1999

Caskey CT, Pizzuti A, Fu YH, Fenwick RG, Nelson DL. Triplet repeat mutations in human disease. Science 256(5058): 784-789, 1992

Chu CQ, Field M, Allard S, Abney E, Feldmann M, Maini RN. Detection of cytokines at the cartilage/pannus junction in patients with rheumatoid arthritis: implications for the role of cytokines in cartilage destruction and repair. Br J Rheumatol. 31: 653-661, 1992

Coley WB, Am J Med Sci. 131: 375-430, 1906

Cominelli F, Nast CC, Duchini A, Lee M. Recombinant Interleukin 1 receptor antagonist blocks the proinflammatory activity of endogenous interleukin –1 in rabbit immune colitis. Gastroenterology 103: 65-71, 1992

Court-Brown WM. And Doll R, Mortality from cancer and other causes after radiotherapy for ankylosing spondylitis, British Medical Journal, 2: 1327-32, 1965

Crilly A, Maiden N, Capell HA, Madhok R. The predictive value of Interleukin 1 gene polymorphisms for surgery. (in press)

D'Amato M, Fiorillo MT, Carcassi C, Mathieu A, Zuccarelli A, Bitti PP, Tosi R, Sorrentino R. Relevance of residue 116 of HLA-B27 in determining susceptibility to ankylosing spondylitis. Eur J Immunol. 25(11): 3199-3201, 1995

Danis VA, Millington M, Hyland VJ, Grennan D. Cytokine production by normal human monocytes: Inter-subject and variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. Clin and Exp Immunol. 99(2): 303-310, 1995

Danis VA, Millington M, Hyland V, Lawford R, Huang Q, Grennan D. Increased frequency of the uncommon allele of a tumour necrosis factor alpha gene polymorphism in rheumatoid arthritis and systemic lupus erythamatous. Disease Markers 12(2), 127-133, 1995

Dawkins RL, Leaver A, Cameron PU, Martin E, Kay PH, Christiansen FT. Some disease-associated ancestral haplotypes carry a polymorphism of TNF. Hum Immunol 42:9-14,1995

Deglie-Esposit MA, Leaver AL, Christiansen FT, Witt CS, Abraham LJ, Dawkins RL. Ancestral haplotypes: conserved population MHC haplotypes. Human Immunology. 26:9-14, 1995

Dinarello CA, Wolff SM. The role of Interleukin-1 in disease. N Engl J Med, 328:106-113, 1993

Djouadi K, Nedelec B, Tamouza R, Genin E, Ramasawmy R, Charron D, Delpech M, Laoussadi S. Interleukin 1 gene cluster polymorphisms in multiplex families with spondylarthropathies. Cytokine 13(2):98-103, 2001

Donn RP, Farhan AJ, Barrett JH, Thomson W, Worthington J, Ollier WE, Absence of association between interleukin 1 alpha and oligarticular juvenile chronic arthritis in UK patients. Rheumatology 38(2): 171-5, 1999

Edwards JCW, Bowness P, Archer JR. Jekyll and Hyde: the transformation of HLA-B27. Immunology Today. 21(6):256-260,2000

Elliot MJ, Maini RN, Feldmann M, Brennan FM, Kalden JR, Aontoni C, Smolen JS, Leeb B, Breedveld JD, Macfarlane JD. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor α (A2) versus placebo in rheumatoid arthritis. Lancet 344:1105-1110,1994

Falkenbach A, Griessmayer H, Tripathi R. The impact of a positive family history on the prognosis in patients with ankylosing spondylitis.Weiner Linische Wochenschrift, 110(1): 20-22, 1998

Feldtkeller E, Disease outcome in male vs female suffers from spondyloarthritis. Aktuelle Rheumatologie, 23(5):145-153, 1998

Ferretti M,Casini-Raggiv, Pizarro TT, Eisenberg SP, Hast CC, Cominelli F. Neutralisation of endogenous Interleukin-1 receptor antagonist exacerbates and prolongs inflammation in rabbit colitis. J Clin Invest 94(1): 449-53, 1994

Field M, Gallagher G, Eskdale J. Tumour necrosis factor locus polymorphisms in rheumatoid arthritis. Tissue Antigens 50:303-7, 1997

Firestein GS, Boyle DL, Yu C, Paine MM, Whisenand TD,Zvaifler NJ, Arend WP. Synovial interleukin-1 receptor antagonist and interleukin-1 balance in rheumatoid arthritis. Arthritis and Rheumatism, 37(5): 644-652, 1994

Fiorilo MT, Greco G, Sorrentino R. the Asp 116-His 116 substitution in a novel HLA B27 subtype influences the acceptance of the peptide C- terminal anchor. Immunogenetics, 41:38-39, 1995

Fraile A, Nieto A, Beraun Y, Vinasco J, Mataran L, Martin J. Tumour necrosis factor gene polymorphisms in ankylosing spondylitis. Tissue Antigens. 51(4): 386-390, 1998

Galbraith GM, Steed RB, Sanders JJ, Pandey JP. Tumour necrosis factor alpha production by oral leukocytes: influences of tumour necrosis factor genotype. J Peridontol. 69(4): 428-433, 1998

Gallagher G, Eskdale J, Oh H-H, Richards SD, Campbell DA, Field M. Polymorphisms in the TNF gene cluster and MHC serotypes in the West of Scotland. Immunogenetics, 45:188-194,1997

Garcia F, Marina A, Lopez de Castro JA. Lack of carboxyl-terminal tyrosine distinguishes the B*2706- bound peptide repertoire from those of B*2704 and other HLA-B27 subtypes associated with Ankylosing spondylitis. Tissue |Antigens. 49:215-221, 1997

Goldfeld AE, Doyle C. and Maniatis T. Human tumour necrosis factor alpha gene regulation by virus and LPS. PNAS, 87(82): 9769-73, 1990

Gratacos J, Collado A, Filella X, Sanmarti R, Canete J, Llena J, Molina R, Ballesta A, MunozGomez J. Serum cytokines (IL-6, TNF-alpha, IL-1beta and IFN-gamma) in ankylosing spondylitis: A close correlation between serum IL-6 and disease activity and severity. Br J Rheumatol. 33(10): 927-931, 1994

Hacker UT, Gomolka M, Keller E, Eigler A, Folwaczny C, Fricke H, Albert E, Loeschke K, Endres S. Lack of association between an interleukin-1 receptor antagonist gene polymorphism and ulcerative colitis. Gut. 40(5): 623-627, 1997

Hajeer AH, Worthington J, Silman AJ, Ollier WE. Association of tumour necrosis factor microsatellite polymorphisms with HLA-DRB1*04-bearing haplotypes in rheumatoid arthritis patients. Arthritis and Rheumatism, 39(7): 1109-14, 1996

Hammer RE, Maika SD, Richardson JA, Tang JP, Taurog JD. Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human β_2m: an animal model of HLA-B27 associated human disorders. Cell 63: 1099-112, 1990

Hill HFH, Hill AGS. And Bodmer JG, Clinical diagnosis of ankylosing spondylitis in women and relation to presence of HLA B27. Annals of Rheumatic Diseases, 35, 267, 1976

Hjelmstrom P, Peacock CS, Giscombe R, Pirskanen R, Lefvert AK, Blackwell JM, Sanjeevi CB, Polymorphism in tumour necrosis factor genes associated with myasthenia gravis. J Neuroimmunol, 88(1-2): 137-43, 1998

Holher T, Schaper T, Schneider PM, Meyer KH, Marker-Hermann E. Association of different tumour necrosis factor a promoter allele frequencies with Ankylosing spondylitis in HLA-B27 positive individuals. Arthritis and Rheumatism. 41(8):1489-1492, 1998

Holzinger I de Baey A, Messer G, Kick G, Zwierzina H, Weiss EH. Cloning and genomic characterization of LST1: A new gene in the human TNF region. Immunogenetics. 42(5), 315-322, 1995

Hurme M, Helminen M. Resistance to human cytomegalovirus infection may be influenced by genetic polymorphisms of the tumour necrosis factor-alpha and interleukin-1 receptor antagonist genes. Scand J Infect Dis. 30(5):447-9, 1998

Hurme M, Santilla S. IL-1 receptor antagonist (IL-1Ra) plasma levels are co-ordinately regulated by both IL-1Ra and IL-1 beta genes. Eur J Immunol. 28(8): 2598-602, 1998

Islam SMM, Numaga J, Fujino Y, Masuda K, Ohda H, Hirata R, Maeda H, Mitsui H. HLA DR8 and acute anterior uveitis in ankylosing spondylitis. Arthritis and Rheumatism. 38(4):547-550, 1995

Jacob CO, Fronek Z, Lewis GD, Koo M, Hansen JA, McDevitt HO, Heritable major histocmpatibility complex class II-associated differences in production of tumour necrosis factor a: relevence to genetic predisposition to systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA, 87:1233-37, 1990

Jacob CO, McDevitt HO. Tumour necrosis factor-a in murine autoimmune lupus nephritis. Nature 331: 356-8, 1988

Jones DE, Watt FE, Grove J, Newton JL, Daly AK, Gregory WL, Day CP, James OF, Bassendine MF. Tumour necrosis factor-alpha promoter polymorphisms in primary biliary cirrhosis. J Heptol. 30(2):232-6, 1999

Jones EY, Stuart DI., Walker ND. Structure of tumour necrosis factor.. Nature 338(6212):225-228, 1989

Jongeneel CV, Briant L, Udalova IA, Sevin A, Nedospasov SA, Cambon-Thomsen A. Extensive genetic polymorphism in the human tumour necrosis factor region and relation to extended HLA haplotypes.Proc Natl Acad Sci USA,88:9717-9721,1991

Jouvenne P, Chaudhary A, Buchs N, Giovine FS, Miossec GW. Possible genetic association between interleukin-1 alpha gene polymorphism and the severity of chronic polyarthritis. Eur Cytokine Netw. 10(1):33-6, 1999

Julkunen H, Synovial inflammatory cell reaction in chronic arthritis. Acta rheumatolgia Scandinavica 12: 188, 1966

Keen RW, Woodford-Richens KL, Lanchbury JS, Spector TD. Allelic variation at the interleukin-1 receptor antagonist gene is associated with early postmenopausal bone loss at the spine. Bone. 23(4):367-71,1998

Kendall MJ, Farr M, Meynell MJ, Hawkins CF. Synovial fluid in ankylosing spondylitis. Annals of the Rheumatic Diseases 32:487,1973

Kennedy LG, Will R, Calin A. Sex ratio in the sponylarthropathies and its relationship to phenotypic expression, mode of inheritance and age at onset. J Rheumatology 20(11):1900-4,1993

Khare SD, Luthra HS, David CS. Spontaneous inflammatory arthritis in HLA-B27 mice lacking b2-microglobulin: a model of human Spondyloarthropathies. J Exp Med. 82:1153-1158,1995

Khan MA, Kushner I, Braun WE, Zachary AA, Steinberg AG. HLA-B27 homozygosity in Ankylosing spondylitis: relationship to risk and severity. Tissue Antigens 11(5):434-8,1978

Kontoyiannis D, Pasparakis M, Pizarro TT, Cominelli F, Kollias G. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity. 10:387-98,1999

Kroeger KM, Abraham LJ. Identification of an AP-2 element in the –323 to –285 region of the TNF-alpha gene. Biochemistry and Molecular Biology International, 40(1):43-51,1996

La Nasa G, Mathieu A, Mulargia M, Carcassi C, Vacca A, Ledda A, Contu L. Association of the HLA-A2, CW2, B27, S31, DR2 haplotype with ankylosing spondylitis. A possible role of non-B27 factors in the disease. Disease Markers. 11(4):191-203,1993

Lee YH, Ji JD, Kim JS, Bak YT, Lee CH, Kim CH, Chae YS, Song GG. Ileocolonoscopic and histologic studies of Korean patients with ankylosing spondylitis. Scandinavian Journal of Rheumatology. 26(6):473-767,1997

Lindheimer F, Milicic A, Laval SH, Rudwaleit M, Hohler T, Wordsworth BP, Brown MA. TNF-alpha associations in British and German caucasians:suggestive evidence of susceptibility loci in ankylosing spondylitis lying centromeric of TNF-alpha. [Abstract], BSR, Brighton, 2000

Lopez LC, J Immunol 150:2675-2686,1993

Lopez LC, Sujirachato K, Mehra NK, Chiewsilp P, Isarangkura D, Kanga U, Domingnez O, Coto E, Pena M, Setien F, GonzalezRoces S. HLA-B27 subtypes in Asian patients with ankylosing spondylitis. Evidence for new associations. Tissue Antigens 45(3):169-176,1995

Loughrey BV, Maxwell PA, Fogarty DG, Middleton D, Harron JC, Patterson CC, Darke C, Savage DA. An interleukin 1B allele, which correlates with a high secretor phenotype, is associated with diabetic nephropathy. Cytokine.10(12):984-988,1998

Louis E, Franchimont D, Piron A, Gevaert Y, Schaaf-Lafontaine N, Roland S, Mahieu P, Malaise M, De Groote D, Louis R, Belaiche J. Tumour necrosis factor (TNF) gene polymorphism influences TNF-alpha production in lipopolysaccaride (LPS)-stimulated whole blood cell culture in healthy humans. Clin Exp Immunol 113(30):401-406,1998

Maksymowych WP,Tao S, Li Y, Wing M, Russell AS. Allelic variations at the TAP 1 locus influences disease phenotype in HLA B27 positive individuals with ankylosing spondylitis. Tissue Antigens 45(5):328-332,1995

Maksymowych WP, Gorodezky C, Olivo A, Alaez C, Wong C, Burgos-Vargas R, Sanchez-Corona J, Ramos-Remus C, Russell AS. HLA-DRB1*08 influences the devlopment of disease in Mexican mestizo with spondyloarthropathy. Journal of Rheumatol, 24(5):904-907,1997

Mansfield JC, Holden H, Tarlow JK, Di Giovine FS, McDowell TL, Wilson AG, Holdsworth CD, Duff GW. Novel genetic association between ulcerative colitis and

the anti-inflammatory cytokine interleukin-1 receptor antagonist. Gastroenterology 106:637-642,1994

Martinez-Borra J, Gonzalez S, Lopez-Larrea C. Genetic factors predisposing to spondylarthropathies. Arthritis and Rheumatism,43(3):485-492,2000

McDowell TL, Symons JA, Ploski R, Forre O, Duff GW. A genetic association between juvenile rheumatoid arthritis and a novel interleukin-1α polymorphism. Arthritis and Rheumatism 38(2):221-228,1995

McGarry F, Cousins L, Sturrock RD, Field M. A polymorphism within the transforming growth beta 1 (TGFb1) gene is associated with Ankylosing spondylitis (AS). BSR (abstract) 2001

McManus R, Wilson AG, Mansfield J, Weir DG, Duff GW, Kelleher D. TNF2 a polymorphism of the tumour necrosis factor alpha gene promoter is a component of the celiac disease major histocompatibility complex haplotype. European Journal of Immunology 26(9):2113-2118,1996

Messer G, Spengler U, Jung MC, Honold G, Blomer K, Pape GR, Riethmuller G, Weiss EH. Polymorphic structure of the tumour necrosis factor (TNF) locus: An Nco.1 polymorphism in the first intron of the human TNF-β gene correlates with avariant amino acid in position 26 and a reduced level of TNF-β production. J Exp Med, 173:209-219,1991

Miehle W, Schattenkirchner M, Albert D, Bunge M. HLA-DR4 in Ankylosing spondylitis with different patterns of joint involvement. Annals of Rheumatic Diseases, 44:39-44,1985

Mielants H, Vey EM, Cuvelier C, De Vos M, Goemaere S, De Clercq L, Schattteman L, Gyselbrecht L. and Elewaut D. The evolution of spondyloarthropathies in relation to gut histology. Journal of rheumatology , 22:2279-2284,1995

Mielants H, Veys EM, Cuvelier C, De Vos M, Course of gut inflammation in spondylarthropathies and therapeutic consequences. Bailliere's Clinical Rheum. 10(1):147-164,1996

Molvig J, Baek L, Christensen P, Manogue KR, Vlassara H, Platz P, Nielsen LS, Svejgaard A, Nerup J. Endotoxin-stimulated human monocyte secretion of Interleukin 1, tumour necrosis factor alpha and prostaglandin E_2 shows stable interindividual differences. Scand J Immunol, 27:705-716,1988

Mu H, Chen JJ, Jiang Y, King MC, Thomson G, Criswell LA. Tumour necrosis factor a microsatellite polymorphism is associated with rheumatoid arthritis severity through an interaction with the HLA-DRB1 shared epitope. Arthritis Rheumatism. 42(3):438-42,1999

Mulcahy B, WaldronLynch F, McDermott MF, Adams C, Amos CI, Zhu DK, Ward RH, Clegg DO, Shanahan F, Molloy MG, Ogara F. Genetic variability in the tumour necrosis factor-lymphotoxin region influences susceptibility to rheumatoid arthritis. American Journal of Human Genetics. 59(3):676-683,1996

Nahal R, Gautreau C, Amor B, Berthelot JM, Combes B, Dougados M. Genetic investigation in familial spondylarthropathy with focus on HLA region using transmission disequilibrium test [abstract]. Arthritis and Rheumatism, 39:suppl9,S122,1996

Nedospasov SA, Udalova IA, Kuprash DV, Turetskaya RL, DNA sequence polymorphism at the human tumour necrosis factor (TNF) locus. Journal of Immunology 147(3):1053-1059, 1991

Nicklin MJ, Weith A, Duff GW. A physical map of the region encompassing the human interleukin-1 alpha, interleukin-1 beta and interleukin-1 receptor antagonist genes. Genomics 19(2):382-384,1994

Ohlsson K, Bjork P, Bergenfeldt M, Hageman R, Thompson RC. IL-1Ra reduces mortality from endotoxin shock. Nature 348(6001):550,1990

Orchard TR, Thiyagaraja S, Welsh KI, Wordsworth BP, Gaston JSH, Jewell DP. Clinical phenotype is related to HLA genotype in the peripheral arthropathies of inflammatory bowel disease. Gastroenterology 118(2):274-278,2000

Perriere S, Coussediere C, Dubost JJ, Albuisson E, Sauvezie B. IL-1 receptor antagonist (IL-1Ra) gene polymorphism in Sjogren's syndrome and rheumatoid arthritis. Clin Immunol Immunopathol. 87(3):309-13,1998

Pettipher ER, Higgs GA, Henderson B. Interleukin 1 induces leukocyte infilatration and cartilage proteoglcan degradation in the synovial joint. PNAS,83(22):8749-8753,1986

Ploski R, Maksymowych W, Forre O. HLA-DR8 and susceptibility to acute anterior uveitis in Ankylosing spondylitis. Arthritis and Rheumatism 39:351,1996

Pociot F, Messer, Derkx HHF, Journal of Endotoxin Research,2:19-25,1995

Pociot F, Molvig J, Wogensen L, Worsaae H, Dalboge H, Baek L, Nerup J, A tumour necrosis factor beta gene polymorphism in relation to monokine secretion and insulin-dependent diabetes mellitus. Scand J Immunol, 33: 37-49,1991

Pociot F, Briant L, Jongeneel CV, Molvig J, Worsaae H, Abbal M, Thomsen M, Nerup J, Cambon-Thomsen A, Association of tumour necrosis factor (TNF) and class II major histocompatibility complex alleles with the secretion of TNF-α and TNF-β by human

mononuclear cells: a possible link to insulin-dependent diabetes mellitus. Eur J Immunol 23:224-231,1993

Rau H, Usadel KH, Ommert S, Badenhoop K, PVU II polymorphism of LST-1 (leucocyte specific transcript-1) in type I diabetes mellitus, Graves' disease and healthy controls. European Journal of Immunogenetics. 22(3):277-282,1995

Ren EC, Koh WH, Sim D, Boey ML, Wee GB, Chan SH, Possible protective role of HLA-B*2706 for ankylosing spondylitis. Tissue Antigens 49:67-69,1997

Repo H, Clin Exp Immunol 72:410-414, 1988

Robinson W,van der Linden Sm, Khan MA, Rentsch HU, Cats A, Russell A. HLA-Bw60 increases susceptibility to Ankylosing spondylitis in HLA-B27+ patients. Arthritis and Rheumatism. 32:1135-41,1989

Rubin LA, Amos CI, Wade JA, Martin JR, Bale SJ, Little AH, Gladman DD, Bonney GE, Rubenstein JD. and Siminovitch KA, Investigating the genetic basis for Ankylosing spondylitis. Linkage studies with the major histocompatibility complex region, Arthritis and Rheumatism 37:1212-1220,1994

Rudwaleit M, Siegert S, Yin Z, Eick J, Theil A, Radbruch A, Seiper J, Braun J. Low T cell production of TNF alpha and IFN gamma in Ankylosing spondylitis: its relation to HLA-B27 and the influence of the TNF –308 gene polymorphism. Ann Rheum Dis. 60(1):36-42,2001

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA. and Arnheim N, Enzymatic amplification of beta globin genomic sequences and restriction site anaylsis for diagnosis of sickle cell anemia. Science 230:1350-4,1985

Salvetti M, Ristori G, Bomprezzi R, Pozzilli P, Leslie RDG. Twins:mirrors of the immune system. Immunology today. 21(7):342-347,2000

Sanmarti R, Eralla MG Brancos MA, Cid MC, Colado A, Rotes-Querol J. HLA class II antigens (DR, DQ loci) and peripheral arthritis in Ankylosing spondylitis. Ann Rheum Dis 46(7):497-500,1987

Santtila S, Savinainen K, Hurme M. Presence of the IL-1Ra allele 2 (IL1RN*2) is associated with enhanced IL-1beta production in vitro. Scand J Immunol. 47(3):195-8,1998

Sham PC. And Curtis D, Monte Carlo tests for associations between disease and alleles at highly polymorphic loci, Ann Hum Genet. 59:97-105,1995

Sims JE, Gayle MA, Slack JL, Alderson MR, Bird TA, Giri JG, Colotta F, Re F, Mantovani A, Shanebeck, K, etal. Interleukin 1 signaling occurs exclusively via the type I receptor. PNAS, 90(13):6155,1993

Smith JD, Brinton EA, Breslow JL, Polymorphism in the human apoliproprotein A-I gene promoter region. Association of the minor allele with decreased production rate in vivo and promoter activity in vitro. J Clin Invest 89(6):1796-1800,1992

Strecher RM, Heredity in Joint Disease, Documenta Rheumatica [Geigy]12:1957

Sullivan KE, Wooten C, Schmeckpeper BJ, Goldman D, Petri MA. A promoter polymorphism of tumour necrosis factor a associated with systemic lupus erythematosus in African-Americans. Arthritis and Rheumatism. (12)2207-2211,1997

Tarlow JK, Blakemore AIF, Lennard A, Solari R, Hughes HN, Steinkasserer A. and Duff GW, Human Genet 91:403-404,1993

Taurog JD, Maika SD, Satumtira N, Dorris ML, McLean IL, Yanagisaawa H, Sayad A, Stagg AJ, Fox GM, Le O'Brien A, Rehman M, Zhou M, Weiner AL, Splawski JB, Richardson JA, Hammer RE. Inflammatory disease in HLA B27 transgenic rats. Immunological Reviews, 169:209-223, 2001-11-16

Tiwana H, Natt RS, Benitez-Brito R, Shah S, Wison C, Bridger S, Harbord M, Sarner M, Ebringer AE. Correlation between the immune reponses to collagens type I,II,IV and V and *Klebsiella pneumoniae* in patients with Crohn's disease and Ankylosing spondylitis. Rheumatology. 40:15-23,2001

Tountas NA, Yang H, Coulter DL, Rotter JL, Cominelli F, Increased carriage of allele 2 of IL-1 receptor antagonist (IL-1Ra) in Jewish populations, Gastroentrology, 110:A1029,1996

Toyoda H, Wang SJ, Yang HY, Redford A, Magalong D, Tyan D, McEldree CK, Pressman SR, Shanahan F, Targan SR, Rotter JI. Distinct associations of HLA class II genes with inflammatory bowel disease. Gastroenterology, 104:741-748,1993

Tuokko J, Koskinen S, Westman P, Yli-Kerttula U, Toivanen A, Ilonen J. Tumour necrosis factor microsatellites in reactive arthritis, British Journal of Rheumatology, 37:1203-1206,1998

Udalova IA, Nedospasov SA, Webb GC, Chaplin DD, Turetskaya RL. Highly informative typing of the human TNF locus using six adjacent polymorphic markers. Genomics 16:180-186,1993

Van de Loo AAJ, J Rheumatology 19:348-56,1990

Van Der Linden SM, Valkenburg HA, de Jongh BM, Cats A. The risk of developing Ankylosing spondylitis in HLA B27 positive individuals. Arthritis and Rheumatism 27(3):241-249,1984

Vencovsky J, ERRW, Oxford 2000, Abstract no. 70

Verjans GMG, van der Linden SM, van Eys GJJM, de Waal LP, Kijlstra A. A restriction fragment length polymorphism of the tumour necrosis region in patients with Ankylosing spondylitis. Arthritis and Rheumatism. 34:486-489,1991

Verjans GMG, Brinkman BMN, Van Doornik CEM, Kijlstra A, Verweij CL. Polymorphism of tumour necrosis factor alpha (TNF-alpha) at position –308 in relation to ankylosing spondylitis. Clin Exp Immunol. 97(1):45-47,1994

Weissensteiner T, Lanchbury JS. An integrated multiplex-PCR and PCR-RFLP typing system foe markers associated with seronegative arthritides. Human Immunology 59(2): 119-132,1998

West HF, The aetiology of ankylosing spondylitis, Annals of the Rheumatic Diseases 8:143,1949

Westman P, Leirisalo-Repo M, Partanen J, Koskimies S. A comparative study of HLA genes in HLA-B27 positive Ankylosing spondylitis and HLA-B27 positive peripheral reactive arthritis. Arthritis and Rheumatism 39(6):943-9,1996

Wilkinson M. and Bywaters EGL. Clinical features and course of AS as seen in a follow up of 222 hospital referred cases. Ann Rheum Dis 17:209-228,1958

Will R, Calin A., Kirwan J. Increasing age at presentation for patients with ankylosing spondylitis. Annals of Rheumatic Diseases, 51:340-342,1992

Wilson AG, di Giovine FS, Blakemore AIF, Duff GW, Single base change in the human tumour necrosis factor alpha (TNFa) gene detectable by NcoI restriction of PCR product. Hum Mol Genet, 1:353,1992

Wilson AG, de Vries N, van de Putte LBA, Duff GW, A tumour necrosis factor a polymorphism is not associated with rheumatoid arthritis. Ann Rheum Diseases, 54:601-603,1995

Wilson AG, Gordon C, di Giovine FS, de Vries N, van de Putte LBA, Duff GW. A genetic association between systemic lupus erythematosus and tumour necrosis factor alpha. Eur J Immunol 24:191-195,1994

Wu DY, Ugozzoli L, Pal BK, Wallace RB. Allele specific enzymatic amplification of b-globin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci USA 86:2757-60,1989

Yamaguchi A, Tsuchiya N, Mitsui H, Shiota M, Ogawa A, Tokunaga K, Yoshinoya S, Juji T, Ito K. Association of HLA-B39 with HLA-B27 negative Ankylosing spondylitis and pauciarticular juvenile rheumatoid arthritis in Japanese patients. Arthritis and Rheumatism,38(11):1672-1677,1995