GENETIC EPIDEMIOLOGY OF ANKYLOSING SPONDYLITIS

By

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Abstract

Introduction: Ankylosing spondylitis (AS) is a chronic inflammatory arthritis and represents the most common seronegative spondyloarthropathy with an approximate prevalence of one in one thousand Caucasians. Clinically, AS commonly presents as a predominantly axial arthritis affecting the spine and girdle joints. AS is a complex genetic disease with multiple susceptibility factors. Though it is known AS susceptibility has a strong genetic component, with a sibling recurrence risk ($\lambda_S$) of 82, the majority of the susceptibility genes are unidentified.

Objective: The objective of this study was to identify and test potential candidate genes for susceptibility to AS in the Newfoundland population. Candidate genes were chosen based on functional relevance to disease phenotype or on positional proximity to known chromosome regions of linkage to AS.

Methods: All patients with AS met the modified New York Criteria and the controls were ethnically matched. A Newfoundland cohort of 101 AS patients and 103 ethnically matched controls were genotyped using the Sequenom MassArray platform. All controls met Hardy Weinberg equilibrium. Analysis was performed using appropriate statistical tests.

Results: The minor allele frequencies for the candidate genes TLR4 Asp299Gly ($p_c$=0.05) and TNF$\alpha$ promoter polymorphisms -308 ($p_c$=0.008), -863/-1031 (in linkage disequilibrium $p_c$=0.000) were found to be associated with AS. Other candidate genes tested showed no association with disease. Minor alleles of the single nucleotide

polymorphisms chosen for the candidate genes FGFR2, TCIRG1, and DLL3 were not present in the Newfoundland population. No secondary associations of phenotype and genotype were noted for disease severity indices, gender or age of onset.

Conclusion: A novel association was noted between TLR4 and AS and the association of promoter polymorphisms of TNFα was validated in the Newfoundland population.

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## 1.1 THE PHENOTYPE

### 1.1.1 Epidemiology

Ankylosing spondylitis (AS) has been present in society since ancient times. Recent studies of X-rays taken of the mummies of Amenhotep II (Greek name Amenophis), Ramses II (Ramses the Great), and Merenptah (son of Ramses II and next Pharaoh of Egypt) show all three had ossification of the spine as is seen in AS (Feldtkeller et al., 2003).

AS is a chronic inflammatory arthritis (Laval et al., 2001), and represents the most common seronegative spondyloarthropathy with an approximate prevalence of one out of one thousand Caucasians (Brown et al., 2003). This prevalence may be underestimated as patients with mild disease are commonly overlooked. Clinically, AS commonly presents as a predominantly axial arthritis affecting the spine and girdle joints. Enthesitis, the inflammation of tendon where it inserts into bone, is a common presenting feature of AS. Peripheral arthritis of distal joints can also occur, but is less common. Extra-articular features include inflammatory bowel disease (IBD) and acute anterior uveitis, also known as iritis.

There are gender differences in AS. It has been reported that the prevalence of the disease is primarily in males with a three to one ratio of male to female AS patients (Brophy et al., 2003). There are differences in presentation of the disease for each gender (Mori et al., 2003). Clinically, male patients tend to have primarily axial involvement, while females are more likely to also have peripheral arthritis symptoms (Mori et al., 2003). Men are generally more likely to have a younger age of onset and a higher

frequency of extra-articular features such as psoriasis and inflammatory bowel disease than women (Breban et al., 2003).

## 1.1.2 Clinical Features

### Spondyloarthropathy

AS is a disease that presents primarily with axial symptoms. Lower back pain is the first symptom in three quarters of AS patients. A distinguishing characteristic of the inflammatory back pain felt with AS over mechanical back pain is the pain tends to improve with exercise and worsen with rest. Suspicion of AS is indicated when the onset is insidious, onset occurs when patient is under the age of 40, and the duration of pain is over three months. In the spinal column, the disease process is one of inflammation at areas of contact between ligament and vertebrae. In areas of enthesitis, surgical samples show edema and inflammation, extending into adjacent bone marrow (Laloux *et al.*, 2001). Cytotoxic CD8+ T cells and macrophages are plentiful, with fewer CD4+ helper T lymphocytes in these areas (McGonagle *et al.*, 2002). Biopsies of the sacroiliac joint show areas of new cartilage formation, osteitis, and subchondral granulation tissue, as well as evidence of both synovitis and enthesitis (Francois *et al.*, 2000).

### Peripheral Joints

Synovial joints are also involved in AS, most commonly in the hip and shoulder joints. Histological examination of samples from AS hip joints reveal fibrin, proliferation of lining cells, inflammatory infiltrate with lymphocytes and large numbers of plasma cells (Chang *et al.*, 1992). Manifestation of synovitis may be by erythema, swelling, and tenderness. These signs are more readily apparent in superficial joints, and may not be

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seen at all in deep joints such as the hip and shoulder, where pain and limited range of motion may be the only symptoms.

**Extra-articular Manifestations**

The eyes, lungs, gastrointestinal tract, and heart can be affected in AS. As well, AS patients can have non-specific systemic signs of inflammation such as mild fever, fatigue and weight loss. Iritis, inflammation of the uveal tract, is the most common extraarticular complication in AS, occurring in twenty five to forty percent of patients (Maksymowych *et al.*, 1995). There is some evidence that AS patients with more peripheral involvement are more likely to develop iritis (Maksymowych *et al.*, 1995). Iritis typically presents as acute unilateral eye pain, sensitivity to light and blurring of vision. Acute episodes may last two to three months, but rarely lead to permanent impairment of vision.

While the presence of diagnosed IBD in AS patients is one to four percent, subclinical inflammation of the gut and bowel is seen in forty to sixty percent of AS patients (Baeten et al., 2002). An estimated ten to fifteen percent of IBD patients have disease complicated by AS (Crane et al., 2002). The diseases seem to be linked epidemiologically through a common aberration of the immune system as both respond to similar therapies, such as the anti-TNFα biologic treatment (as reviewed in Vermiere et al., 2002).

**Prognosis**

Most patients with mild disease and minimal peripheral involvement will be able to maintain most of their joint range of motion, and functional capacity. Disease activity usually varies within the individual patient with "flares" of worsening disease activity.

Symptoms usually persist throughout life, though a small minority will enter a stage of disease "burnout" where the disease does not progress any further (Kennedy *et al.*, 1993). Prognostic indicators of worse disease activity include hip arthritis, dactylitis, poor efficacy of non-steroidal anti-inflammatory drugs (NSAID), high erythrocyte sedimentation rate (ESR) (>30), limitation in range of motion in the lumbar spine, oligoarthritis, and age of onset under 16 (Amor *et al.*, 1994). Smoking has been linked to poorer outcomes. AS does not negatively affect mortality. While quality of life is significantly decreased, lifespan is normal in severe as well as mild cases. The burden of illness in AS is high and may be comparable to that of rheumatoid arthritis (Zink *et al.*, 2000). Pain from spine inflammation is significant and persistent (Scalapino and Davis, 2003). There is impact on employability and quality of life throughout the disease course.

## 1.1.3 Laboratory Studies

Laboratory studies in AS are non-specific and non-diagnostic. ESR is frequently elevated, as are levels of C reactive protein (CRP). Mild anemia may also be seen. Serology for rheumatoid factor is typically negative. Levels of complement may be slightly elevated reflecting an acute inflammatory state. Testing for HLA-B27 has negative predictive value only, as a patient that is HLA-B27 negative is unlikely to have AS unless the clinical symptoms are diagnostic. However, the prevalence of HLA-B27 in the population without AS is high enough that the presence of the allele alone is not diagnostic for the disease, so the positive predictive value is poor.

## 1.1.4 X-Ray Findings

Radiography of the sacroiliac joints is necessary for the definitive diagnosis of AS. Radiographic grading of the changes to the sacroiliac joint progresses through normal, sclerosis with some erosions, severe erosions with widening of joint space, and finally complete ankylosis, or fusion of the joint. To best view the joint, the Ferguson view is commonly used. In this method the patient lies prone and the X-ray is angled thirty degrees towards the patient's head. It is important to recognize that early changes to the sacroiliac joint that occur in the first stages of the disease are not apparent on plain film. As radiographic changes are necessary to diagnose AS, if symptoms continue, repeated X-rays are warranted.

Other changes that can be seen on plain X-ray are changes associated with enthesitis. Erosions or osteitis of the ischial tuberosities, the iliac crest, or the femoral trochanter can be used to determine extent of peripheral involvement. Squaring of the vertebral bodies is an early change that allows suspicion of an AS diagnosis.

Magnetic Resonance Imaging is a better tool for early diagnosis of AS. Early bony changes detected by MRI progress to the radiographic changes diagnostic of AS on later X-ray. While in early cases this is the imaging method of choice, in most centres access to the MRI machine is limited for AS.

Studies are currently underway to determine the efficacy of ultrasound in early diagnosis of AS. To date, this method is infrequently used due to the large variability seen with operator expertise and the imaging itself takes a substantial amount of time with both an affected and unaffected joint for comparison. Bone density scans can be

used to assess osteoporosis levels in the spine and predict patients more likely to undergo vertebral fracture.

### 1.1.5 Diagnostic Criteria

Diagnosis is performed using the standardized modified New York criteria. To be diagnosed with AS by these criteria, patients must have radiological evidence of sacroiliitis (at least grade II bilaterally or grade III-IV unilaterally), and must have at least one of the following clinical criteria: 1) low back pain and stiffness for more than 3 months that improves with exercise but not with rest; 2) limitation of motion of the lumbar spine in both sagittal and frontal planes; 3) limitation of chest expansion relative to normal values corrected for age and sex (Braun 2003 ACR Symposium).

Differential Diagnoses for patients presenting with AS-like symptoms include rheumatoid arthritis, Reiter's syndrome, psoriatic arthritis, and diffuse idiopathic skeletal hyperostosis.

### 1.1.6 Clinical Course, Outcome, and Treatment

AS is characterized by inflammation of the axial skeleton, primarily the spine and sacroiliac joints in the pelvis (Laval et al., 2001). The inflammation is thought to come first in the disease progression, followed by joint erosion and finally ankylosis of the inflamed joints. The final stage of the disease may result in fusion of the entire spine, called bamboo spine, and complete loss of joint (Mori et al., 2003).

Pain from spine inflammation is significant and persistent (Scalapino and Davis, 2003). Pain associated with AS tends to be worse in the morning and after periods of rest

or inactivity and improves with exercise and movement. This pattern is the opposite of mechanical back pain, which improves with rest and intensifies with exercise.

Treatment regimens vary depending on the severity of the disease. NSAIDs in combination with physical therapy and exercise regimens are the standard initial therapy for AS.

Physical therapy has been shown to improve range of motion and maintain affected joint mobility as well as reducing pain symptoms (Uhrin *et al.*, 2000). Standard physical therapy involves postural training, range of motion exercises and can involve hydrotherapy. Swimming is recommended to newly diagnosed patients as a way to maintain range of motion without putting strain on the joints.

NSAIDs are initially effective in the treatment of AS. However, NSAID use may result in gastrointestinal disturbances. It has been reported that approximately 60% of AS patients experience adverse effects of NSAIDs, while 50% do not achieve pain relief while on these medications (Scalapino and Davis, 2003). Selective targeting of the cyclooxygenase-2 pathway with the new COX2 inhibitors have reduced this toxicity but not improved efficacy in all users (Mease, 2003).

When NSAIDs fail to control pain symptoms, disease modifying anti-rheumatic drugs or DMARDs are used as an alternate approach. While these drugs modify the course of inflammation, they often have deleterious side effects and primarily target peripheral joints. AS disease activity is predominantly axial, so for many AS patients, DMARDs are ineffective. All of these drugs treat the symptoms, not the cause, and do not prevent progression of the disease (Braun, 2003). Sulfasalazine and is a common DMARD used in AS for patients who fail NSAID therapy. Sulfasalazine has marginal

effects on axial symptoms, and is more linked to improvement of peripheral involvement. Side effects of sulfasalazine include nausea, dizziness, headache and rash, with more rare severe events of leukopenia and neutropenia. Pamidronate, a bisphosphanate, has shown promise in treating spondylitis in a subset of patients (Maksymowych *et al.*, 2002).

Corticosteroids such as prednisone are also commonly used when NSAIDs fail to relieve symptoms. Long term use is not recommended, however, due to the effects of steroid therapy on bone density. Methotrexate has not been shown to be efficacious in AS.

The biologic compounds are designed to lower circulating levels of the inflammatory cytokine TNFα, and treat the cause of inflammation (Braun, 2003). These agents are the best choice for AS patients who do not respond or have adverse events with conventional treatment. Indicators of a positive response to biologic agents, which was defined as a greater than 50% improvement in disease activity measured by the Bath ankylosing spondylitis disease activity index (BASDAI) within 12 weeks of treatment, included younger age, short disease duration, good functional ability (measured using the Bath ankylosing spondylitis functional index), elevated ESR and CRP, and higher disease activity (measured by BASDAI) (Rudwaleit *et al.*, 2004). Side effects of biologic therapy are those of immunosuppression: opportunistic infections, reactivation of tuberculosis, multiple sclerosis-like symptoms, and malignancy. Also, as these drugs are relatively new, there is little data on effects of long term use.

## 1.2 ETIOLOGY

AS is a complex multifactorial disease. Multiple risk factors with multiplicative effects are responsible for developing this disease. While most of these risk factors are unidentified, it is known that immunology, environment, and genetics all contribute to susceptibility.

Pathologically, there are two key steps in the development of AS. The first is an autoimmune chronic inflammatory response at sites of enthesis. The second is the healing of these lesions by conversion of cartilage to fibrous tissue. Determining the factors responsible for these pathological steps is key to the understanding of the mechanism of AS development.

### 1.2.1 Immunological

What is known about the mechanism of AS points to a role for the innate and acquired immune response in its development. While the components of the immune response contributing to AS susceptibility are currently unknown, this is an area of intense research.

One component of the immune system that has been linked to AS susceptibility is human leukocyte antigen B27. The HLA class I molecules function to present antigens from the cytoplasm to the cytotoxic CD8+ T cells of the immune system (Smith, 2002). The HLA-B27 allele in the major histocompatibility (MHC) region is strongly linked to AS. This HLA molecule has been observed to have more homology to bacterial proteins that the other HLA molecules (Ramos et al., 2002). The homology seen has led to theories of autoimmunity based on immune system stimulation by similar bacterial

peptides (Ramos et al., 2002). The HLA-B27 protein is a heterodimer of $\beta_2$-microglobulin and an $\alpha$-chain (Smith, 2002). The $\alpha$-chain contains the antigen binding cleft used to present antigens to the circulating cytotoxic T-cells. However, it is unknown whether it is a mutation in the HLA-B27 gene that is responsible for AS risk or a mutation in a gene tightly linked to HLA-B27 that is responsible.

## 1.2.2 Environmental

Evidence from animal models of AS and its common comorbidities shows a strong role of infectious agents in the development of disease. When animal models of Crohn's disease are raised in a germ-free environment, they do not develop disease while the litter-mates raised in a normal environment develop symptoms (Bonen et al., 2003). Similarly, HLA-B27 transgenic mice in sterile environments do not develop symptoms; but when removed from sterility, AS-like disease spontaneously develops. The involvement of microorganisms in developing disease implies a potential misregulation of the immune response which is triggered by exposure to a pathogen and leads to the autoimmunity seen in AS. The infection by specific microorganisms, which have yet to be identified, triggers the development of spondyloarthropathy (Rudwaleit et al., 2001).

## 1.2.3 Genetic

There are several lines of evidence that show AS has a strong genetic component. Heritability of AS is 90% as measured by twin studies (reviewed in Brown *et al.*, 2003). Sibling recurrence risk ratio ($\lambda_S$), which is the risk of developing the disease if you have an affected sibling divided by the population prevalence of the disease, is used as a

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measure of the role genetics plays in disease susceptibility. This evidence has been gathered from population based studies, twin studies, association and linkage studies.

**Population based studies:**   Population based studies have determined the risk for developing AS if a sibling is affected is $\lambda_S = 82$ in the British population, which is high compared to other rheumatologic disease such as rheumatoid arthritis ($\lambda_S = 14$) (reviewed in Turesson and Matteson, 2006) and systemic lupus erythematosus ($\lambda_S = 24$) (reviewed in Tsao, 2002). This is Risch's recurrence risk ratio, where the sibling risk ($\lambda_S$) is calculated by determining the prevalence of disease among affected siblings and dividing by the population prevalence, which is often estimated. This estimation of the denominator introduces some error in the calculation as each population has a different prevalence of disease. This figure is dependent on the population being studied, and highly heritable diseases may have low sibling recurrence risks because of this bias. This bias does not apply here as the disease is rare, and thus has a low prevalence (around 1:1000). A Risch's recurrence risk ratio greater than two is considered indicative of a genetic component in the disease etiology. A $\lambda_S$ of 82 is very high, the highest known for any complex rheumatic disease, as most complex genetic diseases have values less than 25. The sibling recurrence rate for the Newfoundland population has not been determined, but as the descent of this population is primarily from Britain and Ireland, the rate is potentially similar to the British population.

**Twin studies:** Twin studies are used to determine whether a disease has genetic susceptibility factors. Concordance rates test the percentage of twins that have or develop

the disease if the other twin has a known diagnosis. Since twins are raised in the same

environment and are exposed to the same extraneous risk factors, if there is a difference

in concordance rates between monozygotic (identical) and dizygotic (fraternal) twins it is

due to the shared genetic risk factors of the monozygotic twins. Twin studies have shown

a concordance rate of 63% for monozygotic twins and only 27% for dizygotic twins,

when both individuals are HLA-B27 positive (Tsui et al., 2003). This can be compared to

concordance rates of 15.4% for monozygotic twins and 3.6% for dizygotic twins for

rheumatoid arthritis (Silman *et al.*, 1993), and 24% and 2% for systemic lupus

erythematosus (Deapen *et al.*, 1992).


**Association:** Association studies using a candidate gene approach have yet to identify

all the genes that contribute to AS susceptibility. HLA-B60/ IL-1 gene cluster/ CYP450/

ANKH have all been linked to AS susceptibility (as reviewed in Wordsworth 1998;

Maksymowych *et al.*, 2006; van der Paardt *et al.*, 2002; Brown *et al.*, 2000; Timms *et al.*,

2003). However these genes are associated with a low relative risk, it is hard to know

significance of the functional consequences, and these results have not been validated.

Association studies take unrelated cases and compare genotype frequencies to that of the

general population for candidate genes chosen arbitrarily based on what is known about

the mechanism of disease. Genes that have been examined with respect to AS to date are

primarily involved in the inflammatory immune response.


**Linkage:** Linkage studies take large families with multiple affected members in

multiple generations and examine if molecular markers are distributed differently in

patients with the disease as compared to those without the disease. The areas determined using genome wide scans, which use many markers over the entire genome in an assay, tend to be broad regions. Fine mapping of these regions can then be performed using many markers with smaller distance between them within the broad region, or a directed linkage scan. Large numbers of patients are required to adequately perform a genome-wide scan that is generalizable to a population. Several chromosome arms outside the major histocompatibility complex region (6p) have been identified as potential linkage sites for AS (van der Paardt et al., 2002). Regions identified as contributing to AS susceptibility are on chromosomes 1p, 2q, 9q, 10q, 16q, and 19q (reviewed in Sims *et al.*, 2004). Other identified areas are linked to severity measures such as the Bath AS scales (the Disease Activity Index, and Functional Index). The BASDAI has been linked to chromosomes 11q, 16p, 18p and 20q. The BASFI was linked with chromosome 2q. Age of onset was linked with chromosome 11q. Regions on 3p, 11p, 11q, 16p, and 18p were linked with more than one phenotype (reviewed in Sims *et al.*, 2004).

## 1.3 IDENTIFICATION OF SUSCEPTIBILITY GENES

Traditionally, there are two main methods in genetic study design: linkage studies and association studies. Linkage studies can be divided by sibling pair or traditional multiplex approach. The traditional multiplex approach uses large families with multiple generations to create a model for the inheritance of the disease. This method is highly successful for monogenic disorders that follow Mendelian transmission. Unfortunately there has been some difficulty adapting this model to analyze susceptibility for complex models of disease that involve more than one genetic loci or varying disease penetrance. Assumptions about candidate gene frequency, mode of transmission, penetrance of disease, phenocopy rate and marker allele frequency are necessary to create a model of inheritance. As the inheritance of the disease grows more complex the more error is introduced in making these assumptions. This method is also dependent on the family being studied and the quality of information obtained about multiple generations. Sibling pair studies are model independent and relatively easy to collect samples for, however, success is modest and for complex diseases with a relative risk less than two, these studies don't produce replicable results. Diseases with a low relative risk are by definition rare as the risk of a random sample developing the disease is low. Linkage studies are very powerful for monogenic diseases, but the power drops as the complexity of the disease increases. Problems arise when using linkage studies to analyze complex diseases in the following situations: incomplete penetrance of the gene, i.e. the phenotype (visible expression) varies despite having the genotype; phenocopies, i.e. the disease phenotype is also associated with factors other than the gene being studied; genetic heterogeneity, i.e. more than one loci independently cause disease; environment interactions, i.e. an

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environmental trigger contributes to disease susceptibility; and insufficient recruitment of family members. These problems can also be seen in association studies but can be more effectively managed with population selection by selecting a population with homogenous genetic heritage and homogenous environment. Recruitment of family members is not an issue in association case control studies as the samples should not be related to maintain independence. As AS is a complex disease with a multigenic etiology and a low relative risk, linkage studies are less powerful for identifying susceptibility genes with the available number of patients.

The benefit of performing an association study for diseases where the relative risk is less than two is that sample sizes do not need to be as large and large families are not required, as illustrated in Figure 1 (Risch, 2000). Association studies can be divided into two main methods; case control, and family based, which is analyzed by transmission disequilibrium tests (TDT). TDT studies analyze trios, an affected child and his/her parents, for transmission of the affected haplotype from parent to child. Case control studies are more representative of the general population studied than TDT or linkage studies, which provide results specific for a single large family. Case control is a good method for diseases with a low relative risk, with smaller sample sizes needed than linkage studies for these diseases (Risch, 2000). Controls should be unrelated individuals from the same population to maximize statistical significance while maintaining smaller sample sizes (Risch, 2000). One disadvantage to using a case control approach is a higher number of false positives when examining genetic association to disease than other study designs, in other words showing association when in truth none exists. However, there

**Figure 1:** Comparison of sample sizes between association and linkage studies by relative risk of disease (from Risch, 2000). The dotted line represents linkage studies while the solid line represents association studies.
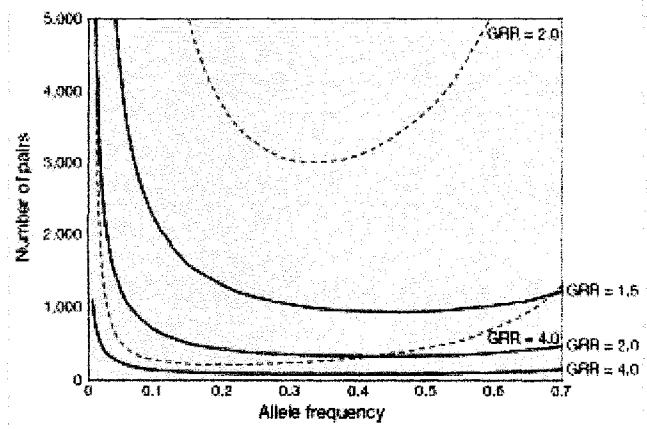
are true positives coming out of these studies and false results can be minimized with careful design and multiple testing statistics.

### 1.3.1 Study Design

To best utilize the Newfoundland AS population to assess genetic association with disease, a case control association study with a candidate gene approach was used. This approach is more feasible in the Newfoundland population because the available affected population is from multiple small families with only one or two generations of affected family members, which would make a linkage study more difficult. As AS has a relative risk in the general population less than two, case control is a good method to use with the sample size available (Risch, 2000). For a genetic study such as this, it is possible to use prevalent cases of the disease. Incident cases are ideal for most epidemiological studies due to the defined onset of disease, and the knowledge that all patients in the case group are at the same stage in disease progression. However, as the genetic makeup of an individual will not change with time or disease stage, it makes no difference how long the patient has had symptoms.

Candidate genes tend to be chosen based on position or function. This means that genes selected for studies are either physically located near genomic regions linked to AS through previous linkage studies, or the gene product has a biological function that logically could link it to the disease. Linkage studies have shown that the MHC region, as well as several areas outside the MHC region are linked to AS. Further discussion of each gene chosen, including the rationale for the polymorphisms examined, is contained in the following section.

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The candidate genes were arbitrarily separated into several categories relating to the pathological steps in development of AS. These are cytokines which link the innate and acquired immune responses, signalling molecules involved in the innate immune response to bacteria, and genes that have been linked to bone disease that are potentially involved in the conversion of cartilage to fibrous tissue.

Cytokines are the messengers of the immune system. As AS is an autoimmune disease, problems within the immune system of these patients exist. The cytokine release pattern in AS is predominantly $T_H2$ with impairment of the $T_H1$ response (Baeten et al., 2001). The $T_H2$ subset of CD3+ T cells are involved with the inflammation seen in allergy.

Evidence from animal models of AS and its common comorbidities shows a role for infectious agents in the development of disease. The involvement of microorganisms in developing disease implies a potential misregulation of the immune response which is triggered by exposure to a pathogen and leads to the autoimmunity seen in AS (Rudwaleit et al., 2001). Signalling molecules involved in stimulating the immune response to microorganisms are thus logical candidates for study in this disease.

The bulk of research published regarding AS targets the inflammation aspect of the disease, as proteins involved in the immune response are the common candidates. However, as previously stated, AS is a disease with two main stages, those of inflammation, which presents first, and ankylosis, which is the end stage of the disease. The ankylosis of AS has not been studied in depth, and logic dictates that susceptibility to AS must include genes that promote ankylosis, as all of the inflammatory arthropathies have inflammation, but only AS has fusion of the inflamed joints. Thus, as a subset of

this project, several candidate genes involved in bone disease were chosen based on locations identified in genome-wide scans to be linked to AS susceptibility.

Analysis of genetic association to disease was performed using single nucleotide polymorphisms (SNPs). SNPs are naturally occurring variants in genes within the human genome. While some SNPs cause functional variants of proteins with varying effects of biological function, most are silent, in that they do not change the ultimate amino acid sequence of the gene product. Functional variants are of higher interest due to these potential effects on biological function which could explain the misregulation that leads to development of disease. Also of interest is SNPs of the promoter regions of genes which could alter the amount of protein being transcribed by interfering with transcription factors and thus mRNA production.

### 1.3.2 Candidate genes

#### 1.3.2.1 Genes within the MHC region

The MHC region on chromosome 6p remains the most strongly linked region to AS in genome wide scans. Over ninety percent of AS patients test positive for the HLA B27 allele (Tsui et al., 2003). The population frequency of this allele is six to nine percent in Caucasian populations. However, only an estimated nine percent of all HLA-B27 positive individuals develop AS. The presence of the HLA-B27 allele only accounts for sixteen percent to thirty three percent of the genetic basis of this disease (Sims *et al.*, 2004; Brophy *et al.*, 2003). Thus, there are more genes to be discovered that confer risk to the development of this disease. The association with chromosome 6p may be due to the presence of HLA-B27, but research has been focused in this region for additional

susceptibility genes for AS. As there is some debate whether or not it is HLA-B27 and not a gene tightly linked to it that confers genetic risk, examining genes in this area is of scientific merit.

### TNF alpha

Tumor necrosis factor alpha (TNFα) is a cytokine with multiple functions that is constitutively expressed by adipose tissue and selectively released by cells of the immune system as part of the immune response (Lee et al., 2000). The major source of TNFα in the body stems from activated macrophages (Field, 2001). Treatment of cells with TNFα results in the activation of NF-κB through dissociation of IκB, the inhibitory subunit of the protein complex (Rosenstiel et al., 2003). TNFα has been shown to upregulate cell signaling molecules such as CARD15 through increased mRNA expression in a manner dependant on time and concentration of TNFα used (Hisamatsu et al., 2003).

The role of TNFα in AS is only now being explored. TNFα levels are elevated in AS patients, and this cytokine is released by the helper T cells that are associated with the disease (Braun et al., 2003). mRNA has been found in the inflamed sacroiliac joint characteristic of AS (Field, 2001). Biopsies of inflamed sacroiliac joints in AS patients have shown increased levels of TNFα around sites of ectopic bone formation (McGarry et al., 2001; Zhang et al., 2003). Genetic studies of TNFα SNPs have been inconclusive in determining association with AS. It is apparent that population plays a role in association as much of the conflicting evidence is from different cultural groups. TNFα would be a logical candidate gene to study due to the marked efficacy of the anti-TNFα therapies in these patients. TNFα is located within an area identified by genome wide scan as linked to AS, 6p21.

Polymorphisms to be examined within this gene are located in the promoter at positions -238, -308, -857, -863, and -1031. The promoter polymorphisms would most likely affect the regulation of the gene. They would not have any effect on function as they are outside of the sequence of the TNFα protein. These polymorphisms were chosen because TNFα protein levels are elevated in AS patients, implying a potential defect in regulation. Polymorphisms at positions -238 and -308 are thought to lead to decreased transcriptional activity of TNFα, and a lower response against antigen stimulation (Kaluza et al., 2000), while polymorphisms at positions -857, -863 and -1031 have been associated with increased TNFα production (Soga et al., 2003). Separately, these polymorphisms have been examined in several populations (Caucasian and Asian primarily) with respect to several immune-mediated diseases, such a celiac disease, IBD, Graves disease, and psoriatic arthritis. While the SNPs at -238 and -308 have been studied before in AS patients of Dutch, Spanish, German, and Scottish descent with conflicting results, the SNPs at the other three positions have not yet been examined in AS (Milicic et al., 2000; Fraile et al., 1998; Hohler et al., 1998; McGarry et al., 1999; Verjans et al., 1994; Field, 2001).

### 1.3.2.2 Genes outside the MHC region

At least half of the genetic risk for developing AS is estimated to come from factors outside the MHC region (Jaakkola et al., 2004). To date, many candidate genes have been examined, but no conclusive susceptibility genes have been named. Much of the MHC region is in linkage disequilibrium with the HLA molecules, where linkage disequilibrium is the inheritance of genes in linked segments. For example, it may be

shown that gene A is associated with AS, but gene A is only inherited along with gene B, which is the true disease susceptibility gene. Since HLA-B27 is known to be associated with AS, any gene that is inherited along with B27 may be associated with AS only due to this linkage as opposed to function. The non-MHC region thus provides an area to study where the results cannot be linked to HLA status and falsely reported as positive associations.

### 1.3.2.2.1 Cytokines

Cytokines are messengers of the immune system that link the innate and acquired immune responses. These small molecules have pleiotrophic effects on several cell types, often inducing a characteristic response in different cells by activating linked second messenger pathways. Cytokines are divided into categories based on similar functions and characteristics. Cytokines that are logical candidates for AS research include proinflammatory cytokines, such as IL-1 and TGF-$\beta$, and anti-inflammatory cytokines, such as IL-10, in which functional variants could affect regulation of the immune response to the responsible microbial trigger of AS.

**IL-1:** The Interleukin-1 gene cluster is located on chromosome 2q14, which has been identified by genome wide scan as a potential linkage region to AS (van der Paardt *et al.*, 2002). One susceptibility loci for AS is located 0.3 centimorgans from the IL-1 gene cluster (Ravindran *et al.*, 2004). Strong linkage disequilibrium exists between genes in this cluster in the Dutch Caucasian population, and associations found in this region are questionable as the polymorphisms may be linked to a disease causing allele (van der Paardt *et al.*, 2002). Interleukin-1 is a potent proinflammatory cytokine and is thought to have a key role in joint inflammation (Cantagrel *et al.*, 1999).

Interleukin-1 α and β are inflammatory cytokines involved in the immune response. Interleukin-1 α and β have been associated with juvenile chronic arthritis and with joint erosion in RA (Djouadi *et al.*, 2001). The proinflammatory cytokines promote the inflammation changes seen in AS, and eventually will lead to destruction of bone and cartilage (Crawley *et al.*, 1999). The gene cluster was recently associated to ankylosing spondylitis in three Canadian populations (Maksymowych *et al.*, 2006). Interleukin–1 β has been previously implicated in destructive joint changes (Maksymowych *et al.*, 2003). Polymorphisms in this gene have been linked to IBD (van der Paardt *et al.*, 2002).

Interleukin-1 receptor antagonist (IL-1Ra) works to antagonize IL-1 signaling by competitive inhibition. This protein provides a natural competitive inhibitor of the IL-1 response. IL-1Ra binds to the Interleukin-1 receptor but does not induce a signaling response. Recombinant IL-1Ra is currently being evaluated at the clinical trial level for treating the inflammatory arthropathies (Cantagrel *et al.*, 1999). Polymorphisms in the IL-1Ra gene have been related to Multiple Sclerosis and IBD, which are both chronic inflammatory diseases (van der Paardt *et al.*, 2002). A recent study found polymorphisms in IL-1Ra associated with AS, including a variable 86 base pair repeat in intron 2 (Maksymowych *et al.*, 2003). The authors concluded that the association was likely due to linkage disequilibrium with an unidentified primary disease locus. Scottish and Dutch studies have shown association with the repeat allele 2 of IL-1Ra and AS, but there have been problems in replicating these findings with other studies (Breban *et al.*, 2003; McGarry *et al.*, 2001). The repeat allele 2 has been reported to increase IL-1Ra production, and has been associated with juvenile idiopathic arthritis, psoriasis, ulcerative

colitis, alopecia areat, lichen sclerosus, systemic lupus erythematosus, and diabetes (Vencovsky *et al.*, 2001; Breban *et al.*, 2003; Djouadi *et al.*, 2001).

**IL-10**: Interleukin 10 is an anti-inflammatory cytokine that acts to inhibit the inflammatory response. This cytokine has been shown to inhibit the antibacterial response by macrophages (Yu and Kuipers, 2003). IL-10 is produced by $T_H2$ T cells to suppress stimulation of the $T_H1$ response. Since the response seen in AS patients is primarily $T_H2$ in nature, cytokines produced by these T cells may be disrupted in AS patients. Interleukin-10 has been linked to joint destruction in RA, though this has been disputed (Huizinga *et al.*, 2000; MacKay *et al.*, 2003). IL-10 was shown to inhibit the production of proinflammatory cytokines and the proliferation of $T_H1$ lymphocytes. The addition of exogenous IL-10 to rodent models of chronic arthritis protected against bone destruction (Huizinga *et al.*, 2000). The production of $TNF\alpha$ and IL-1, other candidate genes being studied in this project, are inhibited by IL-10 *in vivo* (Huizinga *et al.*, 2000). The inhibition of IL-1 is achieved by upregulating release of soluble IL-1Ra (Crawley et al., 1999). $TNF\alpha$ is inhibited by IL-10 action both at the level of synthesis and by inhibiting release of mature $TNF\alpha$ from leukocytes (Crawley *et al.*, 1999). IL-10 is produced by monocytes and B cells, and its release triggers a potent increase in production and differentiation of B cells, characteristic of the humoral response triggered by $T_H2$ signaling (MacKay *et al.,* 2003).

Control of IL-10 production appears to occur at the transcriptional level (Crawley *et al.*, 1999). Promoter polymorphisms are located within putatative transcription factor binding sites (Crawley *et al.*, 1999). In AS patients the level of IL-10 secretion by cytotoxic T cells is significantly higher than in controls regardless of HLA-B27 status

(Goedecke *et al.*, 2003). Studies have suggested a minimal role for IL-10 polymorphisms in AS in the Finnish population, but as the evidence is not supported with replicated studies, examination of potential functional polymorphism variants is warranted.

**TGF-β:**     The TGFβ gene is located on chromosome 19, where a genome wide scan has identified a potential AS candidate region in the English and Finnish populations (Jaakkola *et al.*, 2004). TGFβ is a cytokine that is involved in the inflammatory response, tissue fibrosis and bone remodelling (Jaakkola *et al.*, 2004). TGFβ is also involved in osteoblast development (Zhang *et al.*, 2003). Defective TGFβ1 is associated with Camurati-Engelmann disease, an autosomal dominant disease characterized by hyperostosis and sclerosis of the diaphyses of long bones (Janssens *et al.*, 2003). TGFβ1 is released by macrophages, lymphocytes, and chondrocytes. This production by chondrocytes is intriguing as the conversion of cartilage to fibrous tissue likely occurs in AS. Detectable levels of TGFβ are found in biopsies of AS inflamed sacroiliac joints near sites of ectopic bone formation (Zhang *et al.*, 2003). This evidence suggests a role for TGFβ in the ankylosis of AS. SNPs to be examined for this study include three promoter polymorphisms and one polymorphism within the first exon, all of which theoretically have functional consequences.

### 1.3.2.2.2 Signalling molecules

Molecules involved with the innate immune response to enteric bacteria are a logical choice for study in AS patients for two key reasons. The first is the apparent microbial trigger of AS development (reviewed in Rudwaleit et al., 2001). If the response to the unknown microbe is impaired, the microbe would remain in the system longer and

have access to the mechanisms that lead to autoimmunity. The second reason supporting this area of research is the high prevalence of subclinical inflammatory bowel disease in AS patients (Baeten et al., 2002). This subclinical IBD may result from improper clearance of foreign enteric flora.

**CARD15:** Caspase recruitment domain-containing protein 15 (CARD15) is associated with Crohn's Disease, a common comorbidity of AS. This gene contributes approximately forty percent of the genetic susceptibility to Crohn's Disease (Martin *et al.*, 2002).

The gene for CARD15 is located on chromosome 16 (16p12-q21), which is the chromosomal region commonly identified in genome wide scans for AS susceptibility genes with the highest likelihood of odds (LOD) score of 4.7 (Laval *et al.*, 2001; van der Paardt *et al.*, 2003). Expression of the CARD15 protein is intracellular, with primary expression in monocytes (Bonen *et al.*, 2003). It has been shown that CARD15 mRNA and protein levels are specifically upregulated in intestinal epithelial cells by TNFα (Rosenstiel *et al.*, 2003; Gutierrez *et al.*, 2002). The current consensus is that CARD15 acts to enhance inflammation through activation of Nuclear Factor-kappa B (NF-kB) upon stimulation with bacterial proteins, primarily those of the bacterial coat; lipopolysaccharide (LPS) and peptidoglycan (PGN) (Gutierrez *et al.*, 2002; Inohara *et al.*, 2003). CARD15 is also upregulated by NF-κB, through a NF-κB transcriptional activation sequence in the CARD15 promoter (Gutierrez *et al.*, 2002). The variants of CARD15 determined by Hugot *et al.* to be involved in Crohn's disease are to be studied.

The common CARD15 variants associated with Crohn's Disease are not found in the Asian population (Inoue *et al.*, 2002; Yamazaki *et al.*, 2002). This fact is important to

note for research in genetic susceptibility, as it illustrates genetic linkage to disease varies with ethnic group. Also of interest is the fact that the incidence of Crohn's Disease in the Japanese population is ten times less than the rates in Western populations, but has increased steadily during the past few decades (Inoue *et al.*, 2002). This increase could potentially be due to changes in diet and environmental factors to more closely resemble that of Western populations.

The 1007fs (frameshift mutation at position 1007) variant of CARD15 (also denoted as 3020insC), results in a truncated CARD15 protein at the C-terminal LRR domain (Bonen *et al.*, 2003). CARD15 recognizes the base unit of peptidoglycan (PGN) common to all bacteria, muramyl dipeptide (MDP) (Girardin *et al.*, 2003a). The 1007fs truncated protein is unable to activate NF-kB optimally upon stimulation by lipopolysaccharide (LPS), and is almost entirely unresponsive to MDP *in vitro* (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003b). This reduction in activity is somewhat controversial as studies using colonic tissue from Crohn's Disease patients show increased NF-κB levels (van der Linde *et al.*, 2003). It has also been found that CARD15 molecules lacking the C-terminal LRR domain are still capable of activating NF-kB through unspecific stimuli (Kabesch *et al.*, 2003). This mutation has been associated with significantly higher circulating levels of IgE, the immunoglobulin associated with allergy (Kabesch *et al.*, 2003). With respect to Crohn's Disease, the 1007fs mutation has been associated with fibrostenosing disease regardless of ethnic variation (studied in Jewish and Caucasian populations) and earlier age of onset in the Irish population (Abreu *et al.*, 2002; Bairead *et al.*, 2003). Prevalence of this mutation is three percent in the European population (Marsh and McLeod, 2003).

Two other common variants in CARD15, G908R and R702W have been associated with Crohn's Disease (Bonen *et al.*, 2003). These variants respond to bacterial LPS and PGN, but have impaired NF-kB activation. The R702W mutation has been linked to a two-fold risk of allergic rhinitis, and the G908R mutation has been associated with a three-fold increase in risk of developing allergic rhinitis and a two-fold risk of developing atopic dermatitis as an allergic response (Kabesch *et al.*, 2003). Prevalence of the G908R mutation in the European population is three percent, while prevalence of the R702W mutation in this population is two percent.

The CARD15 variants have been examined for association with AS. No association between these variants and AS has been shown to date (Crane *et al.*, 2002). However, the populations studied have been heterogeneous in nature, and analysis by ethnic group has not been performed.

**TLR4:** Toll-like Receptor 4 (TLR4) is a protein with function similar to CARD15 (Inohara *et al.*, 2003). The two proteins recognize different elements of bacterial antigens and stimulate the innate immune response through NF-κB. Where CARD15 is thought to primarily recognize peptidoglycan, TLR4 primarily recognizes lipopolysaccharide (LPS) (Rehli *et al.*, 2000). TLR4 is located on chromosome 9q32-33, which was identified in genome wide scans for AS susceptibility (reviewed in Sims *et al.*, 2004). The commonly studied polymorphisms Asp299Gly and Thr399Ile will be examined in this study. These single nucleotide changes negatively affect reponsiveness to LPS. This gene has recently been studied with respect to AS (van der Paardt *et al.*, 2005). The Asp299Gly variant was examined in 113 Dutch AS patients. The p-value was not significant, but the authors caution small numbers may be responsible for the lack of significance. The Asp299Gly

variant was recently shown to be associated with protection against Rheumatoid Arthritis but did not affect disease severity (Radstake *et al.,* 2004). The recent study of this gene in AS patients with questionable results supports our choice of these polymorphisms as candidates for AS susceptibility in our study.

TLR4 stimulates the immune response to LPS via stimulation of NF-κB which induces transcription of pro-inflammatory cytokines such as TNFα and activates naïve T cells (Palsson-McDermott and O'Neill, 2004). LPS is a major component of Gram negative cell walls. Aside from LPS, TLR4 also recognizes lipoteichoic acid, endogenous heat shock protein 60 ligands (HSP60), fibronectin, and hyaluronic acid (van der Paardt *et al.,* 2005).

Allele frequency of the Asp299Gly polymorphism was recently found to be associated with Crohn's Disease in the Greek population (Gazouli *et al.,* 2005). Crohn's disease is a common comorbidity of AS. Due to the high prevalence of subclinical gut inflammation in AS patients (60%), looking to genes known to be involved in the pathogenesis of gut inflammation for potential involvement in AS is logical.

### 1.3.2.2.3 Genes Involved in Bone Disease

Most of the research concerning AS to date investigates the inflammatory response in AS. The pathological step involving the conversion of cartilage to fibrous tissue and ectopic bone formation is poorly understood. The mechanism of this pathology is largely unknown. Genes were chosen based on location in susceptibility regions identified by genome wide scans, functional involvement in bone remodeling, or when known mutations are linked to bone disorders. As AS is one of many inflammatory

spondyloarthropathies, but is unique in that there is new bone formation, it is logical that part of the genetic susceptibility to AS development includes genes that predispose to ectopic bone formation.

**FGFR2:**     Fibroblast growth factor receptor 2 (FGFR2) is located on chromosome 10, which has been linked to AS through genome wide scans (reviewed in Sims *et al.*, 2004). This protein is a receptor for fibroblast growth factor and defects in this gene are responsible for the bone disorders Crouzon syndrome, Jackson-Weiss syndrome, Apert syndrome, and Pfeiffer syndrome (MIM:123500; MIM:123150; MIM:101200; MIM:101600). These diseases are all characterized by craniosynostosis, the early fusion of the infant skull. Of interest to this study, Pfeiffer syndrome is also characterized by ankylosis, particularly of the elbow. The SNPs being examined from this gene were chosen based on potential functional consequences of polymorphism, all are located within exons of the gene.

**ALX4:**     Aristaless-like homeobox 4 (ALX4) is a transcription factor located on chromosome 11, which has been linked to AS through genome wide scans, as well as being linked to BASDAI scores and age of onset (reviewed in Sims *et al.*, 2004). This protein is thought to be involved in skull and limb development. Defects in ALX4 are responsible for parietal foramina 2, an autosomal dominant disease characterized by deficient ossification of the parietal notch of the skull (Mavrogiannis *et al.*,2001). Expression of this protein is restricted to bone, making it an attractive candidate for bone disease susceptibility. The SNPs chosen for examination are all located within exons.

**DLL3:**     Delta-like three (DLL3) is a ligand for the Notch receptor located on chromosome 19. Mutations in this gene are responsible for spondylocostal dystosis

(Whittock *et al.*, 2004). The polymorphisms chosen to examine this gene are a frameshift polymorphism in exon 2 and a singe nucleotide polymorphism in exon 6.

**TCIRG1:** T-cell immune regulator 1 (TCIRG1) is located on chromosome 11. Mutations in TCIRG1 have been linked to infantile malignant osteopetrosis, a disease characterized by bone thickening. The TCIRG1 gene is expressed in osteoclastomas and the thymus. A polymorphism of the promoter was found to be associated to lower bone mass in women (Sobacchi *et al.,* 2004). The SNPs chosen for examination in this study have potential functional consequences, rs7479081, rs2471829, and rs1047817 are located within exons.

## 1.4 POTENTIAL BENEFIT

There are three main benefits to pursuing this research. Firstly, identifying genes associated with AS will add to knowledge about the disease once the functional consequences are inserted into the known stages of pathogenesis. Secondly, genes involved may be used to predict course and outcome in individual patients with the disease. Thirdly, identifying susceptibility genes provides potential targets for directed treatments such as the anti-TNFα biologics that may prove to be more effective in treating the disease.

AS is a disease with an early age of onset. This early age often leads to a long delay between symptom onset and diagnosis. This delay has been reported to be an average of 5-7 years (Braun, 2003). Early treatment and education about exercise techniques and posture has been shown to slow the progression of the disease and maintain function of the spine (Scalapino and Davis, 2003). The primary test for diagnosis of AS is a positive X-Ray for inflammation in the sacroiliac joint. This inflammation occurs once the disease has progressed to a certain point, leaving a need for an earlier indicator of disease so that treatment can begin as soon as possible (van der Heijde, 2003). Epidemiological studies show that the principal amount of damage and decline in function happens during the first ten years of disease, especially in those patients with severe involvement (Landewe *et al.*, 2003). The identification of genetic risk factors would help in early diagnosis, and if conducted prior to symptom onset can be used preventatively to spur radiological testing at the first sign of symptoms. This early diagnosis would aid in maintaining function, preventing fusion and improving quality of life in AS patients.

## 1.5  OBJECTIVE

The objective of this study is to identify and test potential candidate genes for susceptibility to AS in the Newfoundland population. Candidate genes were chosen based on functional relevance to the disease phenotype of autoimmune inflammation and bone remodeling or positional proximity to regions previously linked to AS in other studies.

**Chapter 2 Methods**

**2.1 Population**

Choice of population is important in association studies. With respect to genetics, populations often differ in associated risk factors for disease. In AS there are reported differences in the prevalence of HLA-B27 in different ethnic groups. In the Caucasian population, over 90% of AS patients are HLA-B27 positive. In African-Americans this prevalence drops to only approximately 50% of AS patients (Mijiyawa *et al.*, 2000). The disease is phenotypically the same in these two groups, so there must be different genetic risk factors, or at least different risk contributions from genetic risk factors. Population stratification is a common confounder in case control studies. This confounder occurs when the cases and controls are not from the same genetic background. This is less of a concern in a founder population such as Newfoundland.

Newfoundland is a founder population, which means that the population descended for the most part from the same core group of original settlers. From census data, it has been determined that the population of Newfoundland grew from around 20,000 settlers from Ireland and Britain in 1760 to 200,000 in 1890, largely due to natural expansion as the immigration rate was low during this time (Rahman *et al.*, 2003a). Newfoundland communities have historically been geographically isolated with most individuals remaining where they were born. The main advantage of a founder population is a relatively homogenous group genetically which leads to a reduction in the genetic heterogeneity seen from ethnic differences (Rahman *et al.*, 2003a). This reduction in heterogeneity makes the signal of a minor susceptibility gene easier to read and determine to be significant. This advantage has been utilized by several groups to identify

susceptibility genes for many conditions such as Crohn's disease, Bardet-Biedl

syndrome, and hereditary non-polyposis colorectal cancer (reviewed in Rahman *et al.*,

2003a). Incidentally, the first ever linkage study in AS was with a Newfoundland family,

noting linkage to the MHC region (Rubin *et al.*, 1994). Linkage disequilibrium, the non-

random association of genetic alleles, in young founder populations also makes it easier

to find genetic association to disease, as the area that is linked through linkage

disequilibrium tends to be larger in these populations. This linkage works to the

advantage of studies in that it is easier to find a signal in a region in which susceptibility

lies. However, it must be remembered the susceptibility may not be linked to the site

tested, but instead a more distant site that is linked to the tested site through linkage

disequilibrium. Genetic drift is the change in prevalence of genetic alleles often seen in

founder populations. Genetic drift results in increased incidence of genetic disorders in

these populations (reviewed in Rahman *et al.*, 2003a). This increased incidence of disease

makes this population ideal for the study of rare diseases, such as AS, as the patient base

will be higher.

Any population based association study can only claim to identify susceptibility

genes for the population tested. As there are differences between populations,

generalizing results to other ethnically diverse populations cannot be done without further

testing. Thus, a limitation of the Newfoundland population is that the results are only

generalizable to Caucasian populations, specifically of Irish and English origin, and

further study in different ethnicities is needed to verify the findings for other populations.

This limitation is acceptable, however, because it will identify strong potential candidates

for study in an area where few strong candidates are apparent.

The other advantage of using Newfoundland as a study population is the environmental matching of cases and controls. There is relative homogeneity in the environment and living conditions throughout the province. This is an advantage again over a major centre such as Toronto, which would have to deal with potential confounders such as urban pollution when assessing risk.

## 2.2 Analysis Plan

Unless there is selection pressure present in the population, allele frequency is found in an equilibrium defined by $p^2 + 2pq + q^2 = 1$, known as the Hardy Weinberg equilibrium. The first analysis performed on each set of results is a test for Hardy Weinberg equilibrium. The expectation is that controls would satisfy the equilbrium.

The primary outcome is the association of genotype to disease. This analysis is to be performed using the chi-squared statistical test. The number of minor alleles in both groups out of total alleles present will be used to determine association. The test will be two tailed since the minor allele may be associated with the control group or the case group and thus be either a protective factor or risk factor for the disease respectively.

Nonparametric statistics are used to examine the association between a categorical independent variable and nominal or ordinal dependent variables. In the primary outcome of this study both variables were nominal, genotype and disease status. The best test to analyze this data was the chi-square test. If the frequency for any cell was less than 5, Fisher's Exact Test was used. Significant associations will then be corrected for multiple testing using logistic regression which will also ensure that association with one allele was not due to association with another allele through linkage disequilibrium.

If an association of genotype to disease is found, secondary analysis will examine potential association with common phenotypic information, such as gender, age of onset, and disease severity (as measured by the validated disease activity scales). This analysis will be performed using appropriate statistical techniques, primarily logistic regression. Logistic Regression is an extension of multiple regression that can be used when the outcome is categorical.

## 2.3 Clinical

### 2.3.1 Patient selection criteria

To be included in this study, patients had a definite diagnosis of ankylosing spondylitis, were over the age of 18, were at least third generation Newfoundlander, and were able to give informed consent. Patients were excluded if they had family members included in the study, as the samples must be independent. Patients used in this study were enrolled over the period from April 2003 to August 2004. Patients who wished to take part in the study spent approximately 45 minutes completing a questionnaire, medical history, donating a blood sample, and had assessments on their disease status performed by a research nurse and the investigator. Patient questionnaires were collected by Tara Snelgrove, physical data and blood samples were collected by research nurses Yvonne Tobin and Maxine Kelly.

Ankylosing spondylitis was diagnosed in patients using the modified New York criteria. To be diagnosed with ankylosing spondylitis by these criteria, patients must have had radiological evidence of sacroiliitis (at least grade II bilaterally or grade III-IV unilaterally), and must have had at least one of the following clinical criteria: 1) low back

pain and stiffness for more than 3 months that improves with exercise but not with rest; 2) limitation of motion of the lumbar spine in both sagittal and frontal planes; 3) limitation of chest expansion relative to normal values corrected for age and sex (Braun 2003 ACR Symposium).

The samples used as a control population were from healthy volunteers from the Newfoundland population collected for previous research with Dr. Rahman. The volunteers had no evidence of AS and as a group was of similar average age as the case group. Using a population control eliminates Berkson's selection bias in case-control studies, which is a bias in participant selection where the control population is at a different risk of exposure to risk factors for the disease than the cases. Controls will not be matched by case, as there are problems in genetics from over matching. If the difference between groups is too narrow, it becomes easier to miss differences due to disease processes.

### 2.3.2 Ethical Considerations

This study was approved with respect to ethical considerations by the Human Investigations Committee of Memorial University of Newfoundland in 2000 as well as with the author as primary investigator in 2004 (study #04.116). Patients were enrolled after obtaining written informed consent and the wishes of the patient with respect to future use of their DNA was obtained in accordance with standard policy. Patient identifying information was kept in a locked cabinet in a locked room for the duration of the study and storage thereafter.

### 2.3.3 Patient information collected

The questionnaire consists of demographic information, a brief medical history, and validated scales of AS disease measurement. These scales are the BASDAI, the BASFI and the BASMI, as well as a brief quality of life index, the ASQoL. The measures of disease activity, functional index, physical impairments (or metrology index) and quality of life have all been previously validated (Bath Ankylosing Spondylitis Disease Activity Index, Bath Ankylosing Spondylitis Functional Index, Bath Ankylosing Spondylitis Metrology Index, Ankylosing Spondylitis Quality of Life questionnaires respectively). These measures of disease severity and progression are included in the questionnaires so cases can be stratified by severity for phenotypic analysis for further analysis of any noted associations. The questionnaire is attached as Appendix A. A pedigree of affected relatives was obtained from each patient.

### 2.4 Laboratory

#### 2.4.1 DNA extraction

DNA extraction from the white blood cells in the blood sample and analysis was performed blind by Tara Snelgrove except in control data for TNFα, IL-1, IL-10, and CARD15, for which the allele frequencies were provided by personal communication from Dr. Rahman as these SNPs had already been genotyped in the control population by Lynette Peddle. The samples were given a study code and there were no identifiers to link severity of case to sample until after the genetic analysis was performed.

DNA was extracted from 10 mL of whole blood collected in two 5 mL EDTA treated tubes for each participant using the Promega Wizard Extraction Kit. The whole

blood was spun at 2000 rpm for ten minutes. This separated the blood into three layers. The top layer of plasma was discarded and the layer of white blood cells, or buffy coat, was removed and combined for both tubes of blood. The buffy coat was then added to 30 mL of cell lysis solution which breaks up any red blood cells that were extracted with the white blood cells. Red blood cells do not contain nuclei, and are thus unable to be used for extracting DNA. The cell lysis solution mixture is then incubated for ten minutes at room temperature, with gentle mixing to ensure lysis. The mixture is then centrifuged for ten minutes at 3100 rpm. This produces a pellet of white blood cells. The supernatant, which consists of cell lysis solution and lysed red blood cells, is discarded and the pellet is resuspended by vortex. Nuclei Lysis solution is added to each tube (10 mL) and the solution is mixed by pipetting until the solution is homogenous. The solution is then incubated at 37°C for one hour, or until there are no visible clumps of pellet remaining. A protein precipitation solution is then added (3.3 mL) and the solution is vortexed until precipitate is observed. The samples are then centrifuged for ten minutes at 3100 rpm to create a pellet of precipitated protein. The supernatant is transferred to a clean 50 mL tube and 10 mL of isopropanol is added to precipitate out the DNA. The solution is gently mixed until precipitate is observed and centrifuged at 3100 rpm for one minute. The supernatant is removed, and the pellet of DNA is washed with 10 mL of 70% ethanol. The solution is centrifuged at 3100 rpm for one minute and the ethanol wash is removed. The pellet is then incubated at 37°C overnight to dry the pellet and remove any residual alcohol. The dried DNA pellet is then resuspended with DNA rehydration solution, a buffer, by incubation at 60°C until the DNA pellet is no longer visible. The sample is then aliquoted into 1.5 mL tubes for future use.

Concentrations of extracted DNA were determined by spectrophotometry by creating a one in twenty dilution with rehydration solution and assessed for optic density which was then converted via spreadsheet into ng/μL of DNA. Concentrations were used to dilute samples to 2.5 ng/μL for mass array reactions, and 10 ng/μL for gel electrophoresis genotyping.

### 2.4.2 Sequenom MassARRAY SNP Genotyping

The Sequenom system uses matrix-assisted laser desorption ionization (MALDI) coupled with a time of flight (TOF) tube for spectral analysis of single nucleotide polymorphisms in DNA fragments amplified by polymerase chain reaction (PCR). Equipment used includes a Bruker *Biflex* mass spectrometer, a Beckman multimek robot for liquid handling, the custom Spectropoint Pin Tool for high throughput spotting of samples onto the chip for reading, and software to design SNP assays. The software designs multiplex reactions genotyping up to 12 SNPs per well. The procedure followed is detailed below.

### 2.4.3 Assay Design

The goal of designing multiplex reactions is to maximize the potential of the Sequenom mass array system while preventing reactions within the SNP primers being tested. The software lumps assays based on termination mix and tests for primer overlap to prevent cross reaction within and between primers to ensure that the only amplification is of target DNA. There are three different primers designed for each SNP, forward and reverse PCR primers for amplification of a DNA fragment containing the SNP, and a massEXTEND primer that is used to determine which allele is present at the SNP site. The massEXTEND primers are designed to have a melting temperature of 60°C or

greater, which is accomplished with GC content. GC pairs require more energy to break than AT pairs because they contain three hydrogen bonds versus two. To be able to distinguish between multiple reactions the massEXTEND primer mass cannot be identical to any other primer in the multiplex mixture. Termination mix is the replacement of three of the four nucleotides from deoxynucleotides to dideoxynucleotides which cannot be extended and thus terminate the strand. The correct termination mix is determined by the software which ensures that the extensions do not go beyond three bases before terminating.

### 2.4.4 Polymerase Chain Reaction

Template DNA was extracted from case and control samples and diluted to 2.5 ng/µl concentration for use in PCR. Primers were 20-30 nucleotides in length, contained approximately 40-60% G or C nucleotides to induce stable binding of primers without forming secondary structure which would impair binding. A PCR master mix was created containing forward and reverse primers, buffer, 1.5M magnesium chloride, deoxynucleic acids (dATP, dCTP, dGTP, dTTP), and *Taq* DNA polymerase, 4µL of master mix was placed in each sample well, to which 1µL of 2.5ng/µL DNA was added. Standard PCR cycling programs for multiplex reactions were used (95°C for fifteen minutes, 45 cycles of [95 °C for twenty seconds, 56 °C for thirty seconds, 72 °C for one minute], 72 °C for three minutes, 4 °C).

### 2.4.5 SAP Treatment

Two microlitres of a SAP master mix containing sterilized deionized water, homogeneous mass extend buffer and SAP were added to each sample well using the multimek robot pipettor. The reaction including the SAP mixture was incubated in the

PCR machine according to standard protocol (37 °C for twenty minutes, 85 °C for five minutes, 4 °C).

### 2.4.6 Homogenous massEXTEND Reaction

The massEXTEND reaction is an amplification of the polymorphism site in the fragment using PCR. The massEXTEND primers were added to hME buffer and an aliquot of 2μL was added to each sample well by multimek robot pipettor. The PCR reaction times differed if the reaction was multiplexed, assessing several SNPs (94 °C for two minutes, 100 cycles of [94 °C for five seconds, 52 °C for five seconds, 72 °C for five seconds], 4 °C forever); or uniplexed, assessing a single SNP (94 °C for two minutes, 45 cycles of [94 °C for five seconds, 52 °C for five seconds, 72 °C for five seconds], 4 °C).

### 2.4.7 Spectroclean Resin Treatment

Upon completion of the extension reaction, the samples were treated with Spectroclean resin to remove any cations that would interfere with the mass array signal. The resin was mixed with the sample and distilled water using the multimek robot and then centrifuged at high speeds to prevent the resin from being transferred to the reading surface of the chip. A mass array silicon dioxide chip was spotted with the samples from a 384 well plate by the MassARRAY nanodispenser. The chip was then transferred to the reading plate of the Bruker *Biflex* mass spectrometer and sealed. The spotted chip was then read by the mass array under vacuum using a laser to aerosolize the sample and interpret the mass reading into spectra. These spectra were converted into genotype calls for each SNP based on expected masses of allele fragments by the software of the equipment.

### 2.4.8 Analysis of the VNTR of IL-1RA

The IL-1Ra repeat allele was analyzed using PCR, restriction enzymes and gel electrophoresis.

### PCR

The variable number tandem repeat (VNTR) of IL-1RA was amplified by PCR using the a standardized cycling protocol (94 °C for two minutes, 40 cycles of [94 °C for one minute, 58 °C for one minute, 72 °C for one minute], 72 °C for two minutes, 4 °C ). The PCR products were run on a 1% agarose gel. Bands were visualized with ethidium bromide treatment and the size of the VNTR fragment was determined using a molecular weight ladder.

## Chapter 3 Results

### 3.1 Demographics

Demographics of the case and control groups are shown in Table 1. The case group consisted of 101 patients, with mean age of 45.6 years (standard deviation 12.4), mean age of onset at 25 years (11.8). Seventy percent of the case group was male, and 88% of the patients were HLA-B27 positive, which is consistent with standard AS demographics. The control group consisted of 103 volunteers from the community, with mean age of 44 (13.7), and 39% of this group was male.

Eighty seven percent of our AS patients had girdle joint involvement, specifically the hip. Assessment was made of extra-articular involvement in the case group from data obtained in the questionnaire. Achilles tendonitis caused by enthesitis was seen in 38.4% of our patients. Table 2 shows the status of our AS population with respect to extra-articular involvement of disease. Common comorbidities of AS include IBD, iritis, and psoriasis. With respect to IBD in our population, 9.1% of patients were diagnosed with ulcerative colitis, and 4.04% had a diagnosis of Crohn's disease. Iritis was a common extra-articular involvement in our population, with 44.4% of the cases experiencing at least one occurrence. Psoriasis was also present in 20.2% of the case population.

### 3.2 Hardy Weinberg

The control group met Hardy Weinberg equilibrium for all the SNPs examined in which minor alleles were present in the Newfoundland population. The data for controls was entered into a spreadsheet calculation using actual allele frequencies of homozygotes to calculate a theoretical frequency of heterozygotes. This was then multiplied by the sample size to yield a theoretical number of heterozygotes in the population.

**Table 1:**     **Demographic information for case and control groups**

|            | Cases       | Controls    |
| ---------- | ----------- | ----------- |
| N          | 101         | 103         |
| Percent male | 70%       | 39%         |
| Mean age (years) | 45.6 ± 12.4 | 44 ± 13.7 |
| Mean age of onset (years) | 25 ± 11.8 | N/A |
| HLA-B27 Status | 88%     | -           |

**Table 2:** Comorbidity status in ankylosing spondylitis patients examining extent of extra-articular involvement in the patient cohort.

| Comorbidity | Percentage of AS Patients (%) |
|---|---|
| Ulcerative Colitis | 9.1 |
| Crohn's Disease | 4.04 |
| Iritis | 44.4 |
| Psoriasis | 20.2 |

This theoretical value was then compared to the actual observed number of heterozygotes using chi-square analysis to determine if any difference was statistically significant. As differences between theoretical and observed allele frequencies in the control group was not statistically significant at p=0.05, it can be said that Hardy-Weinberg equilibrium was maintained in the population for these alleles, and any difference between case and control groups is not due to inherent differences in the population.

### 3.3 Primary Outcome

With respect to association of gene allele to disease susceptibility, several important results were noted summarized in Table 3. Values marked with a * were provided by Dr. Rahman by personal communication from ongoing research, and values marked with † are statistically significant with α=0.05. On preliminary analysis with chi-square statistics, SNPs from the TNFα, CARD15, and TLR4 genes appeared to be significantly associated with disease status, and SNPs from TGF-β and ALX4 showed trends towards significance. From further analysis of the allele frequencies using logistic regression, the only SNPs that retained statistical significance when corrected for multiple analysis are the SNPs at positions -308, and -863/-1031 in linkage disequilibrium of the TNFα promoter region and the Asp299Gly polymorphism of TLR4. Further analysis of the TLR4 haplotype with Haploview software (done by a statistician for publication of results) revealed an association of the minor allele haplotype with disease (p=0.03) (Snelgrove et al., submitted). The TNFα SNPs were less prevalent in cases than in controls, while the TLR4 variants were more prevalent in the case population. It is also of note that the minor alleles for the SNPs chosen for FGFR2,

**Table 3:** Allele Frequencies and associated P values of SNPs located within 12 candidate genes in ankylosing spondylitis patients and controls from the Newfoundland population.

| Gene | SNP | N Case | Case (%) | N Control | Control (%) | Δ (%) | P value | P cor'd |
|---|---|---|---|---|---|---|---|---|
| TNF-α | 238 (A) | 100 | 4.5 | 103 | 5.33* | -0.8 | 0.8197 | 0.567 |
| | 308 (A) | 100 | 10.0 | 103 | 18.9* | -8.9 | 0.0114 | 0.008[†] |
| | 857 (T) | 100 | 11.0 | 103 | 7.7* | +3.3 | 0.3079 | 0.338 |
| | 863 (A) | 100 | 5.5 | 103 | 12.9* | -7.4 | 0.0002 | 0.000[†] |
| | 1031 (C) | 100 | 5.0 | 103 | 22.8* | -17.8 | <0.0001 | (LD) |
| IL-1 | IL-1α (T) | 100 | 33.0 | 95 | 31.0* | +2.0 | 0.381 | |
| Cluster | IL-1β (T) | 100 | 24.5 | 95 | 25.3* | -1.1 | 0.477 | |
| | IL-1Ra (2) | 75 | 34.0 | 86 | 25.6* | +8.4 | 0.1118 | |
| IL-10 | IL-10 (A) | 100 | 51.0 | 86 | 48.8* | +2.2 | 0.378 | |
| CARD15 | 702 (T) | 101 | 0.0 | 90 | 3.9* | -3.9 | 0.0048 | 0.002[†] |
| | 908 (C) | 100 | 0.5 | 90 | 2.8* | -2.3 | 0.1054 | 0.170 |
| | 1007 (C) | 101 | 2.5 | 89 | 0.56* | +1.9 | 0.2207 | 0.187 |
| TLR4 | 299 (G) | 100 | 7.5 | 98 | 2.55 | +4.95 | 0.0366 | 0.05[†] |
| | 399 (T) | 101 | 7.4 | 100 | 3.0 | +4.4 | 0.0707 | 0.09 |
| TGF-β | 1800471 (C) | 98 | 8.7 | 100 | 6.5 | +2.2 | 0.4518 | 0.430 |
| | 1800468 (A) | 101 | 8.9 | 100 | 11.5 | -2.6 | 0.4142 | 0.456 |
| | 1800469 (T) | 99 | 30.3 | 99 | 36.9 | -6.6 | 0.2016 | 0.216 |
| ALX4 | 3802805 (T) | 101 | 34.6 | 100 | 27.0 | +7.6 | 0.1058 | 0.265 |
| | 3824915 (G) | 93 | 33.9 | 97 | 25.8 | +8.1 | 0.0928 | 0.108 |
| FGFR2 | 3135824 (C/T) | 100 | 0 | 100 | 0 | | | |
| | 3135827 (C/T) | 100 | 0 | 100 | 0 | | | |
| | 3135829 (C/T) | 100 | 0 | 100 | 0 | | | |
| | 1047102 (A/G) | 100 | 0 | 100 | 0 | | | |
| | 4647921 (C/T) | 100 | 0 | 100 | 0 | | | |
| | 4647922 (A/G)) | 100 | 0 | 100 | 0 | | | |
| TCIRG1 | 7479081 (C/T) | 100 | 0 | 100 | 0 | | | |
| | 1047817 (C/T) | 100 | 0 | 100 | 0 | | | |
| | 2471829 (C/G) | 100 | 0 | 100 | 0 | | | |
| DLL3 | 7249157 (C/T) | 100 | 0 | 100 | 0 | | | |
| | 5828039 (-/C) | 100 | 0 | 100 | 0 | | | |

† p<0.05

*personal communication, P. Rahman

TCIRG1, and DLL3 were not present in the Newfoundland population in either cases or controls.

## 3.4 Secondary Outcomes

The TNFα and TLR4 polymorphisms were examined for association with secondary outcomes using logistic regression. No association was found between polymorphism and gender, age of disease onset, BASDAI score greater than 4, or BASFI score greater than 5 which are standard cutoffs of disease severity.

# Chapter 4 Discussion

## 4.1 The benefit of studying the genetic associations of disease susceptibility

AS has a large genetic burden and its sibling recurrence risk is among the highest for any complex disease. Thus, examining the genetic basis of disease is useful as identifying genes involved in susceptibility may allow for elucidation of disease mechanisms that are otherwise difficult to understand. Genetic variants can be used to identify risk early in life allowing for better management and fewer delays in diagnosis for individuals expressing symptoms. Further analysis of the functional consequences of these variants can be potentially used to develop treatment targets for biologic agents that will halt progression of the disease or become therapeutic options to reduce symptomatology.

There are large regions associated with AS susceptibility and a systematic analysis of these areas is necessary to determine the true susceptibility genes. Examining genes within the regions that have been linked to AS by genome wide scan is important even if the results show these candidates are not linked to AS. Since the areas are large there are multiple genes that have potential involvement in susceptibility and reducing the number of genes that must be examined will allow narrowing of the susceptibility regions. As long as the study has the power to detect a significant association, negative results will further narrow the regions of interest.

## 4.2 Challenges in genetic association study design

One of the challenges in identifying genes with small to moderate impact on genetic susceptibility is the inherent heterogeneity in phenotype and genotype for

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complex diseases. To minimize phenotypic heterogeneity, the modified New York criteria standardized method of diagnosis for AS was used as a strict guideline for inclusion in the study. Genetic heterogeneity in complex disease refers to the contribution of multiple genes to susceptibility and subsequent development of disease. Different combinations of genotypes may lead to the same disease phenotype. Using the founder population of Newfoundland minimizes the genotypic heterogeneity of the study population. Reducing these heterogeneities will further enhance the signal of genes contributing small to moderate risk for AS susceptibility.

Linkage disequilibrium is both a challenge and a benefit of using a founder population. Genetically homogenous populations such as founder populations and isolates have larger areas of linkage disequilibrium due to the natural expansion of the population. Large areas of linkage disequilibrium allow for positive associations for polymorphisms distant from the true susceptibility allele but linked evolutionarily. The challenge with linkage disequilibrium is further research is required to determine whether the polymorphism examined is the one conferring risk for the disease or whether it is just linked to an allele that confers risk. The next step in the research instigated by this study is to further analyze regions identifies as warranting closer examination using high marker density methods.

Another challenge in complex disease genetic research is incomplete penetrance. As many genes are likely to be involved in risk and susceptibility with a threshold of alleles necessary for disease development, it is possible that individuals in the "control" population will carry some of risk alleles without developing AS. This frequency of alleles in the control population will reduce the differences between cases and controls,

potentially minimizing the true significance of the alleles. The bias this would add to our study is to mimize the true association, with more likelihood of a false negative than a false positive. With AS, however, the low prevalence of disease makes it unlikely that there are susceptibility alleles in the control population. This risk can also be minimized by controlling the age of the control group. The mean age in this study was over 40, thus, it is highly unlikely there are any people in the control population that have AS and are not yet into the symptomatic disease course.

Environmental factors are also known to play a role in developing AS. As populations can differ with respect to environmental exposure, this fact could impact the genetic research of AS. Theoretically it is possible that members of the control group have the genetic susceptibility factors required to develop AS but have not developed symptoms because they have not been exposed to the environmental trigger. The impact of environmental factors on this study is that it is possible the frequencies of candidate genes in the control population does not accurately reflect frequencies in individuals who are not at risk for developing AS and have been exposed to the environmental trigger. While using a founder population in an environment such as Newfoundland will minimize this problem as exposure rates for most microbes are equal throughout the province, since the microbial trigger of AS is unknown it is impossible to say whether the control and case groups were equally exposed to the environmental factors responsible for AS development.

Population stratification is a challenge in genetic research. Population stratification refers to the potential confounder of genetic factors related to ethnicity on the study. Population stratification is a concern in large heterogeneous populations with

multiple lines of ancestry. Using the founder population of Newfoundland reduces the potential error due to population stratification as the population is relatively homogeneous when compared to other study populations. Using unrelated controls from the same population further reduces the magnitude of this confounding factor.

Potential sources of bias were minimized as much as possible. All interviewers were trained in measurement taking and assessments as a group prior to the first patient. This reduces the interobserver bias. The patient performed the parts of the questionnaire that are subjective responses themselves, with the interviewer explaining the scale but not asking the questions. This reduced any expectation bias on the part of the patient.

## 4.3 Genetic Epidemiology

This field of research is rapidly evolving and until recently study design was quite variable and false positive results were commonly reported due to poorer quality studies. A recent publication of guidelines for genetic association studies will allow more uniform investigations and data interpretation from several population studies will be easier in meta-analysis (Freimer, 2005). As this study was completed prior to the publication of these guidelines, not all objectives listed were achieved. Objectives met by this study include the following. Each candidate chosen for this study was discussed separately as to the reasons for interest in AS, either from previous research, which was cited, or due to what is known about the function of the gene. When discussing candidates for this study, an effort was made to identify the populations in which the prior studies were performed, as population differences may account for differences in results. P-values were corrected for multiple testing within each gene examined. Objectives that were not achieved by this

study were reporting of p values obtained by previous studies, as differences in population prevalence of alleles makes comparison difficult, probability statistics were not utilized to determine whether the list of candidate genes was likely to produce a relevant result, and the significant p value was set at 0.05 not $10^{-7}$. Were this study to be repeated, the suggested guidelines would be integrated into the study design as well as expanding the sample size to remove any issues of power limitations.

## 4.4 Candidate gene selection

The tumor necrosis factor $\alpha$ gene was chosen based on position within the MHC region of chromosome 6. Several previous studies examining two of the SNPs chosen for this study have given different results for different populations (as reviewed in introduction). Three of the SNPs examined have not yet been tested in AS patients. The controversy coupled with the marked effectiveness of the anti-TNF$\alpha$ agents in AS patients make this gene an interesting candidate for this study.

The cytokines interleukin 1, interleukin 10 and tumor growth factor $\beta$ (IL-1, IL-10, TGF$\beta$) were chosen based on position within areas of interest identified on genome wide scan (reviewed in Sims *et al.*, 2004). These cytokines were also chosen due to association with other inflammatory arthropathies or extra-articular manifestations seen in AS, as reviewed in the introduction.

The genes for caspase recruitment domain-containing protein 15 and toll-like receptor 4 (CARD15, TLR4) were chosen based on their location in the chromosome as well as their function as signaling molecules involved in the innate immune response to intestinal flora. The CARD15 gene has recently been associated with psoriatic arthritis in

the Newfoundland population (Rahman *et al.*, 2003b). The TLR4 gene has recently been studied in the Dutch population, as discussed in the introduction.

The genes for fibroblast growth factor receptor 2, aristaless-like homeobox 4, delta-like three, and T-cell immune regulator 1 (FGFR2, ALX4, DLL3, TCIRG1) were all chosen based on their proximity to regions previously associated with AS (reviewed in Sims *et al.*, 2004) and based on function of the gene product. All four of these genes appear to play some role in bone metabolism regulation as mutations lead to diseases involving bone pathology, as discussed in detail in the introduction.

## 4.5 Interpretation of Results in this study

### 4.5.1 Gene Discovery

The toll like receptor 4 (TLR4) gene is within the region on chromosome 9q that was identified as a susceptibility region (reviewed in Sims *et al.*, 2004). TLR4 is a signaling molecule that initiates a response against lipopolysaccharide via stimulation of NF-κB which induces transcription of pro-inflammatory cytokines such as TNFα. Lipopolysaccharide is a major component of Gram negative cell walls. TLR4 also recognizes lipoteichoic acid, endogenous heat shock protein 60 ligands (HSP60), fibronectin, and hyaluronic acid (van der Paardt *et al.*, 2005). The Asp299Gly polymorphism has been shown to reduce this response. The importance of this reduction *in vivo* is still being elucidated. The TLR4 gene is of interest based on both position and function. While the microbial trigger of AS is unknown, it is likely that a decreased response to this pathogen would allow it to remain in the system for longer periods of time before clearance by the immune system and allow it to trigger onset of the disease.

Thus, signaling molecules such as TLR4 are important considerations in understanding how the genotype influences the phenotype and the mechanism of AS susceptibility.

This study noted an increase in prevalence of the minor allele in the case population (p=0.05). The implications of this result involves minor allele functional misregulation of the innate immune system conferring susceptibility to AS. This result could help further elucidate the mechanism of AS onset and the connection between environmental triggers and disease.

Despite a previous negative association study between AS and TLR4, this study found a positive result with both SNP (see Table 3) and haplotype analysis (using Haploview). This may be in part due to the differences in populations used, and again points to the benefits of using a young founder population such as the one in Newfoundland, and it is possible the result of this study is a false positive. False positive results are possible through chance, multiple testing or linkage disequilibrium. The effects of chance were minimized by holding a significant alpha of 0.05 but still are a risk inherent to any statistical technique. Multiple testing was minimized by correcting the p value and using logistic regression for the secondary outcomes.

The next step in the analysis of TLR4 and AS is independent validation in other populations, haplotypes involving more markers, and further functional analysis of the polymorphisms studied. This study proposes that this further investigation is warranted. The association of this gene to disease could provide a further biological target for pharmaceuticals to control disease development and progression.

## 4.5.2 Gene validation

Cytokines are very important in initiating and maintaining the inflammatory response. TNFα is a very potent cytokine with many effector cells. Regulation of TNFα production is thus important to normal function. Control of TNFα production occurs at both the transcriptional and posttranscriptional levels (Wilson et al., 1997). The promoter region of TNFα is key to control at the transcriptional level. There are recognition sequences for several transcription factors in the TNFα promoter, as well as recognition sites for synthesis inhibitors such as the anti-inflammatory cytokines (Liz-Grana et al., 2001). DNase I footprinting has shown the −308 polymorphic site as a hypersensitive site for cleavage with a protected area adjacent (Wilson et al., 1997). The region containing the −1031 and −863 polymorphisms is involved in induction of expression by MKK6(EE) and MLK3 (Hoffmeyer et al., 1999). TNFα has the strongest biological relevance to AS. Strong evidence for a role of TNFα in AS are the effectiveness of the biologic agents.

The findings of this study (see Table 3) have been validated in other populations (Stone et al., in press). The polymorphisms at positions -308, and -863/-1031 in linkage disequilibrium were negatively associated with diseases susceptibility, meaning the presence of the minor alleles may have a protective role in AS onset. The promoter polymorphisms may be in linkage disequilibrium with the true functional polymorphism, or they may themselves have functional consequences by interfering with TNFα regulation at the transcriptional level. Since it appears that the -1031 and -863 polymorphisms are in linkage disequilibrium the former explanation seems likely. Linkage disequilibrium is a challenge, while it does help identify areas of interest in the Newfoundland population, it is difficult to ascertain whether the SNP analyzed is

associated with the disease or just linked to the true susceptibility polymorphism. The true susceptibility polymorphism is elucidated by higher density gene mapping and examining the area for the highest association to susceptibility which would be a future direction of this study.

Further study of these polymorphisms in populations of AS patients being treated with the biologic medications could identify genetic prognosticators of success or failure. The capability to investigate the pharmacogenetics of TNFα and the biologic agents in the Newfoundland population is limited due to the fact that the number of AS patients is small, and the number of these patients on biologic therapy is quite small. These polymorphisms can also be examined in larger centers for prognostic factors of severity and outcome. While no association with current BASDAI and BASFI was found in this study, the sample size may be too small to detect a difference, and as this was a case control study, the data is taken at one point in time, while a cohort design may be a better way to answer the question of prognostic associations.

### 4.5.3 Lack of Disease Association

False negatives are a potential problem of any genetic association study. There are several ways in which a falsely negative result can occur. Power of the study to capture the difference between groups is an important factor. It is possible some of the small differences noted are significant but our sample size is not sufficient to be captured statistically. It is also likely that some of the susceptibility genes are population specific, which would correlate with different disease prevalence by population, as is seen with the CARD15 alleles in the Japanese population. This study was also not designed to accurately capture interactions with environmental and other gene polymorphisms with

the genes being studied. Thus it is possible that a combination of factors is necessary for disease development which may not have been met in the control population who carried the polymorphisms studied. An example is lack of exposure to a pathogenic trigger organism. While it is likely that these results are not false negatives, it is prudent to examine larger studies from other populations before fully disregarding these candidates.

Most of the SNPs examined were not statistically significantly associated with disease. The SNPs that had no minor alleles present in the Newfoundland population were those for the genes FGFR2, TCIRG1, and DLL3 (Table 3). These findings are important for future research in this population as these SNPs were not present in either case or control groups and thus are unlikely to be found in the Newfoundland population as a whole. These genes have not been examined with respect to AS before and this preliminary research suggests that further research involving the regions from which these genes originate is warranted. The examination of other genes involved in bone disease is necessary to elucidate the mechanism of ankylosis in AS.

The negative results in this study, both the genes with no minor alleles present in the population, and other candidates chosen (IL-1, IL-10, ALX4, TGF-β, and CARD15) also show that logical and positional correlation with disease does not translate into genetic association. Searching for genes associated with complex diseases is a complicated process.

## 4.6 Limitations of Study

### 4.6.1 Sample size-power issues

Genetic studies are limited by the populations used. The Newfoundland population used in this study adds power to the study, as the reduction in the signal to noise ratio provided by the Newfoundland population will make any modest gene contributions more readily detectable. However, by limiting the studies to a specific population, we limit the potential sample size of the study. This is a case control study of a disease with a prevalence of only 1-3 out of 1000 in Caucasian populations (Bown *et al.*, 2003). As the Newfoundland island population was estimated by Canadian census to be 482,000 in 2001, the maximum sample size is 482 patients with a 100% participation rate. A further complication to sample size is the issue of independent samples. This disease has a strong genetic component for susceptibility. This genetic component results in multiple affected family members. These samples are not independent as there may be genetic factors other than the ones being studied contributing to risk. This removal of affected family members decreases the available sample pool from which to draw samples. As a result, one goal of this study was to obtain as many independent samples as possible from the population being studied, which for this study was just over 100 unrelated cases.

### 4.6.2 Newfoundland population

Using the Newfoundland population gives an enhanced signal to noise ratio allowing detection of genes with small to moderate effects on risk. This is a benefit of a founder population. A limitation of this population is the relative homogeneity of the population prevents generalization to an admixed population. As a result genes identified

using this population must be validated in larger populations with mixed heritage to truly assign risk. The advantage of using this population is that it can identify genes that warrant extended research in these admixed populations that would not be identified using similar techniques in the heterogeneous population. The results of this study will be used to stimulate further study of the polymorphisms with marginal significance in the Toronto population as a continuation of the work initiated with this thesis.

### 4.6.3 Demographic differences between groups

While the difference in gender between the two groups is statistically significant ($p<0.05$), none of the genes examined are related to the X chromosome or have expressions influenced by sex hormones. It has also been reported that AS is not associated with the X chromosome. However, this difference in group composition, stemming from the nature of the volunteer control population and the higher prevalence of AS in males, was noted.

### 4.6.4 Absence of replication studies

Another limitation of this study is that the polymorphisms found to be significantly associated with AS in the Newfoundland population are not supported by the current literature. While there is some evidence that TNFα is associated with AS, there are as many negative studies as positive ones published and the polymorphisms found to be significant vary from population to population. Some of these discrepancies may be explained by population differences, and that the use of the founder population enables smaller associations to be readily seen.

# Chapter 5 Conclusions

## 5.1 Outcomes

The TNF$\alpha$ and TLR4 genes seem to be associated with Ankylosing Spondylitis in the Newfoundland population. The TNF$\alpha$ promoter polymorphism minor alleles were statistically less prevalent in cases than controls. A novel association was noted in this study. The TLR4 Asp299Gly polymorphism was significantly more prevalent in cases than controls. These polymorphisms were not associated with gender, age of onset, or measures of severity, the BASDAI and BASFI.

## 5.2 Clinical significance of results

While the polymorphisms found were statistically associated with the disease, their use in screening is not warranted. The presence of the TNF$\alpha$ promoter polymorphism minor alleles seem to be protective for the disease, but are still present in the disease population so the presence of the polymorphism cannot be used to rule out a diagnosis of Ankylosing Spondylitis. The TLR4 polymorphism is a risk factor for the disease, but the polymorphism is present in the control population as well so it cannot be used to definitively diagnose AS and will have poor predictive value as a diagnostic test for AS.

## 5.3 Importance of population

This study illustrates the importance of the study population on interpretation of the results of genetic testing. While using a founder population has its advantages, mainly

with detection of genes that would normally be lost in the noise of genetic heterogeneity, the limitation with any genetic study remains that the results are generalizable only to the population being studied. This reduced generalizability is seen with all populations, and thus it is important when conducting literature reviews to note the population being studied.

The founder population of Newfoundland allows for great strides to be made in hypothesis generating investigations by indicating candidate genes that warrant hypothesis testing. Hypothesis testing involves a higher marker density and haplotype analysis, which is costly and time consuming. By doing hypothesis generating studies of single nucleotide polymorphisms that are likely to have functional consequences in a population where the linkage disequilibrium and the signal to noise ratio is high, the genes that make it to the second stage of research have been shown to likely be involved in disease pathogenesis and susceptibility. This allows for more efficient and focused research.

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*October 2003*

**Faculty of Medicine, Schools of Nursing and Pharmacy of Memorial University of Newfoundland; Health Care Corporation, St. John's; Newfoundland Cancer Treatment and Research Foundation**

## Consent to Take Part in Health Research

TITLE:         Genetic Linkage Study of Ankylosing Spondylitis

INVESTIGATOR(S):        Drs. P. Rahman, R. Inman, M. Stone

SPONSOR:         National Institutes of Health

You have been asked to take part in a research study. It is up to you to decide whether to be in the study or not. Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

The researchers will:

- discuss the study with you
- answer your questions
- keep confidential any information which could identify you personally
- be available during the study to deal with problems and answer questions

If you decide not to take part or to leave the study this will not affect your normal treatment

## 1. Introduction/Background:

Ankylosing Spondylitis is a disease that has genetic risk factors. These risk factors have yet to be identified. Finding these risk factors will lead to a faster diagnosis of disease and earlier treatment in patients. This early diagnosis and treatment may help slow the development of the disease.

## 2. Purpose of study:

The purpose of this study is to identify inherited and environmental factors that affect the immune system leading to the development of Ankylosing Spondylitis. This study may eventually lead to a better understanding and treatment of this disease.

## 3. Description of the study procedures and tests:

You will be asked to:
     1. allow the researchers to review your medical records and x-rays
     2. complete a questionnaire about your medical and family history
     3. have a genetic blood test (2 tubes of blood approximately 10 mls)

## 4. Length of time:

You will be asked to complete a questionnaire during one visit taking about 45 minutes

## 5. Possible risks and discomforts:

There is a risk of slight pain or bruising when your blood is drawn.

## 6. Benefits:

It is not known whether this study will benefit you.

## 7. Statement on Genetic studies

In order to interpret the results of the research properly, it is essential to have accurate information concerning parentage. Sometimes the research results may point out discrepancies in parentage (which may occur in case of adoption or a mistake in the identity of a father). If this happens, the information will be kept in the strictest confidence and will not be released to anyone, including family members and yourself.

## 8. Future use of DNA

In order to preserve a valuable resource, your DNA samples may be stored at the end of this research project. It is possible that these samples may be useful in a future research project which may or may not be related to the current research project. Any future research would have to be approved by a Research Ethics Board (REB).

Please tick **one** of the following three options:
- O  1.  I agree that my DNA samples can be used for any REB-approved research project (about inflammatory diseases), including those in which my name is given to the researchers, without obtaining further consent from me.
- O  2.  Specific consent must be obtained from me before using my DNA samples in any future research project in which my name is associated with the sample.
- O  3.  Under no circumstances may my DNA samples be used for any future research project. The samples must be destroyed at the end of the present project.

Signature _____     Date: _____

Witness: _____     Date:_____

## 9. Liability statement:

**Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.**

10. **Questions:**

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. That person is:

<u>Dr Proton Rahman    777-5733</u>

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:

<u>Office of the Human Investigation Committee (HIC) at 709-777-6974</u>
<u>Email: hic@mun.ca</u>

# Signature Page

**Study title:** Genetic Linkage study of Ankylosing Spondylitis

**Name of principal investigator:** Dr. Proton Rahman

**To be filled out and signed by the participant:**

|  | Please check as appropriate: |
|---|---|
| I have read the consent [and information sheet]. | Yes { }    No { } |
| I have had the opportunity to ask questions/to discuss this study. | Yes { }    No { } |
| I have received satisfactory answers to all of my questions. | Yes { }    No { } |
| I have received enough information about the study. | Yes { }    No { } |
| I have spoken to a physician and my questions have been answered | Yes { }    No { } |
| I understand that I am free to withdraw from the study | Yes { }    No { } |

- at any time
- without having to give a reason
- without affecting my future care

I understand that it is my choice to be in the study and that I may not benefit.        Yes { }        No { }

I agree that the study doctor or investigator may read the parts of my hospital        Yes { }        No { }
records which are relevant to the study.

I agree to take part in this study.        Yes { }        No { }


_____        _____
Signature of participant                          Date


_____        _____
Signature of witness                                Date

## To be signed by the person obtaining consent:

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.


_____        _____
Signature of person obtaining consent          Date


Telephone number:   (709)777-5733

# Ankylosing Spondylitis Questionnaire

DATE OF ASSESSMENT (Y/M/D) _____     FAMILY #_____

NAME_____     PID _____

MAIDEN NAME _____     REFERRED BY_____

ADDRESS _____
_____ Tel. (H) _____
_____ (W)_____
_____ Prefer Call:    1.Day  2.Evening

RELATIONSHIP TO PROBAND 1.Proband  2.Parent  3.Sibling  4.Child  5.Other _____

DOB (Y/M/D)_____MCP_____GENDER 1.Male  2.Female

FATHER'S PID _____     MOTHER'S PID _____

FATHER'S RELIGION_____     MOTHER'S RELIGION_____

**ETHNICITY**
1. Caucasian
2. Other          Specify_____

|  |  |
|---|---|
| You | _____ |
| Mother | _____ |
| Father | _____ |
| Maternal GF | _____ |
| Maternal GM | _____ |
| Paternal GF | _____ |
| Paternal GM | _____ |

**MARITAL STATUS**

1.Single          2.Married          3.Widowed          4.Divorced          5.Separated          6.Common Law

**EDUCATION**

1.<grade 8          2.Grade 8          3.Highschool grad          4. College          5. University

**EMPLOYMENT STATUS**

1. Employed          2. Retired          3. Homemaker          4. Student
5. Disabled          6. Sick Leave          7. Looking for Work          8. Other

**CRF collected by the patient**

---

NRS pain
Please tick the box that represents your answer ( i.e. ⊠ )

36. <u>Nocturnal Back Pain</u>
Based on your assessment, please indicate what is the amount of **back pain at night** that you experienced **during the last week?**

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

no pain                                                              most severe pain

37. <u>Total Back Pain</u>
Based on your assessment, please indicate what is the amount of **back pain at any time** that you experienced **during the last week?**

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

no pain                                                              most severe pain

---

NRS patient global disease activity

38. Please tick a box to indicate your overall assessment of your disease activity **during the last week.**

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

none                                                                  severe

---

NRS BAS-G

39. Please tick a box to indicate the effect your disease has had on your well-being over the **last week.**

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

none                                                                  very severe

40. Please indicate the effect your disease has had on your well-being over **the last six months.**

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

none                                                                  very severe

## NRS BASDAI

Please tick the box which represents your answer.
All questions refer to **last week**. (i.e. ☒ )

41. How would you describe the overall level of fatigue/tiredness you have experienced?

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

none                                        very severe

42. How would you describe the overall level of AS **neck, back or hip** pain you have had?

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

none                                        very severe

43. How would you describe the overall level of pain/swelling in joints **other than** neck, back or hips you have had?

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

none                                        very severe

44. How would you describe the overall level of discomfort you have had from any areas tender to touch or pressure?

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

none                                        very severe

45. How would you describe the overall level of morning stiffness you have had from the time you wake up?

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

none                                        very severe

46. How long does your morning stiffness last from the time you wake up?

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

0 hr                            1 hr                           2 or more hrs

## NRS BASFI

Please indicate your level of ability with each of the following activities during **the last week**. (i.e. ⊠ )

(An aid is a piece of equipment which helps you to perform an action or movement)

47. Putting on your socks or tights without help or aids (e.g. sock aid).

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

easy                                                                          impossible

48. Bending forward from the waist to pick up a pen from the floor without an aid.

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

easy                                                                          impossible

49. Reaching up to a high shelf without help or aids (e.g. helping hand).

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

easy                                                                          impossible

50. Getting up out of an armless dining room chair without using your hands or any other help.

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

easy                                                                          impossible

51. Getting up off the floor without help from lying on your back.

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

easy                                                                          impossible

52. Standing unsupported for 10 minutes without discomfort.

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

easy                                                                          impossible

53. Climbing 12-15 steps without using a handrail or walking aid. **One foot at each step.**

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
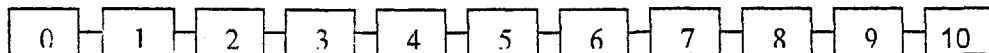
easy                                                                                  impossible

54. Looking over your shoulder without turning your body.

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
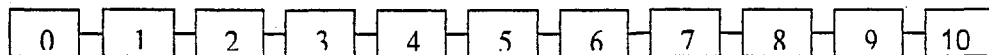
easy                                                                                  impossible

55. Doing physically demanding activities (e.g. physiotherapy exercises, gardening or sports).

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
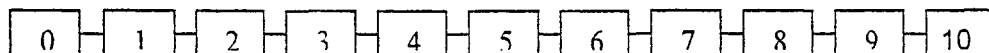
easy                                                                                  impossible

56. Doing a full days activities, whether it be at home or at work.

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
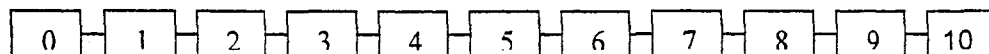
easy                                                                                  impossible

**ASQol**

Below you will find some statements which have been made by people who have Ankylosing Spondylitis.

Please read each statement carefully. We would like you to tick "yes" if you feel the statement applies to you, and tick "no" if it does not.

Tick the **one** response that applies best to you **at the moment.**

| | | |
|---|---|---|
| 57. My condition limits the places I can go | yes | – |
| | no | – |
| 58. I sometimes feel like crying | yes | – |
| | no | – |
| 59. I have difficulty dressing | yes | – |
| | no | – |
| 60. I struggle to do jobs around the house | yes | – |
| | no | – |
| 61. It's impossible to sleep | yes | – |
| | no | – |
| 62. I am unable to join in activities with my friends/family | yes | – |
| | no | – |
| 63. I am tired all the time | yes | – |
| | no | – |
| 64. I have to keep stopping what I am doing to rest | yes | – |
| | no | – |
| 65. I have unbearable pain | yes | – |
| | no | – |
| 66. It takes a long time to get going in the morning | yes | – |
| | no | – |
| 67. I am unable to do jobs around the house | yes | – |
| | no | – |
| 68. I get tired easily | yes | – |
| | no | – |
| 69. I often get frustrated | yes | – |
| | no | – |
| 70. The pain is always there | yes | – |
| | no | – |
| 71. I feel I miss out on a lot | yes | – |
| | no | – |
| 72. I find it difficult to wash my hair | yes | – |
| | no | – |
| 73. My condition gets me down | yes | – |
| | no | – |
| 74. I worry about letting people down | yes | – |
| | no | – |

SYMPTOMS    SHADE CIRCLES FOR <u>ALL</u> THAT APPLY

|  | Yes | No | Date Dx (y/m/d) |
|---|---|---|---|
| **INFLAMMATORY BOWEL** | | | |
| Ulcerative Colitis | O | O | _____ |
| Crohn's Disease | O | O | _____ |
| Uveitis/Iritis | O | O | _____ |
| Psoriasis | O | O | _____ |
| Reiter's Syndrome Or Reactive Arthritis | O | O | _____ |
| Peripheral Arthritis Hips, Shoulders | O | O | _____ |
| Peripheral Arthritis Other | O | O | _____ |
| **JOINT REPLACEMENT** | | | |
| Hip Replacement | O | O | _____ |
| Knee Replacement | O | O | _____ |
| Other Replacement | O | O | _____ |
| **OTHER** | | | |
| Sausage Digits | O | O | _____ |
| Heel Pain (enthesitis) | O | O | _____ |

FUNCTIONAL CLASS          1     2     3     4

GRADE 1:    All activities without pain or handicap
GRADE 2:    Adequate for most ADL but some discomfort or limitation
GRADE 3:    ADL limited to self-care and/or few daily activities
GRADE 4:    Little or no self-care or confined to a bed or wheelchair

X-RAY FINDINGS _____

## MEDICATIONS (CIRCLE ALL THAT APPLY)

### NSAIDS

| | | | |
|---|---|---|---|
| ASA-enteric coated | never | past | present |
| Phenylbutazone | never | past | present |
| Idomethacin (Indocid) | never | past | present |
| Ibuprofen (Motrin) | never | past | present |
| Naproxen (Naprosyn) | never | past | present |
| Tolmetin (Tolectin) | never | past | present |
| Ketoprofen (Oruidis) | never | past | present |
| Surgam | never | past | present |
| Diclofenac (Voltaren) | never | past | present |
| Feldene | never | past | present |
| Other (specify) | never | past | present |

### DMARDS

| | | | |
|---|---|---|---|
| Gold –IM | never | past | present |
| Imuran | never | past | present |
| Sulphasalazine | never | past | present |
| Cyclosporin | never | past | present |
| Oral Steroids | never | past | present |
| Chloroquine | never | past | present |
| Hydroxycloroquine | never | past | present |
| MTX – oral | never | past | present |
| MTX – im/sc | never | past | present |
| Fish Oil | never | past | present |
| Retinoid | never | past | present |
| Other | never | past | present |

## PHYSICAL EXAMINATION

Height_____cm          Weight_____kg          BP____/_____          Pulse_____

**Head and Neck**

| | | |
|---|---|---|
| Abnormal | N | Y |
| Conjunctivitis | N | Y |
| Iritis | N | Y |
| Uveitis | N | Y |
| M. membrane ulcers | N | Y |

**Lungs**

| | | |
|---|---|---|
| Abnormal | N | Y |
| COLD | N | Y |
| other (specify) | N | Y |

**Heart**

| | | |
|---|---|---|
| Abnormal | N | Y |
| Arrythmia | N | Y |
| Heart failure | N | Y |
| Valvular disease | N | Y |

**Abdomen**

| | | |
|---|---|---|
| Abnormal | N | Y |
| Hepatomegaly | N | Y |
| Splenomegaly | N | Y |

**Lymphadenopathy**

| | | |
|---|---|---|
| Cervical | N | Y |
| Axillary | N | Y |
| Inguinal | N | Y |
| Multiple | N | Y |

**Skin**

| | | |
|---|---|---|
| Psoriasis | N | Y |
| Flexural psoriasis | N | Y |
| Guttate | N | Y |
| Erythroderma | N | Y |
| Nail lesions | N | Y |
| Pits | N | Y |
| Onycholysis | N | Y |
| Ridges | N | Y |

**CNS**

| | | |
|---|---|---|
| Abnormal | N | Y |
| Cranial neuropathy | N | Y |
| Peripheral neuropathy | N | Y |
| Other (specify) | N | Y |

**Muscle**

| | | |
|---|---|---|
| Proximal Weakness | N | Y |
| Distal Weakness | N | Y |

HLA – B27     1.Yes     2.No     3.Don't Know

Date (y/m/d)_____
Result   1.Positive          2. Negative

SURGERY FOR ARTHRITIS   1.Yes   2.No   Type_____

OTHER SURGERY              Date (y/m/d)_____Type_____

                                 Date (y/m/d)_____Type_____

                                 Date (y/m/d)_____Type_____

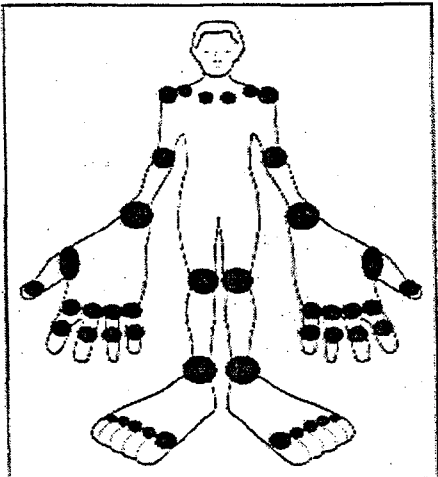AFFECTED          1.Yes          2.No

DNA COLLECTED   1. Yes (y/m/d)_____     2. No


INTERVIEWED BY:_____(Y/M/D)_____

**ISSAS**

**CRF collected for every patient by the metrologist**

| 1. Country | | NL – |
| | | Belgium – |
| | | Germany – |
| | | France – |
| | | Italy – |
| | | Spain – |
| | | Finland – |
| | | Norway – |
| | | UK – |
| | | US – |
| | | Canada – |
| | | Mexico – |
| | | Australia – |
| 2. Date of birth (day/month/year) | | ⊔⊔ /⊔⊔ /⊔⊔ |
| 3. Gender | | male – |
| | | female – |
| 4. Year of symptom onset | | ⊔⊔⊔⊔ |
| 5. Year of diagnosis | | ⊔⊔⊔⊔ |

| 6. Radiographic sacroiliitis | unilateral | bilateral | no | unknown |
|---|---|---|---|---|
| 7. History of peripheral arthritis | | | yes | no |
| 8. History of IBD | | | yes | no |
| 9. Hip involvement | | | yes | no |
| 10. Total hip replacement | unilateral | bilateral | | no |
| 11. Paid job | | | yes | no |
| 12. Current sick leave | | | yes | no |

**Total Swollen Joint Index (44 Joints)**



13. Number of swollen joints: count and mark the swollen joints on the mannequin ⊔ ⊔

21 March 2003

Number of tender enthesis
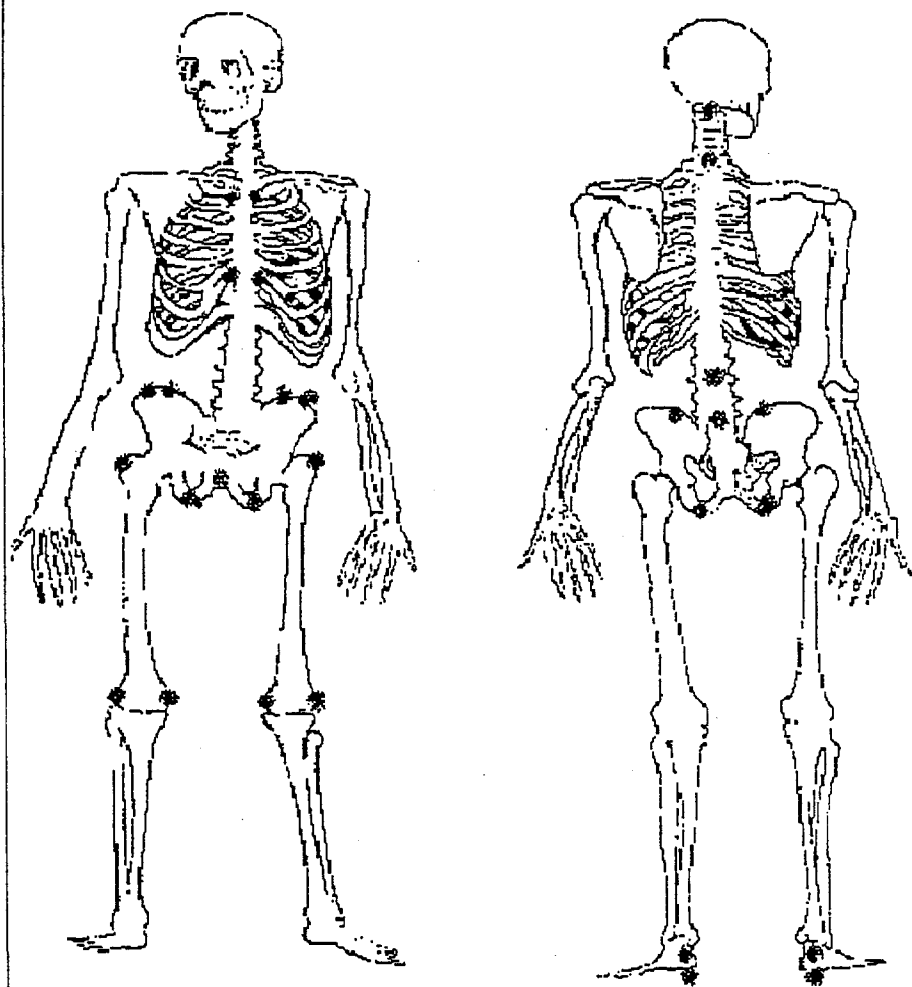


These are the sites marked on the mannequin:

| | |
|---|---|
| C1/C2 | Pelvic adductor origin ri/le |
| C7/T1 | Ischial tuberosities ri/le |
| Costochondral 1 ri/le | Greater Trochanter ri/le |
| Costochondral 7 ri/le | Spina iliaca posterior ri/le |
| T12/L1 | Spina iliaca anterior superior ri/le |
| L5/S1 | Medial femurcondyl ri/le |
| Crista iliaca ri /le | Lateral femurcondyl ri/le |
| Anterior Superior border of iliac crest ri/le | Insertion Achilles tendon ri/le |
| Symphysis pubis | Plantar fascia ri/le |

The patients' response to firm palpation over these entheses.

14. Number of tender enthesis          ⊔ ⊔

21 March 2003

| | |
|---|---|
| 15. Occiput wall distance<br>*(Heels and, if possible, the back against the wall, with the distance measuredin centimeters to the nearest 0.1 cm from the occiput to the wall during maximal effort to touch the head to the wall, without raising the chin above its usually carrying level. The best of two tries should be recorded)* | ⊔ ⊔,⊔ cm |
| 16. Modified Schober test<br>*(This test is performed by marking a point over the spinous process of L5 (found as the first process below the projected line across the back at the level of the top of the iliac crest, although the exact point is not absolutely critical). The second point directly 10 cm above the first, while the patient is extending his lumbar spine in neutral position. The patient then flexes forward as far as possible and the distance between the two points is measured. Normally the 10 cm distance increases to 16 cm or more. The difference should be written (in this case 10/16, while 10/10 would indicate no change on flexion of the lumbar spine). The actual distance in centimeters measured in full flexion should be recorded, rather than the difference between the extension and the flexion of measurements)* | ⊔ ⊔,⊔ cm |
| 17. Lateral spinal flexion<br>*(Is measured by fingertip to floor distance in full lateral flexion without flexing forward or bending the knees. The patient should stand as close to the wall as possible with shoulders level. The distance between patient's middle fingertip and the floor is measured with a tape measure. The patient is asked to bend sideways without bending his knees or lifting his heels and attempting to keep his shoulders in the same place. A second reading is taken and the difference between the two is recorded. The best of two tries is recorded for left and right. The mean of left and right gives the final result for lateral spinal flexion (in cm to the nearest 0.1 cm)* | ⊔ ⊔,⊔ cm |
| 18. Chest expansion<br>*(The difference in centimeters to the nearest 0.1 cm between full expiration and full inspiration, measured at the nipples. The best of two tries should be recorded)* | ⊔ ⊔,⊔ cm |
| 19. Cervical rotation<br>*(Is measured with a gravity action goniometer. The patient lies supine in the neutral position and the goniometer is placed centrally on the forehead. The patient is then asked to turn the head as far as possible to the right and then to the left. The best of two tries for left and right should be recorded. The mean of left and right gives the final result in degrees)* | ⊔ ⊔ ⊔ ° |
| 20. Intermalleolar distance<br>*(The patient supine, the knees straight and the feet pointing straight up. The patient is asked to separate the legs as far as possible and the distance between the medial malleoli is measured (in cm to the nearest cm). The best of two tries should be recorded)* | ⊔ ⊔ ⊔ cm |

| | | | | |
|---|---|---|---|---|
| 21. ESR (within last 3 months) | value | | ⊔⊔⊔ | |
| 22. ESR increased (according to own lab) | yes | no | n.a. | |
| 23. CRP (within last 3 months) | value | | ⊔⊔ | |
| 24. CRP increased (according to own lab) | yes | no | n.a. | |
| 25. Activity on MRI (only if available) | yes | no | n.a. | |
| 26. Rapid radiographic progression (only if available) | yes | no | n.a. | |
| 27. Activity on scintigraphy (only if available) | yes | no | n.a. | |
| 28. Activity on ultrasonography (only if available) | yes | no | n.a. | |

| | |
|---|---|
| 29. Current treatment for AS | Indomethacin ‾ |
| | Diclofenac ‾ |
| | Eterecoxib ‾ |
| | Ibuprofen ‾ |
| | Naproxen ‾ |
| | Piroxicam ‾ |
| | Nebumetone ‾ |
| | Meloxicam ‾ |
| | Rofecoxib ‾ |
| | Celecoxib ‾ |
| | Other ‾ |
| 30. Daily dose of this medication (mgram) | ⊔⊔⊔⊔ |
| 31. Number of previous treatments for AS with NSAIDs other than the present NSAID | 0 ‾ |
| | 1 ‾ |
| | 2 ‾ |
| | > 2 ‾ |
| 32. Maximal dose of these NSAIDS used | yes ‾ |
| | no ‾ |
| 33. Reason for discontinuation | inefficacy ‾ |
| | intolerance ‾ |
| | both ‾ |
| 34. Current treatment with | sulfasalazine ‾ |
| | methotrexate ‾ |
| | biphosphonates ‾ |
| | thalidomide ‾ |
| | TNF-blocker ‾ |
| | systemic corticosteroids ‾ |
| | injections with corticosteroids ‾ |
| | other ‾ |
| 35. Previous treatment with | sulfasalazine ‾ |
| | methotrexate ‾ |
| | biphosphonates ‾ |
| | thalidomide ‾ |
| | TNF-blocker ‾ |
| | systemic corticosteroids ‾ |
| | injections with corticosteroids ‾ |
| | other ‾ |

**ISSAS**

**CRF collected for every patient by the rheumatologist**

| A. | Do you want to start therapy with TNF-blockers in this patient under the folowing assumptions:<br>1. the treatment gives an improvement of at least 50% in at least 50% of the patients;<br>2. the drugs are available and fully reimbursed;<br>3. there may be an increased risk for (serious) infections and little information is available on long term safety.<br>This judgement should be made independent of the patient's wish to start TNF-blockers. |
|---|---|
| | _yes |
| | _no |

| B. | The dominant localisation of symptoms is: | |
|---|---|---|
| | axial | _ |
| | peripheral arthritis | _ |
| | enthesitis | _ |

| C. | This decision is based on: (tick all those that are applicable) | |
|---|---|---|
| | clinical disease activity | _ |
| | disease severity (including structural damage on radiographs, loss of spinal mobility, loss of physical function etc.) | _ |
| | rapid functional decline (in the previous 0-2 years) | _ |
| | hip involvement | _ |
| | peripheral arthritis | _ |
| | other extraspinal manifestations | _ |
| | rapid radiographic progression (in the previous 2-4 years) | _ |
| | disease activity on MRI | _ |
| | increased ESR or CRP | _ |
| | disease inappropriately controlled with present treatment | _ |
| | recurrent infections | _ |
| | past history of tuberculosis | _ |
| | co-morbidity | _ |
| | high risk of adverse events in this patient | _ |
| | expected low compliance in this patient | _ |
| | other reasons, please specify ................................................................................................... | _ |

# Ankylosing Spondylitis Family Study

## DNA Banking for Newfoundland Families

**To**

Dr. R. Inman
Toronto Western Hospital
Fell Pavilion 1-221
399 Bathurst St.
Toronto, ON
M5T 2S8

Telephone: (416) 603-5869

**From**

Dr. Proton Rahman/ Donna Hefferton
1st Floor Morrissey Wing
St. Clare's Mercy Hospital
LeMarchant Rd.
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A1C 5B8

Telephone (709) 777-5733
Email : dheffert@morgan.ucs.mun.ca

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Date Blood Collected _____

Personal Identifying Number _____

Relation to Proband ☐ Proband ☐ Sibling ☐ Parent ☐ Child
☐ Other (specify) _____

Gender ☐ Male ☐ Female

Clinical Diagnosis 1. ☐ Ankylosing Spondylitis

2. ☐ Unaffected

FUTURE USE OF DNA AUTHORIZED BY PARTICIPANT:

☐ Any REB-Approved research project including those in which participant's name is used.
☐ Need participant's consent for studies in which participant's name is associated with the sample.
☐ Not to be used for any future project, destroy samples at the end of the present project.

    3 sodium-heparinized tubes
    2 EDTA tubes

# Ankylosing Spondylitis Family Study

## DNA Banking for Newfoundland Families

| To | From |
|---|---|
| **Dr. R. Inman** | **Dr. Proton Rahman/ Donna Hefferton** |
| **Toronto Western Hospital** | **1st Floor Morrissey Wing** |
| **Fell Pavilion 1-221** | **St. Clare's Mercy Hospital** |
| **399 Bathurst St.** | **LeMarchant Rd.** |
| **Toronto, ON** | **St. John's, NF** |
| **M5T 2S8** | **A1C 5B8** |
| | |
| **Telephone: (416) 603-5869** | **Telephone (709) 777-5733** |
| | **Email : dheffert@morgan.ucs.mun.ca** |

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Date  Blood Collected          _____


Personal Identifying Number     _____

Relation to Proband        ❑ Proband ❑ Sibling ❑ Parent ❑ Child
                           ❑ Other  (specify) _____

Gender                     ❑ Male        ❑ Female

Clinical Diagnosis    1.   ❑ Ankylosing Spondylitis

                      2.   ❑ Unaffected

FUTURE USE OF DNA AUTHORIZED BY PARTICIPANT:

❑ Any REB-Approved research project including those in which participant's name is used.
❑ Need participant's consent for studies in which participant's name is associated with the
  sample.
❑ Not to be used for any future project, destroy samples at the end of the present project.

    3 sodium heparinized tubes
  - 2 EDTA tubes

**Faculty of Medicine, Schools of Nursing and Pharmacy of Memorial
University of Newfoundland; Health Care Corporation, St. John's; Newfoundland Cancer
Treatment and Research Foundation**

## Consent to Take Part in Health Research

TITLE:        Genetic Linkage Study of Ankylosing Spondylitis

INVESTIGATOR(S):        Drs. P. Rahman, R. Inman, M. Stone

SPONSOR:        National Institutes of Health

You have been asked to take part in a research study. It is up to you to decide whether to be in the study or not. Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

The researchers will:

* discuss the study with you
* answer your questions
* keep confidential any information which could identify you personally
* be available during the study to deal with problems and answer questions

If you decide not to take part or to leave the study this will not affect your normal treatment

1.    Introduction/Background:

Ankylosing Spondylitis is a disease that has genetic risk factors. These risk factors have yet to be identified. Finding these risk factors will lead to a faster diagnosis of disease and earlier treatment in patients. This early diagnosis and treatment may help slow the development of the disease.

2.    Purpose of study:

The purpose of this study is to identify inherited and environmental factors that affect the immune system leading to the development of Ankylosing Spondylitis. This study may eventually lead to a better understanding and treatment of this disease.

3.    Description of the study procedures and tests:

You will be asked to:
1. allow the researchers to review your medical records and x-rays
2. complete a questionnaire about your medical and family history
3. have a genetic blood test (2 tubes of blood approximately 10 mls)

4.  **Length of time:**

You will be asked to complete a questionnaire during one visit taking about 45 minutes

5.  **Possible risks and discomforts:**

There is a risk of slight pain or bruising when your blood is drawn.

6.  **Benefits:**

It is not known whether this study will benefit you.

7.  **Statement on Genetic studies**

In order to interpret the results of the research properly, it is essential to have accurate information concerning parentage. Sometimes the research results may point out discrepancies in parentage (which may occur in case of adoption or a mistake in the identity of a father). If this happens, the information will be kept in the strictest confidence and will not be released to anyone, including family members and yourself.

8.  **Future use of DNA**

In order to preserve a valuable resource, your DNA samples may be stored at the end of this research project. It is possible that these samples may be useful in a future research project which may or may not be related to the current research project. Any future research would have to be approved by a Research Ethics Board (REB).

> Please tick **one** of the following three options:
> O 1.  I agree that my DNA samples can be used for **any** REB-approved research project (about inflammatory diseases), including those in which my name is given to the researchers, without obtaining further consent from me.
> O 2.  Specific consent must be obtained from me before using my DNA samples in any future research project in which my name is associated with the sample.
> O 3.  Under no circumstances may my DNA samples be used for any future research project. The samples must be destroyed at the end of the present project.

> Signature _____    Date: _____
>
> Witness: _____    Date:_____

9.  **Liability statement:**

**Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.**

10. **Questions:**

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. That person is:

<u>Dr Proton Rahman    777-5733</u>

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:

<u>Office of the Human Investigation Committee (HIC) at 709-777-6974</u>
<u>Email:  hic@mun.ca</u>

# Signature Page

**Study title:** Genetic Linkage study of Ankylosing Spondylitis

**Name of principal investigator:** Dr. Proton Rahman

**To be filled out and signed by the participant:**

Please check as appropriate:

| | |
|---|---|
| I have read the consent [and information sheet]. | Yes { } No { } |
| I have had the opportunity to ask questions/to discuss this study. | Yes { } No { } |
| I have received satisfactory answers to all of my questions. | Yes { } No { } |
| I have received enough information about the study. | Yes { } No { } |
| I have spoken to a physician and my questions have been answered | Yes { } No { } |
| I understand that I am free to withdraw from the study | Yes { } No { } |

- at any time
- without having to give a reason
- without affecting my future care

I understand that it is my choice to be in the study and that I may not benefit.　　Yes { }　　No { }

I agree that the study doctor or investigator may read the parts of my hospital　　Yes { }　　No { }
records which are relevant to the study.

I agree to take part in this study.　　Yes { }　　No { }

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Signature of participant　　　　　　　　　Date

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Signature of witness　　　　　　　　　　Date

## To be signed by the person obtaining consent:

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

---

Signature of person obtaining consent　　　Date

Telephone number:　(709)777-5733