THE ROLE OF THE PROTEIN TYROSINE PHOSPHATASE NON-RECEPTOR TYPE 22 GENE POLYMORPHISM IN DISEASE SUSCEPTIBILITY AND SEVERITY IN BLACK SOUTH AFRICANS WITH RHEUMATOID ARTHRITIS

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of

Master of Medicine in the branch of Internal Medicine

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DECLARATION

I, Nimmisha Harilall Govind declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Internal Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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.....day of.....2011

To my loving parents, Harilall and Tara Govind

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS PROJECT

Publications:

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Poster presentation:

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ABSTRACT

BACKGROUND: The protein tyrosine phosphatase non receptor type 22 (*PTPN22*) gene inhibits T-cell activation. A functional single nucleotide polymorphism (SNP) Arg620Trp (rs2476601), resulting from a C→T substitution at nucleotide position 1858, is a significant risk factor for rheumatoid arthritis (RA) in European populations. The variant allele results in a gain of function that alters the threshold for T-cell signalling and abnormal T regulatory cell function.

AIM: To investigate the role of the *PTPN22* R620W polymorphism in disease susceptibility and severity in Black South Africans (BSA) with RA.

METHODS: A cohort of 187 BSA patients with RA and 93 ethnically matched Black and 60 White controls with no history of RA or other autoimmune diseases were studied. Genotyping was performed by the polymerase chain reaction and pyrosequencing.

RESULTS: The rs2476601 SNP was nonpolymorphic in both Black patients and Black control subjects with total absence of the variant 'T' allele. In White control subjects the frequency of the 'T' allele was 0.092, with T/T, C/T and C/C genotype frequencies of 0.00, 0.183, and 0.817, respectively.

CONCLUSION: This study shows that the rs2476601 SNP of the *PTPN22* gene is nonpolymorphic in BSA and therefore not associated with RA but the minor 'T' allele frequency in White South Africans is similar to that in other European populations. However, since variations in the rest of the gene were not investigated, these results do not exclude other *PTPN22* polymorphisms from playing a role in RA susceptibility in BSA.

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NOMENCLATURE

Anti-CCP	anti-cyclic citrullinated peptide antibody
ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatology
BSA	Black South African
Csk	c-terminal Src kinase
HLA	human leukocyte antigen
GM-CSF	granulocyte-macrophage colony-stimulating factor
GWAS	genome wide association study
Lck	lymphocyte specific protein tyrosine kinase
IL	interleukin
IFN	interferon
Lyp	lymphoid specific phosphatase
mHAQ	modified health assessment questionnaire
MHC	major histocompatibility complex
PCR	polymerase chain reaction
PTPN22	protein tyrosine phosphatase non receptor type 22
RA	rheumatoid arthritis
RF	rheumatoid factor

SE	shared epitope
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
TNF	tumour necrosis factor
Treg	T regulatory cells
TGFβ	transforming growth factor beta

Chapter 1. INTRODUCTION AND LITERATURE REVIEW

1.1 History of rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory arthritis of unknown aetiology. The history of RA is unclear. There is a suggestion that this disease dates back to the 1500s. Historical characters such as Erasmus, considered as the 'Prince of Humanism', Louis XIV, Kant and others had suffered some form of arthritis. The paintings of Rubens, Renoir, Klee and Dufy depict physical changes which many physicians have described as being consistent with 'modern day' RA (Dieppe and Rogers, 1986).

The first recognized description of RA was made in 1800 by the French physician, Dr Augustin Jacob Landre-Beauvais who was based in the famed Salpetriere Hospital in Paris. The name 'rheumatoid arthritis' itself was coined in 1859 by a British rheumatologist, Dr Alfred Baring Garrod (Keitel, 2009).

1.2 Epidemiology and clinical features

RA is the most common autoimmune disease, afflicting 0.5-1% of adults worldwide. RA affects twice as many females than males. The peak age of onset is the 4^{th}-5^{th} decade of life. The prevalence increases after the age of 60 years. The peak age of incidence is between the 4^{th} and 6^{th} decade of life (Gabriel et al., 1999).

Epidemiological studies done in black South Africans (BSA) in the 1970s showed that the prevalence of confirmed RA was 0.12% in the rural Tswana (Beighton et al., 1975). This is in contrast to the prevalence of 1% documented in Caucasians. Furthermore, the severity of RA is thought to be less in Africans (Adebajo and Reid, 1991,Chikanza et al., 1994). However, a study done in urban blacks of Soweto showed that the prevalence was estimated to be 0.9-1.4% and urban dwellers had more severe disease and resembled features of the classical RA observed in the Caucasian population (Solomon et al., 1975).

More recent studies done in Johannesburg show that the disease is frequently severe and can be associated with severe disability (Tikly et al., 2003,Solomon et al., 2005).

RA is characterised by persistent synovitis usually involving peripheral joints in a symmetrical distribution. RA can potentially affect any synovial joint. Classical RA causes swelling of the small joints of the hand as depicted in Figure 1.1.

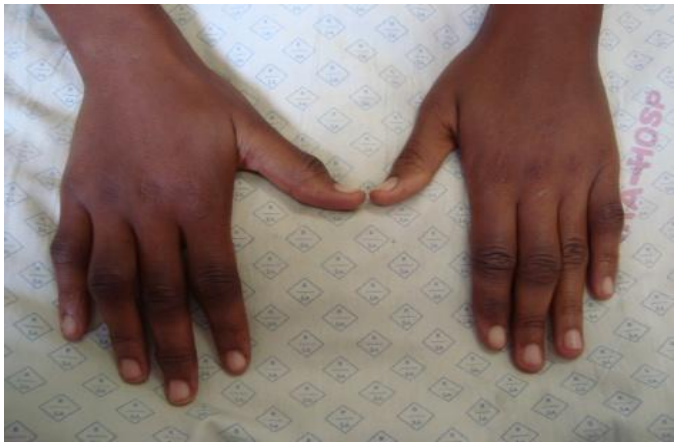

Figure 1.1 Hand involvement with swelling of the proximal interphalangeal joints in RA

However, an African variant has been described which affects predominately the wrists and spares the small joints of the hands (Maritz et al., 2003).

Inflammation of the organs may occur, predominately in rheumatoid factor (RF) positive patients. Rheumatoid nodules are a common manifestation of RA. Other extra articular features include rheumatoid lung (interstitial lung disease and bronchiolitis), keratoconjunctivitis sicca, scleritis, serositis, anaemia of chronic diseases and vasculitis. Risk factors for severe RA include RF and anti- citrullinated protein antibody (ACPA) positivity (Niewold et al., 2007), genetic susceptibility, smoking (Baka et al., 2009, Nyhall-Wahlin et al., 2009). Poor prognostic features of RA are poor functional class at presentation, poor socioeconomic background, older age at presentation, erosive disease and the presence of extra- articular disease.

1.3 Health and economic burden

Cost-of-illness studies have shown that RA is associated with a high health and economic burden (Boonen and Mau, 2009). The economic 'burden' of disease relates to both direct and indirect costs.

The direct cost relates to the actual payment made for diagnosis, therapy, complications and prevention of the disease. The direct costs of RA are 2-3 times higher than the average cost for individuals of a similar age and gender (Lubeck, 2001).

The indirect costs of RA are substantial and relate to loss of resources to the individual, their family and society. These costs relate to work disability and early retirement. Approximately a quarter of patients will discontinue paid work within 2 years of RA diagnosis (Kvien, 2004). A South African study showed that only 24% of RA patients were employed and 35% were dependent on financial support from the state (Mody et al., 1988).

Indirect cost of RA is also linked to an increased mortality due to RA. Life expectancy is decreased by 5-10 years. The five-year survival of patients in the American College of Rheumatology (ACR) functional class four is similar to those patients of triple vessel disease or stage IV Hodgkin's lymphoma (Pincus et al., 1984). The major causes for death in RA are cardiovascular and cerebrovascular disease (Douglas et al., 2006, Meune et al., 2009).

The psychosocial burden of the disease includes loss of quality of life, pain, frustration and lack of self esteem. Studies done in South Africa showed that RA has a significant effect on health-related quality of life (Benitha and Tikly, 2007) and it is also associated with a negative impact on the individual's psychological and social functioning (Schneider et al., 2008).

1.4 Pathogenesis

During normal embryonic development, the immune system is tolerized to self antigen. T-cells in the thymus undergo positive and negative selection to only recognize non-self antigen in the context of major histocompatibility complex (MHC) molecules. Autoreactive T-cells that escape this process are controlled by peripheral regulatory T cells (Tregs). The early breach in T-cell and B-cell tolerance that occurs in RA is poorly understood. It is probable that there is both aberrant thymic selection and peripheral tolerance. Naturally occurring Tregs seem to have impaired regulatory function (McInnes and Schett, 2007).

The T-cell phenotype in RA has been thought to be T helper 1(T_H1) cell type however recent animal studies implicate T_H17 as the key effector cell subtype (Cooles and Isaacs, 2011). T_H17 cell production can be induced by interleukin (IL)-6, transforming growth factor beta (TGFβ) and IL-23. Synovial T cells contribute to synovitis through enhancing T-cell activation, endothelial permeability, plasma cell hyperactivity, macrophage/antigen presenting cell activation, and through the production of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-10, tumour necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Pratt et al., 2009). Angiogenesis, the formation of high endothelial venules and the expression of endothelial adhesion molecules promote migration of T-cells into synovial tissue.

The role of B cells in the pathogenesis of RA is through the production of autoimmune antibodies, cytokines and chemokines such as IL-6, IL-10 and LTβ production and the ability to present antigen. Plasma cells secrete immunoglobulins e.g. RF and ACPA. Various RF isotypes form immune complexes which are capable of complement fixation and neutrophil activation.

Macrophages are key to the production of inflammatory cytokines. They are activated through pattern-recognition receptors. Macrophages produce cytokines, of which TNF is of primary importance. TNF induces leukocyte, endothelial cell activation and synovial fibroblast activation. TNF also induces angiogenesis and cytokine production such as IL-6, IL-15, and IL-1.

Osteoclast differentiation is achieved by the actions of TNF and IL-1, as well as of IL-17, produced by T_H17 cells, and IL-7, produced by synovial fibroblasts. By contrast, IL-4 and IL-10, which are produced by T_H2 cells, and GM-CSF and interferon-γ (IFNγ), which are produced by T_H1 cells, inhibit osteoclast differentiation.

Cartilage degradation is a multistep process based on the release of matrix-degrading enzymes such as aggrecanases (ADAMTS) and matrix metalloproteinases. Cytokines such as IL-1 and IL-17 induce a switch in the synthesis pattern of chondrocytes from matrix molecules to matrix-degrading enzymes. In addition, synovial fibroblasts produce matrix-degrading enzymes and invade cartilage when activated by cytokines such as TNF and IL-1. Chondrocyte death is another feature of cartilage damage, it leads to the formation of empty lacunae and deprives cartilage from the ability to replenish matrix.

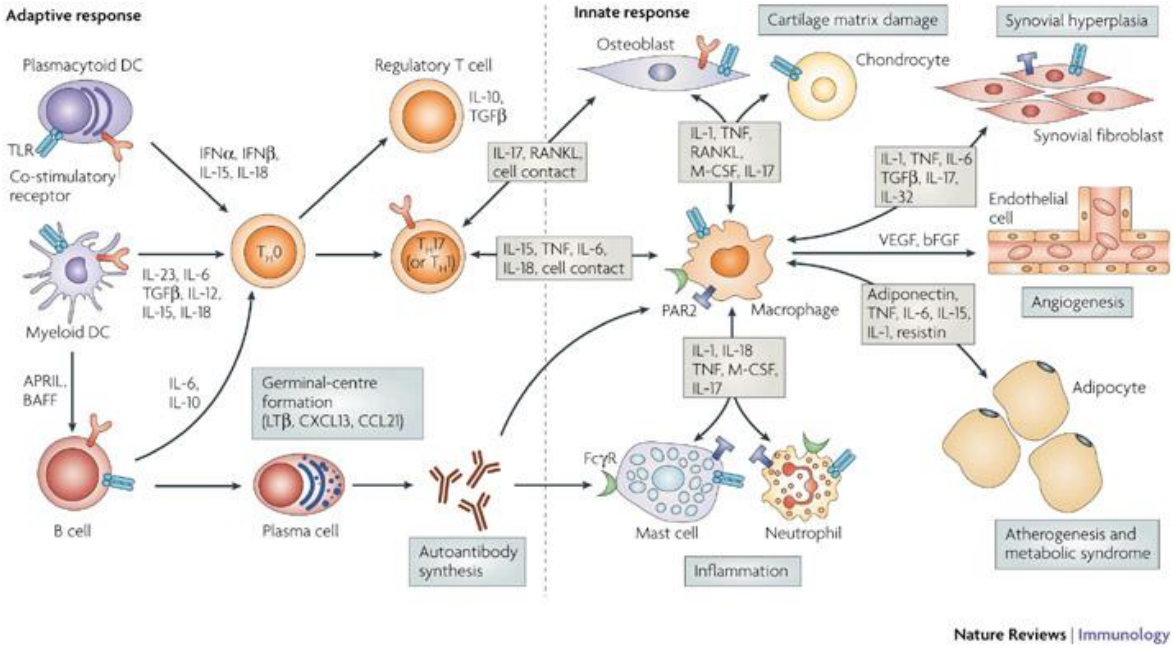

Figure 1.2 Pathogenesis of RA (McInnes and Schett, 2007)

1.5 Aetiology

The aetiology of RA remains unknown. RA is thought to occur in a genetically susceptible host that is then exposed to infectious, environmental, hormonal or other yet unidentified triggers.

Infections such as Epstein-Barr virus (Ollier, 2000, Saal et al., 1999), Parvovirus B19 and mycobacteria probably cause disease through molecular mimicry of specific microbial peptides with autologous molecules such as the rheumatoid or cartilage-derived epitopes. However, convincing evidence of a specific pathogen-derived antigen or of cross-reactivity of self-antigen-specific T or B cells with pathogen derived peptides is lacking.

Smoking confers not only an increased risk of developing RA, but also influences the course of the disease. Smoking is the most prominent example of a gene-environment interaction in RA pathogenesis. A meta-analysis revealed that smoking is a risk factor for RA, especially RF+ males and heavy smokers (Sugiyama et al., 2010). Tobacco exposure

increases the risk for ACPA+ RA, but only in shared epitope positive patients (Linn-Rasker et al., 2006).

RA occurs more frequently in women than men. Oestrogen exerts a stimulatory effect on the immune system by inhibiting T cell suppressor function and facilitating T cell maturation (Cutolo, 2004). Women with older age at menarche (>15) have an almost 2-fold increased risk of developing RA as compared to women with early menarche (<12). Pregnancy increases the risk of developing RA. Twelve percent of women with RA develop RA within a year after pregnancy. Females having multiple pregnancies are at risk of severe RA. Longer duration of breastfeeding increases the risk of RA in individuals who are ACPA positive in the presence of the PTPN22 risk allele (Berglin et al., 2010). The use of the oral contraceptive pill delays onset of disease rather than protect against its occurrence (Doran et al., 2004).

Occupational exposure to mineral oils was found to be of an increased risk of RA in a Swedish study (Sverdrup et al., 2005). A diet low in antioxidants, e.g. vitamin C and β-cryptoxanthin, has been found to increase the risk of RA, as has excessive coffee consumption, however only in ACPA + RA (Pattison et al., 2005).

1.6 The Genetics of RA

The evidence for a significant genetic component to RA susceptibility was shown by the excess disease concordance in monozygotic twins (15%) compared to dizygotic twins (3.6%) (MacGregor et al., 2000). The increased risk of the disease in siblings of patients with RA compared with that of the general population has been estimated to be between 2 and 17 fold.

1.6.1 The HLA genes and RA

It is estimated that the heritability for RA is 50-60% (MacGregor et al., 2000), a third of which is accounted for by polymorphisms of the *HLA-DRB1* gene. The strongest link between genetic susceptibility and RA is the association of the disease with an epitope in the third hypervariable region of β-chain of the HLA-DR molecule, also known as the 'shared epitope' (SE) (Gregersen et al., 1987). SE alleles are found in ~60-70% of European ancestry subjects with RA, compared to ~40% of controls. The *DRB1*0401* allele confers a relative risk (RR) of 5 for RA, more than any other single gene or allele so far identified. Studies in BSA with RA showed a strong association with *HLA-DR4* haplotypes, *DR1* and *DR4* (Martell et al., 1989, Mody et al., 1989, Pile et al., 1992). Furthermore, certain alleles, *HLA-DRB1*0401* and **0404*, were associated with severe erosive disease (Meyers et al., 2004). It is thought that additional risk alleles within the major histocompatibility complex (MHC) exist, but these alleles remain to be pinpointed precisely. The SE still remains the only genetic risk that confers susceptibility for RA across all ethnic groups. Non HLA susceptibility genes, however have failed to show consistency between different populations.

1.6.2 Non HLA genes and RA

In total, the HLA genes only contribute 30-50% of the genetic variability of RA. In the European population, the second strongest genetic risk outside of the HLA region is a functional single nucleotide polymorphism (SNP) in the *PTPN22* gene which is a C→T substitution at nucleotide position 1858 (rs2476601), which results in a substitution of trytophan (W) for arginine (R) at codon 620 (Begovich et al., 2004). In European populations T allele carrier rate in RA patients is 1.38-2.04 fold compared to the general

populations. However, the rs2476601 SNP was not found to be associated with RA in the Asian population (Lee et al., 2009, Ikari et al., 2006).

Ethnic differences in genetic susceptibility in RA between European and Asian populations are evident with the *PADI4* gene. Polymorphisms in the *PADI4* gene are associated with the second strongest genetic risk outside of the HLA region in Asians (Iwamoto et al., 2006). This association has not been found in the European populations (Burr et al., 2010) . Lee et al studied genetic variants at 4q27, 6q23, CCL21, TRAF1/C5, and CD40 in Koreans. None of the susceptibility loci identified in Caucasian patients with RA contributed significantly to disease in Koreans (Lee et al., 2009).

In patients of African ancestry ethnic differences are evident in differential susceptibility of the interleukin 10 (IL10) (MacKay et al., 2003) and the corticotrophin releasing hormone (CRH) gene (Baerwald et al., 2000) compared to Caucasians. STAT4 variants have been associated with RA in Asians and Europeans populations but not in African Americans (Kelley et al., 2010). The association was previously confirmed in two other studies in Asians (Lee et al., 2007, Kobayashi et al., 2008)

Although there is much emerging data, the genome wide association studies (GWAS) have been done primarily in European-Caucasian patients and there is limited data relating to susceptibility and severity gene variants in patients of African descent.

1.6.3 Genetic variants associated with RA severity

Genes associated with severity of disease have mostly been studied in Caucasian populations. Apart from the HLA SE alleles, only 6 other loci have been associated with RA radiographic severity: *CD40 (rs4810485), KIF5A/PIP4K2C (rs1678542), CDK6 (rs42041), CCL21 (rs2812378), PRKCQ (rs4750316), PTPN22 (rs2476601) and MMEL/TNFRSF14 (rs3890745)* (van der Linden et al., 2009, Orozco et al., 2010., Marinou

et al., 2010, Steer et al., 2005). There is a paucity of information relating to the genetic risk loci associated with severe RA in African patients. This is in part, due to the lack of identified susceptibility genes and of appropriate clinical, serological and radiological databases.

1.6.4 Genome wide association studies (GWAS) and candidate gene analysis

Most genetic studies in RA have been single candidate gene studies. They involve investigating genes that have known function or association with RA or other autoimmune disease. Candidate genes have usually been selected based on biological studies of disease pathogenesis.

Advances in high throughput technology now allow identification of a large number SNPs simultaneously and have facilitated large case control collections or GWAS (Raychaudhuri, 2010). The GWAS approach is revolutionary because it permits interrogation of SNPs throughout the entire human genome in thousands of unrelated individuals without any prior hypotheses regarding genetic association with diseases.

There are limitations to both the GWAS and candidate gene approaches. In GWAS, because of the massive number of statistical tests performed there is potential for high rates of false positive results. There is also lack of information on gene function, insensitivity to rare variants and structural variants, requirements for large sample sizes, and possible biases due to case and control selection and genotyping errors (Newton-Cheh and Hirschhorn, 2005,Pearson and Manolio, 2008).

.There is often limited information available about environmental exposure and other non-genetic risk factors in GWA studies. This makes it difficult to identify gene-environment interactions or modification of gene-disease association in the presence of environmental factors.

GWAS has advantages over the candidate gene approach which uses costly genotyping methods. This limits the number of variants assayed to at most several hundreds. The candidate genes are selected based on an imperfect understanding of biological pathways. Many candidate gene studies have failed to replicate in subsequent studies.

In the last few years genome-wide association studies and other large scale genetic studies have identified as many as 31 risk RA loci outside of the HLA region (figure 1.2) (de Vries, 2011).

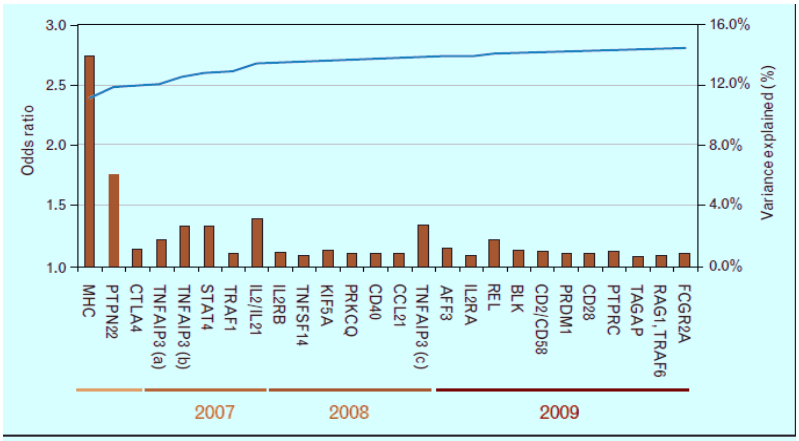

Figure 1.3 Odds ratio of the risk allele for RA (left axis and bars) and cumulative genetic variance explained by loci (right axis and line) (Raychaudhuri, 2010)

1.6.5 The protein tyrosine phosphatase non receptor type 22 gene *(PTPN22)*

1.6.5.1 The single nucleotide polymorphism (rs2476601)

As mentioned earlier, a functional SNP R620W is the strongest genetic association outside of the HLA region in Caucasian populations. In 2004, a candidate gene approach study showed that the minor allele T confers a predisposition to type 1 diabetes in American and Italian populations (Bottini et al., 2004). Begovich et al. reported the same association in RA using firstly a screening strategy of examining 16 000 potential functional SNPs in

genes that were candidates for RA or were located in linkage regions identified in RA whole-genome screening, followed by fine mapping (Begovich et al., 2004).

1.6.5.2 The role of the PTPN22 gene

The *PTPN22* gene maps to chromosome 1p13.3-p13.1. The gene encodes a lymphoid-specific phosphatase (Lyp). Lyp is expressed in haemopoietic tissues, thymus, spleen and bone marrow as well as peripheral blood mononuclear cells.

Lyp binds to C-terminal Src kinase (Csk), an intracellular tyrosine kinase, through a proline rich binding site, SH3 (Bowes and Barton, 2008). Tyrosine phosphatases (TP) function in a coordinated manner with protein tyrosine kinases in the regulation of signalling responses. Kinases are involved with controlling the amplitude of a signalling response and phosphatases are important for controlling the rate and duration of the response. The biological mechanisms underlying the association between *PTPN22* and RA are incompletely understood. The polymorphism leads to a gain of function. Enzyme activation is thought to lead to an increase in the threshold for T-cell receptor signalling.

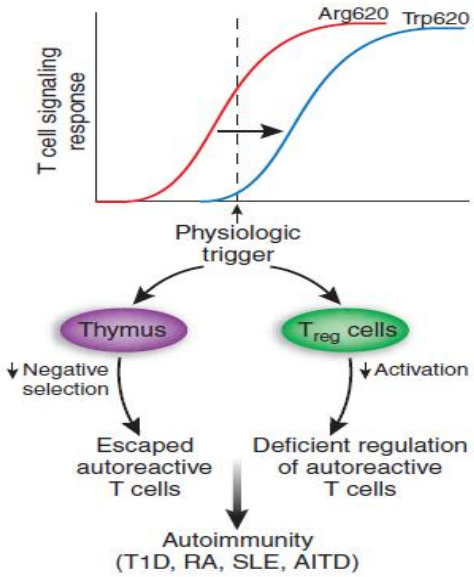

Figure 1.4 *PTPN22* polymorphism: The proposed mechanism of autoimmunity (Gregersen, 2005)

Although the exact mechanism of autoimmunity is not known, it is thought to have effects on the thymus and T reg cells. In the thymus, this altered signalling could result in positive selection of thymocytes that would normally have been removed and this in turn leads to an increase in the number of autoreactive T cells in the periphery. A second mechanism of autoimmunity is altered signalling in Treg cells which lead to altered surveillance and an increase in autoregulatory T cells.

1.6.5.3 PTPN22 polymorphism and ethnic populations

The increase in T allele frequency in RA cases compared with controls has been replicated in numerous European populations (Seldin et al., 2005, Plenge et al., 2005, Pierer et al., 2006). Although the *PTPN22* has consistently shown to be associated with RA in Caucasians, the R620W risk allele in this gene is not found in Asian populations. The R620W SNP was nonpolymorphic in both Japanese (Ikari et al., 2006) and Korean (Lee et al., 2009) populations. This highlights that susceptibility genes associated with disease in Caucasians do not always play a role in other ethnic populations.

1.6.5.4 PTPN22 polymorphism and autoimmunity

Apart from the HLA region, one of the best examples of a genetic contribution to autoimmunity is the *PTPN22* gene. Several studies have confirmed its association with type I diabetes (Smyth et al., 2004, Zheng and She, 2005), systemic lupus erythematosus (Orozco et al., 2005, Kyogoku et al., 2004), and Graves' disease (Velaga et al., 2004, Skorka et al., 2005).

1.6.5.5 The PTPN22 polymorphism and RA severity

There is evidence that the *PTPN22* polymorphism is associated with disease severity. In a British cohort disease occurred earlier and was associated with more erosive disease (Steer et al., 2005). However, other studies did not observe an association between the *PTPN22*

risk variant and radiological damage (Wesoly et al., 2005,Karlson et al., 2008). In the presence of ACPA, disease occurred even earlier (Kokkonen et al., 2007).

1.6.5.6 The PTPN22 polymorphism and predicting RA

The *PTPN22* R620W variant has been found to be a predictive marker for the development of RA. The combination of this variant and ACPA antibodies was found to have a specificity of 100% for diagnosing future onset RA (Johansson et al., 2006). In patients with undifferentiated arthritis the R620W did not predict RA development over the predictive value of ACPA alone (Feitsma et al., 2007).

1.7 Aim of the study

To investigate the role of the PTPN22 R620W polymorphism in disease susceptibility and severity in black South Africans with rheumatoid arthritis.

Chapter 2. PATIENTS AND METHODS

2.1 Study population

The study population comprised of a sample of 187 consecutive, unselected, unrelated, consenting BSA patients with RA attending the Arthritis Clinic of the Chris Hani Baragwanath Hospital. All patients with RA were older than 18 years of age at disease onset and met the American College of Rheumatology (ACR) 1987 revised criteria for the classification of RA (Arnett et al., 1988) (APPENDIX A). Assessment of severity was assessed by the presence of extra-articular manifestations, mean CRP at baseline, functional disability as measured by the modified health assessment questionnaire (mHAQ) (Pincus et al., 1983), (APPENDIX B), history of joint replacement and radiological assessment using the modified Larsens score (Wick et al., 2003).

Radiographic damage is assessed by the modified Larsen method. The joints that are assessed include the 2 interphalangeal joints of the big toe, 10 metatarsophalangeal joints, 4 quadrants of the 2 wrists, 2 interphalangeal joints of the thumb, 8 proximal interphalangeal joints and 10 metacarpophalangeal joints. The maximum possible Larsen damage score (LDS) is 200.

The mHAQ comprises 8 domains relating to different aspects of daily activity. Each domain has questions that are scored out of 3, 0 equates to no assistance required while 3 to almost complete dependence on others to perform daily activities.

They were compared to 93 geographically and ethnically matched Black controls and 60 White controls for which no history of RA or other autoimmune diseases were noted in the self reported questionnaire at the time of enrolment. Patients and controls who were self confessed Africans were regarded as being Black if all four grandparents were of African descent. The Black controls were mainly staff members of the hospital or patients admitted

to casualty with minor injuries. The DNA samples of White controls were made available from the molecular genetics laboratory at the National Health Laboratory Service.

Informed consent was obtained from every subject. This study was approved by the University of the Witwatersrand Human Research Ethics Committee (APPENDIX C).

2.2 Laboratory investigations

2.2.1 Serology

Rheumatoid factor and anti-cyclic peptide antibodies (anti-CCP), a subgroup of ACPA were measured by the enzyme-linked immunosorbent assay. Individuals with RF > 11IU/ml and anti-CCP> 10 IU/ml were regarded as positive.

2.2.2 Genotyping

2.2.2.1 DNA extraction

The DNA was extracted using the salting-out method that was first described by (Miller et al., 1988). Patient's blood was collected in EDTA tubes to prevent clotting and was stored at -20ºC until it was required for extraction. The blood was thawed prior to proceeding with DNA extraction and thereafter approximately 10ml of blood was transferred to NUNC tubes. The tube was filled with Sucrose-Triton X(S-T X) Lysing buffer, which lyses the blood cells. The tubes were inverted a few times to assist mixing. The tubes were centrifuged for 10 minutes at 2300rpm, thereafter the supernatant was removed and a red pellet remained. The pellet was resuspended in S-T X lysing buffer and placed on ice for 5 minutes. The mixture was then centrifuged again for 5 minutes at 2300rpm .The supernatant was again discarded and the pellet mixed with 1.5ml of T20E5 solution. Thereafter 100µl of 10% SDS and 250µl of Proteinase K was added to the solution. The mixture was incubated for 24 hours, allowing the Proteinase K to digest the remaining protein.

After 24 hours 1ml of saturated NaCl solution was added. The mixture was vigorously shaken for 15 seconds and then placed on ice for 5 minutes. Thereafter the mixture was centrifuged for 30 minutes at 2500rpm.The supernatant containing the DNA was transferred to a NUNC tube. Two volumes of absolute ethanol were added to the solution to allow the DNA to precipitate out. DNA was visible as a stringy-like substance, was fished out using a pipette tip, and then washed in 500µl of 70% ice cold ethanol to remove excess salt. The DNA was air dried and resuspended in 1xTRIS-EDTA buffer. The DNA was stored at 4ºC.

2.2.2.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is an *in-vitro* technique that catalyses DNA synthesis to create many identical copies of a sequence of template DNA. Primers were designed by the Assay Design Software of the pyrosequencer. The PCR primers were as follows:

Forward primer: 5'TCACCAGCTTCCTCAACCACAATA

Reverse primer which contains the tag that binds to the universal primer: 5' GACGGGACACCGCTGATCGTTTAGATGATGAAATCCCCCCTCC3') with a sequence specific primer (5'CACACTAAATGATTCAGGTG 3').

The two PCR primers, that were complementary to the DNA sequences flanking the target DNA fragment, were added to the DNA template and in the presence of dNTPs buffer, $MgCl_2$ and Taq polymerase; the complementary DNA sequence was synthesized. The enzyme responsible for this extension is DNA polymerase. The PCR cycle consists of denaturation of the double stranded DNA by heating the reaction to 95ºC, annealing the primers at a temperature specific to the primer sets and extension of the primers by the DNA polymerase at 72ºC.

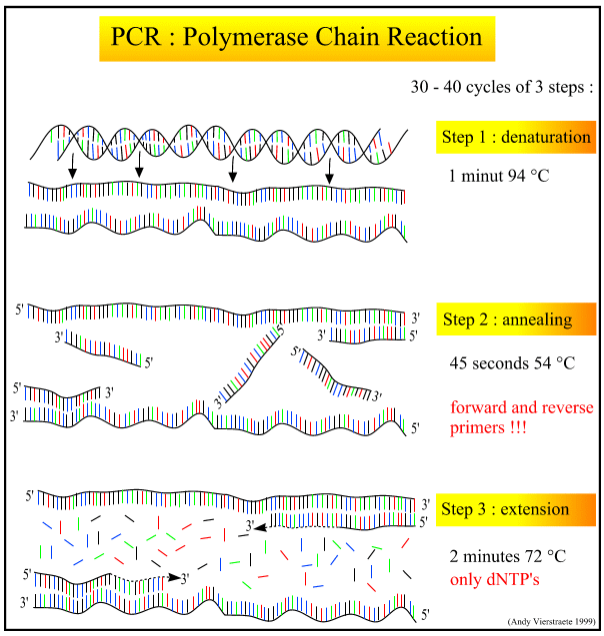

Figure 2.1 Polymerase chain reaction method (Vierstraete 1999)

2.2.2.3 Checking the PCR product

DNA fragments can be separated, identified and purified by means of gel electrophoresis. This mechanism relies on the negative charge of DNA. When an electric field, 125V-140V, is applied across the gel, the negatively charged DNA migrates through the gel toward the anode. The speed and distance migrated by the DNA is influenced by size of the DNA fragments. Using a molecular weight marker, the product can be sized accurately.

2.2.2.4 Pyrosequencing

Pyrosequencing is a DNA sequencing technique that is based on the detection of released pyrophosphate (PPi) during DNA synthesis. To 20µl of PCR product, 20µl of ddH20 was added. A solution of 3µl sepharose beads and 40µl binding buffer was made for each sample. 43µl was added to each sample. The 96 well plates were left on the shaker for >5mins so that the beads did not settle on the bottom of the wells.1.6µl of 10Um sequencing primer and 38.4µl of annealing buffer was added to each sample. The vacuum on the Pyrosequencer (PSQ) was applied to the 96 well plates. The vacuum device with the

attached beads was then moved to 70% ethanol trough for 5 seconds, then to the denaturing buffer for 5 seconds and then to the wash buffer for 5 seconds. The beads and purified PCR products were released into 96 well plates.

The samples were then ready to be analyzed. The cascade starts with the annealing of the sequencing primer and a nucleic acid polymerization reaction. Upon binding of the nucleotide acid to its complementary base and attachment by polymerase, there is a release of inorganic PPi. The PPi is converted to ATP by ATP sulfurylase, which provides the energy to luciferase to oxidize luciferin and generate light.

This reaction generates visible light that is proportional to the number of nucleotides incorporated. Because the added nucleotide is known, the sequence of the template can be determined.

2.3 Statistical Methods

Each group was tested for compliance with Hardy-Weinberg Equilibrium to assess potential bias in sampling or possible genotyping errors. Once the genotypes were scored and allele frequencies calculated, a Chi-squared test was performed to determine if the difference in allele and genotype frequencies between patients and controls was statistically significant.

In the Chi-squared test, the value of the test-statistic is

$$\mathrm{X}^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i},$$

where : X^2 = chi-square, O_i = an observed frequency; E_i = an expected (theoretical) frequency, asserted by the null hypothesis

Chapter 3. RESULTS

3.1 Demographic and clinical features of RA patients

Of the 187 RA patients 83% were females, the mean age at disease onset was 46.7 years. Only a minority had nodulosis and 4.8% had serious extra-articular manifestations (1 interstitial lung disease, 1 pyoderma gangerosum, 1 vasculitis 5 scleritis). Of those tested, 83% were RF positive and 79% were anti-CCP positive (Table 3.1).

Table 3.1 Demographic and clinical features of the 187 Black rheumatoid arthritis patients.

Variable	
Female (%)	156 (83)
Age at disease onset in years, mean (SD)	46.7 (13.6)
Disease duration in years, mean (SD)	10.6 (7.3)
History of joint replacement	8/103 (7.7%)
Swollen joint count, mean (SD)	7.9 (6.3)
Nodulosis	14/165 (8.4%)
Serious extra-articular manifestations	8/165 (4.8%)
mHAQ, mean (SD)	0.87 (0.36)
CRP at baseline, mean (SD)	34.7 (41.1)
Anti-CCP +	72/91 (79%)
RF +	156 (83%)
Larsen Score mean (SD)	53.9 (34.8)

Patients had moderate disease activity as evidenced by the mean swollen joint count, CRP at baseline, history of joint replacement, mean mHAQ and Larsen scores.

3.2 Genotype and allele frequencies

The functional R620W SNP was nonpolymorphic in both the Black patients and controls (Table 3.2). The R620W was polymorphic in the White control subjects, in whom the frequency of the 'T' allele was 0.092. Figures 3.1 and 3.2 show the pyrogram differences between a Black patient with a C/C homozygous genotype and and White controls with a C/T heterozygous genotype.

Table 3.2 Genotypes and allele frequencies in patients and control subjects

	N	**Genotypes**			**Alleles (frequency)**	
		C/C	T/C	T/T	C	T
Black RA patients	187	187	0	0	374 (1.000)	0
Black controls	93	93	0	0	186 (1.000)	0
White controls	60	49	11	0	109 (0.908)	11 (0.092)

N*= number of subjects studied*

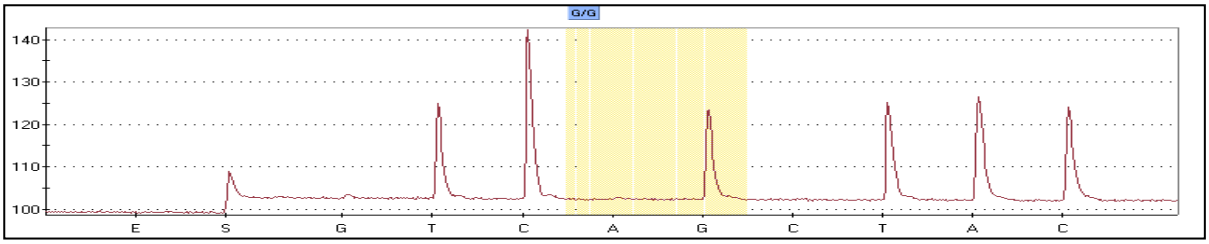

Figure 3.1 Pyrogram of a Black patient with an absent A peak and G peak only (shaded area), implying a C/C genotype.

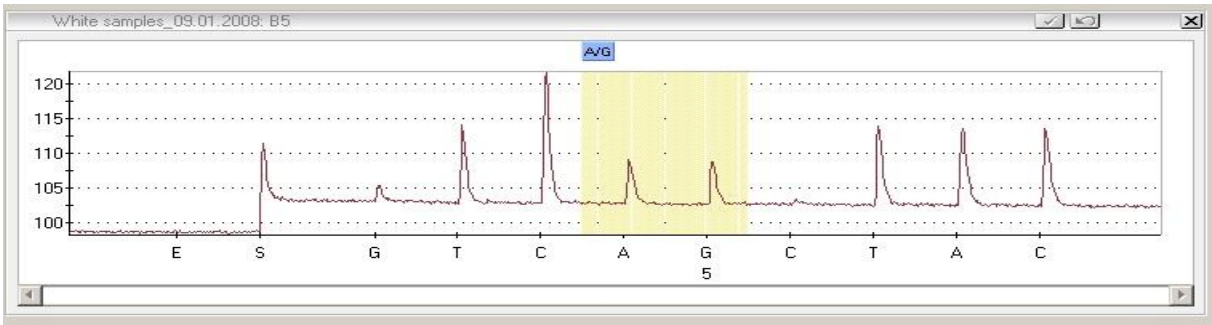

Figure 3.2 Pyrogram of a White control subject with both A and G peaks (shaded area), implying a C/T genotype.

Genotypes were in Hardy-Weinberg equilibrium for the White controls as shown below:

$(p+q)^2 = p^2 + 2pq + q^2$

p= 0.908 (C allele frequency)

q= 0.092 (T allele frequency)

Table 3.3 Observed and expected genotypes in White controls

	C/C	**C/T**	**T/T**	**Total**
Observed	49	11	0	60
Expected	49	11	0	60

The chi test was calculated in the White controls.

In the white controls the genotype was in Hardy-Weinberg equilibrium suggesting no bias in sampling or possible genotyping errors. The p value is not significant. The polymorphism was not present in the Black patients and controls; therefore statistical analysis was not necessary.

Chapter 4. DISCUSSION

In this study, the role of a missense SNP in a gene encoding *PTPN22* was studied for its role in RA disease susceptibility and severity in Black South Africans. The genetic variant was found to be nonpolymorphic in BSA. By contrast, the White controls, which served as a positive control group, had a 'T' allele frequency very much in line with studies in other Caucasian populations (Plenge et al., 2005). Studies done in the Asian population showed, that similar to BSA, this SNP is nonpolymorphic and therefore not associated with RA.

However, this does not mean that aberrant *PTPN22* function does not play a role in RA in BSA with RA. Functional polymorphisms of the *PTPN22*, other than rs2476601, may exist in the BSA population. In the Japanese, *PTPN22* allelic heterogeneity was studied, but none of the 8 other SNPs present in the European populations, were found to be polymorphic (Ikari et al., 2006).

Another explanation may be that the variants in the *PTPN22* gene may reflect genuine differences in pathogenic processes between European and BSA patients. However, this can only be said with certainty once all variants of the gene have been studied. This study shows further evidence of inter-ethnic genetic variability in RA at non-HLA loci. The inconsistent associations might reflect differences in sample size, although this is unlikely in this study as the rs2476601 was nonpolymorphic in both Black patients and controls. These differences might also relate to clinical phenotypic differences between populations. For example, Maritz et al have shown that BSA patients have relative sparing of the small joints of the hands (Maritz et al., 2003).

Genotype-phenotype studies provide evidence that genetic factors modify the serological and clinical expression of the disease. RA is categorised into two distinct subsets, ACPA positive (+) and ACPA negative (-). These autoantibodies are good serological markers for

RA, with specificity of >95% and a sensitivity comparable to that of RF (80%) (Whiting et al., 2010, Hodkinson et al., 2010). The majority of the identified susceptibility genes confer risk to ACPA+ RA and only two genes confer risk to ACPA- RA (van der Helm-van Mil and Huizinga, 2008). The *HLA DRB1* genes associate exclusively with ACPA+ disease. In most studies, variants in the *PTPN22* gene have also been found to be associated with ACPA + however in on study the variant was associated with ACPA negative disease (Pierer et al., 2006). In the Asian population the *PADI4* gene variant has not been conclusively found to be associated with ACPA status (Suzuki et al., 2006). In a study done in Cameroon, there was a discrepancy between the high prevalence of ACPA+ patients and the relative low numbers of SE positive cases. This suggests that other genes that have not yet been identified are associated with ACPA+ RA in African RA patients (Singwe-Ngandeu et al., 2010). In this study majority of patients were RF positive. ACPA testing in South Africa has only recently become available, thus accounting for the low percentage of the test done in this study population.

Studies of genetic predictors of individual RA phenotypes have suggested association between *PTPN22* variants and antibody status (ACPA and RF), erosive disease and age of onset of RA. It is associated with an earlier age of disease onset (8.6 years earlier onset in homozygotes of the high risk allele and 4.7 years earlier onset on heterozygotes) (Karlson et al., 2008).

In this study, the prevalence of antibodies was similar to those reported in most other studies. The presence of ACPA in the absence of the *PTPN22* high risk allele demonstrates the importance of ACPA in the pathogenesis of joint damage in BSAs with RA as well as the possibility of the role of other genetic variants not yet identified in Africans. A hypothesis for the disease mechanism of ACPA positive RA is as follows; citrullination possibly due to smoking, provokes ACPA production in individuals who have

susceptibility alleles of genes including HLA, followed by joint inflammation in an auto-antibody dependant manner (Kallberg et al., 2007).

4.1 **Limitations of the study**

One of the limitations of the study is that the sample size could have been too small to demonstrate the polymorphism if it has a modest effect in BSA. Another limitation of the study is that the other variants of the gene were not studied. The smoking histories of most patients are unknown. The percentage of patients tested for ACPA was only 48%.

Chapter 5. CONCLUSION

The present findings are further evidence that non-HLA genetic risk is inconsistent across ethnic groups and that among the genetic predisposition factors for RA identified to date, some variants have been found to be restricted to specific ethnic groups. This might reflect migration history and the natural selection that shaped genetic variation in these populations. Additional genetic factors may also have exerted different magnitudes of risk for the disease among the different populations, which might be explained by their interactions with other genetic and environmental factors. Current evidence suggests that substantial heterogeneity exists in the genetics underlying autoimmunity among different ethnic populations.

The findings show conclusively that the functional R620W variant, a major risk factor for RA in Caucasian populations, is not present and therefore does not play a role in the pathogenesis of RA in BSA. The verdict is still out as to whether *PTPN22* has a role in RA susceptibility in African populations and this question could be addressed by targeted resequencing of *PTPN22* and its flanking sequences or by quantitative mRNA studies or functional studies.

5.1 The way forward

The aim of future studies could be to resequence the *PTPN22* gene in BSA individuals to discover additional SNPs which could then be assessed for functionality and tested in a case control association study. Another approach would be to carry out a comprehensive association study using tagging SNPs across the entire region to examine association within different linkage disequilibrium blocks in and around the *PTPN22* gene. Potentially HapMap data could be used to identify suitable tag SNPs in people of African ancestry.

More extensive phenotyping could be done to stratify the patient group into ACPA+ and ACPA- RA.

Whole genome association studies in African populations would most likely reveal additional susceptibility loci but would require large sample sizes. This could help explain differences in the clinical expression of the disease and would lead to a better understanding of the pathogenesis of RA and in so doing identify newer targets of therapy.

APPENDIX A

AMERICAN COLLEGE OF RHEUMATOLOGY CRITERIA FOR THE DIAGNOSIS OF RHEUMATOID ARTHRITIS, 1987

The diagnosis of rheumatoid arthritis requires the presence of at least four of the following seven features:

1. Stiffness in and around the joint that last for 1 hour before maximal improvement
2. Arthritis (soft tissue swelling or fluid) in three or more joint areas simultaneously (observed by the physician)
3. Arthritis involving the proximal interphalangeal, metacarpophalangeal, or wrist joints
4. Symmetric arthritis
5. Rheumatoid nodules (subcutaneous, over bony prominences or extensor surfaces)
6. Positive serum rheumatoid factor test
7. Radiographic changes of rheumatoid arthritis (erosions, periarticular osteopenia)

The diagnosis of rheumatoid arthritis requires that four of seven criteria be observed and that the first four (1 through 4) must be present for at least 6 weeks.

APPENDIX B

NAME:_____ **DATE:** _____

HEALTH ASSESSMENT QUESTIONNAIRE

We are interested in learning how your illness affects your ability to function in daily life. Please feel free to add any comments at the end of this form.

Please tick the one response, which best describes your usual abilities over the past week.

	Without ANY Difficulty	*With SOME difficulty*	*With MUCH difficulty*	*Unable to do*
1. *DRESSING AND GROOMING*				
Are you able to:				
Dress yourself, including tying shoelaces and doing buttons?	_____	_____	_____	_____
Shampoo your hair?	_____	_____	_____	_____
2. *RISING*				
Are you able to:				
Stand up from an armless straight chair?	_____	_____	_____	_____
Get in and out of bed?	_____	_____	_____	_____
3. *EATING*				
Are you able to:				
Cut your meat?	_____	_____	_____	_____
Lift a full cup or glass to your mouth?	_____	_____	_____	_____
Open a new carton of milk or soap powder?	_____	_____	_____	_____
4. *WALKING*				
Are you able to:				
Walk outdoors on flat ground?	_____	_____	_____	_____
Climb up five steps?	_____	_____	_____	_____

Please tick any aids or devices that you usually use for any of these activities:

_____ *Cane*
_____ *Walking frame*
_____ *Crutches*
_____ *Wheelchair*
_____ *Devices used for dressing (button hook, zipper pull, long handled shoe horn, etc.)*
_____ *Built-up or special utensils*
_____ *Special or build-up chair*
Other (Specify): _____

Please tick any categories for which you usually need help from another person:

_____ *Dressing and Grooming* _____ *Eating* _____ *Rising* _____ *Walking*

Page 1

Please tick the one response, which best describes your usual abilities over the past week.

	Without ANY Difficulty	*With SOME difficulty*	*With MUCH difficulty*	*Unable to do*
5. *HYGIENE*				
Are you able to:				
Wash and dry your entire body?	_____	_____	_____	_____
Take a bath?	_____	_____	_____	_____
Get on and off the toilet?	_____	_____	_____	_____
6. *REACH*				
Are you able to:				
Reach and get down a 2 kg object (e.g. bag of potatoes) From just above your head?	_____	_____	_____	_____
Bend down to pick up clothing from the floor?	_____	_____	_____	_____
7. *GRIP*				
Are you able to:				
Open car doors?	_____	_____	_____	_____
Open jars, which have been previously opened?	_____	_____	_____	_____
Turn taps on and off?	_____	_____	_____	_____
8. *ACTIVITIES*				
Are you able to:				
Run errands and shop?	_____	_____	_____	_____
Get in and out of a car?	_____	_____	_____	_____
Do chores such as vacuuming, housework or light gardening?	_____	_____	_____	_____

Please tick any aids or devices that you usually use for any of these activities:

_____ *Raised toilet seat* _____ *Bath rail*
_____ *Bath seat* _____ *Long handled appliances for reach*
_____ *Jar opened (for jars previously opened)*
*Other (Specify):*_____

Please tick any categories for which you usually need help from another person:

_____ *Hygiene* _____ *Gripping and opening things* _____ *Reach* _____ *Errand and housework*

APPENDIX C: ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Govind

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M070417**
PROJECT	The role of the protein Tyrosine Phosphatase Non Receptor type 22 (PTPN22) gene polymorphism in disease susceptibility
INVESTIGATORS	Dr N Govind
DEPARTMENT	Department of Rheumatology
DATE CONSIDERED	07.05.04
DECISION OF THE COMMITTEE*	APPROVED UNCONDITIONALLY

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 07.09.03 **CHAIRPERSON** .....
(Professors PE Cleaton-Jones, A Dhai, M Vorster, C Feldman, A Woodiwiss)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Tikly M Prof

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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