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WITWATERSRAND,
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FC GAMMA RECEPTOR III POLYMORPHISMS AS RISK FACTORS FOR SYSTEMIC LUPUS ERYTHEMATOSUS IN BLACK SOUTH AFRICAN PATIENTS

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, June 2017

Declaration

I, Nerissa Wendy Bloch, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Nerissa W. Bloch

20 June 2017

To all female scientists: past and present.

“Science and everyday life cannot and should not be separated.”

Rosalind Franklin

(1920- 1958)

Poster Presentations

- Young Researchers Forum, August 2015, University of Pretoria, South Africa
- SASHG Congress, August 2015, Centurion, South Africa
- Molecular Biosciences Research Thrust (MBRT) Annual Research Day, December 2015, University of the Witwatersrand, South Africa
- Accepted to present a poster at the African Society of Human Genetics Conference (Dakar, Senegal) but was unable to attend due to lack of funding.
- Faculty of Health Sciences Research Day, September 2016, University of the Witwatersrand, South Africa
- Molecular Biosciences Research Thrust (MBRT) Annual Research Day, December 2016, University of the Witwatersrand, South Africa
- South African Rheumatology and Arthritis Foundation Congress, February 2017, Sandton, South Africa
- Accepted to present a poster at the European Society of Human Genetics Conference, May 2017, Copenhagen, Denmark

Abstract

Introduction: Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease of unknown aetiology. There is growing evidence environmental factor(s) trigger the disease in the genetically susceptible host. Fragment crystallisable receptor (FCR) genes encode receptors that recognise the fragment crystallisable (Fc) portion of immunoglobulins (IgG) play an important role in the removal of antigen-antibody complexes from the circulation. Genes that code for these receptors have shown to be associated with susceptibility to SLE in various populations. The aim of the present study is to determine the role of single nucleotide polymorphisms (SNPs), allotypes and copy number variations of FC Gamma receptor genes IIIA and IIIB in susceptibility for the Black South Africans with SLE.

Methods: DNA from 162 Black South African SLE patients and 155 matched controls were investigated using Taqman assays to determine SNP genotyping differences (*FCGRIIIA*) and copy number variation (CNV) number (*FCGRIIIB*). A PCR was optimised in order to determine the allotype differences (*FCGRIIIB*) via agarose gel electrophoresis. Statistical analyses were then performed on the data to see if the results displayed significance in susceptibility to SLE.

Results: The minor allele of the allotypes (*FCGRIIIB*) and the rs396991 SNP (*FCGRIIIA*) were not statistically significant in conferring susceptibility to SLE in cases or controls. The rs10127393 SNP (*FCGRIIIA*) was shown to be monomorphic within both cases and controls for the T allele and is not associated with SLE. The cumulative percentage of copy numbers (*FCGRIIIB*) ≤2 copies were 0.6% larger in cases than seen in controls. Although this was not significant, this was what has been previously suggested in the literature. Almost half of the cases (43.8%) had lupus nephritis (LN). Upon investigation the NA1/NA2 alleles were found to confer susceptibility to LN (p=0.018), whereas the rs396991 G allele did not (p=0.643).

Conclusion: In this study the allotypes, SNPs and CNV investigated were not found to confer susceptibility to SLE. However, subtle trends suggest that further studies

are required with larger sample sizes to acquire more data. Almost half of the cases were diagnosed with LN and the NA2 allele was shown to be a risk factor in developing LN.

Acknowledgements

My supervisor, Dr Jacqueline Frost, thank you for your constant support, unbelievable patience and advice. Thank you for helping me to learn and to grow.

My co-supervisors, Dr Nimmisha Govind and Professor Mohammed Tikly, thank you for your guidance, knowledge and for all your help.

Dr Wesley van Hougenhouck-Tulleken for allowing me to use your lupus blood samples that took many months to collect.

The Connective Tissue Disease Research Fund and the Faculty Research Committee Grant for funding this project.

My twin sister, Lara Bloch, thank you for being my better, beautiful half. You have never faltered to be there for me and you keep me sane. I am so blessed to have had you with me on this journey.

Mom and Dad, thank you for your ongoing support, financially and emotionally. You always pushed me to achieve the goals I set for myself and I am so grateful you were there every step of the way. I love you both more than you know!

Yair Mark, my wonderful boyfriend, thank you for your positivity and kindness. You never got irritated with me once and showed me love throughout. You are the shining light in my life.

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Abbreviations

α	alpha
A	adenine
ACR	American College of Rheumatology
ADCC	antibody-dependent cell-mediated cytotoxicity
ANA	anti-nuclear antibody
β	beta
bp	base pairs
°C	degrees Celsius
C3	complement 3
C4	complement 4
CHBAH	Chris Hani Baragwanath Academic Hospital
CI	confidence interval
CNS	central nervous system
CNV	copy number variation
dbSNP	single nucleotide polymorphism database
DNA	deoxyribose nucleic acid
dsDNA	double stranded DNA
EBV	Epstein-Barr Virus
ELISA	enzyme-linked immunosorbent assay
F	phenylalanine
FCGRIII	fragment crystallisable gamma receptor III gene
FDA	The Food and Drug Administration
G	guanine
GPI	glycosylphosphatidylinositol
GWAS	genome-wide association studies
H	histidine
H1RNA	ribonuclease P RNA component H1 gene
HLA	human leucocyte antigen
HRQOL	health-related quality of life

HWE	Hardy-Weinberg equilibrium
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitor motif
IU/ml	international Units per millilitre
kb	kilobase
L	leucine
LN	lupus nephritis
MAF	minor allele frequency
MGB	minor groove binder
MHC	major histocompatibility complex
ml	millilitre
MLPA	multiplex ligation-dependent probe amplification
µl	microlitre
NA	neutrophil-antigen
ng/µl	nanograms per microlitre
OR	odds ratio
%	percentage
PCR	polymerase chain reaction
R	arginine
RA	rheumatoid arthritis
RNP	ribonucleoprotein
SD	standard deviation
SLE	systemic lupus erythematosus
SLEDAI	SLE disease activity index
SLICC	Systemic Lupus International Collaborating Clinics
SNP	single nucleotide polymorphism
T	thymine

TB	tuberculosis
TLR	toll-like receptor
UPCR	urine protein-to-creatinine ratio
UTI	urinary tract infection
UV	ultraviolet
V	valine
>	greater than
≤	less than or equals to

Chapter 1

Introduction

1.1. Definition

Systemic lupus erythematosus (SLE) is a multi-systemic autoimmune disease with varied clinical features. The disease itself is characterised by autoantibodies against nuclear antigens (Tsokos, 2011). These antibody-antigen immune complexes deposit into target tissues leading to inflammation and end-organ disease.

1.2. Prevalence and Population Dynamics

SLE has a prevalence that ranges from 20-150 cases in every 100 000 people. European populations have a prevalence of 40-50 in 100 000 whereas non-European populations have a prevalence that is three to four times higher (Hopkinson et al., 1994; Gill et al., 2003; Guerra et al., 2012; Tiffin et al., 2013). However, the incidence of SLE has tripled in the past few decades probably due to improved diagnostic measures (Bertsias et al., 2012).

It has been found that people of African, Hispanic and Asian ancestry tend to have an increased prevalence. The prevalence of SLE in Afro-Caribbean women, as estimated in a study, is 206 in 100 000 people (D'Cruz, 2006). The affected patients tend to have greater vital organ involvement. Previous studies have shown greater cardiac and renal involvement in non-European (60%) than in European patients (31%) (Alarcon et al., 1999; Fernandez et al., 2007). Black South Africans have a prevalence of about 12 in 100 000 (Tikly et al., 1996).

The ratio of people being affected with SLE with regard to gender is 9 females to 1 male. The fact that females are more affected is most likely due to the fact that they

have two X chromosomes, containing genes linked to the immune system (Elkon and Santer, 2012).

1.3. History of SLE

SLE was first recognised during the Middle Ages, when the dermatological presentation of the disease was described. During the 13th century, a physician called Rogerius described the characteristic “butterfly rash” (Robinson et al., 2011). The first illustrations of SLE were published in the *Atlas of Skin Diseases* in 1856 by Ferdinand von Hebra (Bertsias et al., 2012). The term lupus was given in the 19th century, when the word, lupus (Latin for ‘wolf’) was used to describe the red pattern on the patients’ faces (Tsokos, 2011).

In 1872, Moritz Kaposi considered lupus to be a multiphenotypic disease (Rudofsky and Lawrence, 1999). However, only in the 20th century was it discovered that the disease is in fact systemic and not localised to the skin. Sequira (1903) suggested that the disorder may have a genetic link, and he reported on two affected siblings who were not twins. Gallo and Forde (1966) wrongly proposed that the disease was inherited via a dominant gene and two years later Rowell and Burch (1968) constructed mathematical analyses in order to support the hypothesis that SLE is genetically based.

Human leucocyte antigen (HLA) research done in the 1970’s concluded that certain genes in the major histocompatibility complex (MHC) were linked to SLE, mainly the HLA class I B8, and this observation was supported by the addition of linkage and association studies (Sestak et al., 2011). Bias et al. (1986) were the first group to perform a linkage study on human SLE. It was found that the association is in fact stronger between HLA class II DR2 and DR3 (Millard and McGregor, 2001). The current picture supports the hypothesis that SLE is not dominantly inherited, but is a polygenic disease in which many genes at different loci interact in order to produce the final phenotype (Tan and Arnett, 1998). Some genes and specific alleles have

been identified as linked more to SLE disease susceptibility than others, although other factors such as sex and environment also impact the emergence of the disease

1.4. Clinical Features

The most common manifestations of SLE are cutaneous, arthritis and mild haematological derangements, however in severe disease the central nervous system, kidneys and lungs may be involved (Bronson et al., 2012) (Figure 1.1). The aetiology of SLE is complex and it is caused by both genetic and environmental factors (Boackle, 2013).

SLE presents with variable symptoms that affect many systems. Symptoms can be mild to severe and some patients may have an isolated form such as cutaneous lupus erythematosus which only affects the skin (D'Cruz, 2006).

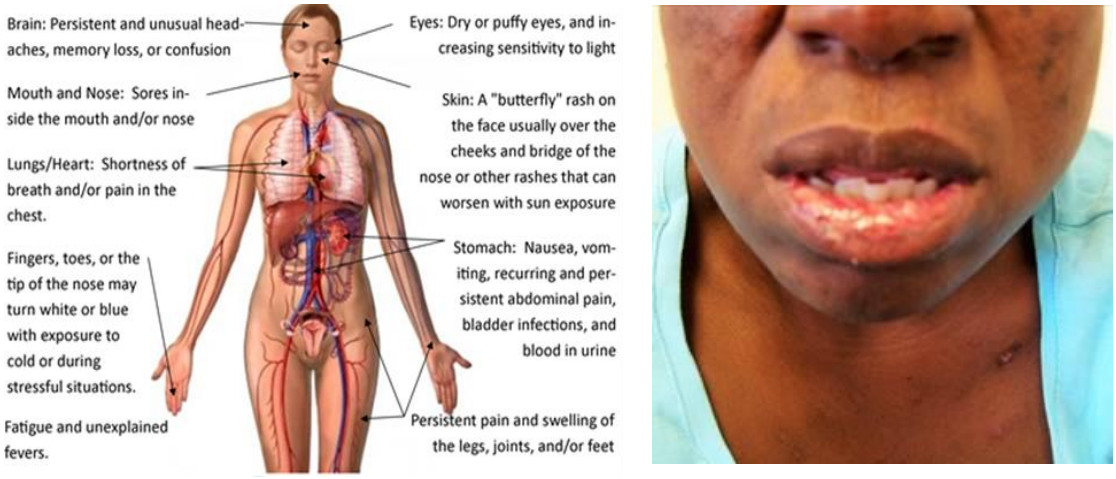


Figure 1.1: The symptoms and clinical features present in systemic lupus erythematosus (SLE) (Rhodus et al., 1990).

(Left) Symptoms may vary between individuals and many tissues and systems may be affected. (Right) The typical “butterfly” rash is present on the face of a female diagnosed with SLE.

In 80% of cases, patients will experience joint pain, arthritis and stiffness as seen in rheumatoid arthritis. Skin lesions as well as puffy hands and feet are also a common

presentation (Doria et al., 2006). The classic “butterfly” rash or malar rash is seen on the cheeks and nose, and it presents as a flat or elevated rash. Organ damage may occur due to persistent inflammation; 61% of SLE patients develop such organ damage within seven years of diagnosis. The most commonly affected organ systems to be damaged are neurological, musculoskeletal and renal (Gill et al., 2003).

1.5. Etiology of SLE

1.5.1. Genetic Risk Factors

Many genes have been found to be linked with SLE using techniques such as candidate gene approaches and genome-wide association studies (GWAS). Genes both within and outside of the major histocompatibilty complex (MHC) have proven relevant genetic markers for SLE (D'Cruz, 2006). GWAS takes note of a marker that is always inherited with a disease within families. The marker and disease gene are therefore strongly linked. Twin pair studies are a good indicator that SLE has a genetic component. Monozygotic twins have a 25%-57% concordance rate of SLE whereas dizygotic twins have a 4% rate. Twelve percent of SLE patients have a first degree relative with SLE and 3% of SLE first degree relatives of probands will develop SLE compared to 0.3% of people with healthy probands (Tan and Arnett, 1998).

A candidate gene approach compares gene variations within SLE patients and controls in order to ascertain specific disease alleles (Sestak et al., 2007). Genes that have been found to be involved in SLE, predominantly function within the immune system (Figure 1.2). Murine genetic models have been created in order to determine genes that result in lupus-like disease. The most well studied model is the New Zealand hybrid model, in which the New Zealand black (NZB) mouse and New Zealand white (NZW) mouse develop lupus-like disease (Vyse and Kotzin, 1998).

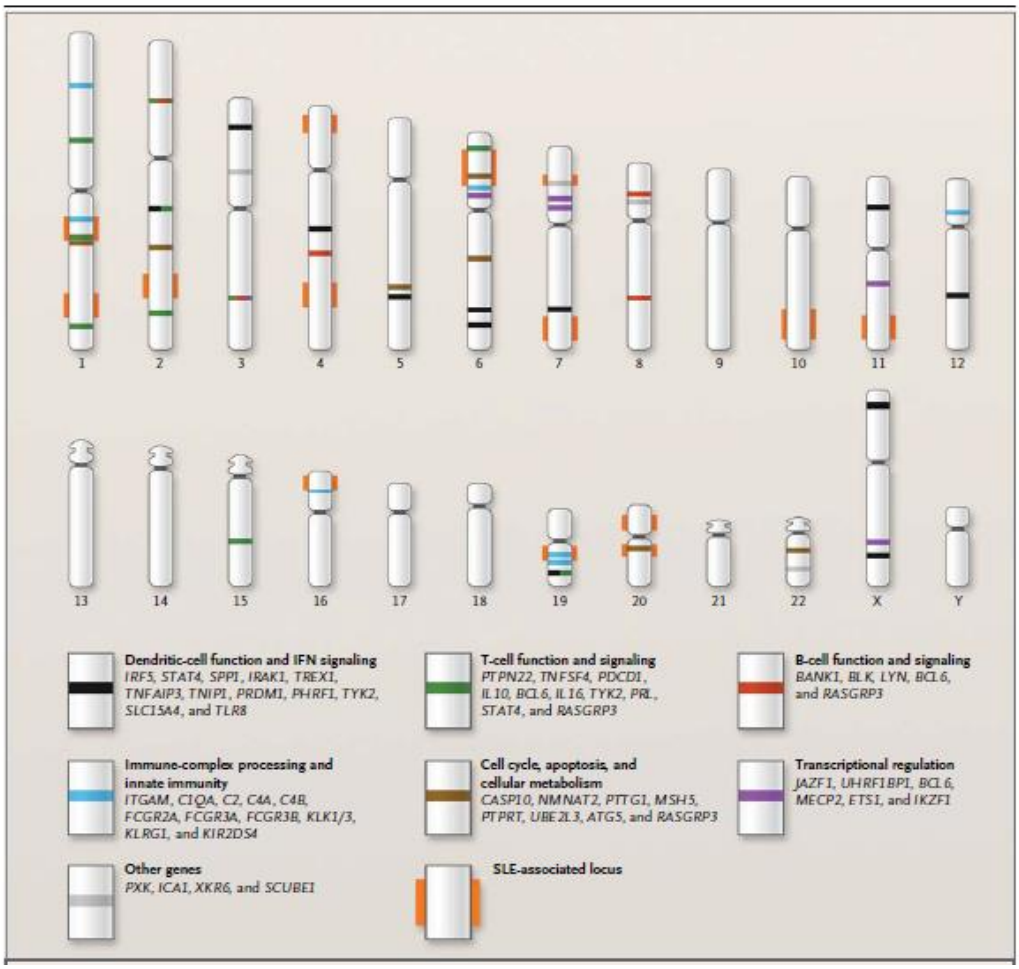


Figure 1.2: Chromosome loci and genes associated with SLE (Tsokos, 2011)

The approximate locations on the chromosomes of the genes associated with SLE are depicted. The genes are divided into six categories according to the main known function of the gene. Each category is represented by a different colour. An additional category (gray) includes genes that do not belong in these functional groups. Chromosome loci with orange bars on both sides indicate large SLE-associated loci.

1.5.2. Epigenetics

Epigenetics refers to the physical alterations in the structure of DNA. These changes affect gene expression by either activating or inactivating genes via mechanisms such as methylation or acetylation (Tsokos, 2011). Epigenetics plays a major role in SLE. Evidence has shown high levels of hypomethylated DNA in T cells allowing increased activation of transcription and thus increased disease activity (Costa-Reis and Sullivan, 2013). Hypomethylation or the inhibition of DNA methylation by drugs such as hydralazine and procainamide may result in drug-induced SLE. Certain methylation-sensitive genes, such *as ITGAL, CD40LG, CD70* and *PPP2CA*, involved

in SLE have been shown to be hypomethylated, and therefore overexpressed, upon investigation (Tsokos, 2011). This overexpression has resulted in T cell autoreactivity contributing to SLE pathogenesis (AlFadhli, 2012, Sestak *et al*., 2007). Pro-inflammatory immune genes such as TNF-α are regulated at a chromatin level and changes may result in inflammation of tissues and organs (Costa-Reis and Sullivan, 2013).

1.5.3. Environmental Risk Factors

In the presence of certain susceptibility genes, there are external factors that can initiate SLE or worsen symptoms. Possible SLE triggers include viruses, ultraviolet radiation, smoking and certain drugs. The disease is more commonly seen in patients from urban areas rather than from rural areas. This suggests that particle pollution in the air is another trigger of the disease (Sestak et al., 2011).

Previous research proposes a link between the Epstein-Barr virus (EBV) and a greater risk of developing SLE. The similarity between the viral nuclear antigen 1 and the SLE autoantigen Ro support this idea. Ultraviolet (UV) rays, found in sunlight, and smoking are known SLE triggers and smoking may further damage affected organs (Millard and McGregor, 2001).

Hormones such as oestrogen are found in high levels in females, and the hormone is suggested to enhance autoimmunity, while androgens and progestins suppress the immune response (Mok and Lau, 2003). Blood samples of SLE patients show low levels of androgens in the body. In addition, SLE patients are able to convert androgens to oestrogen at high rates resulting in high oestrogen concentration in the body. The fact that women of child-bearing age are often diagnosed with SLE suggests strongly that oestrogen may act as a trigger (Tsokos, 2011). Therefore, women with a high risk of developing SLE are advised to avoid oral contraceptives that contain oestrogen as they result in a 1.9 times higher chance of developing the disease (Sestak et al., 2007).

1.6. Diagnosis

The American College of Rheumatology (ACR) in 1971 developed eleven criteria in order to classify SLE in a clinical setting. Four of the eleven criteria must be present in order to make a positive diagnosis (Heinlen et al., 2007).

Since 1971, the ACR criteria have been revised in 1982 and in 1997 as the original list involved too many cutaneous conditions and too few criteria for organ involvement (Tan et al., 1982; Hochberg, 1997). The Systemic Lupus International Collaborating Clinics (SLICC) formulated SLICC criteria in 2012 which further modify the ACR criteria in order to make the diagnosis highly reliable (Yu et al., 2014).

In order to make diagnosis more reliable, sensitive, specific and more timeously, the ACR and SLICC criteria were combined into a single table in 2015 (Salehi-Abari, 2015). Each SLE criterion is designated a certain amount of points (as seen in Table 1.1). A patient with 4 points out of 16, has a definite SLE diagnosis, a patient with three points is suggestive of SLE, while two points suggests probable SLE.

There are two major scoring systems to evaluate the activity of SLE in clinical studies. The most frequently used measurement of lupus activity is called the SLE Disease Activity Index (SLEDAI) (Arbuckle et al., 2003). This index consists of 24 criteria, of which 16 are clinical criteria and 8 are laboratory results. These criteria are scored based on whether these manifestations are present or absent in the patient in the previous 10 days (Bertsias et al., 2012).

Table 1.1: 2015 Revised ACR/SLICC criteria for the diagnosis of SLE (Salehi-Abari, 2015)

Acute/subacute cutaneous lupus rash	Up to 2 points
• Malar rash	2.p
• Subacute cutaneous Lupus erythematosus (SCLE) rash • Palpable purpura or urticarial vasculitis	1.p 1.p
• Photosensitivity	1.p
Discoid lupus erythematosus (DLE) rash or hypertrophic Lupus rash	1.p
Non-scarring frank alopecia [a]	1.p
Oral/nasal ulcers	1.p
Joint disease	1.p
Pleurisy and/or pericarditis	1.p
Psychosis and/or seizure and/or acute confusion	1.p
Kidney involvement	Up to 2 points
• proteinuria≥ 3+ or ≥ 500 mg/day or urinary casts	1.p
• Biopsy-proven nephritis compatible with SLE	2.p
Hematologic	Up to 3 points
• WBC count < 4000/mm^3 or lymphocyte count < 1500/mm^3 on ≥ 2 occasions or WBC count < 4000/mm^3 along with lymphocyte count < 1500/mm^3 in one occasion	1.p
• Thrombocytopenia < 100,000/mm^3	1.p
• Hemolytic Anemia	1.p
Serologic tests	Up to 3 points
• Low titer positive ANA	1.p
• High titer FANA with homogenous or rim pattern	2.p
• Positive anti-ds DNA	2.p
• Positive anti-Sm	2.p
• Anti-phospholipid antibodies (aPLs)	1.p
• Low serum complement (C_3 and/or C_4 and/or CH_{50})	1.p

1.7. Prognosis and Treatment

Historically SLE had a very poor prognosis and was often fatal. In 1955 SLE had a five year survival rate after diagnosis but recently with a better understanding of the disease, earlier diagnosis, better understanding of the genetics and novel treatments, this disease has a good prognosis of a 70% 10 year survival rate (Doria *et al.*, 2006, Tsokos, 2011). Males tend to develop more severe SLE disease, which includes renal and neurological symptoms. This results in a lower survival rate than is seen in females (Robinson et al., 2011).

Mortality is mostly due to active SLE which results in renal, pulmonary and intestinal manifestations (Doria et al., 2006). Infection is also a large contributor to causes of death (Cervera et al., 2003). Infections include tuberculosis (TB), pneumonia, urinary tract infections (UTIs) and sepsis. Mortality is often higher in patients with renal involvement.

There are few effective therapies available to treat SLE. Medications such as corticosteroids were used as treatment for SLE from the middle of the 20th century. However, currently, SLE patients are given corticosteroids, anti-malarials and immunosuppressant drugs depending on the severity of the disease and the affected organs. Immunosuppressant drugs such as Mycophenolate mofetil (MMF) are used after organ transplants to prevent tissue rejection and are effective in treating lupus nephritis (Sestak et al., 2011). It is now possible to develop more effective therapies with the recent discovery of genetic loci and pathways. In the last twenty years certain target biologic agents have been created such as monoclonal antibodies, protein inhibitors and signalling modulators. B cells, T cells, cytokines and complement pathways can be targeted in order to treat SLE (Sestak et al., 2011). Clinical trials are in progress that genotype specific genetic polymorphisms in order to create specific therapeutic agents.

A drug called Rituximab, which is a human-mouse monoclonal antibody directed against CD-20 on B cells (D'Cruz, 2006), has been shown to be effective in a certain subset of patients, often if used in combination with MMF. The Food and Drug Administration (FDA) of the USA approved eculizumab in 2007 which acts against the complementary protein C5 (Navarra, 2008). In 2012, the FDA approved the drug Belimumab which is a human monoclonal antibody that inhibits BLyS, the B-lymphocyte stimulator (Bronson et al., 2012). Belimumab was shown to have significant effects within the first year of treatment. Cytokines initiate inflammatory effects, therefore effective treatments working against cytokines IL-10 and IL-18 (Sestak et al., 2011) are available. In a study of six people with SLE who were treated with IL-10 antibody, the IL-10 levels within the patients decreased greatly and were sustained over six months. Thus, although there is no cure for SLE, certain

drugs are available that can offer relief and control symptoms. However, in South Africa, at present, these treatments are too costly and are not generally available to the public.

1.8. Pathogenesis

The genes involved in SLE code for proteins involved in B and T cell function, antigen presentation, the type I interferon/toll-like receptor pathway, apoptosis and immune complex clearance (Deng and Tsao, 2010; Costa-Reis and Sullivan, 2013). Immune complexes function in immunoregulation which includes enhancement as well as suppression (Nimmerjahn and Ravetch, 2006). The absence of immune tolerance, surplus T helper cell load, faulty B cell activation and autoantibody production contribute to SLE pathogenesis (Mok and Lau, 2003).

1.8.1. T and B cell Signalling

Certain genes associated with SLE regulate the function and activation of B cells (*BLK, BANK1 and LYN*) and T cells (*STAT4 and PTPN22*) (Deng and Tsao, 2010, Guerra *et al*., 2012).

SLE patients tend to have increased numbers of T cells and T helper cells. These T cells lead to increased secretion of inflammatory cytokines, activation of B cells and dendritic cells and tissue damage (Figure 1.3). These T cells undertake a rapid rate of apoptosis, thus further contributing to SLE pathogenesis (Guerra et al., 2012).

Hyperactive B cells present autoantigens and produce pathogenic autoantibodies (Figure 1.3), thus inducing T helper cells and inhibiting T regulatory cells. This process leads to secretion of pro-inflammatory cytokines such as IL-10 and IL-23, which inhibits T regulatory cells.

1.8.2 TLR/IFN Signalling

Toll-like receptors (TLRs) activate the production of type I interferons (IFN) (Elkon and Casali, 2008) (Figure 1.3). Type I interferons are powerful cytokines (IFNα and IFNβ) that mediate the T helper cell response, sustain activated T cells and lower the B cell activation threshold. Impairment of this pathway results in secretion of pro-inflammatory cytokines and thus chronic inflammation (Guerra et al., 2012).

1.8.3. Autoantibody Production

Autoantibody production is the major disease basis in SLE as well as raised levels of IgG antinuclear antibodies (Vyse and Kotzin, 1998). IgG antibodies are antibodies present in the body that bind to autoantigens on nuclei of mammalian cells such as double-stranded DNA, membrane phospholipids and nuclear proteins. Disease-causing IgGs are produced by B cells and the disease involves the emergence and activation of both autoreactive T cells and B cells. Autoreactive cells become large as a result of antigen stimulation and act against own tissues or cells.

1.8.4. Immune Complex Clearance

SLE patients have defective phagocytic cells that cannot clear immune complexes. Defective clearance results from inadequate phagocytosis of IgG2 and IgG3 containing complexes. Allelic polymorphisms of the IgG receptor genes and *ITGAM* have been described. Some of the polymorphic alleles are associated with lower binding of the Fc portions of IgG2 and IgG3, and hence impaired clearance of immune complexes (Cui et al., 2013).

1.8.5. Apoptosis

Apoptosis, programmed cell death, is defective in SLE and apoptotic cells are not efficiently removed by the immune system, leading to a further transformation of necrotic cell death (Costa-Reis and Sullivan, 2013). Apoptotic cells change their morphology and envelop self-antigens thus forming apoptotic blebs (Guerra et al.,

2012). These blebs carry proteins on their surface which act as a further source of autoantigens. This triggers inflammatory cytokine and interferon-α production resulting in further inflammation.

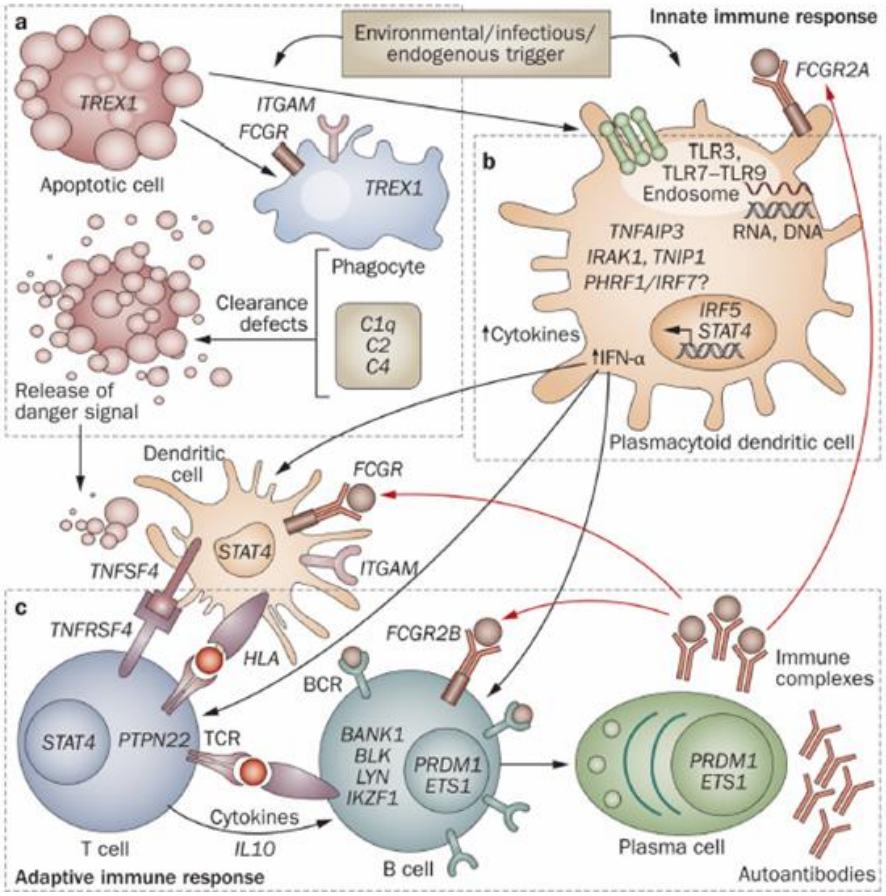


Figure 1.3: The pathogenesis of SLE (Deng and Tsao, 2010).

a. Abnormal processing and clearance of immune complexes and apoptotic blebs via phagocytic cells.
b. TLR–IFN signalling Activated TLRs result in secretion of type I IFN cytokines.
c. T-cell activation via cytokines (IFNα and IFNβ) results in hyperactivation of B cells that produce autoantibodies.

Abbreviations: IFN= interferon; TLR= Toll-like receptor

1.9. SLE in South Africa

SLE is considered rare in tropical Africa yet people with African ancestry living away from the tropics, such as African-Americans, have the highest prevalence rates of 12.2 in 100 000, associated with a more aggressive disease (Tikly *et al*., 1996, Wadee *et al.,* 2007). Literature regarding SLE in South Africa is limited, leaving a gap in current knowledge, and highlighting the need for future studies.

Benitha and Tikly (2007) conducted a study to ascertain the quality of life in 50 Black SLE South African patients. Questionnaires were distributed to these patients relating to functional disability and health related quality of life (HRQOL). It was found that both socioeconomic and ethnic factors influenced the quality of life in South African patients with SLE. Another important conclusion that came out of this research is that state funded healthcare is extremely under resourced which results in delayed diagnosis and therefore delayed therapy. This also impacts negatively on the quality of life for these patients, as some patients are unable to work without therapy and therefore a reduced income is being brought into these households (Harris et al., 2011).

In a developing, middle-income country such as South Africa the main causes of death in patients with SLE are infections, such as tuberculosis (TB) and pneumonia (45%), renal failure (26%) and CNS associated lupus (13%). Overall, infection and active lupus account for 92.3% of deaths in SLE patients (Patel and Mody, 2014). Renal involvement and severe inflammation of body tissue lining the lungs and heart are more common in Black patients (present in 70% of black patients) (Mody et al., 1994). Renal involvement results when immune complexes deposit themselves within the glomerulus. This leads to progressive disruption of the glomerular basement membrane, leading to proteinuria, which will eventually progress to end stage renal disease if not treated (Bertsias et al., 2012).

Dubula and Mody (2015) led a study that showed that 167 SLE patients had 327 hospital admissions. This study was conducted in a Durban hospital in South Africa over a 79 month period. The reasons for admissions were divided into two categories, one being due to active disease, which accounted for 66.7% of admissions and the second being due to infection. Infections included pneumonia, sepsis, urinary tract infections (UTIs) and TB. TB accounted for 13.5% of admissions in this study and it is found to be prevalent in SLE patients (Simmons et al., 2008). This infection rate is due to immune system abnormalities such as complement deficiencies as well as drug therapy. Since SLE is treated with immunosuppressive drugs, which result in a weakened immune system, patients are more susceptible to contracting TB (Wadee et al., 2007).

Tikly et al. (1996) identified the autoantibodies present in Black South African patients. Ro antibodies were seen in 60.5% of patients, La antibodies in 28.4%, and Sm antibodies in 44.2%. Anti- ribonucleoprotein (RNP) antibodies are also more commonly found in Black patients compared to White patients (Tikly *et al*., 1996, Mody *et al*., 1994).

In comparison to studies done on White patients it was shown that Black patients produce higher levels of La antibodies (31% in Black patients vs. 9% in White patients). Anti-dsDNA antibodies are frequent more often in Black patients (41%) than in White patients (21%) (Alarcon et al., 1999).

The results of these studies propose that the disease outcome of SLE patients in Southern Africa is considerably worse than patients in better developed countries (Lalloo et al., 2004). The fact that Black patients tend to have a more aggressive disease course and that South Africa lacks suitable healthcare facilities causes a higher mortality rate (Wadee et al., 2007). This situation again highlights the need for further studies to be conducted in South Africa to better understand the pathogenesis and genetics of the disease.

1.10. FC Gamma Receptor IIIA and IIIB Genes

Fragment crystallisable receptor (*FCGR*) genes encode a structurally and functionally diverse family of receptors that recognise the fragment crystallisable (Fc) portion of immunoglobulins (IgG) and enable the effectiveness of antibody-antigen interactions (Brown *et al*., 2007, AlFadhli, 2012).

Fc gamma receptors recognise immunoglobulin G (IgG) antibodies and are involved in the interaction and removal of antigen-antibody immune complexes by phagocytic cells, mainly macrophages (Brown *et al*., 2007, Tan and Arnett, 1998). Other functions include enhancement of antigen presentation, antibody-dependent cell-mediated cytotoxicity (ADCC), degranulation, regulation of antibody production and

inflammatory cell activation (Gessner et al., 1998). These receptors play a role in SLE susceptibility as well as lupus nephritis (Tsao, 2004).

There are three classes of Fc gamma receptors: FCGRI, FCGRII and FCGRIII, encoded by eight genes (Brown et al., 2007). These genes can be subdivided into isoforms such as FCGRIIA, FCGRIIB, FCGRIIIA, FCGRIIIB and FCGRIIIC (Figure 1.4). This dissertation focuses solely on the isoforms FCGRIIIA and FCGRIIIB.

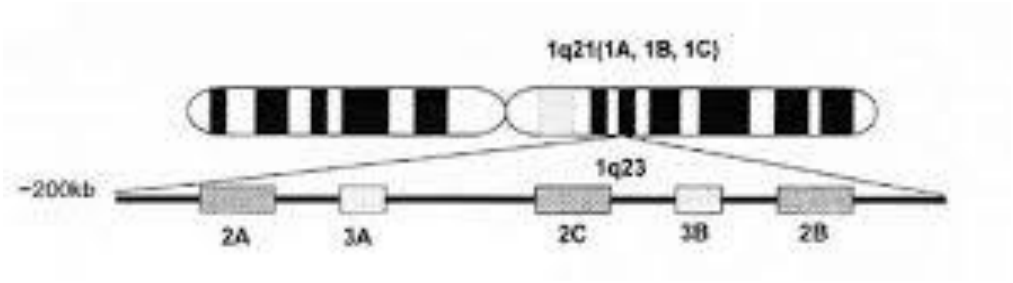


Figure 1.4: The Fc gamma receptor gene cluster on Chromosome 1q21: a susceptibility locus for SLE (Pradhan et al., 2008).

Fc gamma receptors can be classified into high or low affinity receptors based on their affinity for the monomeric IgG antibody or they can be classified into either activating or inhibitory receptors based on their signalling activity and functions. The receptors transmit their signals via immunoreceptor tyrosine-based activation (ITAM) or inhibitor motifs (ITIM) (Nimmerjahn and Ravetch, 2006). Receptor functions include phagocytosis, B cell activation, cytokine production and immune complex uptake and clearance (Morgan et al., 2006; AlFadhli, 2012; Costa-Reis and Sullivan, 2013). Both FCGRIIIA and FCGRIIIB are activating receptors and bind IgG1 and IgG3 differentially (Figure 1.5). IgG3 is bound with greater affinity.

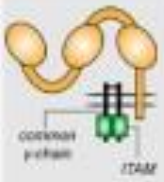
Name	FcγRI CD64	FcγRIIa CD32a	FcγRIIb C32b	FcγRIIc CD32c	FcγRIIIa CD16a	FcγRIIIb CD16b
Structure						
Function	Activating	Activating	Inhibitory	Activating	Activating	Activating
Affinity	High	Low	Low	Low	Low	Low

Figure 1.5: The three classes of Fc gamma receptors.

The red block highlights Fc gamma receptors IIIA and IIIB. FCGRIIIA is attached to the cell surface via α and β chains whereas FCGRIIIB is attached via a GPI anchor. Both receptors are activating in function and low in affinity to IgG1 and IgG3 (Vogelpoel et al., 2015).

FCGRIIIA receptors are present on monocytes, macrophages, cytotoxic lymphocyte cells and dendritic cells, whereas FCGRIIIB receptors are present on neutrophils and a subset of basophils (Lehrnbecher et al., 1999). FCGRIIIB is unique in its attachment to the cell via a glycosylphosphatidylinositol (GPI) anchor, whereas other Fc gamma receptors are attached to the cell membrane via ligand binding α chains and/or signal transducing β chains (Nimmerjahn and Ravetch, 2008). This attachment variation occurs via an amino acid change at position 203 from a phenylalanine to a serine (Ravetch and Kinet, 1991). Variations in these genes have been found to lead to ineffective clearance of immune complexes from the circulation therefore resulting in deposition in tissues contributing to inflammation (AlFadhli, 2012). The lower ability to remove immune complexes from the circulation can affect disease progression and outcome. Immune complexes are mostly cleared from the spleen and liver (Manger et al., 2002). In previous studies, Fc gamma receptors have been shown to be involved in autoimmune diseases as well as immune dysregulation (Hart et al., 1983; Tsokos, 2001; Rioux and Abbas, 2005; Rhodes and Vyse, 2008).

Allelic variation and copy number variation within these genes have been found to confer variable risk for SLE in Caucasian and Hispanic patients (Brown *et al*., 2007, Mok and Lau, 2003). Variation has also been shown to affect disease progression and presentation, not only disease susceptibility (Pradhan et al., 2010; Costa-Reis

and Sullivan, 2013). However, there are very few data for Black South African SLE patients at the Fc gamma receptor locus (Lassauniere and Tiemessen, 2016).

1.10.1. FCGRIIIA

Two missense single nucleotide polymorphisms (SNP) are present within the *FCGRIIIA* gene. The FCGRIIIA SNP (rs10127939) is a tri-allelic and the SNP (rs396991) is a bi-allelic polymorphism (Lehrnbecher et al., 1999).

The rs10127939 tri-allelic single nucleotide polymorphism results in either a thymine to adenine or guanine base change at position 230 (c. 230T>G>A). This base change will also result in an amino acid change at position 48 (p. L48R/H). A tri-allelic SNP exists when three different base variations coexist within a population. This SNP was first described by de Haas et al. (1996) and is found in the membrane distal IgG-like domain of FCGRIIIA (Koene et al., 1997).

The 48H allele is linked to the severe immunodeficiency of natural killer (de Vries et al., 1996). These authors describe a patient who suffered from recurrent viral respiratory tract infections who was found to have a base change from T to A at position 230 in both alleles, resulting in a leucine to histidine amino acid change at position 48 (de Vries *et al*., 1996).

Jawahar et al. (1996) also identified a patient with recurrent herpes infection to have reduced circulation of cytotoxic lymphocyte cells. This patient was homozygous for the 48H allele. Cytotoxic lymphocyte cells perform a role in protection against viruses and bacteria and therefore play an important part in infection rates. This is significant as infections are commonly seen in Black South African SLE patients and therefore this SNP may be linked.

However, in past SLE studies the 48L allele was more frequently seen in SLE patients than in controls (Koene et al., 1998). Interestingly, linkage has been shown between the rs396991 176F allele and the rs10127939 48L allele in SLE patients. This may be due to the link between the IgG binding of these polymorphisms as 176F and 48L bind less efficiently (Koene *et al*., 1997, de Haas *et al.,* 1996). In most cases 176V/V is seen together with 48H/H, whereby a study was done on two children with recurrent infections. Upon genotyping these individuals were both found to be homozygous for the 176V and 48H alleles (de Vries et al., 1996; Koene et al., 1997; Van der Pol and Van de Winkel, 1998).

The genotype frequencies of rs10127939 in Caucasian SLE patients is TT (78.7%), TG (7.3%), TA (12.8%), GG (0.4%), AG (0.4%) and AA (0.5%). The genotype frequencies in African American SLE patients is TT (94.3%), TG (2.3%), TA (2.9%), GG (0.0%), AG (0.2%) and AA (0.3%) (Dong et al., 2014).

The allele frequencies in Caucasian control studies have found T at 0.86, G at 0.06 and A at 0.08 (de Vries *et al*., 1996, de Haas *et al*., 1996). On the genetic variation database, dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/), the allele frequencies of this SNP are given for various populations. In the Yoruba population, the T allele is found at 0.91, G at 0.09 and the A at 0.00. This strongly suggests that the A allele may not be present within the South African Black population. However, no data exist on allele or genotype frequencies in the healthy Black South African population.

The rs396991 single nucleotide polymorphism results in a thymine to guanine base change at position 559 (c. 559T>G). This will also result in an amino acid change at position 176 (p. F176V). This SNP was first described by Wu et al. (1997) and is found in the membrane- proximal IgG-like loop of FCGRIIIA (Koene et al., 1997).

The low affinity phenylalanine allele (176F) results in reduced binding capacity of the receptor to certain IgG antibody subclasses to clear immune complexes such as

IgG1 and IgG3 thus resulting in increased inflammation (Tanaka et al., 2005; Li et al., 2009; Deng and Tsao, 2010; Sestak et al., 2011). 176F does not have the capacity to bind IgG4 (Li et al., 2014). Individuals homozygous for 176F/F were found to have higher titres of anti-dsDNA antibodies (Lee et al., 2002).

The higher affinity valine allele (V176) binds more efficiently to IgG1, IgG3 and IgG4 immune complexes resulting in more effective clearance (Karassa et al., 2003; Li et al., 2014). 176V/V cells have increased calcium influx, greater CD25 expression and more efficient apoptosis (Li et al., 2014).

The 176F allele confers a 1.2 fold risk increase for developing lupus nephritis in Europeans, Africans and Asians, as immune complexes deposit in renal glomeruli (Alarcon et al., 1999; Karassa et al., 2003; Kyogoku et al., 2004; Alizadeh et al., 2007; Brown et al., 2007; Sestak et al., 2011). Previous studies have shown that this allele has a strong association with lupus nephritis in African Americans and SLE in Caucasians, thus highlighting the impact it has on SLE (Gessner et al., 1998; Koene et al., 1998; Seligman et al., 2001; Edberg et al., 2002; Karassa et al., 2003; Sullivan et al., 2003; Dong et al., 2014). A study found that the 176F allele has a strong association with SLE and lupus nephritis in Asian patients (Kyogoku et al., 2002; Li et al., 2010). Caucasian studies have shown that the 176F/F genotype is associated with arthritis and serositis disease symptoms (Dijstelbloem et al., 2000). The 176V allele has been shown to have a protective effect against lupus nephritis (Lee et al., 2003). This is interesting as the 176V allele is more commonly seen in Caucasians and they tend not to suffer from lupus nephritis as greatly as non-Caucasians (Zuniga et al., 2001; Fernandez et al., 2007).

This association is not seen in rheumatoid arthritis studies; hence it may not be a susceptibility locus for all autoimmune diseases. In fact there has been an overrepresentation of the 176V allele in rheumatoid arthritis patients in previous studies (Kastbom et al., 2005). However, a link has been found between the 176F allele and SLE in Spanish rheumatoid arthritis patients (Nieto et al., 2000), but no

association has been found between this SNP and SLE in Mexican patients (Brambila-Tapia et al., 2011).

A previous study conducted on Korean SLE patients by Lee *et al.* (2003) found that the frequency of GG homozygotes was observed at 15.4%, TT homozygotes were seen at 30.1% and TG heterozygotes at 54.5%. These data were also supported by a study done by Yun et al. (2001). However, in both studies no association was found between the 176F allele and SLE.

A study on African American SLE patients saw GG homozygotes at 8%, TT homozygotes at 42% and TG heterozygotes at 50%. Caucasian SLE patients were seen at GG (11%), TT (50%) and TG (39%) (Lehrnbecher *et al*., 1999, van der Pol and van de Winkel, 1998).

In summary, GG homozygotes are seen at a lower frequency in African Americans than are seen in Caucasians and Asians correlating with the higher frequency of lupus nephritis in African Americans as the G allele is protective against lupus nephritis. TT homozygotes are seen at a higher frequency in Caucasians than are seen in African Americans and Asians.

The genotype frequency, as seen in the healthy Black South African population, is TT (39.7%), TG (50%) and GG (10.3%). The allele frequency of T is 0.63 and G is 0.37 (Lassauniere and Tiemessen, 2016). Another study using Caucasian controls found allele frequencies of T and G to be 0.74 and 0.26 respectively (Koene *et al*., 1998, Lehrnbecher *et al*., 1999). A previous study by Cunniffe (2011) using Black South African SLE patients could not find an association between the rs396991 SNP and SLE susceptibility. However, the study sample size of patients and controls was small and other techniques, pyrosequencing and High Resolution Melt Analysis, were chosen for genotyping, which may have proven unreliable. On the genetic variation database, dbSNP, the allele frequencies of this SNP are given for various

populations. In the Yoruba population the G allele is found at 0.04 and the T allele at 0.96.

In summary, these SNPs (rs10127939 and rs396991) result in reduced efficiency of the monocytes and macrophages to clear IgG immune complexes and both SNPs have been found to have strong associations with SLE in African Americans.

1.10.2. FCGRIIIB

Two allelic variants or alloantigens are present within exon 3 of *FCGRIIIB*, namely FCGRIIIB-NA1 and FCGRIIIB-NA2. These alleles differ by 5 nucleotides causing a change of 4 amino acids. This results in four N-linked glycosylation sites in NA1 (FCGRIIIB*01), whereas NA2 (FCGRIIIB*02) has six sites (Brown et al., 2007; Moritz et al., 2008; Willcocks et al., 2009). The molecular weight of NA1 is 50-65 kD whereas NA2 is 65-80kD. The difference in molecular weight is most probably due to the different glycosylation patterns (Bux et al., 1997).

A third allele, SH, has been described in the literature. SH differs from NA2 by a single nucleotide change of a cytosine to an adenine at position 266 (Lehrnbecher et al., 1999). SH occurs in Caucasians at 4% and in Africans at 15% (Kissel et al., 2000; Kyogoku et al., 2004). This translates to there being three copies of the *FCGRIIIB* gene in some individuals (Brown *et al*., 2007, Lehrnbecher *et al*., 1999). Each gene will express a combination of NA1, NA2 and SH in these cases (Bux *et al*., 1997, Moritz *et al*., 2008). Kyogoku et al. (2004) found a similar SH allele frequency in Caucasian patients as seen in healthy Causcasians of other studies. This study concluded that the function of SH is still unknown, but it is thought to not be associated with SLE. However, given how prevalent this allotype is in Africans as compared to Caucasians, the study provides strong rationale to investigate SH in the South African population and its possible association with SLE. However, this allotype was not investigated in this study.

A small percentage of the European population (0.1%) and African population (2%) do not display either NA1 or NA2 on their neutrophils and are said to be NA-null, whereby they are deficient in the *FCGRIIIB* gene (Muniz- Diaz et al., 1995; Kissel et al., 2000; Matsuo et al., 2000; Siriboonrit et al., 2003; Kyogoku et al., 2004; Willcocks et al., 2008). The Asian population has been shown have no NA-null individuals (Kyogoku et al., 2004). These individuals rarely show any signs of autoimmune disease and do not suffer from repeated bacterial infections. These individuals may however cause alloimmune neutropenia in their neonate (Clark et al., 1990; de Haas et al., 1995; Bux et al., 1997; Fanciulli et al., 2007; Moritz et al., 2008). Information regarding NA-null individuals in other ethnic groups is lacking.

The NA2 allele is associated with low function and results in reduced binding efficacy of antigen-antibody complexes and lower phagocytic capacity, whereas the NA1 allele binds IgG1 and IgG3 more efficiently (Gessner et al., 1998; Hatta et al., 1999; Lehrnbecher et al., 2000; Tanaka et al., 2005; Morris et al., 2010). NA1 possesses a higher phagocytic capacity and increases neutrophil- mediated phagocytosis (Salmon *et al*., 1990; Bredius *et al.*, 1994)

Previous Asian studies have found that the NA1 allele is overrepresented at a frequency of 0.66, whereas the NA2 allele is underrepresented at a frequency of 0.34 (Rascu et al., 1997). The NA2 allele has been shown to be a susceptibility allele for SLE in Asians (Li *et al*., 2009, Siriboonrit *et al*., 2003).

Contrary to that Asian report, the NA2 allele is more commonly seen in Caucasians at 0.7 whereas NA1 is seen at 0.3 (Van de Winkel and Capel, 1993; Muniz- Diaz et al., 1995; Bux et al., 1997; Hatta et al., 1999; Lehrnbecher et al., 1999; Edberg et al., 2002; Gonzalez-Escribano et al., 2002; Kelly et al., 2002; Hong et al., 2005; Brown et al., 2007; Moritz et al., 2008; Nielsen et al., 2012).

The NA1 allele is over represented in the healthy Black South African population and its frequency is 0.51, the NA2 allele occurs at a frequency of 0.34 and SH at a

frequency of 0.16 in healthy Black individuals (Lassauniere and Tiemessen, 2016). However, studies on other African populations show the NA1 allele to be at a frequency of 0.32 and NA2 at 0.68 (Hessner et al., 1996; Hessner et al., 1999; Lehrnbecher et al., 1999; Kissel et al., 2000; Matsuo et al., 2000; Moritz et al., 2008).

FCGRIIIB is highly expressed and there are 150 000- 200 000 receptors per neutrophil (Li et al., 2009). A low copy number of the *FCGR3B* gene reduces *FCGR3B* membrane expression on neutrophils and results in decreased clearance of immune complexes (Breunis et al., 2009; Boackle, 2013). A copy number of less than two is associated with SLE, lupus nephritis, higher autoantibody titres and higher disease activity scores (SLEDAI), predominantly in Caucasians (Aitman et al., 2006; Fanciulli et al., 2007; Hollox et al., 2009; Li et al., 2009; Morris et al., 2010; Mueller et al., 2013; Nossent et al., 2013). Statistical analysis has found association between 0 to 1 *FCGRIIIB* copies and SLE (OR= 1.59; p=9.1 x 10-7) (McKinney and Merriman, 2012). In fact, it has been shown that ≥2 copies of FCGRIIIB have a protective effect against autoimmune disease (Tiffin *et al*., 2013, McKinney and Merriman, 2012). A median copy number of two is found in most study controls (Fanciulli et al., 2007). In a previous study the SLE cases were shown to have an average copy number of 1.9, whereas controls had an average copy number of 2.1 (Morris et al., 2010). Lassauniere and Tiemessen (2016) found that copy number variation (CNV) within FCGRIIIB is at a frequency of 26% in the South African Black population. Black individuals were found to have one gene copy of FCGRIIIB at 7.6%, two gene copies at 74%, three gene copies at 15.3% and four gene copies at 3.1%.

1.11. Clinical Variables

Clinical variables will be investigated according to gender, age of onset, the SLICC disease index, presence of certain autoantibodies, the levels of the C3 and C4 complement proteins that may result in hypocomplementemia (low levels of both C3 and C4), presence or absence of lupus nephritis and previous tuberculosis infection.

1.12. Study Objective and Aims

The primary objective of this study is to determine if single nucleotide polymorphisms (SNPs), allotypes and copy number variation, present within the Fc gamma receptor genes IIIA and IIIB, contribute to the susceptibility of systemic lupus erythematosus (SLE) within the Black South African population. The three specific aims are:

Aim 1: To ascertain if there is a significant association with the various SNPs, allotypes and copy number variation and the SLE phenotype.

Aim 2: To ascertain if there is a significant association of the various SNPs, allotypes and copy number variation with clinical variables with particular interest in lupus nephritis.

Aim 3: To ascertain the association of the various genetic variants with an increased risk of infections, including tuberculosis (TB) in Black South Africans with SLE.

Chapter 2

Subjects and Methods

2.1. Subjects

2.1.1. Patients

A total of 162 consenting black South African patients from the Chris Hani Baragwanath Academic Hospital (CHBAH), >18 years of age at disease onset and fulfilling the 1997 American College of Rheumatology classification criteria (Hochberg, 1997) for SLE were included in the sample. Only patients with four or more SLICC (Systemic Lupus International Collaborating Clinics) criteria (Heinlen et al., 2007) have been included. Patients were considered as Black, if they reported that all four of their grandparents were 'Black South Africans'.

Certain clinical and laboratory information on the patients was documented at the time of blood collection. This information included: gender and age of the patient, age at the time of disease onset, the number of SLICC criteria present, TB status, C3 and C4 levels, the presence of anti-dsDNA and anti-Sm antibodies and the grade of lupus nephritis, if present. The stage of lupus nephritis was determined by a renal biopsy. Other information was acquired via blood test results and by consulting with the patient. The Human Research Ethics Medical Committee of the University of the Witwatersrand gave ethics clearance with ethics clearance certificate number M150624 (Appendix A).

2.1.2. Controls

A total of 155 healthy control samples matched to the experimental group in age, ethnicity and gender, who display no symptoms of SLE were used for comparison. Blood samples collected from these controls have been used in previous

autoimmune studies (Govind et al., 2014). These DNA samples are stored in the Division of Human Genetics, National Health Laboratory Service.

Quanto (USC Biostats) was used to calculate the power of this study, and it was found to be 48%, whereas the preferred power would be 80%. However, the markers that are being investigated may function in a dominant or recessive manner whereas this power calculation assumes an additive model. The fact that the power of this study is low does not mean that significant associations will not be detected. Information regarding the second SNP is not available and if this SNP has a minor allele frequency (MAF) of 5%, the study may be more adequately powered.

To increase the power of the findings a total of 317 samples (162 patients and 155 controls) were investigated. Only patients who have been informed of the research and who have signed consent forms for the retrieval and use of their blood samples (Appendix B) were included in this study. Patients were excluded if they are not of African ethnicity or if they had mixed ancestry as lupus presents with different severities in other ethnicities.

2.2. Clinical Variables

Clinical variables that were investigated included gender, age of disease onset, the SLICC disease index, presence of anti-dsDNA and anti-Sm antibodies, the levels of the C3 and C4, presence or absence of lupus nephritis and previous tuberculosis infection.

Patient clinical, immunologic and demographic information were obtained from patient files, and results of blood samples, urine samples and kidney biopsies. The data were collected by a physician, Dr Wesley van Hougenhouck-Tulleken at CHBAH as part of his Masters of medicine research project.

Clinical and immunologic features, relevant to this study, that were documented were the following:

SLICC Score

The SLICC criteria are a list of criteria used in the diagnosis of SLE. SLICC criteria can be divided into those that are clinical or immunologic criteria. Clinical criteria include acute cutaneous lupus, chronic cutaneous lupus, oral or nasal ulcers, non-scarring alopecia, arthritis, serositis, renal involvement, neurologic involvement, hemolytic anaemia, leukopenia and thrombocytopenia. Immunologic criteria include antinuclear antibodies (ANA), anti-dsDNA antibodies, anti-Sm antibodies, antiphospholipid antibodies, low complement C3, C4 and CH50 levels and the direct Coombs' Test. Four SLICC criteria in a patient are required for making a diagnosis of SLE including at least one clinical and one immunological criterion (Bertsias et al., 2012).

Lupus Nephritis

Certain tests were performed, such as the urine to protein creatinine (UPCR) test, to determine renal function. This test gives the protein to creatinine ratio found in the urine. A result that is greater than 300g/mmol creatinine translates to proteinuria (high levels of protein in the urine) (Bertsias et al., 2012).

If a nephritis was suspected, a kidney biopsy was performed. From this the type of lupus nephritis could be determined. There are five classes of lupus nephritis: class I relates to minimal mesangial lupus nephritis; class II to mesangial proliferative lupus nephritis; class III to focal nephritis; class IV to diffuse nephritis; and class V to membranous nephritis. Class III, IV and V are the most severe types of lupus nephritis.

Tuberculosis (TB)

Previous TB infection was determined from the patient's medical records. If the patient had had TB previously the date of diagnosis and site (pulmonary and extrapulmonary) was noted.

Complement C3 and C4 Levels

C3 and C4 serum levels are determined via stimulation by a substance to produce an antibody using a turbidimetric technique. This technique measures the loss of light intensity passing through the solution based on its cloudiness. Low levels are considered below C3 0.2 and C4 -1.0 (Bertsias et al., 2012).

Anti-dsDNA and Anti-Sm Antibodies

Enzyme-linked immunosorbent assays (ELISA) are performed on blood samples to detect autoantibodies. Patients with anti-dsDNA antibody levels greater than 75 IU/ml and anti-Sm antibody levels greater or equal to 1 IU/ml together have a higher probability of having SLE, however, a negative anti-dsDNA antibody results does not exclude the diagnosis of SLE (Bertsias et al., 2012)

2.3. Laboratory Methods

2.3.1. Collection of Samples

Blood samples were collected from each patient and control at the Chris Hani Baragwanath Academic Hospital by a trained research nurse. The blood samples were collected and stored in EDTA blood collection tubes to avoid coagulation of the blood and kept at -40°C.

2.3.2. DNA Extraction

Two EDTA tubes of blood were taken from each individual as this material and amount is optimal for DNA extraction. The blood samples were then frozen prior to extraction. The DNA was extracted using the Salting-Out method that was first described by (Miller *et al*., 1988). Approximately 10ml of blood is poured into a centrifuge tube and centrifuged for 10 minutes and the supernatant containing the plasma removed, the tube is then filled with a Sucrose-Triton X Lysing buffer, which lyses the blood cells. After mixing, by inverting the tube several times, centrifuge for 10 minutes at 2300rpm after which the supernatant is discarded and a red pellet remains. The pellet is then resuspended in 20ml Sucrose-Triton X Lysing buffer and placed on ice for 5 minutes and then centrifuged again for 5 minutes at 2300rpm. The supernatant is again discarded and 3ml T20E5 (which chalates the Mg2+ molecules needed for DNA activity), 200µl 10% SDS (denature proteins) and 500µl Proteinase K solution (digest the proteins) is added to the pellet. After 24 hour incubation at 42ºC 1ml of saturated NaCl solution is added. The NaCl salts out the proteins and forms a concentration gradient. The tube is centrifuged for 30 minutes at 2300rpm after which the supernatant, which contains the DNA, should be poured into a clean tube. 2 Volumes of absolute alcohol is used to precipitate the DNA which is then air dried. The extracted DNA is then re-suspended in 1x TE buffer and stored in the fridge.

2.3.3. Primer Design

A PCR based method was used to determine the NA1 and NA2 alleles within samples from the SLE patients and controls. Primers were previously designed by Hong et al. (2005) and are shown in Table 2.1.

The primers were ordered and purchased from Whitehead Scientific and manufactured by IDT (San Diego, United States of America).

Table 2.1: Primer sequences to amplify gDNA samples using PCR

Gene	Sequence (5'→3')	Annealing temp (˚C)	Product size (bp)
NA1 (F)	CTCAATGGTACAGGGTGCTC	57.5	118
NA1 (R)	GGCCTGGCTTGAGATGAGGT		
NA2 (F)	CTCAATGGTACAGCGTGCTT	57.8	171
NA2 (R)	CACCTGTACTCTCCACTGTGTCGTT		
GAPDH (F)	CTCCTTTTGCAGACCACAGTCC	57.5	350
GAPDH (R)	CTGTTGAAGTCAGAGGAGACCAC		

2.3.4. Allotyping Studies (NA1 and NA2)

Genotyping was performed on each case and control sample. Genomic DNA fragments were amplified via PCR using specific amounts of the above-mentioned primers and other PCR components to a total reaction volume of 20µl (Table 2.2). The Bio Rad T100 Thermal Cycler machine was used. Twenty eight cycles were performed consisting of 93°C for 1 minute, 60 °C for 1 minute and 72°C for 1 minute and 30 seconds. Extension occurred for 10 minutes at 72°C after which PCR was terminated. A 20µl PCR product was run on a 2% agarose gel stained with ethidium bromide and visualised using a UV illumination system (Geldoc, Vacutec).

Table 2.2: Standard PCR protocol for NA1 and NA2 allele determination

PCR Components	Stock Concentration	Final Concentration	1X Mix (µl)
DNA			2µl
Kapa Taq Ready Mix			10µl
NA1_F	10pmol/µl	5pmol	1.5µl
NA1_R	10pmol/µl	5pmol	1.5µl
NA2_F	10pmol/µl	5pmol	1.5µl
NA2_R	10pmol/µl	5pmol	1.5µl
GAPDH_F	10pmol/µl	5pmol	0.5µl
GAPDH_R	10pmol/µl	5pmol	0.5µl
ddH_20			1µl
Total			**20µl**

2.3.5. Real-Time PCR

All samples prior to real-time PCR were normalised to 20ng/µl using distilled water. This was confirmed using the NanoDrop spectrophotometry system whereby any read that was below 50ng/µl was considered acceptable.

2.3.6. SNP Genotyping Assays

SNP genotyping of the two *FCGRIIIA* gene SNPs rs10127939 and rs396991 were carried out using pre-designed TaqMan assays (Life Technologies) whereby allelic discrimination was used to determine the SNPs utilising allele specific probe chemistry.

The SNP genotyping assays have been designed to detect single nucleotide polymorphisms (SNPs) within a genome. The specific TaqMan probes have a minor groove binder (MGB) on the 3' part of the probe which binds to the minor groove of the DNA. The 5' part of the probe contains a dye label such as VIC or FAM and on the 3' side of the probe there is a quencher which quenches any fluorescence. There are two probes within the reaction. The first will be specific to a certain SNP and the other specific to another SNP. Each probe will contain a specific dye of different colours. Once the probe has bound and has been fragmented by the polymerase the specific colour fluorescence produced directly correlates to the SNP present (Thermo Fisher, 2013)

The SNP genotyping assays were received at a concentration of 40X and were stored at -20°C in dark conditions. This assay was diluted down to a concentration of 20X using 1 X TE buffer as per manufacturer instructions. Aliquots of the 20X working stock were prepared. The PCR reaction setup involved preparing the reaction mix and the reaction plate. The reaction mix consisted of 2.5µl TaqMan Genotyping Master Mix and 0.25µl of 20X TaqMan genotyping assay. The reaction mix calculations were adjusted in order for there to be sufficient volume for each well of a 384-well plate. Each well was required to have 2.75µl of reaction mix. Once the reaction mix was complete, 2.75µl of reaction mix were added to each well of the reaction plate. DNA from each case and control (2.25µl) were added to the separate wells. Therefore each well of the reaction plate had a final volume of 5µl.

The plate was covered in an adhesive film and centrifuged briefly. The reaction plate included samples of known genotype, samples of unknown genotype and two no

template controls (distilled water). Samples of known genotype were determined via sequencing whereby 10 samples were randomly chosen to be genotyped.

The plate document was set up and labelled and then PCR was performed using the 7900HT Fast System real-time PCR system. PCR conditions were as follows: 95°C for 10 minutes once only, 95°C for 15 seconds and 60°C for 1 minute for a total of 40 cycles. Thereafter, PCR was terminated, the plate was removed and the alleles were determined via fluorescence measurements made during the plate read. Software was then used for interpretation of results. This was done for both SNPs that were required to be genotyped in this study.

2.3.7. Reproducibility

Assay reproducibility was performed for the genotypes obtained during the TaqMan assays to determine the reliability of the assays. 30 SLE samples (20%) were re-run using the same TaqMan assays previously used. Samples were chosen at random but included all genotypes previously called.

2.3.8. Copy Number Variation (CNV) Assay

A TaqMan copy number assay specific to the *FCGRIIIB* gene was used to determine copy number variation. The TaqMan copy number assay uses relative quantification whereby the reference assay detects a sequence that is known to be present in two copies in the diploid genome. The RNase P reference assay was used. This assay detects the Ribonuclease P RNA component H1 (H1RNA) gene (RPPH1) on chromosome 14.

The CNV assays were received at a concentration of 20X and were stored at -20°C in dark conditions. Aliquots of the 20X working stock were prepared. The PCR reaction setup involved preparing the reaction mix and the reaction plate. The reaction mix consisted of 5µl TaqMan Gene Expression Master Mix, 0.5µl of 20X

TaqMan CNV assay and 0.5µl of TaqMan Copy Number Reference Assay. The reaction mix calculations were adjusted in order for there to be sufficient volume for each well of a 384-well plate. Each well was required to have 8µl of reaction mix. Once the reaction mix was complete, 8µl of reaction mix were added to each well of the reaction plate. DNA from each case and control (2µl) were added to the separate wells. Therefore each well of the reaction plate had a final volume of 10µl.

The plate was covered in an adhesive film and centrifuged briefly. The reaction plate included samples of unknown copy number, a sample of known copy number (calibrator sample) and two no template controls (distilled water).

The plate document was set up and labelled and then PCR was performed using the Applied Biosystems 7900HT Fast System real-time PCR system. PCR conditions were as follows: 95°C for 10 minutes once only, 95°C for 15 seconds and 60°C for 60 seconds for a total of 40 cycles. Thereafter, PCR was terminated, the plate was removed and the copy numbers of each sample were determined. Software was then used for interpretation of results.

2.3.9. Analysis of Real-Time PCR

The genotyping and copy number assay results were analysed using their corresponding analysis softwares. TaqMan Genotyper, for the SNP genotyping assays and Copy Caller, for the CNV analysis. These are free for download from the Life Technologies website.

2.4. Statistical Analyses

Pearson's chi-squared ($\chi 2$) test was used to determine whether proportions of genotypes were consistent with Hardy-Weinberg Equilibrium using a significance threshold of $p<0.05$. The chi-squared ($\chi 2$) test determines the amount of deviation between the observed genotype frequencies and those expected under HWE.

Genotype and allele frequencies were calculated for all cases and controls, these allele frequencies were compared using Pearson's chi-squared ($\chi 2$) test with a statistical significance level threshold of $p<0.05$, to test for association with SLE. In addition, lupus nephritis cases underwent further statistical analyses.

Two sample t-test was used to compare percentages obtained from the copy number studies. Standard statistical software such as stata or graphpad prism was used.

Chapter 3

Results

3.1. Patient Demographics and Clinical Variables

The majority of patients were females (93.8%). The mean disease duration was 6.7 years. Lupus nephritis (LN) severity was graded into categories by a nephrologist. The majority of the patients biopsied had LN class III and class V. Approximately a quarter of patents had a history of TB. Two thirds of the patients had low complement levels. The demographic, clinical and laboratory data for the patient group are summarised in Table 3.1.

Table 3.1: Mean values for the demographic, clinical and laboratory data for the SLE

Clinical Feature	Patients (n= 162)
Female: Male ratio	13.5 : 1
Age of patient in years- mean (SD)	39.1 (12.4)
Age of disease onset in years- mean (SD)	32.4 (15.3)
SLICC Score- mean (SD)	8 (2.2)
Lupus nephritis	43.8% (n=71)
Lupus nephritis Class I	2.5% (n=4)
Lupus nephritis Class II	4.3% (n=7)
Lupus nephritis Class III	12.3% (n=20)
Lupus nephritis Class IV	4.9% (n=8)
Lupus nephritis Class V	19.8% (n=32)
Previous TB infection	23.5% (n=38)
Low C3 and C4 levels	66.7% (n=108)
Anti-dsDNA Antibodies	42% (n=68)
Anti-Sm Antibodies	72.8% (n=118)

3.2. Allotype Studies

Figure 3.1 represents the allelic and genotypic frequencies of the NA1 and NA2 alleles in SLE patients and controls. The 118bp PCR product represented the NA1 allele and the 171bp PCR product represented the NA2 allele (Figure 3.2). The genotype frequencies in controls (χ^2= 0.87; p = 0.3438) were found to be in agreement with HWE expectations. Conversely, the genotype frequencies in SLE patients (χ^2= 11.75; p = 0.0006) were not in agreement with HWE expectations.

NA1 and NA2 allelic association in SLE is shown in Table 3.2. The minor allele NA2 does not confer susceptibility to SLE (OR = 1.281, 95% CI: 0.902-1.817, p =0.166), with a minor allele frequency of 31% in cases and 37% in controls. The NA2 allotype exceeded the threshold p<0.05 and therefore is not statistically significant in conferring susceptibility to SLE in Black South Africans.

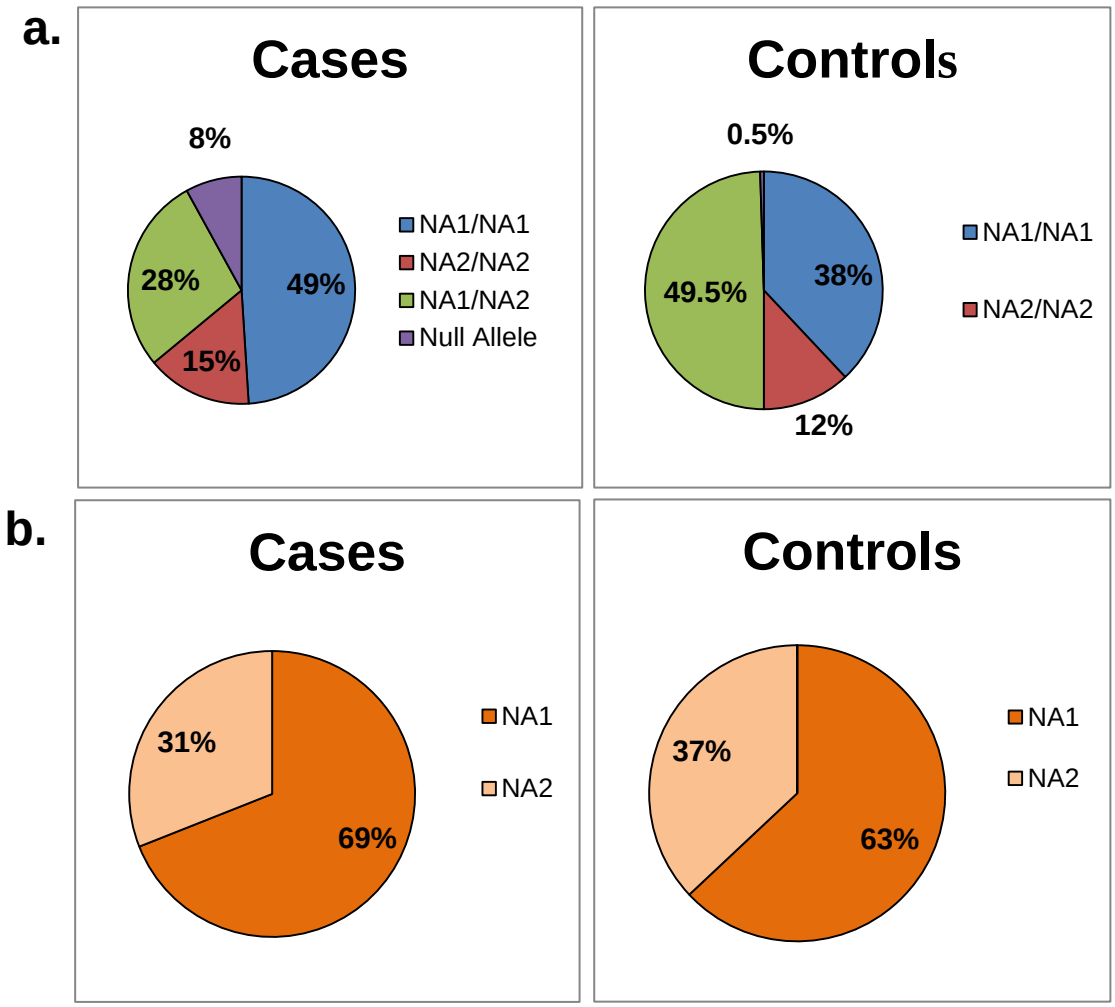


Figure 3.1: Pie charts illustrating the observed genotypes (a) and allele frequencies (b) of NA1 and NA2

Genotype frequencies and allele frequencies in 141 cases and 155 controls.

Table 3.2: Statistical analysis of results from the NA1 and NA2 alleles

Major allele	Minor allele	Case MAF	Control MAF	OR (95% CI)	Chi-square value	P-value
NA1	NA2	0.31	0.37	1.281 (0.902-1.817)	1.92	0.166

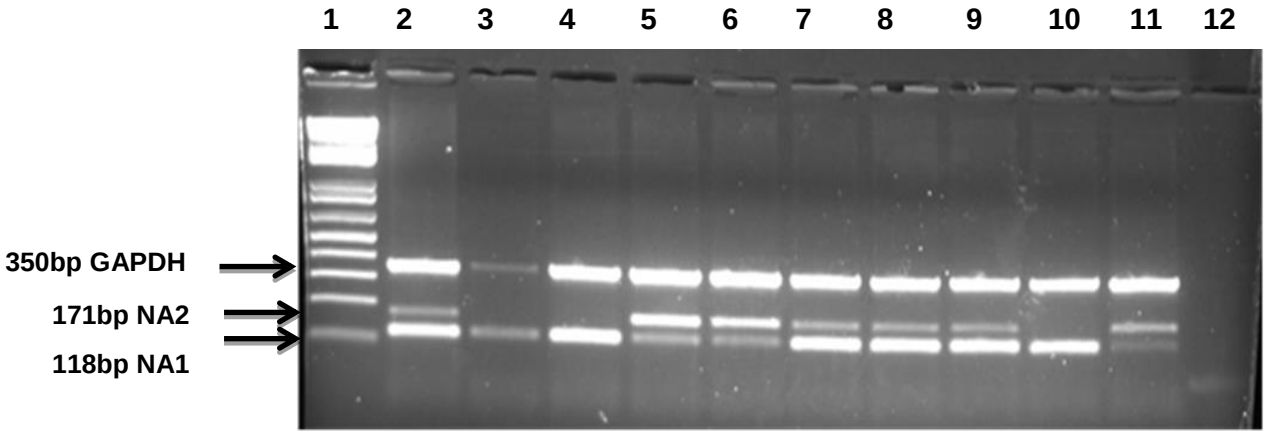


Figure 3.2: PCR amplification via agarose gel electrophoresis of allotypes NA1 and NA2

Lane 1: 1kb molecular weight marker; lanes 2- 6: SLE cases; lanes 7- 11: controls; lane 12: blank
NA1 can be seen as a product of 118bp, NA2 of 171bp and the internal control, GAPDH, at 350bp.

3.3 SNP Genotyping

3.3.1. rs10127939

All SLE cases and controls were analysed using the TaqMan assay designed specifically for the tri allelic SNP rs10127939. Firstly an assay to detect the presence of the T and A allele was run. No amplification was seen for the A allele in both cases and controls (Figure 3.3c). Only the T allele showed amplification (Figure 3.3b). Secondly, an assay to detect the presence of the T and G allele in the same samples

was run. This assay again showed no amplification of the G allele and only the T allele amplified in both cases and controls. Validation of the genotyping confirmed the presence of only the T allele in these samples (Figure 3.3d). One can conclude that because neither the cases nor controls carried the A and G variants, that the A variant is monomorphic within the South African population. Therefore, the polymorphism does not confer susceptibility to SLE in the Black South African population.

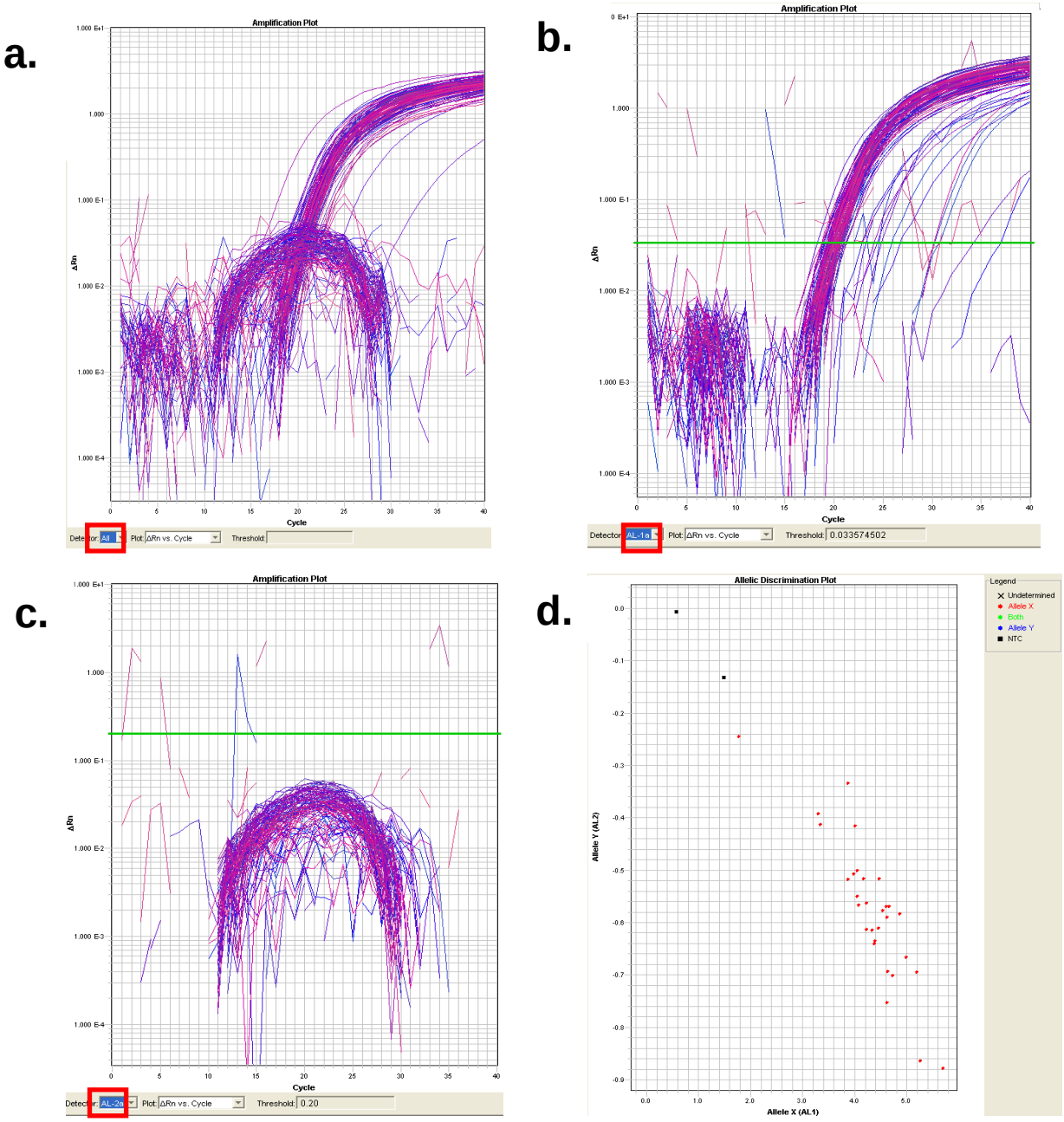


Figure 3.3: Amplification plot and allelic discrimination plot results of rs10127939

(a) Amplification plot of both alleles.
(b) Amplification plot of allele 1 showing amplification
(c) Amplification plot of allele 2 showing no amplification
(d) Allelic discrimination plot showing only allele 1 is present in the samples run

3.3.2. rs396991

Allelic discrimination for the G and T allele in SNP rs396991 was again performed using TaqMan technologies. Figure 3.4 shows an example of one of the allelic discrimination plots created whereby the three genotypes are plotted against the x and y-axes. Figure 3.5 represents the frequencies of the alleles and genotypes of the rs396991 SNP as calculated in the SLE patients and controls. The genotype frequencies in SLE cases (χ^2= 0.2; p = 0.65) and controls (χ^2= 0.11; p = 0.74) were both in agreement with HWE expectations.

T and G allelic association in SLE is shown in Table 3.3. The minor G allele does not confer susceptibility to SLE (OR = 1.135, 95% CI: 0.811-1.588, p =0.654), with a minor allele frequency of 32% in cases and 35% in controls. This G allele exceeded the threshold p<0.05 and therefore is not statistically significant in conferring susceptibility to SLE in Black South Africans.

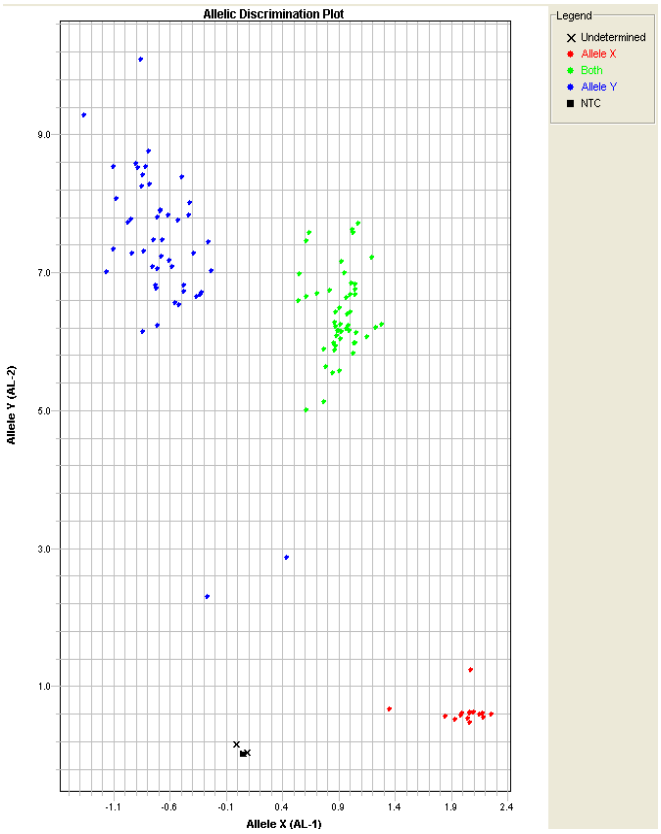
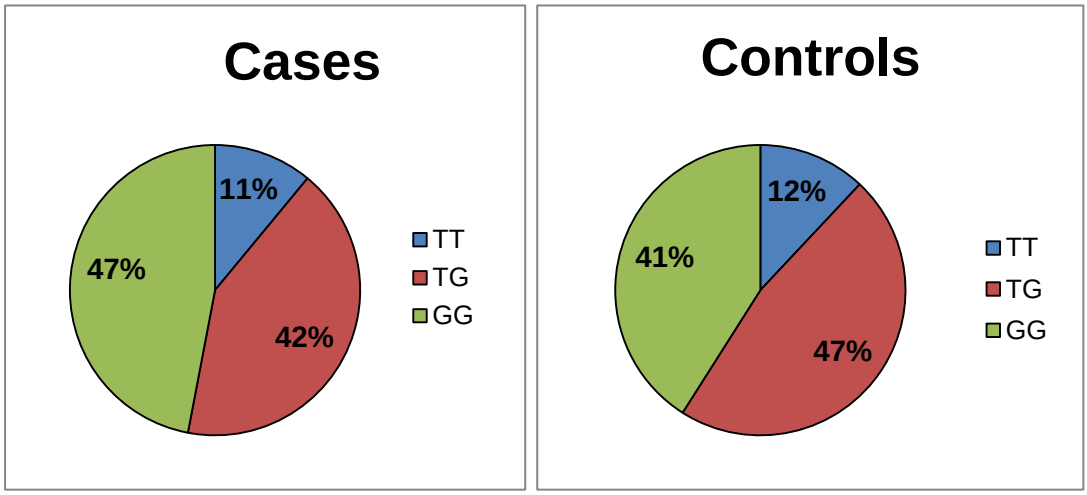


Figure 3.4: Allelic discrimination plot of selected SLE samples and controls for rs396991

Red dots denote individuals with TT genotypes, green dots with TG genotypes and blue dots with GG genotypes. The no template controls (NTC) can be seen as two black squares. Samples that could not be determined can be seen as black crosses. The different genotypes cluster together along the x and y-axes.

a.



b.

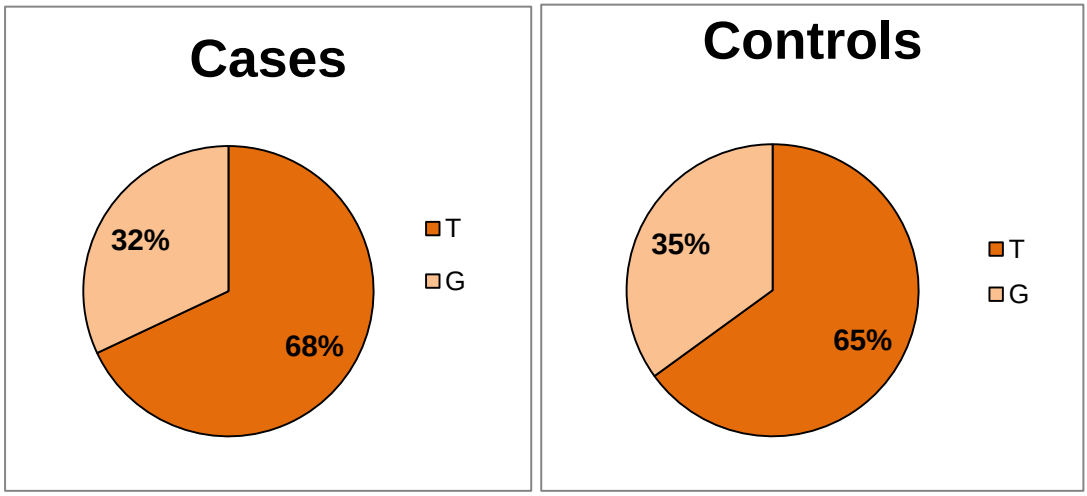


Figure 3.5: Pie charts illustrating the observed genotypes (a) and allele frequencies (b) of rs396991

Genotype frequencies and allele frequencies in 152 cases and 154 controls.

Table 3.3: Statistical analysis of results from rs396991

Major allele	Minor allele	Case MAF	Control MAF	OR (95% CI)	Chi-square value	P-value
T	G	0.32	0.35	1.135 (0.811-1.588)	0.85	0.654

3.4. Lupus Nephritis

Lupus nephritis (LN) was present in 43.8% (n=71) of cases. Majority had the severe forms of nephritis III, IV and V (84%). The allele frequencies of the allotypes and the SNP rs396991 were calculated for LN cases and non-LN cases (Figure 3.6). The allele frequency of NA1 in LN cases was 0.73 and NA2 was 0.27, whereas the allele frequency in non-LN cases was NA1 at 0.59 and NA2 at 0.41. The FCGRIIIB NA1/NA2 does confer susceptibility to LN (OR = 1.879, 95% CI: 1.109-3.184, X^2= 5.57, p =0.018).

The allele frequency of T in LN cases was 0.31 and G was 0.69, whereas the T allele frequency in non-LN cases was 0.28 and G at 0.72. The minor allele G does not confer susceptibility to LN (OR = 1.121, 95% CI: 0.693-1.813, X^2= 0.215, p =0.643), with a minor allele frequency of 69% in cases and 72% in controls.

Of the LN cases, 22.4% had null-alleles whereas 7.37% of non-LN cases had null-alleles. Null alleles in this study refers to the absence of the *FCGRIIIB* gene. This can indicate that low or no copy number of *FCGRIIIB* is associated with lupus nephritis, as seen in other population groups (Niederer *et al*., 2010). A two-sample t-test was performed between the percentages of null-allele individuals at an alpha level of 0.05 and degrees of freedom of 160. The t-statistic calculated was 2.75 and the p-value was 0.007.

The mean copy number of FCGRIIIB was calculated for LN cases and was found to be 1.995. This is below 2 copies and is consistent with previous studies that a copy number of ≤2 confers susceptibility to LN.

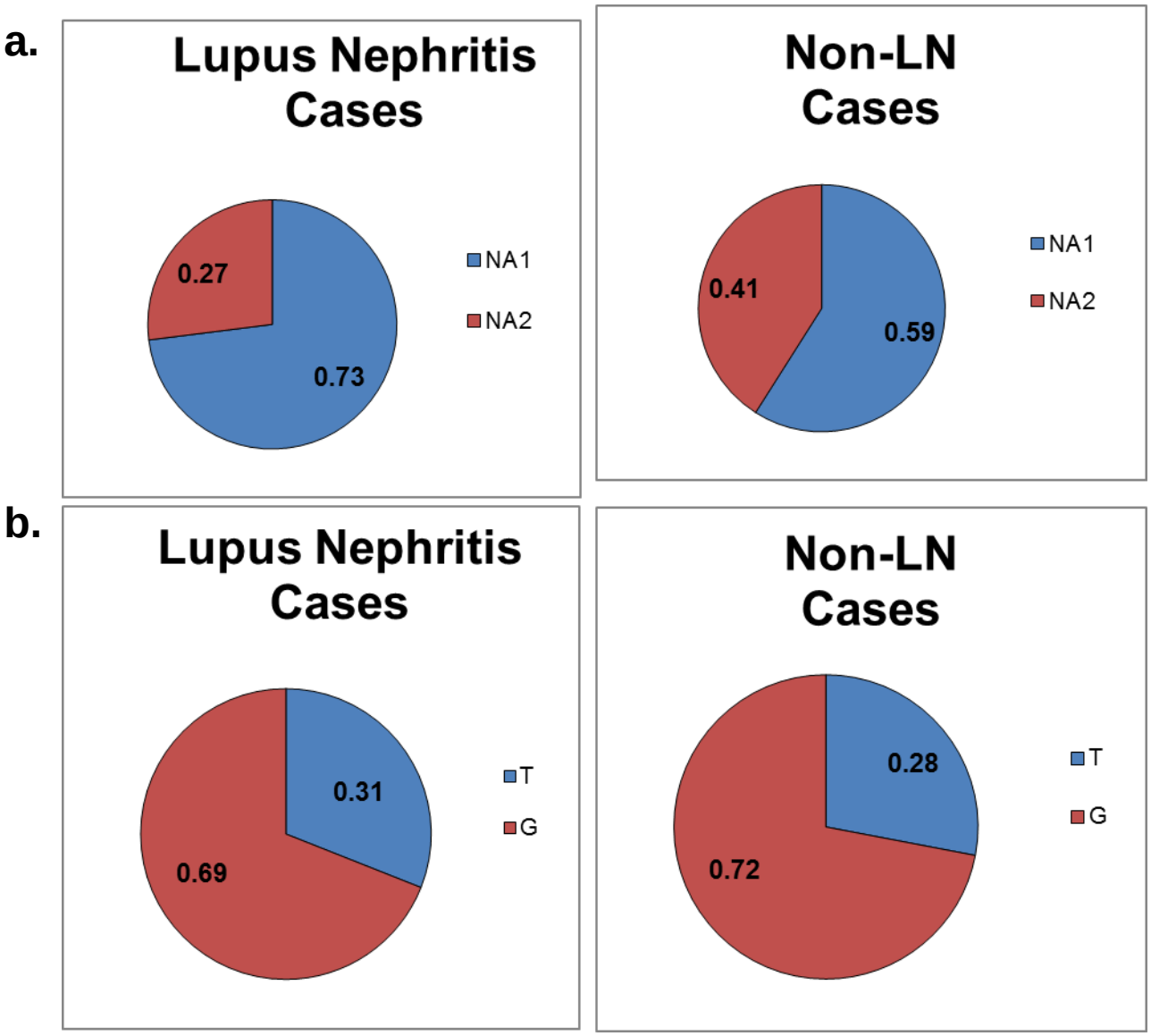


Figure 3.6: Pie charts illustrating the allele frequencies of NA1/NA2 allotypes in LN and non-LN cases (a) and allele frequencies of rs396991 in LN and non-LN cases

3.5. Copy Number Variation

Copy numbers of cases and controls were calculated using the CopyCaller software (Figure 3.7). Each sample was analysed in duplicate with the average of these two values used to calculate the percentage of copy numbers (Figure 3.8a). Copy numbers were categorized as either 0, 1, 2, 3 or 4 copies. The cumulative percentages of ≤2 copies and >2 copies were then calculated for cases and controls as this was the focus of the study (Figure 3.8b).

A two sample t-test between percents was performed to see if there was a significant difference between ≤2 copies and >2 copies seen in cases and controls (Quirks Stats Calculator). At an alpha level of 0.05 and degrees of freedom of 307, the t-statistic was 0.219 and the p-value was 0.826. Due to the p-value being greater than 0.05, the difference is not significant.

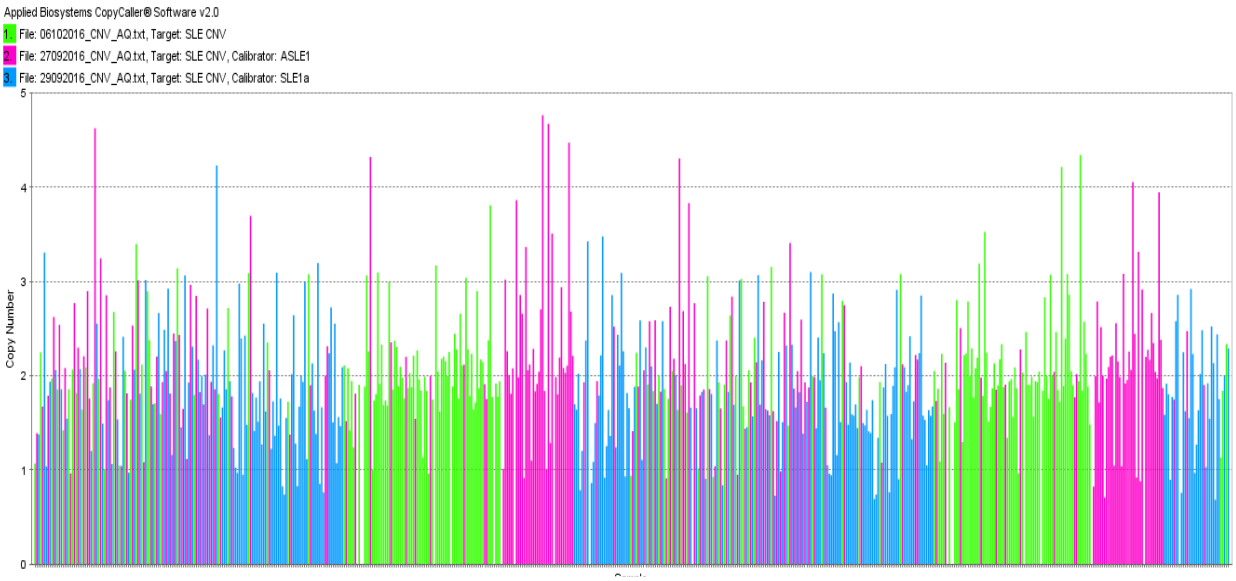


Figure 3.7: Bar graph representing copy number variation found in the *FCGRIIIB* gene in SLE cases and controls

Each line on the x-axis represents a sample that was run. The lines are colour coded into green, purple and blue to represent the three different plates run as all samples could not fit onto one plate. The y-axis represents the number of copy numbers and is numbered from 0 copies to 5 copies.

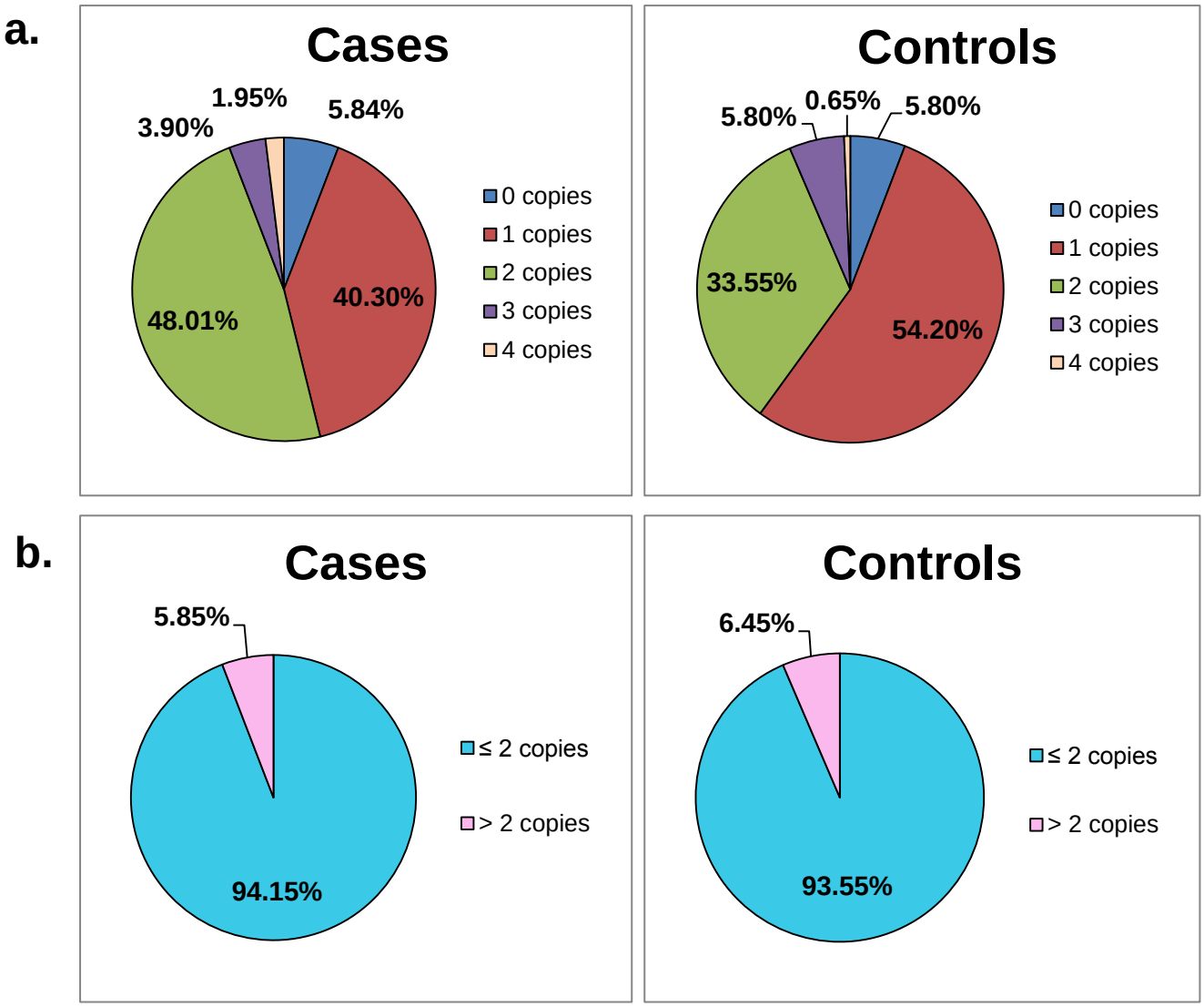


Figure 3.8: Pie charts illustrating the observed copy numbers of *FCGRIIIB* in cases and controls

(a) Copy number variation categorised as 0- 4 copies in 154 cases and 155 controls
(b) Copy number variation categorised as ≤ 2 copies and > 2 copies in 154 cases and 155 controls

Chapter 4

Discussion

4.1. Clinical Variables

Analysis of clinical variables revealed that the majority of the SLE patients were female (93.8%). The mean age of the patient at diagnosis was 32.4 years and the mean current age of the patients was 39.1 years. This translates to the mean disease duration being 6.7 years. The SLICC criteria further make diagnosis more accurate. There are 17 criteria whereby four must be present to make a positive diagnosis (Yu *et al.,* 2014, Bertsias *et al.,* 2012). In this study, the mean SLICC score was eight. Lupus nephritis was present in 43.8% of patients and this percentage was broken down into the five classes of lupus nephritis. 23.5% of patients were recorded to have previous TB infection. Immunologic factors were investigated such as low levels of complement 3 and 4 proteins (66.7%), presence of anti-ds DNA antibodies (42%) and presence of anti-Sm antibodies (72.8%).

The female to male ratio in this study was 13.5 females to 1 male. This correlates to numerous previous studies of 9 females to 1 male ratio (Elkon and Santer, 2012). A previous South African study performed at the same location as this study, Chris Hani Baragwanath Academic Hospital, also had this female to male ratio (Benitha and Tikly, 2007). The fact that the female ratio is slightly higher, than seen in populations in this study, may be due to gender bias whereby certain restrictions according to age, ethnicity and SLICC criteria excluded more male patients present at the clinic.

The mean age of patients at diagnosis was 32.4 years. In previous studies this mean age was 31.5 years (Benitha and Tikly, 2007, Mody *et al*., 1994, Wadee *et al*., 2007). Benitha and Tikly (2007) presented evidence that 84% of patients are diagnosed with SLE before 40 years of age. The data in this study agree with this statement. The mean age of patients when recruited for this study was 39.1 years. This is only

slightly higher than previous studies performed at Chris Hani Baragwanath Academic Hospital in Johannesburg and King Edward VIII Hospital and Inkosi Albert Luthuli Hospital in Durban, whereby the current mean age at recruitment was 36 years (Benith and Tikly, 2007, Patel and Mody, 2013).

Disease duration is considered from the date of diagnosis. The mean length of disease duration was 6.7 years. Studies conducted in hospitals in Johannesburg and Durban, South Africa showed the mean disease duration to be 4.5 years (Benitha and Tikly, 2007, Dubula and Mody, 2015). The 2.2 year discrepancy could be attributed to delayed diagnosis or patients who do not and cannot afford to seek help. 27.1% of South Africans are unemployed and these people cannot afford transport to travel to state hospitals (Stats SA, 2016). People who are employed are often not able to take off work to visit state hospitals on the designated clinic days.

People with African ancestry with SLE often exhibit a more aggressive disease (Tikly *et al*., 1996, Wadee *et al.,* 2007). This involves more renal involvement and infection. The mean SLICC criteria in this study, which are criteria involved in SLE diagnosis, was eight. This translates to 67% of criteria being met in this patient cohort. Criteria involve clinical and immunologic aspects. This study examined three immunologic criteria, these being: anti-ds DNA antibodies, anti-Sm antibodies and low complement levels. These three criteria are specific to autoimmune diseases and are important in the positive diagnosis of SLE (Bertsias *et al*., 2012).

42% of patients in this study had anti-ds DNA antibodies and 72.8% of patients had anti-Sm antibodies present. Tikly et al. (1996) identified the autoantibodies present in Black South African patients. Ro antibodies were seen in 60.5% of patients, La antibodies in 28.4%, Sm antibodies in 44.2% and anti-ds DNA antibodies at 40.8%. Anti-Sm and anti-RNP antibodies are the most commonly seen autoantibodies in Black patients (Mody et al., 1994). In this study a far greater percentage of patients had anti-Sm antibodies than seen in previous studies. This could be attributed to better testing methods and diagnosis. Anti-ds DNA antibodies in this cohort correlate to what has been previously seen.

A low complement level of C3 and C4 indicate a defective complement pathway and autoimmune diseases such as SLE. In this study 66.7% of patients had low complement levels. However, no published South African data exist regarding previous studies on complement levels. Therefore one cannot compare the values obtained in this study to previous values.

Lupus nephritis (LN) and infection are major contributors to mortality in Black South African SLE patients (Dubula and Mody, 2015, Mody *et al*., 1994, Patel and Mody, 2013). Lupus nephritis, once diagnosed, can be categorised into five grades. Grade I is considered the least severe while grade V is considered the most severe. In this study 43.8% of patients had LN. This accounts to just under half of the cohort having LN and emphasises the importance of diagnosing and treating LN in South Africa.

Of the 43.8%, 2.5% had grade I LN, 4.3% had grade II, 12.3% had grade III, 4.9% had grade IV and 19.8% had grade V. The majority of LN patients had grade III and grade V, the higher level grades of LN. This correlates to previous data whereby LN is a main cause for mortality in SLE. Previous studies have shown that grade III and grade V LN is increasing whereas grade II is decreasing. This trend is seen in these data whereby the majority of patients were grade III (12.3%) and grade V (19.8%). Grade II was seen at a lower frequency of 4.3%.

Lupus nephritis affects renal function whereby antigen-antibody complexes accumulate in the kidneys causing glomerulonephritis (Brown *et al.*, 2007, Karassa *et al*., 2003, Kyogoku *et al*., 2004). This results in kidney damage and loss of filter function. The fact that people of African ancestry tend to experience a more severe disease agrees with the high rate of LN present in Black SLE patients in South Africa. The allele frequencies and copy number variation were investigated in LN and non-LN cases and will be discussed in the respective sections.

Another consequence of SLE is infection. Infection susceptibility increases if the immune system is compromised due to autoantibody status, disease duration and

immunosuppressive medication use (Cervera et al., 2003). SLE patients have higher infection rates than seen in the healthy population (Dubula and Mody, 2015). Infections such as TB, pneumonia, sepsis and urinary tract infections (UTIs) contribute to SLE patient mortality. TB infection can be divided into pulmonary and extra-pulmonary TB although this study did not take this into account. Previous TB infection was present in this study cohort at 23.5%. This is a large percentage when taking into account that other infections were not documented. Therefore, one can conclude that there is a link between SLE and infections such as TB. In previous studies TB was seen in 20% of patients. However, taking into account the other infections, this number rose to 35.2% (Dubula and Mody, 2015).

From this study, one can see that the majority of South African SLE patients are female. The disease status is also more aggressive by the mean amount of SLICC criteria present as well as the immunologic statistics. Lupus nephritis and TB infection is prevalent in Black South African patients translating to poor disease prognosis.

4.2. Allotype Studies

The allotypes or allelic variants investigated in this study were NA1 and NA2 which are present on neutrophils and are encoded by the *FCGRIIIB* gene. The NA2 allele is associated with reduced binding efficiency of antigen-antibody complexes, whereas the NA1 allele has a higher binding capacity (Gessner *et al.*, 1998, Hatta *et al.,* 1999, Lehrnbecher *et al*., 2000, Morris *et al.,* 2010, Tanaka *et al*., 2005). Some studies have shown the NA1 allele to be overrepresented in healthy Black South Africans (Lassauniere and Tiemessen, 2016). However, studies on other African populations show the NA1 allele to be underrepresented (Hessner *et al.,* 1996, Hessner *et al.,* 1998, Kissel *et al.,* 2000, Lehrnbecher *et al*., 1999, Matsuo *et al*., 2000, Moritz *et al.,* 2008).

After genotyping using target-specific PCR primers, the genotype and allele frequencies were calculated. The allele frequencies of the cases were NA1 at 0.69 and NA2 at 0.31 and the controls were NA1 at 0.63 and NA2 at 0.37. The genotype

frequencies of the cases and controls were tested using Pearson's chi-squared ($\chi 2$) test to determine whether proportions of genotypes are consistent with Hardy-Weinberg Equilibrium using a significance threshold of $p<0.05$. It was found that controls ($X^2=0.87$; $p=0.3438$) were in agreement with HWE whereas cases ($X^2=11.75$; $p=0.0006$) were not. This is most likely due to sample bias whereby the cases are enriched for a certain trait (Salanti and Ioannidis, 2008).

The allele frequencies calculated were compared using Pearson's chi-squared ($\chi 2$) test, to test for association with SLE. The minor allele frequencies (MAF) were compared. An odds ratio of 1.281 was calculated at a 95% confidence interval (0.902; 1.817). The p value of 0.166 exceeded the threshold of $p<0.05$ and therefore the NA2 allele is not statistically significant in conferring susceptibility to SLE. However, the odds ratio value is >1 which translates to this allele being associated with SLE. The fact that the p value is >0.05 means that the probability that this allele is associated with SLE is more due to chance than it actually being associated with the disease (Gianfrancesco et al., 2016). If the p value were to be <0.05 and the OR>1 one could state that the NA2 allele is associated with SLE in Black South Africans.

It is interesting to note that there was a frequency of 0.08 of NA-null genotypes in cases and a frequency of 0.005 of NA-null genotypes in controls. These figures were however, not included in the data analysis. There is a higher frequency of NA-null in cases which means that these patients are deficient in the *FCGRIIIB* gene. Having no NA allele may confer susceptibility to SLE, although this was not statistically determined in this study due to the low numbers of patients. However, this idea corresponds to the low copy number of *FCGRIIIB* in cases that have been seen in previous SLE studies.

In LN cases the allele frequency of NA1 is 0.73 and NA2 is 0.27. In the literature, the low binding NA2 allele is said to be associated with SLE and LN (Gessner *et al*., 1998, Hatta *et al*., 1999, Lehrnbecher *et al*., 2000, Morris *et al.*, 2010, Tanaka *et al.,* 2005). After statistical analysis, NA1/NA2 was shown to confer susceptibility to LN in

this study and this cohort (Lee *et al*., 2009). Non-LN cases had an NA1 allele frequency of 0.59 and NA2 of 0.41.

4.3. SNP Genotyping

4.3.1. rs10127939

The rs10127939 SNP is a tri-allelic SNP that results in either a thymine to adenine or guanine base change at position 230 (c. 230T>G>A). This base change will also result in an amino acid change at position 48 (p. L48R/H). Previous studies have shown that being homozygous for the A allele results in severe immunodeficiency and high infection rates (de Vries et al., 1996). This is important as this study looks at SLE, an autoimmune disease and infections are commonly seen in Black South African SLE patients. Therefore this SNP was hypothesised to confer susceptibility to SLE in this population.

The allele frequencies in Caucasian control studies have found T at 0.86, G at 0.06 and A at 0.08 (de Haas *et al*., 1996, de Vries *et al*., 1996). The Yoruba of Nigeria studied in the HapMap project have the T allele at a frequency of 0.91, G at 0.09 and the A at 0.00. To date there is no data available of the genotype frequencies of this SNP in Black South Africans.

In both Caucasians and the Yoruba, the T allele is present at a very high frequency and the G allele is seen at a low frequency. The A allele is also present at a very low frequency and is not seen at all in the Yoruba. This strongly suggests that the A allele may also not be present in the Black South African SLE patients or controls.

Upon investigation, whereby all three alleles were genotyped for, the G and A allele were not present in either cases or controls. All cases and controls were genotyped to be homozygous for the T allele. Previous studies have described and confirmed this whereby this SNP is not polymorphic in Black South Africans (Lassauniere *et al*.,

2013; Lassauniere and Tiemessen, 2016). Therefore, the rs10127939 SNP is non-polymorphic or monomorphic in Black South Africans, as was seen in a similar study for a different SNP (Tikly et al., 2010). As both cases and controls are monomorphic for the T allele, one can conclude that this SNP confers no susceptibility to SLE in Black South Africans. Perhaps a bigger sample size could completely prove that the G and A allele are not present within Black South Africans.

4.3.2. rs396991

The rs396991 single nucleotide polymorphism results in a thymine to guanine base change at position 559 (c. 559T>G). This will also result in an amino acid change at position 176 (p. F176V). The T allele results in reduced binding capacity of the receptor to clear immune complexes thus resulting in increased inflammation (Deng and Tsao, 2010, Li *et al*., 2009, Sestak *et al*., 2011, Tanaka *et al.,* 2005).

Previous studies have shown the T allele confers a 1.2 fold risk increase for developing lupus nephritis in Europeans, Africans and Asians (AlFadhli, 2012, Alizadeh *et al*., 2007, Brown *et al.*, 2007, Karassa *et al.*, 2003, Kyogoku *et al*., 2004, Sestak *et al*., 2007). This study aims to determine if this SNP confers susceptibility to SLE in Black South Africans.

The allele frequencies in Black South African controls have found the T allele at 0.63 and the G allele at 0.37 (Lassauniere and Tiemessen, 2016). Other studies using Caucasian controls have found the T allele at 0.74 and the G allele at 0.26 (Koene *et al.*, 1998, Lehrnbecher *et al.,* 1999). The Yoruba of Nigeria studied in the HapMap project see the T allele is found at 0.96 and the G allele at 0.04. As with the previous SNP one can see that the allele frequencies of the Yoruba and Black South Africans are not comparable and are in fact very different.

In this study, the allele frequencies of the cases were T at 0.68 and G at 0.32 and the controls were T at 0.65 and G at 0.35. This sample and these values underwent

statistical analysis. The genotype frequencies of the cases and controls were tested using Pearson's chi-squared (χ2) test to determine whether proportions of genotypes are consistent with Hardy-Weinberg Equilibrium using a significance threshold of $p<0.05$. It was found that both cases ($X^2=0.2$; $p=0.65$) and controls ($X^2=0.11$; $p=0.74$) were in HWE. Controls should always be in HWE as they should represent the greater population. There are however ongoing debates as to whether cases, which are enriched with a certain trait, should or should not be present in HWE (Salanti and Ioannidis, 2008).

The allele frequencies calculated were compared using Pearson's chi-squared (χ2) test, to test for association with SLE. The minor allele frequencies (MAF) were compared. An odds ratio of 1.135 was calculated at a 95% confidence interval (0.811; 1.588). The p value of 0.654 exceeded the threshold of $p<0.05$ and therefore the G allele is not statistically significant is conferring susceptibility to SLE

However, even though this SNP is not statistically significant, one can still see that the low-binding T allele was seen in cases at a 0.03 higher frequency than in controls. This may translate to an association in future studies. Perhaps a bigger sample size would be required to be able to see such an association.

The allele frequencies were calculated in LN and non-LN cases. In LN cases the T allele was seen at 0.31 and the G allele at 0.69. In non-LN cases the T allele was seen at 0.28 and G allele at 0.72. There was no large difference between allele frequencies in both groups and after statistical analysis it was seen that the minor allele, G, does not confer susceptibility to LN. This SNP therefore does not act as risk factor for developing LN in Black South African SLE patients.

4.4. Copy Number Variation

A low copy number and therefore a deficiency in the *FCGRIIIB* gene has been linked to systemic autoimmunity (Fanciulli et al., 2007). In a study performed by Morris et al.

(2010) on Caucasian SLE patients showed the mean copy number in cases to be 1.981 copies and in controls to be 2.052 copies. This trend has been seen in studies of different population groups.

Lassauniere and Tiemessen (2016) found that healthy Black individuals were found to have one gene copy of *FCGRIIIB* at 7.6%, two gene copies at 74%, three gene copies at 15.3% and four gene copies at 3.1%. Therefore, 81.6% of these healthy individuals have ≤2 copies. However, these values were not compared to SLE cases. In the present study, 5.84% of cases had 0 copies, 40.3% had 1 copy, 48.01% had 2 copies, 3.9% had 3 copies and 1.95% had 4 copies. The cumulative percentages of copies ≤2 was 94.15% and copies >2 was 5.85%. In the controls, 5.8% of controls had 0 copies, 54.2% had 1 copy, 33.55% had 2 copies, 5.8% had 3 copies and 0.65% had 4 copies. The cumulative percentages of copies ≤2 was 93.55% and copies >2 was 6.45%.

This study shows that 54.1% of controls have one copy, whereas only 7.6% of healthy Black South Africans in the Lassauniere and Tiemessen (2015) study have one copy. This large discrepancy highlights the inaccuracy in which this data was generated. The level of accuracy of the assays and method of data interpretation may have been a factor in assigning different copy numbers. In fact, other ways in which to interpret the data is not to calculate the mean of copy numbers but to take the relative values as a continuum. It is recommended in future studies that multiplex ligation probe assays (MLPA) should be used, instead of the TaqMan assay. MLPA is more accurate and would be more effective in giving confident results.

It is interesting to note that the SLE cases had almost 2% of individuals having 4 copies. Previous studies have shown that having more copies of FCGRIIIB acts as a protective effect against developing LN and SLE but in this study this was not seen. Both cases and controls had approximately 5.8% of individuals who had 0 copies yet the controls did not have SLE. This could mean that having no copy of *FCGRIIIB* cannot always translate to developing an autoimmune disorder or perhaps those control individuals will develop symptoms over a period of time. Once the copy

number was cumulatively calculated, one can see that SLE cases had a larger number of individuals who have ≤2 copies. This corresponds to what is seen in the literature as having fewer copies results in SLE.

A two-sample t-test was performed between the cumulative percentages at an alpha level 0f 0.05 and degrees of freedom of 307. The t-statistic calculated was 0.219 and the p-value was 0.826. The p-value is greater than 0.05 and therefore there is no significant difference between percentages in cases and controls. Although this cannot be proven statistically, the trend that SLE cases had more individuals with lower copy numbers shows that in this cohort, low copy number is still a risk in developing SLE. Therefore, even if not significant, there is still a 0.6% increase of ≤2 copies seen in cases compared to controls. This correlates to what is seen in the literature.

Copy number variation was calculated in lupus nephritis (LN) and non-LN cases to see if this type of variation could be a risk factor in developing LN. 43.8% of cases had LN and the mean copy number within these individuals was 1.995 copies. Copy number of ≤2 has been linked to SLE and kidney disease and therefore one can suggest that a lower copy number can increase the risk of developing LN. Previous studies on rats have shown that a low copy number has been associated with glomerulonephritis in SLE (Aitman et al., 2006). Non-LN cases (56.2%) had a mean copy number of 2.102 copies. This number is above 2 copies and therefore suggests that a higher copy number lowers the risk of developing LN. In fact, having ≥2 copies has a protective effect against developing LN (McKinney and Merriman, 2012, Tiffin *et al*., 2013). Although the differences of copy number in LN cases and non-LN cases is not very large, there is still obvious dissimilarity that should not be overlooked.

4.5. Strengths and Weaknesses of Study

The strengths of this study include the amount of clinical data obtained at the time of the blood extraction. Many clinical and immunological variables, including lupus nephritis, were included which added further value to this study. The genotyping method that was used for the two SNPs as well as the CNV study was TaqMan technology. This technology is known as the gold standard in quantitative genomics and confidently calls results. This eliminates the chance that human error could influence the results.

Demographics of the controls were not available, except for the race of the controls. It would be preferable to match the cases and controls as closely as possible to gender ratio and age in order to have no variability within the sample. Some DNA samples that were extracted from the blood of patients were not of good quality. This affected the ability for them to be used in certain study methods and they had to be excluded. This impacted further on the sample size. The sample size was not very large and this could have affected some results. The power of the study is therefore not as strong as one would want. The allotype determination was performed using PCR. This may cause the results from the study to have some level of human error as the amplification products were interpreted manually. Genotyping reproducibility only tested 20% of the SLE cases. This percentage may be too low to confidently validate the data that were produced. These genotyping results should have been validated using another genotyping method or by running the assay in another laboratory. The SH allotype was not genotyped and certain studies have shown that the SH allotype would be assigned as NA2 allotypes, if not genotyped. This therefore has major potential to confound results making interpretation of the data dubious. The function of SH is not known in the literature and the genotyping of SH in the Black South African population would result in novel data.

4.6. Significance of Study

The present study integrates and analyses immunologic, clinical and genetic data. This is the first study to investigate these specific genetic polymorphisms in the South African population and the results aid in a better understanding of the genetic aetiology of SLE in black South African patients. Of particular interest were those SLE patients who developed Lupus nephritis (LN), as recurrent infections in these patients are a major cause of mortality. This study looked at the percentage of patients with LN and previous TB infection and concluded that these particular patients are at a greater risk of infection than those patients who do not develop LN. These data will allow clinicians to better treat LN patients thereby improving current treatment strategies. Therefore use of both comprehensive clinical data as well as genetic data enables a better understating of the disease pathogenesis and progression in this unique population as well as highlights the importance of a multidisciplinary approach to genetic and clinical research. Finally, the results from this study once again emphasise the uniqueness of our population and highlights the continued need for further studies to better understand the pathogenesis of disease in our population.

4.7. Further Research

Firstly, the genotyping data obtained were from a relatively small sample size. Genotyping results should be reassessed by using a larger sample size of both cases and controls. This would increase the power of the study and would give more accurate results. The allotype studies should be considered being done using a more reliable genotyping method such as TaqMan genotyping assays instead of using agarose gel electrophoresis in which the interpretation may be inaccurate. In this study two SNPs were investigated. In future studies it would be beneficial to look at more SNPs within the *FCGRIIIA* and *FCGRIIIB* genes as this population is unique and data pertaining to these genes is scarce. In order to determine CNV, an MLPA kit should be used instead of Taqman assays as it is more accurate and allows for the detection of other *FCGR* variants (Lassauniere *et al.*, 2013).

Secondly, one could investigate gene expression within these genes in addition to genotyping. Gene expression could use biomarkers in the diagnosis of patients. These biomarkers could be tested for and could be used in a more timeously diagnosis. One could investigate gene expression in other genes linked to SLE such as *BANK1, ITGAM, PTPN22, STAT4* and *PDCD1.*

Sequencing of the FC gamma receptor gene sequences in patients, such as the introns, exons and promoter sequences could identify further variants that may cause susceptibility to SLE in this population. As discussed, epigenetics plays a role in SLE. Methylation and acetylation studies could be applied to see the role epigenetics plays within this population and SLE.

Finally, the clinical and immunologic data could be further investigated in its link to the genetic results. Molecular and clinical studies are often separated but with future research these two components could be studied together in order to confidently identify an association.

Conclusion

This study focused on the genotypes of two SNPs (rs396991 and rs10127939) and the neutrophil-antigen allotypes as well as the copy number variation within the genes *FCGRIIIA* and *FCGRIIIB.* The methods were performed on DNA from both SLE cases and matching controls. In South Africa there is a paucity of data relating to the genetic mechanism of SLE in black South Africans. This study helps in further understanding the genetic basis of this disease in this population.

It was found that the allotypes, SNPs and copy number variation statistically do not confer susceptibility to SLE in this Black South African patient cohort. However, it

was found that NA1/NA2 is statistically significant in conferring susceptibility to LN in this cohort, which is a major cause of death in this population.

Clinical variables were investigated and it was noteworthy that 43.8% of patients had LN and 23.5% of patients were recorded to have had previous TB infection. This again highlights the need to treat infections timeously and for there to be accurate diagnosis of LN. Other immunologic and clinical criteria were also examined and they were found to be in line with values seen in previous studies in South Africa. This study emphasises the need for further research of SLE genetics in this unique study cohort.

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Appendices

Appendix A – Ethics Approval Certificate

R14/49 Miss Nerissa Wendy Bloch

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150624

NAME: (Principal Investigator)	Miss Nerissa Wendy Bloch
DEPARTMENT:	School of Pathology Division of Human Genetics , NHLS
PROJECT TITLE:	Fc Gamma Receptor III Polymorphisms and Copy Number Variations as Risk Factors for Systemic Lupus Erythematosus in Black South Africa Patients (BEC20150805)
DATE CONSIDERED:	21/08/2015
DECISION:	Approved unconditionally
CONDITIONS:	Application for Collection and Storage of Samples at Sidney Brenner Institute for Molecular Bioscience (BEC20140302)
SUPERVISOR:	Ms J Frost, Dr N Govind and Prof M Tikly
APPROVED BY:	Professor A Woodiwiss, Co-Chairperson, HREC (Medical) Prof A Dhai, Chairperson, BEC
DATE OF APPROVAL:	26/08/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature _____ Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B- Information and Consent Forms

INVITATION TO PARTICIPATE IN A STUDY ON SYSTEMIC LUPUS ERYTHEMATOSUS GENETICS

Patient Information Sheet

Dear Sir/Madam,

My name is Wesley Tulleken. You have been diagnosed with systemic lupus erythematosus (SLE) and I would like to invite you to take part in a study that we are doing in the Rheumatology Clinic at Chris Hani Baragwanath Academic Hospital. We are investigating the role of genetic factors in Black South African patients who have SLE. We plan to look at five genetic regions (rs1143679, rs3131379, rs132771130, rs10516487 and rs4963128) in your DNA. In the future, we would like to look at other genetic regions associated with SLE (about 200 000 sites). This will allow us to better understand the causes of this very complex disease.

The study will not involve any costs to you and participation is completely voluntary. You can withdraw at any time, and you will not be disadvantaged in any way if you refuse. Non-participation will not result in any prejudice of clinical management or treatment. Participation in the study does not advantage you in any way. You will receive treatment as normal.

If you agree to take part in the study, we will do a clinical examination, take 15ml (3 teaspoons) of blood for the DNA studies and document which antibodies you have, and if you have kidney problems from SLE. This procedure may result in discomfort and pain but the procedure is short and the pain and discomfort will not linger afterwards. The blood sample will be stored for ten (10) years and may be used for further genetic studies on SLE in the future. The samples will be anonymously labelled and will be stored in the Division of Human Genetics, National Health Laboratory Service. Any further studies other than the current study using your DNA will need to reapply for ethics approval. The results of the study will be handled confidentially and used by me and my fellow researchers solely for research purposes. We plan to publish the results in a medical journal. We will not publish anything identifying any study participant.

This study has been approved by the Human Research Ethics Committee at the University of the Witwatersrand.

If you would like more information or would like to withdraw from the study, please contact me or the following doctors

Dr W. Tulleken
083 311 5076
Chris Hani Baragwanath Hospital
University of the Witwatersrand

Professor Mohammed Tikly
(011) 933-9577
Chris Hani Baragwanath Hospital
University of the Witwatersrand

Professor Peter Cleaton-Jones
Chairman of the Human Research Ethics Committee
University of the Witwatersrand
(011) 717-2301

INFORMED CONSENT TO PARTICIPATE IN A STUDY ON SYSTEMIC LUPUS ERYTHEMATOSUS GENETICS

You have been asked to participate in a research study which will look at the genetics of systemic lupus erythematosus (SLE).

Have you been informed about the purpose of the study?	**YES**	**NO**
Have you been informed about the procedures involved in the study?	**YES**	**NO**
Do you agree and consent to give a blood sample for use in the study?	**YES**	**NO**
Do you understand that the results will not be made available to you?	**YES**	**NO**
Do you agree and consent to having your blood sample stored for 10 years?	**YES**	**NO**

The research study, including all information, has been described to me. I understand what my involvement in the study means and I voluntarily agree to participate.

Full name of participant:_____

Signature of Participant:_____

Date:_____

Appendix C- Table showing the Allele and Genotype Frequencies of rs396991 And Allotypes NA1/NA2

	LN (n=71)	Non-LN (n=91)	OR (95% CI)	p-value	Overall cases (n=162)	Controls (n=155)	OR (95% CI)	p-value
T	0.31	0.28	1.121 (0.693-1.813)	0.643	0.32	0.35	1.135 (0.811-1.588)	0.654
G	0.69	0.72			0.68	0.65		
TT	8.9% (6)	7.8% (7)			11% (18)	12% (19)		
TG	44.7% (32)	42.5% (39)			42% (68)	47% (73)		
GG	46.3% (33)	49.7% (45)			47% (76)	41% (63)		
NA1	0.73	0.59	1.879 (1.109-3.184)	0.018	0.69	0.63	1.281 (0.902-1.817)	0.166
NA2	0.27	0.41			0.31	0.37		
Null Allele	0.22	0.07						
NA1/NA1	43% (30)	44% (40)			49% (80)	38% (59)		
NA1/NA2	26.6% (19)	21.6% (20)			28% (45)	49.5% (77)		
NA2/NA2	7% (6)	27.03% (25)			15% (24)	12% (18)		
Null/null	22.4% (16)	7.37% (6)			8% (13)	0.5% (1)		

Appendix D- Demographic and Clinical Data of the SLE Patients

SLE #	Gender	Age	Age of Onset	SLICC	LN	LN I	LN II	LN III	LN IV	LN V	TB	↓ C3 and C4	Anti-dsDNA	Anti-Sm
SLE001	F	69	63	9	0	0	0	0	0	0	1	1	0	1
SLE002	F	44	27	4	0	0	0	0	0	0	1	0	0	0
SLE003	F	26	24	7	0	0	0	0	0	0	0	0	0	0
SLE004	F	39	32	6	0	0	0	0	0	0	0	0	0	1
SLE007	F	22	21	13	1	0	1	0	0	0	0	1	0	1
SLE008	F	20	16	11	1	0	0	0	0	1	0	1	1	1
SLE009	F	45	41	4	0	0	0	0	0	0	0	1	0	0
SLE010	F	50	31	9	1	0	0	0	0	1	1	1	1	0
SLE011	M	30	25	7	0	0	0	0	0	0	1	1	1	1
SLE012	F	42	33	6	0	0	0	0	0	0	0	1	0	0
SLE013	F	39	38	7	0	0	0	0	0	0	0	1	0	1
SLE014	F	26	25	10	1	0	0	1	0	0	0	1	1	0
SLE015	F	29	29	6	0	0	0	0	0	0	0	0	0	1
SLE016	F	58	50	6	0	0	0	0	0	0	0	1	0	1
SLE017	F	46	44	8	0	0	0	0	0	0	0	1	0	1
SLE019	F	32	31	6	1	0	0	0	0	1	0	1	0	1
SLE020	F	24	24	8	1	0	0	0	0	1	1	1	0	1
SLE021	F	26	23	8	0	0	0	0	0	0	0	1	1	1
SLE022	F	32	29	7	0	0	0	0	0	0	0	1	1	0
SLE023	F	29	15	10	1	0	0	1	0	0	0	1	1	1
SLE025	F	34	27	10	0	0	0	0	0	0	1	1	0	1
SLE026	F	67	56	4	0	0	0	0	0	0	0	0	0	1
SLE027	F	55	52	5	0	0	0	0	0	0	1	0	0	0

SLE028	F	54	49	6	0	0	0	0	0	0	0	1	0	0
SLE029	F	41	34	8	0	0	0	0	0	0	0	1	0	1
SLE030	F	30	28	6	0	0	0	0	0	0	0	1	0	1
SLE031	F	44	33	6	0	0	0	0	0	0	0	1	1	0
SLE032	F	43	41	9	0	0	0	0	0	0	0	1	0	1
SLE033	F	35	24	12	0	0	0	0	0	0	1	1	1	1
SLE035	F	56	46	4	0	0	0	0	0	0	1	0	0	0
SLE036	F	28	27	9	0	0	0	0	0	0	0	1	0	1
SLE037	F	30	27	11	1	0	0	0	0	1	0	1	1	1
SLE038	F	31	23	10	1	0	0	0	0	0	1	1	0	1
SLE039	F	19	19	5	1	0	0	0	1	0	0	1	0	0
SLE040	F	28	22	9	1	0	1	0	0	0	0	0	0	0
SLE041	M	27	21	8	1	0	0	0	0	1	0	1	1	1
SLE042	F	33	32	4	0	0	0	0	0	0	0	0	0	0
SLE043	F	57	34	5	0	0	0	0	0	0	0	0	0	0
SLE046	F	56	36	9	0	0	0	0	0	0	1	1	1	0
SLE047	F	23	14	9	0	0	0	0	0	0	1	1	1	1
SLE048	F	46	38	11	0	0	0	0	0	0	0	1	1	1
SLE049	F	34	28	4	0	0	0	0	0	0	0	1	1	0
SLE050	F	23	22	7	1	0	0	0	1	0	1	1	1	1
SLE051	F	35	35	8	0	0	0	0	0	0	0	0	0	1
SLE052	F	36	29	9	0	0	0	0	0	0	0	1	0	1
SLE053	F	25	22	9	0	0	0	0	0	0	0	1	0	1
SLE054	F	24	23	12	0	0	0	0	0	0	0	1	0	1
SLE056	M	19	18	14	1	0	0	0	0	1	0	1	1	1
SLE057	F	57	54	8	0	0	0	0	0	0	0	0	1	1
SLE058	F	43	38	8	1	0	0	0	0	0	1	1	0	1
SLE060	F	39	23	10	0	0	0	0	0	0	0	1	1	0
SLE061	F	24	19	9	1	0	0	1	0	0	1	1	1	1

SLE062	F	36	34	5	1	0	0	1	0	1	0	0	0	1
SLE063	F	43	32	10	0	0	0	0	0	0	1	1	0	1
SLE064	F	74	50	4	0	0	0	0	0	0	0	0	0	0
SLE066	F	29	29	8	1	0	1	0	0	0	0	1	1	1
SLE067	F	54	37	4	0	0	0	0	0	0	0	1	0	0
SLE068	F	31	25	11	1	0	0	0	0	1	0	1	0	1
SLE069	F	44	40	9	1	0	0	1	0	0	0	1	1	0
SLE070	F	27	21	8	1	0	0	0	0	1	1	1	1	0
SLE071	M	43	38	7	0	0	0	0	0	0	1	1	0	1
SLE072	F	24	21	13	1	0	0	1	0	0	1	1	1	0
SLE076	F	67	61	7	0	0	0	0	0	0	0	0	0	1
SLE078	F	32	29	8	1	0	0	0	1	0	0	1	1	1
SLE079	F	40	31	6	0	0	0	0	0	0	0	0	0	0
SLE081	F	51	34	12	1	0	0	0	0	1	0	1	1	1
SLE083	M	40	37	4	0	0	0	0	0	0	0	1	0	1
SLE084	F	27	22	9	0	0	0	0	0	0	0	1	1	1
SLE085	F	38	38	12	1	0	0	0	0	1	0	1	1	1
SLE086	F	39	33	10	0	0	0	0	0	0	0	1	1	1
SLE087	F	24	24	9	1	0	1	0	0	0	0	1	1	1
SLE088	F	59	41	13	0	0	0	0	0	0	0	1	1	1
SLE089	M	35	15	13	1	0	1	0	0	0	0	1	1	1
SLE091	F	23	13	7	0	0	0	0	0	0	0	0	0	1
SLE092	F	37	35	7	0	0	0	0	0	0	0	1	0	1
SLE094	F	39	38	8	1	0	0	1	0	0	1	1	0	1
SLE095	F	31	30	12	1	0	0	1	0	0	0	1	0	1
SLE097	F	55	49	12	0	0	0	0	0	0	1	1	1	1
SLE098	F	29	25	11	1	0	0	1	0	0	0	1	1	1
SLE099	F	42	40	4	1	0	0	0	0	1	0	0	0	1
SLE100	M	46	46	6	0	0	0	0	0	0	1	1	0	1

SLE101	F	37	37	7	1	1	0	0	0	0	0	1	0	1
SLE102	F	37	37	11	1	0	1	0	0	1	0	1	1	1
SLE103	F	44	31	9	1	0	0	0	0	1	0	1	0	1
SLE104	F	56	32	9	0	0	0	0	0	0	0	1	1	1
SLE106	F	55	46	4	1	0	0	0	0	1	0	0	0	0
SLE107	F	32	25	7	0	0	0	0	0	0	1	0	1	1
SLE108	F	33	33	8	1	0	0	0	0	1	0	1	0	1
SLE109	F	20	20	8	1	0	0	1	0	0	1	1	0	1
SLE113	F	56	40	8	0	0	0	0	0	0	1	1	0	0
SLE114	F	28	16	10	0	0	0	0	0	0	0	1	1	1
SLE116	F	35	21	12	1	0	0	0	0	1	0	1	1	1
SLE118	F	33	25	6	0	0	0	0	0	0	0	0	0	0
SLE122	F	27	26	9	0	0	0	0	0	0	0	1	0	1
SLE123	F	17	17	9	1	0	0	0	0	1	0	1	1	1
SLE124	F	46	29	11	1	0	0	0	0	1	1	1	0	1
SLE125	F	63	58	5	0	0	0	0	0	0	0	0	0	1
SLE127	F	16	15	8	0	0	0	0	0	0	0	1	1	1
SLE128	F	36	33	9	1	0	0	1	0	0	0	1	0	1
SLE129	F	20	11	10	1	0	0	0	0	1	0	1	1	0
SLE130	F	53	51	7	1	0	0	0	1	0	0	1	0	1
SLE131	F	65	61	4	0	0	0	0	0	0	1	0	0	1
SLE132	F	51	38	4	0	0	0	0	0	0	0	0	0	0
SLE133	M	69	54	8	0	0	0	0	0	0	0	0	0	1
SLE134	F	44	28	11	1	0	0	0	0	1	1	1	0	1
SLE135	F	31	28	9	0	0	0	0	0	0	0	1	1	1
SLE136	F	33	26	7	0	0	0	0	0	0	0	1	0	1
SLE137	F	22	19	7	0	0	0	0	0	0	0	1	0	1
SLE138	F	39	30	5	1	0	0	0	0	1	0	1	1	0
SLE139	F	42	42	10	0	0	0	0	0	0	0	1	0	1

SLE140	F	20	20	4	0	0	0	0	0	0	0	0	0	1
SLE141	F	51	41	9	0	0	0	0	0	0	1	0	0	1
SLE142	F	25	17	8	0	0	0	0	0	0	0	1	0	1
SLE145	M	35	33	5	1	0	0	0	1	0	0	1	0	1
SLE146	F	44	40	8	1	0	0	0	0	1	0	1	0	1
SLE147	F	54	48	5	1	0	0	0	0	1	0	0	0	0
SLE148	F	43	29	5	0	0	0	0	0	0	0	1	0	0
SLE151	F	41	41	6	1	0	0	0	0	0	1	0	0	0
SLE152	F	28	28	11	1	0	0	0	1	0	0	1	1	1
SLE153	F	34	34	9	0	0	0	0	0	0	0	1	1	1
SLE156	F	76	53	11	0	0	0	0	0	0	0	1	1	0
SLE160	F	29	23	12	0	0	0	0	0	0	0	1	1	1
SLE161	F	41	38	10	0	0	0	0	0	0	1	1	1	1
SLE162	F	59	43	6	1	0	0	1	0	0	0	0	1	1
SLE165	F	56	56	4	0	0	0	0	0	0	0	0	0	0
SLE166	F	60	39	9	0	0	0	0	0	0	0	1	1	1
SLE167	F	38	33	10	0	0	0	0	0	0	0	1	1	1
SLE169	F	23	21	8	0	0	0	0	0	0	0	1	0	1
SLE170	F	59	50	7	0	0	0	0	0	0	0	0	0	0
SLE171	F	53	52	11	0	0	0	0	0	0	0	1	0	1
SLE172	F	66	42	5	0	0	0	0	0	0	0	1	0	0
SLE173	F	60	42	8	0	0	0	0	0	0	0	1	1	1
SLE174	F	46	23	5	0	0	0	0	0	0	0	1	0	0
SLE175	F	41	30	10	0	0	0	0	0	0	1	1	0	1
SLE176	F	41	19	14	1	0	0	1	0	0	0	1	1	1
SLE177	F	46	45	7	0	0	0	0	0	0	0	1	0	1
SLE178	F	41	41	6	0	0	0	0	0	0	0	0	0	1
SLE179	M	21	21	9	1	0	0	1	0	0	1	1	1	0
SLE180	F	37	33	10	1	0	0	1	0	0	0	1	1	1

SLE 182	F	30	24	12	1	0	0	1	0	1	0	1	1	1
SLE 183	F	38	27	10	1	0	0	0	0	1	1	1	1	0
SLE 184	F	31	25	11	1	0	0	0	1	0	1	1	1	0
SLE 185	F	14	14	7	1	1	0	0	0	0	0	1	0	1
SLE 187	F	39	39	10	1	0	0	1	1	0	1	1	0	1
SLE 189	F	34	23	10	1	0	0	1	0	1	0	1	1	1
SLE 190	F	34	25	9	1	0	1	0	0	1	0	1	1	1
SLE 191	F	43	30	4	0	0	0	0	0	0	0	0	1	0
SLE 192	F	34	23	9	1	0	0	0	0	1	0	0	1	1
SLE 193	F	50	32	4	0	0	0	0	0	0	1	1	0	0
SLE 194	F	36	27	11	0	0	0	0	0	0	1	1	1	1
SLE 195	F	43	38	6	0	0	0	0	0	0	0	0	1	1
SLE 196	F	54	54	5	0	0	0	0	0	0	0	0	0	1
SLE 197	F	59	46	9	1	0	0	0	0	1	0	0	0	1
SLE 198	F	39	34	6	1	0	0	1	0	0	0	0	0	0
SLE 199	M	54	48	8	0	0	0	0	0	0	0	1	1	1
SLE 200	F	15	13	6	0	0	0	0	0	0	0	0	0	1
SLE 201	F	54	42	6	0	0	0	0	0	0	0	0	0	1
SLE 202	F	21	19	10	1	1	0	0	0	0	0	0	0	1
SLE 203	F	22	21	9	1	0	0	1	0	0	0	1	1	1
SLE 204	M	30	30	5	0	0	0	0	0	0	0	0	0	1
SLE205	F	17	17	7	1	1	0	0	0	1	1	0	0	1
SLE206	F	30	30	10	0	0	0	0	0	0	0	1	1	1

Appendix E- Plagiarism Declaration and Turnitin Report

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PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Nerissa Bloch (Student number: 456102) am a student registered for the degree of Master of Science in Medicine in the academic year 2017.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

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