The role of the *APOL1* genetic variants in nephritis susceptibility in Black South Africans with Systemic Lupus Erythematosus

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A research report submitted to the University of the Witwatersrand, Johannesburg in part fulfillment for the requirements of the degree of Master of Medicine (Internal Medicine)

Declaration

I, Wesley van Hougenhouck-Tulleken, declare that this research report is my own work which is being submitted for the degree Master of Medicine (in the submissible format with my protocol and extended literature review) in the branch of Internal Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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20 July 2016

Dedication

This work is dedicated to my wife Jessica, and my family, for all their continued support. Without you, it would not have been possible.

Acknowledgements

I would like to acknowledge the invaluable assistance of the following people, without whom this report would not have been possible:

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A	Adenosine
ACR	American College of Rheumatology
adsDNA	Anti double stranded deoxyribose nucleic acid
ANA	Antinuclear antigen
APOL1	Apolipoprotein L - 1
BANK1	B-cell scaffold protein with ankyrin repeats 1
BLAST	Basic local alignment search tool
BLK	BLK proto-oncogene, Src family tyrosine kinase
bp	base pairs
^{o}C	Degrees Celsius
C	Cytosine
CI	Confidence interval
CKD	Chronic kidney disease
Del	Deletion
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated glomerular filtration rate
ESRD	End stage renal disease
FSGS	Focal segmental glomerular sclerosis
G	Guanine
G0	Wild type *APOL1* allele
G1	*APOL1* compound allele, comprising two variant single nucleotide polymorphisms (rs73885319 [G allele] and rs60910145 [G allele])
G2	Deletion allele (TTATAA) of *APOL1* insertion/deletion polymorphism (rs71785313)
GN	Glomerular nephritis
H-ESKD	Hypertension end stage kidney disease
HDL3	High density lipoprotein fraction 3
HIVAN	HIV-associated nephropathy

HPR	Haptoglobin-related protein
HWE	Hardy-Weinberg equilibrium
IgA	Immunoglobulin class A
IgM	Immunoglobulin class M
Ins	Insertion
IRF5	Interferon regulatory factor 5
ITGAM	Integrin, alpha M
kb	Kilobases
kDa	KiloDaltons
KIAA1542	PHD and ring finger domains 1
KDIGO	Kidney Disease | Improving Global Outcomes
LN	Lupus nephritis
MDRD	Modification of Diet in Renal Disease
P	Probability
OR	Odds ratio
PCR	Polymerase chain reaction
PXK	PX domain containing serine/threonine kinase
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute
SD	Standard deviation
SLE	Systemic lupus erythematosus
SLICC	SLE international collaborating clinic
SNP	Single nucleotide polymorphism
SRA	Serum resistance-associated
T	Thymidine
TbHpHbR	TLF1 bound HPR–haemoglobin complex receptor.
TLF1	Trypanosome lytic factor 1
TLF2	Trypanosome lytic factor 2
TNFAIP3	Tumour necrosis factor, alpha-induced protein 3

Chapter 1: Abstract

1.1 Aim

To assess whether Apolipoprotein L-I (APOL1) G1 and G2 genotypes are associated with renal disease or Systemic Lupus Erythematosus (SLE) in a cohort of Black South African patients with SLE.

1.2 Methods

One hundred and seventy eight unrelated Black South African patients with SLE were enrolled into the study. All patients fulfilled the Systemic Lupus International Collaborating Clinics (SLICC) 2012 criteria. The patients were recruited from the Chris Hani Baragwanath Academic Hospital Rheumatology clinic. One hundred and eight Black South African individuals with no known renal or connective tissue disease were used as controls. APOL1 G1 and G2 alleles were discerned using in-house restriction fragment length polymorphism analysis and fluorescently labelled primer PCR fragment length determination, respectively.

1.3 Results

APOL1 was successfully genotyped for G0, G1 and G2 in 165 (92.6%) of the samples obtained. The APOL1 genotypes G1 and G2 were associated with SLE (OR 7.42 (2.11 - 26.06), $P = 4.09 \times 10^{-4}$), Lupus nephritis (LN) (5.83 (1.41 – 24.18), $P = 1.30 \times 10^{-2}$) and chronic kidney disease (CKD) (8.38 (2.67 – 30.95), $P = 2.97 \times 10^{-4}$). However, stage 3 renal disease (defined as an estimated glomerular filtration rate (eGFR) < 60ml/min/1.73m^2), showed the strongest association (OR 19.44 (3.71 – 101.91), $P = 6.88 \times 10^{-4}$).

1.4 Conclusion

The APOL1 alleles G1 and G2 show an associated with SLE, LN and CKD. However, the greatest association seen was with stage 3 renal disease. This suggests that the APOL1 alleles are risk factors for SLE and LN, and are strongly associated with progression to CKD in SLE.

Chapter 2: Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder of uncertain aetiology, characterized by a hyperactive humoral immune system producing multiple autoantibodies, subsequent immune complex deposition and ultimately tissue inflammation and destruction. Females and people of African descent are more commonly and severely affected by SLE. The prevalence of SLE varies between populations, with reported ranges of 20 to 150 per 100 000. A female to male ratio of 9:1 is noted (Bertsias et al., 2012).

Systemic lupus erythematosus is a clinically heterogeneous disease which may affect any and multiple organs. Its presentation and course are highly variable, ranging from indolent to fulminant. The most common pattern is a combination of constitutional complaints with skin, musculoskeletal, mild haematologic and serologic involvement. However, some patients have severe disease with predominately haematologic, renal or central nervous system manifestations. In certain instances, the initial presentation will be end stage renal disease (ESRD), with the diagnosis of SLE only being made after renal biopsy. Symptoms wax and wane over time, with multiple organ systems can be affected at any one time (Bernatsky et al., 2006).

The clinical manifestations of SLE are vast. Recently, the Systemic Lupus International Collaborating Clinics (SLICC) proposed a revision of the 1997 American College of Rheumatology (ACR) classification criteria for SLE by including several more clinical and laboratory features in the criteria. To classify a patient as having SLE, a patient must either have biopsy proven lupus nephritis (with positive antinuclear antigen or anti-dsDNA) or satisfy four of the SLICC criteria, with at least one clinical and one immunologic criteria (Appendix 1 – Clinical and immunologic

criteria used in the SLICC classification criteria.). These revised criteria have a sensitivity of 94% and a specificity of 92% (Petri et al., 2012).

2.1 Pathogenesis of Systemic Lupus Erythematosus

The pathogenesis of SLE is multifactorial, compromising genetic factors, epigenetic factors (e.g. DNA methylation status), environmental factors (e.g. ultraviolet light), certain drugs and hormonal factors (e.g. high levels of oestrogen can precipitate SLE in murine models). The various factors combine synergistically, causing irreversible loss of immunological self-tolerance, especially with respect to nuclear antigens. Both aberrations of innate and adaptive immunity are responsible for the inflammation and tissue damage in SLE: Autoantigens released by apoptotic cells are phagocytosed by dendritic cells, then processed and presented to T lymphocytes, activating them. The activated T lymphocytes stimulate B lymphocytes by releasing various cytokines, causing the B lymphocytes to produce autoantibodies. Additionally, B lymphocyte endosomal Toll-like receptors sensitive to necrotic cell products (nucleic-acid, high-mobility group protein B1 and heat shock proteins) can amplify the B lymphocytes autoantigen production, fuelling the immunological storm seen in SLE (Ma et al., 2015, Rahman and Eisenberg, 2006). The autoantibodies mediate disease by immune-complex deposition and subsequent inflammation, or by direct antibody mediated tissue destruction. In addition, deposited immune complexes are not cleared efficiently in individuals with SLE, leading to further tissue damage (Bertsias et al., 2012). Autoimmunity alone is unlikely to lead to the SLE phenotype. Additional risk factors, such as tissue or organ susceptibility phenotypes and abnormal immune complex clearance, are likely required for the development of SLE. The interaction between autoimmunity, immune dysfunction and tissue susceptibility are fundamental to the understanding

of SLE, and the genetic heterogeneity between different populations are likely to assist in shedding light on the pathogenesis of SLE.

2.2 Genetics of Systemic Lupus Erythematosus

While the exact aetiology of SLE is unknown, it is clear that multiple factors are involved. There is a strong genetic contribution to the development of SLE, as evidenced by a high concordance rate between monozygotic twins (24 - 57%) and 5 - 12% of first degree relatives develop SLE (Block et al., 1975, Deapen et al., 1992, Arnett et al., 1984). Early studies focussed on the role of MHC genes as risk factors for SLE. In Caucasians the HLA-A1, B8, Cw7, DR3, DR2, DQ2, and C4A DE haplotype has been recognised as being a more common in SLE patients (Bettinotti et al., 1993). More recently studies have focused on non-HLA genes: Genome wide association studies have identified over twenty genetic loci with alleles showing association with the SLE phenotype. Examples include *ITGAM*, *BLK*, *BANK1*, *KIAA1542*, *PXK*, and *TNFAIP3* (Harley et al., 2009, Sanchez et al., 2011) . The majority of these genes have been implicated in immune complex processing, toll-like receptor function/type 1 interferon production or immune signal transduction in lymphocytes (Figure 2-1) (Harley et al., 2009).

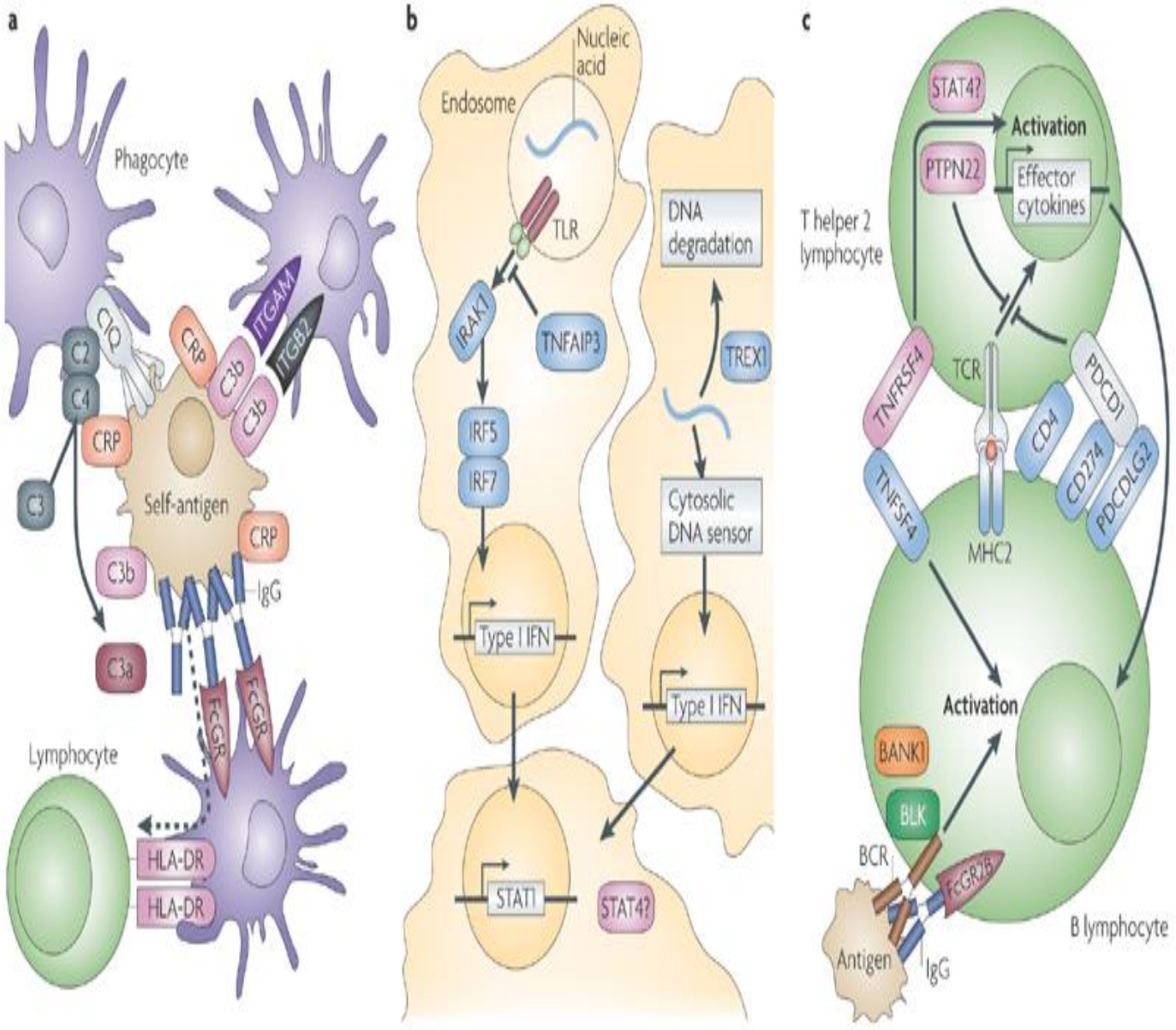

Figure 2-1: Putative SLE pathways

A: Phagocytosis. A trigger, such as UV irradiation, viral infection or dysregulated apoptosis leads to activation of antigen-presenting cells (APC). These cells phagocytose self-antigen that is coated by opsonin molecules (for example, C3b), which are bound by their receptors (for example, ITGAM and ITGB2), leading to subsequent APC activation and presentation of self-antigen to host lymphocytes. Hyper-activation of APC leads to loss of self-tolerance, while autoantibodies are produced when immune complexes are not cleared.

B: Type I interferon production. TREX1 digests cytosolic DNA and prevents activation of a cell-intrinsic type I interferon response pathway. Similarly, activation of Toll-like receptors (TLr7, TLr8, and TLr9) on ligand recognition (CpG DNA or ssRNA) leads to the production of type I interferon by immune cells, particularly plasmacytoid dendritic cells, and the interferon responsive gene expression signature that is observed in SLE serum.

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C: Immune signal transduction. Various stages in the life cycle of lymphocytes are important for the development of the autoreactive B cell clones, which produce the pathological autoantibodies observed in SLE. Self-antigen recognition by B cells starts at the B cell receptor (membrane IgM), where the balance of positive signals (B cell receptor crosslinking) and negative signals (FCGR2B ligation) are transduced by intracellular kinases, such as BLK and BANK1, leading to B cell activation. A similar process, leading to T cell activation, occurs after uptake of the self-antigen and presentation on a class II major histocompatibility complex (MHC) molecule, such as HLA-Dr, to a CD4+ T lymphocyte, which subsequently provides 'help' to B lymphocytes. It should be noted that autoreactive clones must avoid deletion before activation events can lead to florid autoimmunity.

BANK1, B-cell scaffold protein with ankyrin repeats 1; BCr, B cell receptor; BLK, B lymphoid tyrosine kinase; C1Q, complement component 1, subcomponent q; C2, complement component 2; C3, complement component 3; C3a, C3 cleavage product a; C3, C3b, cleavage product b; C4, complement component 4; CD274, programmed cell death 1 ligand 1 precursor; CD4, CD4 molecule; CRP, C-reactive protein; FCGR, Fc fragment of IgG receptor; HLA-DR, major histocompatibility complex, class II, DR; IFN, interferon; IgG, immunoglobulin G; IRAK1, interleukin 1 receptor associated kinase-1; IRF, interferon regulatory factor; ITGAM: integrin alpha M; ITGB2, integrin, beta 2; MHC2, CD74 molecule, major histocompatibility complex, class II invariant chain; PDCD1, programmed cell death 1; PDCD1LG2, programmed cell death 1 ligand 2 precursor; PTPN22, protein tyrosine phosphatase, non-receptor type 22; STAT, signal transducer and activator of transcription; TCR, T cell receptor; TLR, Toll-like receptor; TNFAIP3, tumour necrosis factor-α induced protein 3; TNFrSF4, tumour necrosis factor receptor superfamily, member 4; TNFSF4, tumour necrosis factor superfamily, member 4; TREX1, three prime repair exonuclease 1. Image from Harley et al., 2009.

The majority of genetic susceptibility studies have been conducted on European populations. The few studies of non-European populations show marked differences between risk loci (Niewold, 2015). An example of this is the interferon regulatory factor 5 (*IRF5*) gene, where a single nucleotide polymorphism (SNP) (rs2004640) results in elevated interferon levels, and is strongly associated with SLE in European populations (Graham et al., 2006). However, in SLE patients of African ancestry (American and South African) elevated type 1 interferon levels were noted, but the rs2004640 SNP did not show association (Niewold et al., 2012). This is in contrast to the association between the SNP rs1143679 in *ITGAM* and SLE. rs1143679 harbours an allele that is a risk factor for SLE common to both European and African populations (Nath et al., 2008). This suggests that while there is genetic heterogeneity between different ethnic groups with respect to certain associated loci, other loci are risk factors for SLE regardless of ethnicity.

2.3 Mortality in Systemic Lupus Erythematosus

All-cause mortality risk in people with SLE is approximately 2.4 times higher than the average population (Bernatsky et al., 2006). SLE is commoner in African or Asian

populations and is more severe in these populations. In developing countries, SLE is associated with a higher risk of mortality than in developed countries. A propensity for infections, especially tuberculosis, is noted (Tikly and Navarra, 2008). In developed countries, the major cause of mortality in SLE is disease activity and cardiovascular disease, while in contrast infections and renal disease account for the majority of mortality in Black South Africans (Wadee et al., 2007).

2.4 Lupus Nephritis

Renal involvement occurs in up to 70% of SLE patients, and is one of the leading causes of morbidity and mortality. Immune complex deposition within the glomerulus causes both inflammation and cellular proliferation within the glomerulus. This leads to progressive disruption of the glomerular basement membrane, leading to proteinuria, which in many cases progresses to end stage renal disease (ESRD), if not aggressively treated with immunosuppressive therapy (Bertsias et al., 2012). Lupus nephritis (LN) is divided into six classes, depending on histopathological findings (Appendix 2 - The 2003 International Society of Nephrology/Renal Pathology Society classification of lupus nephritis).

2.5 Renal disease in patients of African ancestry

In South Africa, renal disease is the 11th most common cause of non-communicable disease, accounting for 5.1% of all deaths in 2013 (Statistics South Africa, 2014). Populations of African descent, including African Americans, across all socio-economic classes, suffer from renal disease earlier, and have more severe disease (Naicker, 2013, Mayosi et al., 2009, Genovese et al., 2010). While lifestyle, hypertension and diabetes are well established risk factors for the development of chronic kidney disease (CKD) across all populations, the finding of polymorphisms in people of African ancestry that increase their risk for various types of CKD, such as

Apolipoprotein L-1 (*APOL1*), provides some evidence of why CKD is more common in this group (Genovese et al., 2010, Kasembeli et al., 2015). However, this cannot fully explain the increased rate of renal disease in patients of African origin.

2.6 The *APOL1* gene

The *APOL1* gene is found on chromosome 22q12.1-q13.1, spans 14 kilobases, is comprised of six exons and encodes the Apolipoprotein L-I protein (APOL1) (Duchateau et al., 2001, Uniprotkb - O14791 (APOL1_HUMAN), 2015). APOL1 is comprised of 398 amino acids, has a weight of 44 kDa and is the trypanosomal lytic component of the trypanosome lytic factors (TLF) types one and two (Pays et al., 2014, 2015). It is thought to lyse trypanosomic parasites by forming a pore within the trypanosome endosome, allowing chloride ions to flood into the parasite, resulting in lysis of the parasite (Pays et al., 2014, Uniprotkb - O14791 (APOL1_HUMAN), 2015). Through this mechanism, humans are thought to have been able to become resistant to human sleeping sickness (HSS) caused by *Trypanosoma brucei brucei* infections.

The APOL1 protein has four domains; an N terminal signalling unit, a multi-helical anion pore-forming domain, a membrane-addressing domain and a long carboxy-terminal amphipathic α-helix that may control the activity of APOL1 (Figure 2-2) (Pays et al., 2014). The N terminal signalling allows for extracellular secretion of APOL1, which is then packaged into high density lipoprotein particles, forming TLF1. Trypanosome lytic factor type 2 is lipid poor, but comprises APOL1 and IgM moieties. Figure 2-3 outlines APOL1 entry into the trypanosomal endosome, leading to trypanosomal lysis (Pays et al., 2014).

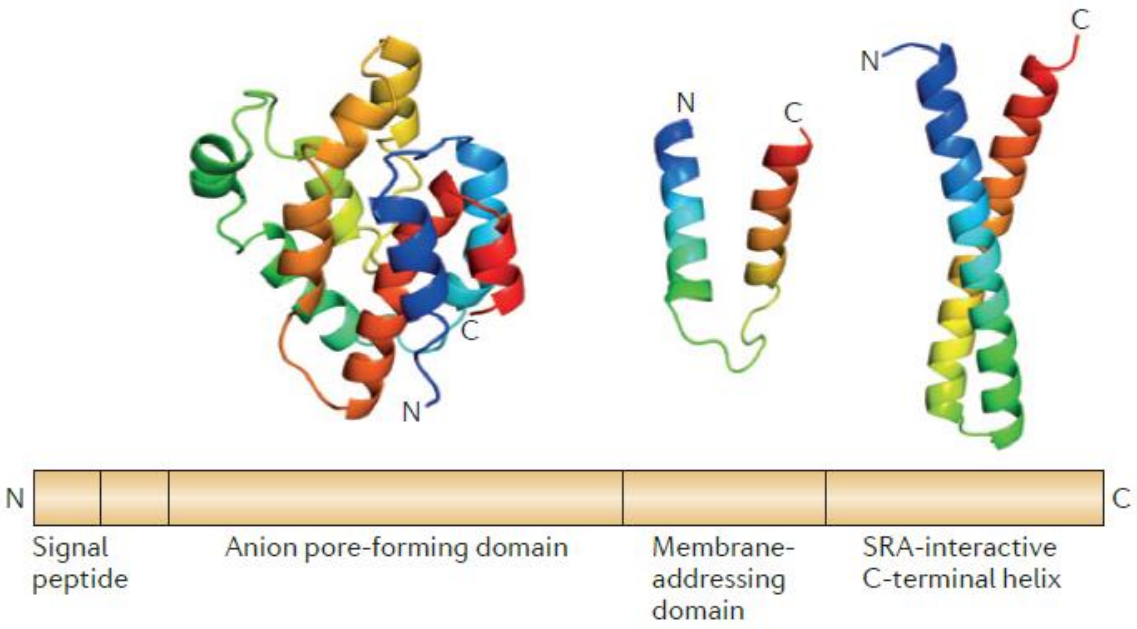

Figure 2-2: Protein domains of APOL1

APOL1 is thought to contain three domains: a multihelical anion pore-forming domain; a double-helical membrane-addressing domain; and a long carboxy-terminal amphipathic α-helix that may control APOL1 activity. This last domain is targeted by the *T. brucei rhodesiense* serum resistance-associated (SRA) protein. Image from Pays et al., 2014.

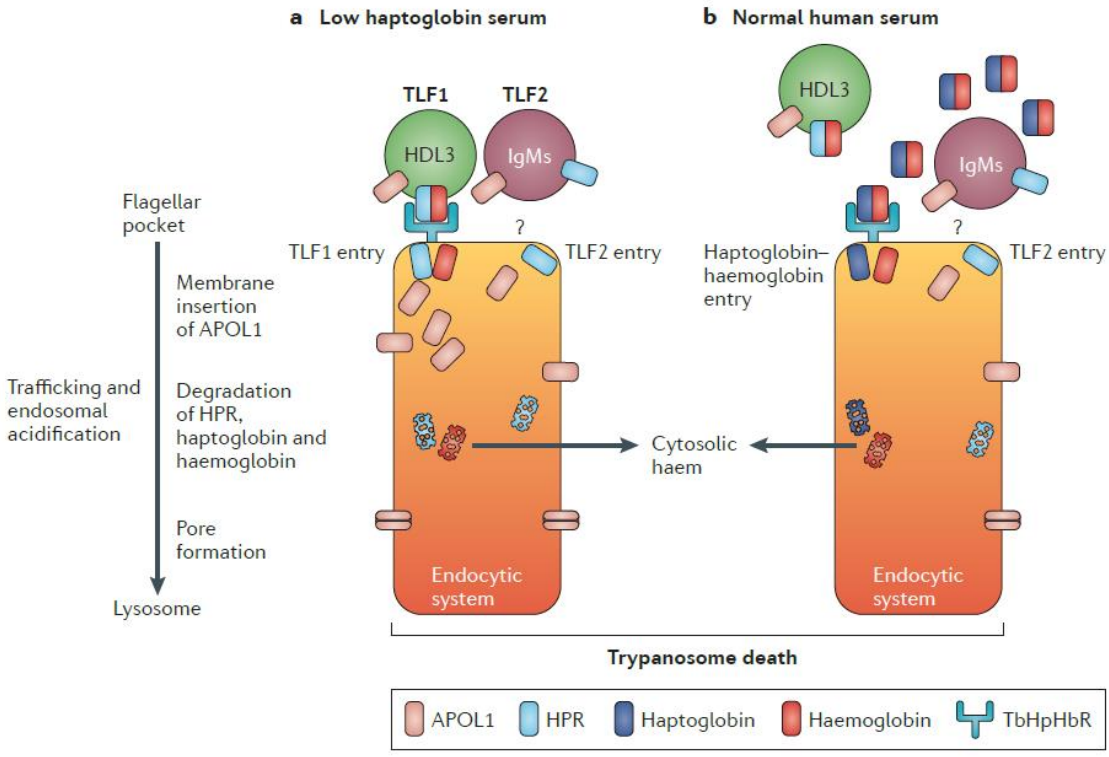

Figure 2-3: Entry of TLF1 and TLF2 into Trypanosomal endosomes

APOL1 gains entry into the trypanosomal cell via either Trypanosome lytic factor 1 (TLF1) or TLF2. TLF1 is a high density lipoprotein fraction 3 (HDL3) molecule that in part is composed of APOL1 and a haemoglobin (HB) - haptoglobin related protein (HPR) heterodimer; while TLF2 is a immunoglobulin class M (IgMs) molecule with APOL1 and HPR molecules attached. **Continued on next page.**

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TLF1 related APOL1 entry into the trypanosomal cell occurs via receptor mediated endocytosis when the HB-HPR heterodimer binds the *T. b. bruceii* haptoglobin–haemoglobin receptor (TbHpHbR). Once within the endosome, the HB and HPR are degraded, and the digestate moves into the cytosol for further metabolism, while the remaining APOL1 inserts into the endosomal membrane, and forms an anionic pore. The APOL1 pore allows for anion flux into the cytosol, resulting in apoptosis of the trypanosome cell. This occurs in low serum haptoglobin levels (a). The mechanism of TLF2 entry into the endosome in normal human serum is unknown (b), but the APOL1 anion pore mediated apoptosis is similar. Image from Pays et al., 2014.

2.6.1 Trypanosomal resistance to APOL1

A closely related species of *T. b. brucei*, namely *T. b. rhodesiense*, is able to overcome APOL1 mediated lysis, and hence can cause HSS. *T. b. rhodesiense* has a novel variant surface glycoprotein, termed serum resistance-associated (SRA), that is unable to localise into the trypanosome plasma membrane. Instead, it is found within the trypanosomal endosome, where it binds APOL1 strongly, disallowing APOL1 insertion into the endosomal membrane. This ultimately leads to APOL1 degradation and loss of APOL1 activity which protects the trypanosome from lysis (Figure 2-4).

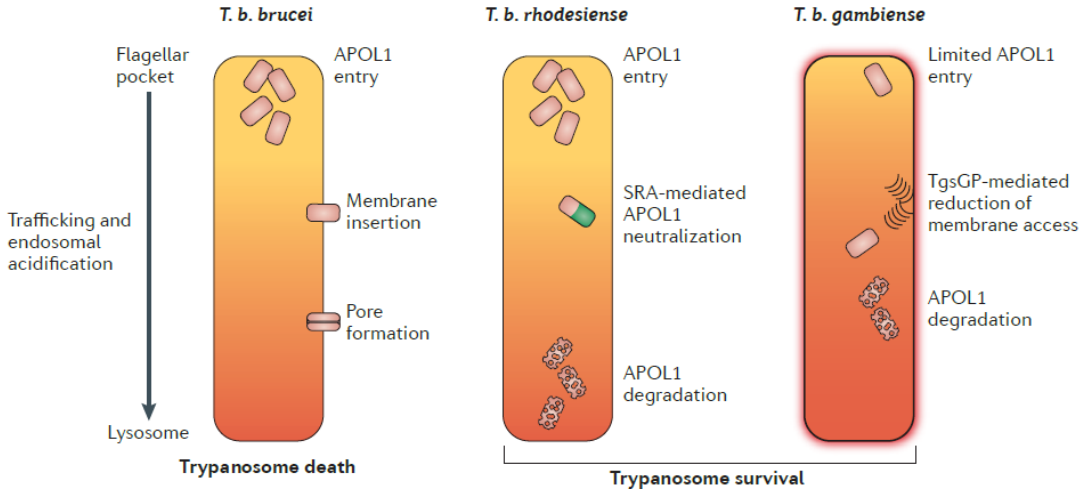

Figure 2-4: Trypanosomal resistance to APOL1 mediated lysis

In *T. b.* brucei, APOL1 is able to insert into the endosome membrane, creating an anionic pore, resulting in apoptosis. In *T. b.* rhodesiense, the variant surface glycoprotein (VSG)-derived serum resistance-associated (SRA) binds APOL1, disallowing APOL1 to insert into the endosome membrane, allowing for survival, while in *T. b. gambiense*, the VSG-derived *T. b. gambiense*-specific glycoprotein (TgsGP) stiffens the endosomal membrane, again disallowing APOL1 insertion, allowing for survival. Image from Pays et al., 2014.

2.6.2 Human resistance to *T. b. rhodesiense*

As SRA interaction with APOL1 is necessary for trypanosomal survival, Lecordier et al. found that certain APOL1 variants could avoid SRA binding, and reinstate trypanosomal lysis (Pays et al., 2014, Lecordier et al., 2009). Interestingly, two human polymorphic alleles, similar to those found in the baboon *Papio anubis* (which is resistant to trypanosomic infection) were found to confer resistance to *T. b. rhodesiense* via inhibition of SRA binding to APOL1 (Genovese et al., 2010). The first allele, termed G1, is actually a haplotype composed of two missense mutations in perfect linkage disequilibrium (rs73885319 [G allele] and rs60910145 [G allele]). The second allele, G2, is a 6–base pair (bp) deletion (rs71785313 insertion/deletion polymorphism [TTATAA/-], with the insertion allele being the wild type allele) and removes amino acids N388 and Y389 from the C terminus of *APOL1*. As rs73885319, rs60910145, and rs71785313 are very close to each other (all three polymorphisms are within 146bp of each other) recombination between G1 and G2 alleles is thought to be very unlikely (Figure 2-5) (Genovese et al., 2010, Kasembeli et al., 2015). These two alleles, G1 and G2, differ from the wild type allele G0 in that they remove the SRA binding site from APOL1, reinstating APOL1 activity and allowing APOL1 to localise into the endosomal membrane, ultimately leading to lysis of the trypanosome.

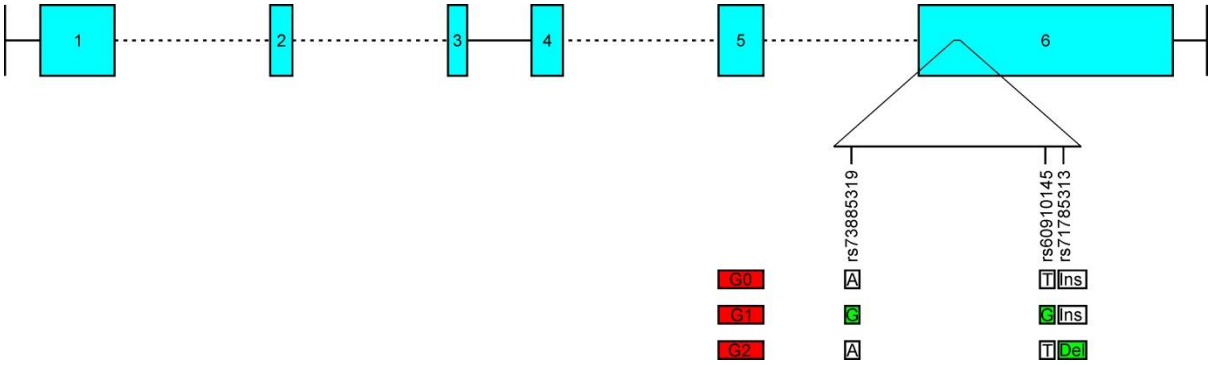

Figure 2-5: *APOL1* showing position of alleles G0, G1 and G2

Schematic representation of APOL1, showing the localisation of rs73885319, rs60910145, and rs71785313 within the sixth exon. Of note, the G0, G1 and G2 "alleles" are actually genotypes, comprised of alleles from rs73885319, rs60910145, and rs71785313, which all lie within 146bp of each other. The G0 allele is the wild type allele with normal function, while the G1 and G2 alleles are risk alleles for renal disease. A: Adenosine. T: Thymidine. G: Guanine. Ins: 6bp insertion, or wild type allele. Del: 6bp deletion allele. 1 to 6: Exons one to six. Dotted lines indicate areas not drawn to scale.

2.6.3 APOL1 and renal disease

In 2010 Genovese et al. discovered that while the G1 and G2 *APOL1* alleles conferred resistance to *T. b. rhodesiense* infection in African Americans, the alleles were also strongly associated with renal disease in this population, specifically focal segmental glomerulosclerosis (FSGS), HIV-associated nephropathy (HIVAN), and hypertension-attributed end-stage kidney disease (H-ESKD) (Genovese et al., 2010). In the presence of one risk allele (G1 or G2), the risk of renal disease was modestly elevated (OR = 1.26, CI 1.01 to 1.56), while the presence of two risk alleles (G1 and G2) yielded an OR of 7.3 (CI 5.6 to 9.5). This effect was mirrored in a study by Kisembeli et al. on South Africa Black patients with chronic kidney disease where two risk alleles were strongly associated with HIVAN (OR 89, CI 17.7 to 912), but interestingly not with FSGS, HIV-associated immune complex kidney disease or glomerular nephritides (GN) such as membranoproliferative GN, membranoproliferative GN consistent with C3 glomerulopathy, membranous GN, IgA nephropathy or Lupus nephritis (LN) (Kasembeli et al., 2015).

2.6.4 Lupus Nephritis and *APOL1*

The association of the *APOL1* polymorphisms with FSGS, HIVAN and H-ESKD in patients of African ancestry (African Americans and Black South Africans) makes G1 and G2 attractive candidates as risk alleles for renal disease in SLE (Genovese et al., 2010, Kasembeli et al., 2015). Both SLE and LN are more common in patients of African ancestry and the finding that the *APOL1* alleles G1 and G2 are strong risk factors for CKD in patients of African ancestry, it would seem possible that there could be an association between LN and G1, G2 or both. This has partially been investigated, and has resulted in apparently contradictory results. Freedman et al., in a study of 1389 patients found a strong association between end stage renal disease (ESRD) due to LN and the *APOL1* G1/G2 alleles in an African American population (OR 2.57, 95% CI 1.89 - 3.50, P =1.49 x 10^{-9}). In addition to this, when two risk alleles were present, the time to develop ESRD was decreased by two years, suggesting that the *APOL1* risk alleles accelerated renal damage, decreasing the time to ESRD. Freedman et al. also noted that the presence of two G1/G2 alleles allowed for a weak trend towards an earlier onset of SLE, suggesting that *APOL1* could also play a role in the pathogenesis of SLE (Freedman et al., 2014).These findings are however offset by the studies by Lin et al. (N = 407) and Kasembeli et al. (N = 3), who both found no association between the *APOL1* risk alleles and LN in patients of African ancestry (Lin et al., 2012, Kasembeli et al., 2015). Further investigation is needed to clarify the association between *APOL1*, SLE and LN.

2.7 Hypothesis

The null hypothesis for this study was that the *APOL1* risk alleles G1 and G2 are not associated with renal disease (LN, CKD or stage 3 renal failure) in Black South

African patients with SLE, while the alternate hypothesis was that the risk alleles G1 and G2 are associated with renal disease in Black South African patients with SLE.

2.8 Aims and Objectives

The primary objective was to genotype *APOL1* for rs73885319, rs60910145, and rs71785313 in Black South African patients with SLE. This would be compared to similarly genotyped healthy controls of African origin in a case-control analysis.

Secondary objectives were to assess the *APOL1* genotypes within subgroups of Black South African patients with SLE, namely those with LN, CKD and stage 3 renal failure (defined as an estimated glomerular filtration rate (eGFR) < 60ml/min/1.73m^2), and compare the sub groups to the healthy control group (Eknoyan, 2013).

Chapter 3: Patients and methods

3.1 Ethics

Ethical approval for this study was obtained from the University of the Witwatersrand Ethics committee (Certificates M10707 and M130727).

3.2 Patients and controls

One hundred and seventy eight Black South African patients with SLE were enrolled into the study. All patients fulfilled the Systemic Lupus International Collaborating Clinics (SLICC) 2012 classification criteria for SLE (Petri et al., 2012). The patients were recruited from the Chris Hani Baragwanath Academic Hospital Rheumatology clinic sequentially, after informed consent was obtained. Patients were excluded if they did not fulfil the SLICC criteria or declined to partake in the study. Name, date of birth, sex, hospital number, date of SLE diagnosis, previous renal abnormalities, average creatinine since diagnosis, urine protein:creatinine ratio, serological data, SLICC score and previous renal biopsy results were recorded. Race was determined by asking for the patient's parents and grandparent's race. Average estimated glomerular filtration rate (eGFR) was calculated using the MDRD formula and each patient was staged according to the KDIGO guidelines (Eknoyan, 2013). Chronic kidney disease is defined as either any kidney damage or an average eGFR < 60ml/min/1.73m^2 (stage 3 or worse renal failure) over three or more months. Kidney damage is defined as any pathologic abnormalities or markers of damage, including blood, urine or imaging investigations (Eknoyan, 2013). One hundred and eight Black South African individuals without any known connective tissue or renal disease were used as controls. The control samples were sourced from the Division of Human Genetics, National Health Laboratory Service, School of Pathology, Faculty

of Health Sciences, University of the Witwatersrand, and have been reported by Kasembeli et al., 2015.

All data for each patient was entered onto an anonymized database. A unique identifier was assigned to each individual and subsequently the only identifier used in subsequent analyses. The primary investigator was the only person with access to the patient's full details.

3.3 Sample preparation

Two samples of venous blood in EDTA tubes were extracted from each patient. The samples were centrifuged at 10 000rpm for ten minutes. The supernatant was pipetted off and stored at -20^{o}C for future studies. The remaining precipitate was stored at -20^{o}C until the DNA was extracted. DNA was extracted using the salting out method (Miller et al., 1988).

3.4 rs73885319 and rs60910145 analysis

The single nucleotide polymorphisms (SNPs) rs73885319 and rs60910145, which are situated close to each other in the *APOL1* gene, were analysed by polymerase chain reaction (PCR) amplification, and then restriction fragment length polymorphism (RFLP) analysis. Primers for the PCR reaction were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Details of the primer sequences and PCR conditions are shown in Figure 3-1 and Figure 3-2.

GACACAAGCCCAAGCCCACGACCTGGTCATCAAAAGCCTTGACAAATTGAAGGAGGTGAGGGAGTTTTTGGGTGAGA
ACATATCCAACTTTCTTTCCTTAGCTGGCAATACTTACCAACTCACACGAGGCATTGGGAAGGACATCCGTGCCCTCAG
ACGAGCCAGAGCCAATCTTCAGTCAGTACCGCATGCCTCAGCCTCACGCCCCCGGGTCACTGAGCCAATCTCAGCTG
AAAGCGGTGAACAGGTGGAGAGGGTTAATGAACCCAGCATCCTGGAAATGAGCAGAGGAGTCAAGCTCACGGATGTG
GCCCCTGTA[A/G]GCTTCTTTCTTGTGCTGGATGTAGTCTACCTCGTGTACGAATCAAAGCACTTACATGAGGGGGCAA
AGTCAGAGACAGCTGAGGAGCTGAAGAAGGTGGCTCAGGAGCTGGAGGAGAAGCTAAACAT[T/G]CTCAACAATAATT
ATAAGATTCTGCAGGCGGACCAAGAACTGTGACCACAGGGCAGGGCAGCCACCAGGAGAGATATGCCTGGCAGGGG
CCAGGAC

Figure 3-1: Genomic DNA segment of *APOL1* containing rs73885319 and rs60910145

Partial genomic DNA sequence of *APOL1* exon 6 indicating the rs73885319 and rs60910145 SNPs (green and red highlights respectively).Yellow highlight indicates the forward primer while blue indicates the reverse primer sequence. The expected PCR amplicon between the two primers is 539bp.

95°C	3 min	
95°C	30 sec	Repeat 30x
65°C	30 sec	
72°C	1 min	
72°C	5 mins	
12°C	Hold	

Figure 3-2: PCR conditions for rs73885319 and rs60910145

rs73885319 is a di-allelic polymorphism, with either an adenosine (A) or a guanine (G) residue present. The PCR product (539bp) was incubated with *HindIII* restriction endonuclease at 37°C for 1 -16 hours, according to the manufacturers' specifications (Thermo Scientific #ER0501). The amplicon with the A allele has one *HindIII* restriction site, and generates two fragments of 316bp and 223bp when incubated with *HindIII*, while the G allele abolishes the *HindIII* restriction site, leaving the PCR amplicon intact after *HindIII* digestion (Table 3-1).

rs60910145 is also a di-allelic polymorphism, with either a thymidine (T) residue or a G residue present. The PCR product was incubated with *NspI* restriction endonuclease at 37°C for 1 - 16 hours, according to the manufacturers' specifications (Thermo Scientific #ER1472). The amplicon with the T allele has one *NspI* restriction site and generates two fragments (351bp and 188bp) when digested

with *NspI,* while the G allele generates a second *NspI* restriction site, generating three fragments (257bp, 188bp and 94bp) upon digestion (Table 3-1).

Table 3-1: RFLP conditions for rs73885319 and rs60910145

SNP	Allele	Restriction Enzyme	Temperature	Expected Fragment Sizes
rs73885319	Adenosine (A)	*HindIII*	37^{o}C	316bp
				223bp
	Guanine (G)	*HindIII*	37^{o}C	539bp
rs60910145	Thymidine (T)	*NspI*	37^{o}C	351bp
				188bp
	Guanine (G)	*NspI*	37^{o}C	257bp
				188bp
				94bp

After incubation with either *HindIII* or *NspI*, the products were electrophoresed on a 3% agarose gel with a DNA molecular marker ladder (100bp ladder, Lonza Rockland Inc., Rockland, USA). Each fragment was sized, and alleles assigned based on the electrophoresis pattern.

3.5 rs71785313 analysis

rs71785313, an insertion/deletion polymorphism with either a 6bp (TTATAA) being present or absent (Figure 3-3), was detected by PCR amplification with fluorescently labelled primers and then electrophoresed on an ABI 3130XL capillary sequencer, with internal size standards. The expected sizes were 164bp or 158bp. Details of the primer sequences and PCR conditions are shown in Figure 3-3 and Figure 3-4.

TCGTGTACGAATCAAAGCACTTACATGAGGGGGCAAAGTCAGAGACAGCTGAGGAGCTGAAGAAGGTGGCTCAGGA
GCTGGAGGAGAAGCTAAACATTCTCAACAATAA[_/TTATAA]GATTCTGCAGGCGGACCAAGAACTGTGACCACAGGG
CAGGGCAGCCACCAGGAGAGATATGCCTGGCAG

Figure 3-3: Genomic DNA segment of *APOL1* containing rs71785313

Partial genomic DNA sequence of APOL1 exon 6 indicating the rs71785313 polymorphism (green highlight).Yellow highlight indicates the forward primer while blue indicates the reverse primer sequence. The expected PCR amplicon between the two primers is 164bp for the wild type allele, and 158bp for the deletion allele. Yellow highlight indicates the forward primer while blue indicates the reverse primer sequence.

95°C	3 min	
95°C	30 sec	
60°C	30 sec	Repeat 30x
72°C	1 min	
72°C	5 mins	
12°C	Hold	

Figure 3-4: PCR conditions for rs71785313

3.6 APOL1 Genotypes

Each sample was genotyped for rs73885319, rs60910145 and rs7178531, and the resultant data was entered into a database (Table 3-2 and Figure 2-5). It should be noted that while G0, G1 and G2 are referred to alleles in the literature, they are actually genotypes. However, as all three loci are located within 146bp of each other, and have been noted to be in perfect linkage disequilibrium with each other, they have been analysed as three alleles of a single locus. (Figure 2-5, Pays et al., 2014). Both the G1 and G2 alleles impair the functionality of the SRA region of APOL1, and are thought to be detrimental to renal function. The effect on the kidney appears to be dose dependant, with two dysfunctional alleles (G1 or G2) appearing to inflict more damage on the renal system than when a single copy of either is present (Pays et al., 2014).

Table 3-2: Genotypic scoring of *APOL1* alleles

	rs73885319	rs60910145	rs71785313
G0	A (Wild Type)	T (Wild Type)	Wild type
G1	G (Alternative allele)	G (Alternative allele)	Wild type
G2	A (Wild Type)	T (Wild Type)	Any deletion

Allelic data from rs73885319, rs60910145 and rs71785313 were scored, and genotypes for each patient were calculated. The genotypes G0, G1 and G2 are referred to in the literature as alleles, as rs73885319, rs60910145 and rs71785313 are perfect linkage disequilibrium (Pays et al., 2014.). Subsequently, in this study G0, G1 and G2 are referred to as alleles. The G0 allele is not associated with risk to renal function, whereas G1 and G2 are associated with risk for renal disease. A: Adenosine. T: Thymidine. G: Guanine

3.7 Statistical analysis

Allelic frequencies were calculated for G0, G1 and G2 for all cases and controls. Odds ratios with 95% confidence intervals were calculated and tested for significance using Fisher's Exact Probability test or Pearsons Chi squared test. Association was sought with SLE, CKD and LN. All data derived from this study were analysed using either the Stata/IC 13.1, Statistica version 12, or Epi info^{tm} 7.1.4.0 statistical analysis programs. A P value of < 0.05 was considered significant.

Chapter 4: Results

4.1 Patient demographics and clinical features

Patient and control characteristics are shown in Table 4-1. The control data are from Kasembeli et al. (with permission). The controls were matched for race, but not age or gender. Of the study population, females comprised the majority of the 178 SLE patients (92.7%), with a female:male ratio of 13:1. LN was seen in 72 patients (40.4%), with LN grade 3 and 5 most frequently seen on renal histology (Figure 4-1). Stage 3 CKD or worse (eGFR < 60 ml/min/1.73m^2) was rare in our study group, being seen in 20 of the SLE patients (11.3%), while CKD (any eGFR with either an abnormal urine analysis, serological or radiological evidence of renal damage) was common, being seen in 107 patients (60.1%).

Table 4-1: Characteristics of study and control populations

Characteristic	**SLE** (N = 178)	**CKD** (N = 107)	**eGFR < 60** (N = 20)	**LN** (N = 72)	**Control** (N = 108)
Female N (%)	165 (92.7)	99 (92.5)	16 (80.0)	66 (91.7)	69 (63.9)
Mean age (±SD)	39.2 (13.7)	37.0 (13.0)	36.5 (12.2)	33.6 (10.7)	38.7 (7.9)
Mean age at diagnosis (±SD)	32.4 (11.3)	31.2 (10.9)	31.4 (11.1)	28.1 (8.8)	N/A
Mean follow up period, years (±SD)	6.8 (6.5)	5.8 (6.3)	5.2 (6.5)	5.4 (6.0)	N/A
Mean SLICC criteria (±SD)	8.1 (2.5)	8.9 (2.5)	9.8 (2.4)	9.0 (2.5)	N/A
Acute Cutaneous Lupus (%)	94 (52.8)	55 (51.4)	12 (60.0)	37 (51.4)	N/A
Chronic Cutaneous Lupus (%)	92 (51.7)	62 (57.9)	13 (65.0)	37 (51.4)	N/A
Oral or Nasal Ulcers (%)	43 (24.2)	26 (24.3)	6 (30.0)	15 (20.8)	N/A
Non-Scarring Alopecia (%)	42 (23.6)	28 (26.2)	6 (30.0)	17 (23.6)	N/A
Synovitis (%)	116 (65.2)	62 (57.9)	12 (60.0)	42 (58.3)	N/A
Serositis (%)	44 (24.7)	31 (29)	9 (45.0)	26 (36.1)	N/A
Renal Disease (%)	107 (60.1)	107 (100.0)	20 (100.0)	72 (100.0)	N/A
Neurologic Disease (%)	28 (15.7)	22 (20.6)	4 (20.0)	12 (16.7)	N/A
Hemolyticanemia (%)	9 (5.1)	7 (6.5)	4 (20.0)	3 (4.2)	N/A
Leukopenia (%)	131 (73.6)	84 (78.5)	14 (70.0)	54 (75.0)	N/A
Thrombocytopenia (%)	40 (22.5)	25 (23.4)	7 (35.0)	15 (20.8)	N/A
ANA Positive (%)	173 (97.2)	104 (97.2)	19 (95.0)	69 (95.8)	N/A
Anti-dsDNA Positive (%)	75 (42.1)	53 (49.5)	14 (70.0)	40 (55.6)	N/A
aSm Positive (%)	129 (72.5)	85 (79.4)	15 (75.0)	56 (77.8)	N/A
aPL Positive (%)	117 (65.1)	76 (71.0)	14 (70.0)	53 (73.6)	N/A
Low Serum C3/C4 complement (%)	128 (71.9)	85 (79.4)	18 (90.0)	60 (83.3)	N/A
Direct Coombs positive	98 (55.1)	65 (60.7)	13 (65.0)	43 (59.7)	N/A
eGFR (ml/min/1.73m^2) (±SD)	110.00 (48.1)	103.9 (54.3)	36.7 (15.5)	103.20 (53.7)	N/A
Average UPCR (g/mmolcreat)(±SD)	0.150 (0.46)	0.241 (0.52)	0.221 (0.22)	0.290 (0.61)	N/A

Basic characteristics of the study and control populations studied. Systemic Lupus Erythematosus (SLE) is the main group studied, and comprises other subgroups, namely Chronic Kidney Disease (CKD),estimated Glomerular filtration rate less than 60ml/min/1.73m^2(eGFR < 60) or Lupus nephritis (LN, Either biopsy proven (N = 67) or

Continued on next page

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with clinical suspicion and awaiting biopsy (N = 5)). CKD, eGFR < 60 and LN are not mutually exclusive. The control data is taken from Kasembeli et al. 2015 and was not matched for age or sex, but was matched for race. SD: Standard deviation. SLICC: SLE international collaborating clinic. ANA: Anti-nuclear antibody. Anti-dsDNA: Anti Double Stranded DNA antibody. aSm: Anti Smith Antibody. aPL: Antiphospholipid antibodies . eGFR: Estimated Glomerular Filtration Rate. N/A: Not Available.

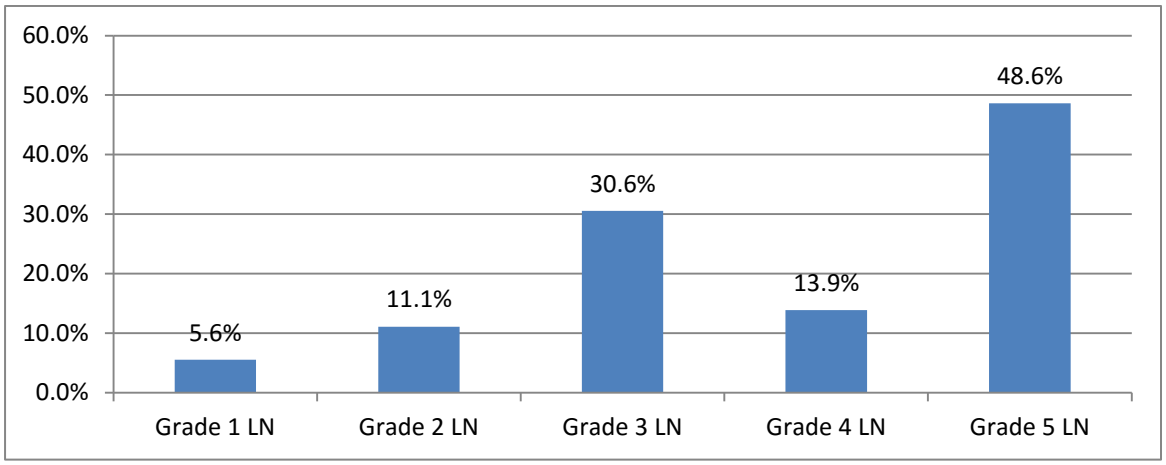

Figure 4-1: Frequency of Lupus Nephritis

Summary of the different grades of lupus nephritis found during the study. N = 72. LN: Lupus nephritis

4.2 rs73885319 and rs60910145 Analysis

The digested PCR products of rs73885319 and rs60910145 were electrophoresed on a 3% agarose gel and an example is shown in Figure 4-2.

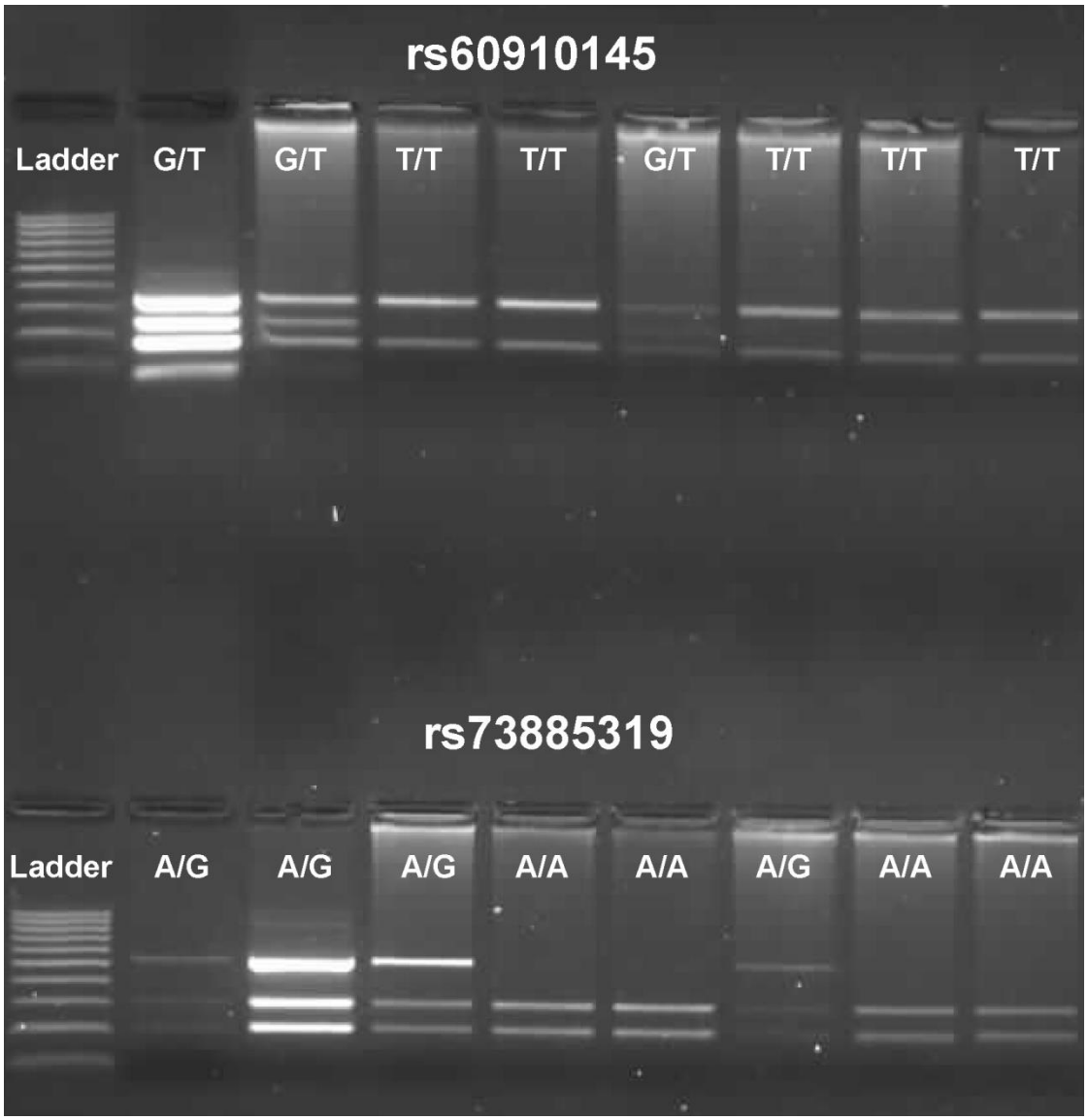

Figure 4-2: RFLP analysis of rs60910145 and rs73885319

Image of a 3% agarose gel after electrophoresis of RFLP digestion of the 539bp PCR amplicon. This allowed for differentiation of the different alleles at rs60910145 and rs73885319.

Ladder:	100bp ladder (Lonza Rockland Inc., Rockland, USA), used as a size standard. Lowest band 100bp. Each successive band increasing by 100bp, until 1000bp.
rs60910145:	G/T band pattern: This represents a patient heterozygous at the rs60510145 locus, for the Guanine (G) and Thymidine (T) alleles. Sizes from lowest to highest: 94bp, 188bp, 257bp and 351bp.
	T/T band pattern: This represents a patient homozygous at the rs60510145 locus for the Thymidine (T). Sizes from lowest to highest: 188bp and 351bp.
rs73885319:	A/G band pattern: This represents a patient heterozygous at the rs73885319 locus for the Adenosine (A) and Guanine (G) alleles. Sizes from lowest to highest: 223bp, 316bp and 539bp.
	A/A band pattern: This represents a patient homozygous at the rs73885319 locus for the Adenosine (A) allele. Sizes from lowest to highest: 223bp and 316bp.

4.3 rs71785313 Analysis

rs71785313, an insertion/deletion polymorphism, was determined by PCR amplification with a fluorescently labelled primer. Resultant amplicons were sized on an ABI 3130xl sequencer. Figure 4-3 shows the resultant electropherogram with the different sized alleles found.

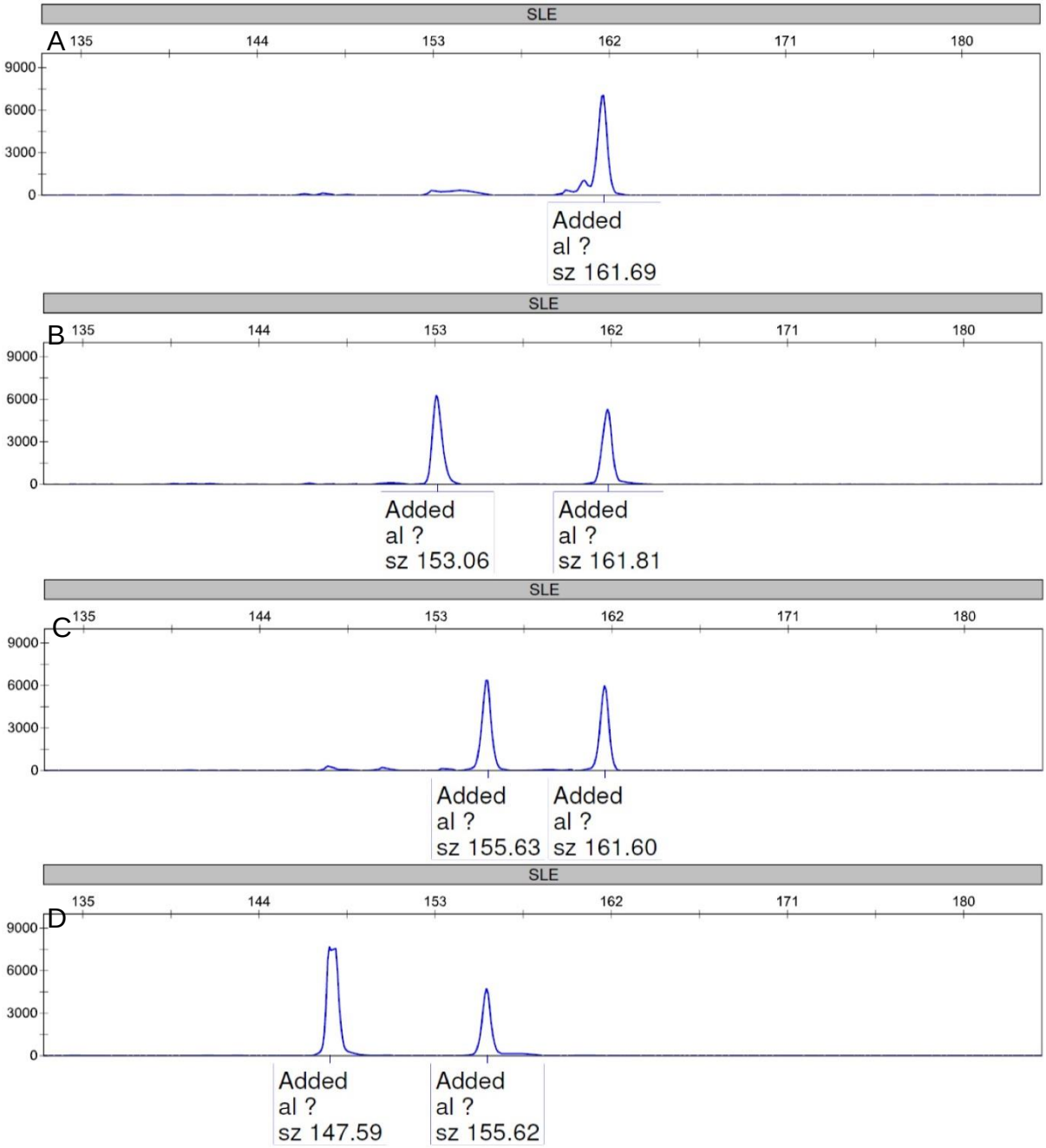

Figure 4-3: Èlectropherogram of the rs71785313 alleles

Examples of the different electropherograms generated while analysing the rs71785313 locus.

A: Homozygous for the 162bp allele. B: Heterozygous for the 153/162 alleles. C: Heterozygous for the 156/162bp alleles. D: Heterozygous for the 148/156 alleles

The expected alleles, according to current protocol design were164bp (wild type allele) or 158bp (deletion allele), but we observed allele sizes of 162bp, 156bp, 153bp and 148bp. The 162bp and 156bp alleles most likely represent the 164bp and 158bp alleles respectively. The 148bp and 153bp alleles are unexpected and could be novel alleles or experimental artefacts. Unfortunately, funding was not sufficient to allow for sequencing of these potentially novel alleles, or to confirm that the 162bp and 156bp alleles actually correspond to the predicted 164bp and 158bp alleles, respectively. For the purpose of this study, the 162bp allele was interpreted as the 164bp or wild type allele, while the smaller alleles (148bp, 153bp and 156bp) were classed as a deletion.

To our knowledge, the smaller rs71785313 alleles have never been reported in the literature. This introduces an unexpected variable into the data, and effectively invalidates further analysis until further investigations have been carried out, to elucidate the cause, and possible effect of the novel alleles on APOL1 function. Therefore, validation is essential before drawing any conclusions about their novelty or association with any disease (SLE, renal or other). As seen in Table 4-2, the novel alleles account for a significant proportion (58%) of the deletion alleles seen at rs71785313, and undoubtedly impact further analysis. As the rs71785313 polymorphism is integral to the *APOL1* haplotype analysis and every rs71785313 deletion allele (G2) is potentially suspect, all subsequent data and analyses should be viewed with caution and all conclusions as tentative.

Table 4-2: Summary of the rs71785313 alleles detected

rs71785313				
148bp Allele	**153bp Allele**	**156bp Allele**	**Any Deletion**	**162bp Allele**
2 (0.6%)	45 (13.6%)	34 (10.3%)	81 (24.6%)	249 (75.5%)

Summary of the deletion alleles (148bp, 153bp and 156bp) and the wild type allele (162bp), highlighting that the novel deletion alleles (148bp and 153bp) account for the majority of the deletions seen.

4.4 *APOL1* Genotypic Analysis

APOL1 genotypes were determined in 92.6% (165) of the SLE subjects, and are summarised in Table 4-3. The G0, G1 and G2 allele frequencies in the SLE patient group and the CKD, eGFR and LN subgroups are summarised in Table 4-4.

Table 4-3: *APOL1* genotypes

Genotype	SLE (%)	CKD (%)	eGFR < 60 (%)	LN (%)	Control (%)
0 risk alleles					
G0/G0	66 (40.0)	39 (40.2)	6 (35.3)	28 (42.4)	70 (64.8)
1 risk allele					
G0/G1	17 (10.3)	8 (8.2)	2 (11.8)	5 (7.6)	11 (10.2)
G0/G2	61 (37.0)	36 (37.1)	4 (23.5)	26 (39.4)	24 (22.2)
2 risk alleles					
G1/G1	1 (0.6)	1 (1.0)	1 (5.9)	1 (1.0)	0 (0.0)
G1/G2	17 (10.3)	12 (12.4)	4 (23.5)	6 (6.1)	0 (0.0)
G2/G2	3 (1.8)	1 (1.0)	0 (0.0)	0 (0.0)	3 (2.8)
Total	165	97	17	66	108

Summary of *APOL1* genotyping results for the entire SLE population studied (N = 165) and various subgroups of the SLE population: those with CKD (N = 97), those with an eGFR < 60 (N = 17) and those with LN (N = 66). The subgroups are not mutually exclusive. Risk alleles are defined as the presence of either the G1 or G2 allele. The number of risk alleles is totalled for each patient, and should be 0, 1 or 2, with the presence of 2 risk alleles theoretically representing the highest risk for renal impairment, 1 an intermediate risk and 0 for the lowest risk.

4.5 Statistical Analysis of the *APOL1* alleles G0, G1 and G2

To ascertain if the *APOL1* alleles carried risk for SLE, CKS, an eGFR < 60 or LN, the allele frequencies were calculated for each group and subgroup, and then tested for significant difference against the allele frequencies in the control group (Table 4-4)

Table 4-4: G0, G1 and G2 allele frequencies in the SLE, CKD, eGFR < 60 and LN groups compared to controls

	SLE	P	CKD	P	eGFR < 60	P	LN	P	Control
G0 (%)	210 (63.6)	**$< 1.0 \times 10^{-5}$**	122 (61.6)	**$< 1.0 \times 10^{-5}$**	18 (52.9)	**1.4×10^{-4}**	87 (65.9)	**7.6×10^{-4}**	175 (81.0)
G1 (%)	36 (10.9)	**8.9×10^{-4}**	22 (11.1)	**1.0×10^{-2}**	8 (23.5)	**8.0×10^{-5}**	12 (9.1)	0.0722	11 (5.1)
G2 (%)	84 (25.5)	**5.8×10^{-4}**	50 (25.3)	**1.2×10^{-3}**	8 (23.5)	0.0722	33 (25.0)	**4.5×10^{-3}**	30 (13.9)
Total	330		194		34		132		216

Testing for significant difference between the G0, G1 and G2 allele frequencies in the SLE group, and the SLE subgroups of CKD, eGFR < 60 and LN, relative to the control group. P values calculated using the Z test. A P value of < 0.05 was considered significant. Greyed out blocks indicate P < 0.05.

As seen in Table 4-4, significant differences exist between the G0, G1 and G2 allele frequencies in all groups relative to the control group, except for the G2 allele in the eGFR < 60 and the G1 allele in the LN group, which show borderline non-significance. This may be due to the small sample sizes used.

Hardy–Weinberg equilibrium (HWE) tests were performed for the SLE patient group and the sub groups, although some of the sample sizes were small and may not be expected to constitute a “randomly mating population” (

Table 4-5). As expected, the control alleles were in HWE, while the alleles in the SLE, CKD and LN were not. Interestingly, the G0, G1 and G2 alleles in the eGFR < 60 group were in HWE. This was not expected, but as the sample size is small, reanalysis with a larger sample size may yield different results.

Table 4-5: Hardy–Weinberg equilibrium calculations within each of the groups studied

		G0/G0	G0/G1	G1/G1	G1/G2	G2/G2	G0/G2	P	HWE
SLE (N = 165)	**Observed**	66	17	1	17	3	61	**$P = 1.6 \times 10^{-3}$**	No
	Expected	66.8	22.9	2.0	9.2	10.7	53.5		
CKD (N = 97)	**Observed**	39	8	1	12	1	36	**$P = 2.0 \times 10^{-3}$**	No
	Expected	38.4	13.8	1.3	5.7	6.4	31.4		
eGFR < 60 (N = 17)	**Observed**	6	2	1	4	0	4	P = 0.18	Yes
	Expected	5.3	3.9	0.7	1.7	0.9	4.5		
LN (N = 66)	**Observed**	28	5	1	6	0	26	**P = 0.026**	No
	Expected	29.3	8.0	0.6	2.9	3.9	21.3		
Control	**Observed**	70	11	0	0	3	24	P = 0.438	Yes
	Expected	70.9	8.9	0.3	1.5	2.1	24.3		

Testing for Hardy–Weinberg Equilibrium (HWE) of the G0, G1 and G2 alleles in the SLE population, and the SLE subgroups of CKD, eGFR < 60 and LN. P values were calculated using the Chi squared test. A P value of < 0.05 was considered significant and greyed out blocks indicate P < 0.05.

To determine the level of risk that the G1 and G2 alleles pose for SLE, CKD, stage 3 renal failure (eGFR < 60) and LN, odd ratios were calculated for the presence of 0, 1 or 2 risk alleles (G1 or G2) in each group. These were calculated compared to the control samples, and were tested for significance using Pearsons Chi squared test or Fishers exact test. The results are summarised in Table 4-6.

Strong association was found with two risk alleles vs no risk alleles in all groups studied. The strongest association was with stage 3 renal disease (eGFR < 60) (OR 19.44 (3.71 – 101.91), $P = 6.88 \times 10^{-4}$) and CKD (OR 8.38 (2.67 – 30.95), $P = 2.97 \times 10^{-4}$). Weak association was found for one risk allele in SLE, CKD and LN. Stage 3 renal disease with one risk allele vs no risk alleles failed to reach significance.

Table 4-6: *APOL1* risk alleles and association with renal disease in SLE

	Risk alleles (%)			OR 2 vs. 0	P Value	OR 1 vs. 0	P Value
	0	1	2	(95% CI)		(95% CI)	
Controls (N = 108)	70 (64.8)	35 (32.4)	3 (2.8)	–	–	–	–
SLE (N = 165)	66 (40.0)	78 (47.3)	21 (12.7)	7.42 (2.11 - 26.06)	**$P = 4.09 \times 10^{-4}$**	2.36 (1.40 – 3.98)	**$P = 1.11 \times 10^{-3}$**
CKD (N = 97)	39 (39.4)	44 (44.4)	14 (14.1)	8.38 (2.67 – 30.95)	**$P = 2.97 \times 10^{-4}$**	2.26 (1.25 – 4.08)	**$P = 6.63 \times 10^{-3}$**
eGFR < 60 (N = 17)	6 (35.3)	6 (35.3)	5 (29.4)	19.44 (3.71 – 101.91)	**$P = 6.88 \times 10^{-4}$**	2.00 (0.60 – 6.65)	P = 0.20
LN (N = 66)	28 (42.4)	31 (47.0)	7 (10.6)	5.83 (1.41 – 24.18)	**$P = 1.3 \times 10^{-2}$**	2.21 (1.15 - 4.25)	**$P = 1.60 \times 10^{-2}$**
LN 1 (N = 4)	0 (0.0)	4 (100.0)	0 (0.0)	N/A	N/A	N/A	N/A
LN 2 (N = 7)	5 (71.4)	1 (14.3)	1 (14.3)	4.67 (0.41 - 53.45)	P = 0.28	0.40 (0.05 - 3.56)	P = 0.36
LN 3 (N = 20)	5 (25.0)	12 (60.0)	3 (15.0)	14.0 (2.22 – 88.12)	**$P = 1.13 \times 10^{-2}$**	4.80 (1.57 – 14.70)	**$P = 3.41 \times 10^{-3}$**
LN 4 (N = 8)	3 (37.5)	4 (50.0)	1 (12.5)	7.78 (0.61 - 98.74)	P = 0.20	2.67 (0.57-12.58)	P = 0.19
LN 5 (N = 33)	16 (48.5)	14 (42.4)	3 (9.1)	4.38 (0.81-23.71)	P = 0.10	1.75 (0.77-3.99)	P = 0.18

To determine if the risk alleles (G1 or G2) were risk factors for the groups studied (SLE, CKD, eGFR <60 or LN (LN here is subdivided into grades 1 – 5)), the number of risk alleles were totalled for each group and odds ratios (OR) were calculated. Odds ratios of two against no risk alleles (OR 2 vs. 0) and one against no risk allele (OR 1 vs. 0) were calculated. Significance was tested using Pearsons Chi squared test or Fishers exact test if all expected cell frequencies are < 5. A P value of < 0.05 was considered significant, and are greyed out if below this value. N/A: Not applicable, data points are too few for meaningful calculation. LN1 – LN5: Lupus Nephritis grades one to five. Sum of LN1 to LN5 (72) is greater than total LN (66) due to dual LN seen in six biopsies.

Chapter 5: Discussion

Systemic Lupus Erythematosus is a poorly studied disease in patients from developing nations. Unsurprisingly, mortality in SLE patients from developing nations is higher (up to 2.4 times more frequent), and more often due to communicable diseases and disease activity (Tikly and Navarra, 2008, Wadee et al., 2007, Bernatsky et al., 2006). While late presentation, limited access to healthcare, healthcare provider unfamiliarity and poor infrastructure undoubtedly lead to this increased mortality, a genetic predisposition to SLE and poorer outcomes in patients of African origin has been appreciated (Niewold, 2015). In addition, the scarcity of data on SLE in the African populations leaves clinicians with little choice but to base clinical decisions on data extrapolated from other populations.

This study provides data that describes some of the clinical characteristics of SLE patients of African origin. Inasmuch, this study corroborates previous findings on patients with SLE of African origin or descent, and contrasts this to SLE in European populations (Table 5-1) (Wadee et al., 2007, Cervera et al., 1993). Interestingly, the majority of criteria seen in Table 5-1 are similar between the different populations, suggesting that there is an overall similarity in pathogenesis and disease progression of SLE, despite the underlying genetic differences seen between the populations (Niewold et al., 2012). The differences in clinical characteristics are difficult to explain, as they may, at least in part, be attributable to the dissimilarities in the diagnostic classifications used between 1993 and those used currently. Concurrent high quality descriptive studies of the various SLE populations around the world are needed to definitively dissect out the various clinical manifestations and to determine if the differences seen are real or not.

Table 5-1: Comparison of SLE characteristics between African and European populations

	Current Study (N = 165)	**Waddee et al. 2007** (N = 226)	**Cervera et al. 1993** (N = 1000)
Population	South African Black population	South African mixed population	European mixed population
African origin (%)	100.0	92.9	2.0
Female (%)	92.7	94.2	91.0
Female:Male ratio	13:1	18:1	10:1
Age at presentation, years (±SD)	32.4 (±11.3)	34.0 (±12.5)	29.0 (±13.0)
Acute Cutaneous Lupus (%)	52.8	58.4	58.0
Chronic Cutaneous Lupus (%)	51.7	41.5	10.0
Oral or Nasal Ulcers (%)	24.2	38.5	24.0
Synovitis (%)	65.2	70.4	84.0
Serositis (%)	24.7	18.1	36.0
Renal Disease (%)	51.1	43.8	39.0
Neurologic Disease (%)	15.7	15.9	2.0
Thrombocytopenia (%)	22.5	12.8	22.0
ANA Positive (%)	97.2	99.1	96.0
Anti-dsDNA Positive (%)	42.1	55.3	78.0
aSm Positive (%)	72.5	40.7	10.0

SD: Standard deviation. ANA: Anti-nuclear antibody. Anti-dsDNA: Anti Double Stranded DNA antibody. aSm: Anti Smith Antibody.

5.1 *APOL1* Genotypes in SLE renal disease

Lupus nephritis is one of the most dreaded and potentially deadly complications of SLE. As LN can be clinically silent until ESRD is established, active screening is needed at every visit for early detection. Furthermore, as patients of African ancestry have a poorer outcome with SLE compared to other population groups, and ESRD develops earlier and more rapidly in this population; early identification and aggressive treatment is warranted (Tikly and Navarra, 2008, Wadee et al., 2007, Freedman et al., 2014, Genovese et al., 2010, Lin et al., 2012). The association of the *APOL1* polymorphisms with FSGS, HIVAN and H-ESKD in patients of African ancestry (African Americans and Black South Africans) makes the G1 and G2 alleles attractive candidates as risk alleles for LN in SLE (Genovese et al., 2010, Kasembeli et al., 2015). This assumption is corroborated by Freedman et al., who describe the presence of the two *APOL1* risk alleles as being a significant prognosticator for ESRD in SLE patients of African ancestry and accelerating progression to ESRD by as much as two years (Freedman et al., 2014). In addition to this, Freedman et al. found that the presence of two G1/G2 alleles allowed for a weak trend towards an earlier onset of SLE (Freedman et al., 2014). These findings are offset by Lin et al., who find no association with LN and the *APOL1* gene in patients of African ancestry (Lin et al., 2012). In a sub-analysis, Lin et al. note a marginally significant association ($P = 0.0418$) between rs71785313 (allele G2) and LN. They argue that as they were powered to detect a p-value of 0.01 in 85% of cases, a p value of 0.0418 suggests there is no effect of *APOL1* in LN ESRD patients (Lin et al., 2012).

Our results need to be viewed with extreme caution and should at best be considered preliminary, as the assignment of the G2 allele was not validated and is not in keeping with the international and national literature. Nonetheless, our results

show association between SLE, CKD, stage 3 renal failure, LN and the presence of two *APOL1* risk alleles, not in keeping with Lin et al.'s findings. Our findings are in keeping with Freedman et al.'s, especially as our highest odds ratios (OR) are found in the CKD and low GFR groups (stage 3 renal failure) (Freedman et al., 2014). However, our stage 3 renal failure sample size was modest in our cohort (N = 17), and as such caution must be used when interpreting these results.

Systemic lupus erythematosus and LN are heterogeneous disease processes, allowing for a wide range of clinical and biochemical presentations (Bertsias et al., 2012). The initiating event for LN is immune mediated, but the exact mechanism is unclear. Proposed mechanisms range from preformed immune complexes deposited within the glomerulus, de novo antibody-antigen complex formation within the glomerular apparatus from deposited antigens and lastly due to antibodies binding to intrinsic glomerular antigens. These mechanisms are not mutually exclusive (Niewold, 2015, Seshan and Jennette, 2009). Our finding that the *APOL1* risk alleles are associated with SLE and hence may have an influence on development of the SLE phenotype is interesting. It was previously noted that SLE disease activity becomes progressively quiescent as ESRD develops (Mojcik and Klippel, 1996). If the *APOL1* G1 and G2 alleles are risk factors for developing SLE, LN and CKD, a possible unifying explanation could be that the G1 or G2 alleles induce novel antigenic sites in the APOL1 protein within the kidney, allowing for humoral activation in a SLE-susceptible individual. The humoral activation and subsequent inflammation would cause local tissue damage, exposing nuclear antigens to the immune system, priming the humoral immune system against nuclear antigens. The resultant cascade of immunological events may lead to the SLE phenotype in a susceptible individual, and possibly drive the autoimmune process while there is still APOL1

antigen present. This would theoretically continue until there is global sclerosis of the kidney, heralding ESRD and resulting in insufficient APOL1 antigen to drive the autoimmune process. This theory could explain not only the association of SLE, LN and CKD with the *APOL1* G1 and G2 alleles, but also why SLE tends to become relatively quiescent once ESRD develops.

A second possibility could be that *APOL1* G1 and G2 are needed to progress from LN to CKD. While the initiating event in LN is an inflammatory response, this response does not necessarily lead to CKD (Oates and Gilkeson, 2002). A “second hit” may be needed, either to allow progression to CKD, or expedite it. The *APOL1* G1/G2 alleles may function as such a “second hit”, requiring an initial insult such as HIV, hypertension, LN or other, and the G1/G2 alleles subsequently sanctions the progression to CKD. Evidence for this was scanty, until recently. Previous understanding of APOL1 protein function was that it was a constituent of HDL, and conveys resistance to HSS (Pays et al., 2014, 2015). Recently, Lan et al. have shown that expression of *APOL1* G1 or G2 genotypes in healthy podocytes leads to apoptosis via increased lysomal permeability (Lan et al., 2014). This may be analogous to the trypanosomal toxicity due to pore formation within the trypanosomal endosome (Pays et al., 2014). Additionally, the APOL1 G0 protein can also cause podocyte injury, but only at supra-physiological concentrations, triggered by incubating the podocytes with interferon γ. This was thought to mimic inflammation within the glomerulus (Lan et al., 2014). Therefore, this information lends itself to the hypothesis that inflammation within the glomerulus, such as that seen within LN, may lead to upregulation of APOL1, resulting in increased podocyte apoptosis. In the presence of a single G1 or G2 allele, the podocyte destruction is likely increased, while with two alleles, the destruction is likely to be relatively catastrophic, resulting

in rapid establishment of renal dysfunction, and rapid progression to CKD. This is corroborated by Anderson et al., who have shown that the zebra fish protein *apol1*, which is orthologous to human APOL1, is needed for normal kidney function in zebra fish embryos. Additionally, in *apol1* knockdown zebra fish embryo's, kidney function is rescued by introduction of wild type human APOL1 (G0) into the embryo, but not by APOL1 G1 or G2 protein (Anderson et al., 2015).

The mechanism whereby the G1 and G2 alleles predispose to renal disease is unknown, but as they both occur in the long carboxy‑terminal amphipathic α-helix that is thought to control the activity of APOL1, the G1 and G2 alleles may upregulate APOL1 activity, leading to renal disease (Anderson et al., 2015 and Pays et al., 2014). As mentioned above, the potentially novel alleles seen in rs71785313 have yet to be confirmed, but we could speculate that they may function in a similar fashion to the G2 allele. If the assumption is made that the 162bp allele represents the expected 164bp allele, and similarly the 156bp allele the 158bp allele, the 153bp allele represents a 9bp deletion, while the 148bp allele represents a 14bp deletion. The 153bp allele would therefore result in a three amino acid in-frame deletion from the APOL1 protein, and may function in a similar fashion to the 156bp (G2) allele. In contrast, the 148bp would result in a 14bp deletion, which is a frameshift mutation. This would most likely have a drastic effect on APOL1 function, most likely detrimental and could partially explain the scarcity of 148bp allele seen, due to a presumed strong negative selective pressure.

In summary, this study provides tentative evidence that the *APOL1* alleles G1 and G2 are risk alleles for SLE and LN. In addition, G1 and G2 are likely to be associated with progression to CKD and/or a low eGFR, in that these two alleles are most likely

the “second hit” that allows progression to CKD, or that the G1/G2 alleles create novel antigens that may prime the humoral immune system and initiate autoimmunity. This is in keeping with Freedman et al., who have demonstrated a strong association with ESRD and the *APOL1* G1/G2 compound heterozygous state in LN (Freedman et al., 2014). As Anderson et al. have shown rescue of *apol1* knockdown zebra fish embryo’s with APOL1 G0, APOL1 G0 could perhaps be considered as a novel therapy in G1/G2 compound heterozygote patients with early nephropathies, such as FSGS, HIVAN, hypertensive nephropathy or even LN (Anderson et al., 2015, 2015).

5.2 Limitations and strengths

This study is limited by the fact that the rs71785313 insertion/deletion alleles were not sequenced, and hence could not be scored with confidence. While the 162bp/156bp alleles most likely correspond to the expected 164bp/158bp alleles, only by sequencing can this confirmed. Additionally, the significance of the potentially novel alleles cannot be defined without sequencing. This has a profound impact on this study, as every haplotype is a composite of the proper scoring of the rs71785313 allele in each sample.

A second limitation is the small sample sizes seen in the subgroups. In particular, the number of SLE patients with stage 3 renal failure (N = 17) is small. Patient ascertainment bias is likely to be the cause of the low number of patients with stage 3 or worse renal failure seen in our cohort, as SLE patients with any evidence of renal disease are urgently referred for renal assessment. Due to large patient loads and extended waiting times in our setting, patients are often only able to follow up at a single clinic. Based on the hypothesis that the G1 and G2 alleles are associated with renal disease, recruiting patients from the Rheumatology clinic may have

caused us to select patients without renal disease, possibly biasing our sample against these patients and underestimating the G1/G2 genotype frequencies. In addition to this, the higher mortality seen in CKD in developing countries such as South Africa, may also partially explain the low number of patients in our cohort with a low eGFR and ESRD (Bernatsky et al., 2006, Tikly and Navarra, 2008, Wadee et al., 2007).

Strengths of this study include that it was a sequential prospective case control study of patients with SLE. This has allowed for a diverse and representative sample of Black SLE patients to be described, a rarely studied group. Additionally, this study yields information on both SLE and renal disease in a South African Black population, in which these diseases are known to carry a far worse prognosis than in other populations studied (Naicker, 2013, Genovese et al., 2010, Duchateau et al., 2001).

Chapter 6: Conclusions and furture studies

From this study it can be tentatively concluded that the G1 and G2 alleles in *APOL1* are likely associated with SLE, LN and CKD in the South African Black SLE population. While the exact pathological method is not fully understood, it appears that APOL1 is involved in podocyte activity within the glomerulus, and alterations in APOL1 function are detrimental to glomerular function (Anderson et al., 2015). Additionally, as the G0 APOL1 protein has been shown to rescue *apol1* knockdown zebra fish embryos from renal dysfunction, G0 APOL1 may prove to be therapeutic in diseases such as FSGS, HIVAN, hypertension-attributed end-stage kidney disease and even LN. This would have to be elucidated using both proteomics and functional studies of APOL1 and its various domains.

The next logical steps from this study would be to validate the rs71785313 deletion alleles before re-analysing the data to determine whether the associations hold true. Further to this, the effect of the polymorphisms at each of rs73885319, rs60910145 and rs71785313 on APOL1 protein function would need to be elucidated using functional studies of APOL1 in its entirety, and in part via analysis of APOL1s' various domains, specifically with the membrane assessing domain and the SRA-interactive C-terminal helix.

Chapter 7: References

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Chapter 8: Proposed manuscript for publication in the journal Arthritis & Rheumatology: The role of APOL1 polymorphisms in Systemic Lupus Erythematosus Nephritis in Black South Africans

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8.1 Abstract

8.1.1 Objective

To assess whether Apolipoprotein L-I (*APOL1*) G1 and G2 genotypes are associated with developing renal disease in a cohort of Black South African patients with Systemic Lupus Erythematosus (SLE).

8.1.2 Methods

One hundred and seventy eight unrelated Black South African patients with SLE were enrolled into the study. All patients fulfilled the Systemic Lupus International Collaborating Clinics (SLICC) 2012 criteria. The patients were recruited from the Chris Hani Baragwanath Academic Hospital Rheumatology clinic. One hundred and eight Black South African individuals with no known renal or connective tissue disease were used as controls. *APOL1* G1 and G2 alleles were discerned using in-house restriction fragment length polymorphism analysis and fluorescently labelled primer PCR fragment length determination, respectively.

8.1.3 Results

APOL1 was successfully genotyped for G0, G1 and G2 in 165 (92.6%) of the samples obtained. The *APOL1* genotypes G1 and G2 were associated with SLE (OR 7.42 (2.11 - 26.06), $P = 4.09 \times 10^{-4}$), Lupus nephritis (LN) (5.83 (1.41 – 24.18), $P = 1.30 \times 10^{-2}$) and chronic kidney disease (CKD) (8.38 (2.67 – 30.95), $P = 2.97 \times 10^{-4}$). However, stage 3 renal disease (defined as an estimated glomerular filtration rate (eGFR) < 60ml/min/1.73m^2), showed the strongest association (OR 19.44 (3.71 – 101.91), $P = 6.88 \times 10^{-4}$).

8.1.4 Conclusion

The *APOL1* alleles G1 and G2 show an associated with SLE, LN and CKD. However, the greatest association seen was with stage 3 renal disease. This suggests that the *APOL1* alleles are risk factors for SLE and LN, and are strongly associated with progression to CKD in SLE.

8.2 Introduction

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disorder, characterised by an overactive humoral immune system, with loss of self-tolerance core to this disease. Clinical manifestations are protean, and range from relatively minor complaints such as isolated arthralgia, to life threatening complications such as end stage renal disease (ESRD) and complex neurological disorders (Bertsias et al., 2012). As the clinical presentation is highly heterogeneous, and early diagnosis is imperative to expedite treatment and avoid life-threatening complications, internationally standardised diagnostic criteria were developed: The SLE international collaborating clinic (SLICC) (revised from the American College of Rheumatology criteria). This has a sensitivity of 94% and a specificity of 92% in diagnosing SLE (Petri et al., 2012).

Mortality risk in SLE is increased up to 2.4 times more than in the general population (Bernatsky et al., 2006). In addition, mortality risk is divided along socio-economic lines. In developed countries, patients with SLE are more likely to demise from non-communicable causes such cardiovascular diseases (25%) and malignancies (predominantly lymphoma and lung carcinoma) (9%) whereas infections are a relatively minor cause of death in these countries (3.5%) (Bernatsky et al., 2006). In contrast, mortality in developing nations is driven predominantly by communicable diseases, with sepsis (18%) identified as the main cause. Other causes, such as renal disease (9%), active SLE disease (7%) and miscellaneous causes (7%) are other common causes (Wadee et al., 2007, Tikly and Navarra, 2008). Worryingly, mortality risk in patients with Lupus nephritis (LN) is markedly increased in developing countries as compared to developed countries (9.0% vs. 2.7%) (Tikly and Navarra, 2008, Wadee et al., 2007, Bernatsky et al., 2006). While this undoubtedly is

partially due to late presentation, poor access to healthcare facilities, lack of diagnostic ability and limited resources available to treat LN in developing countries, genetic susceptibility to renal disease appears to play a role in patients with an African ancestry (Naicker, 2013, Kasembeli et al., 2015, Genovese et al., 2010).

Recently, two *APOL1* alleles in individuals of African descent (African American and Black African populations) have shown strong associations with chronic kidney disease (CKD), specifically in focal segmental glomerulosclerosis (FSGS), HIV-associated nephropathy (HIVAN), and hypertension-attributed end-stage kidney disease (H-ESKD) (Freedman et al., 2014, Kasembeli et al., 2015).The first allele, G1, is characterised by two missense mutations in perfect linkage disequilibrium (rs73885319 [G allele] and rs60910145 [G allele]). The second allele, G2, is a 6–base pair (bp) deletion (rs71785313) and removes amino acids N388 and Y389 from the C terminus of APOL1. As rs73885319, rs60910145, and rs71785313 are very close to each other (all three polymorphisms lie within 146bp of each other), recombination between G1 and G2 alleles is a rare event. Hence G1 and G2 are considered alleles of the same locus (Genovese et al., 2010, Kasembeli et al., 2015). G1 and G2 behave in a recessive-like manner, hence two alleles are considered a necessary condition to confer susceptibility to renal disease.

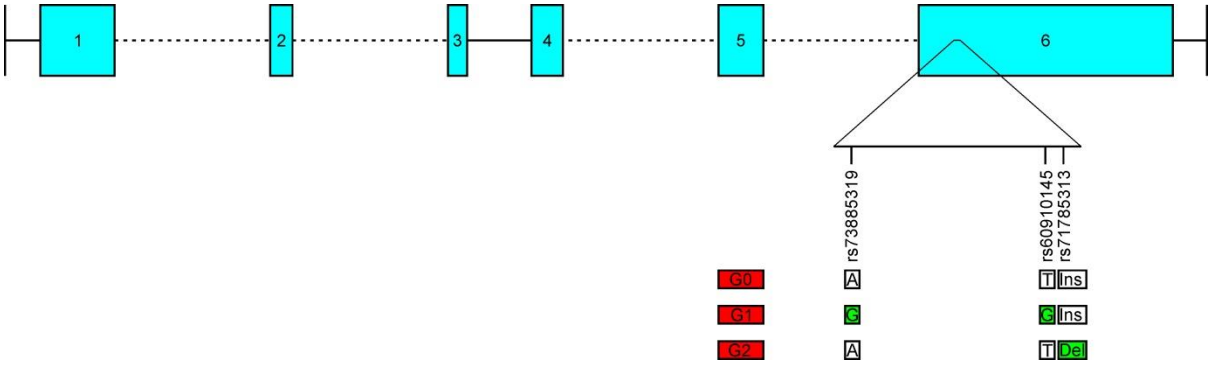

Figure 8-1: APOL1 showing position of alleles G0, G1 and G2

Schematic representation of APOL1, showing the localisation of rs73885319, rs60910145, and rs71785313 within the sixth exon. Of note, the G0, G1 and G2 "alleles" are actually genotypes, comprised al alleles from rs73885319, rs60910145, and rs71785313, which all lie within 146bp of each other. The G0 allele is the wild type allele with normal function, while the G1 and G2 alleles are risk alleles for renal disease. A: Adenosine. T: Thymidine. G: Guanine. Ins: 6bp insertion, or wild type allele. Del: Six bp deletion allele. 1 to 6: Exons one to six. Dotted lines indicate areas not drawn to scale.

APOL1 G1 and G2 alleles have been selected for relatively recently (in the last 10 000 years) in African populations where Trypanosomiasis is endemic. The alleles have evolved as a protective measure against Human Sleeping Sickness (HSS) (Genovese et al., 2010, Pays et al., 2014). Wild type (G0) APOL1 is effective only against HSS caused by *Trypanosoma brucei brucei* and lyses *T. b. brucei* by creating pores within the trypanosomal endosome, thereby allowing efflux of chloride ions, which is fatal to the parasite. However, in *T. b. rhodesiense,* the APOL1 protein is bound by a protein known as serum resistance-associated (SRA), found within the trypanosome endosome. This renders APOL1 ineffective and allows for *T. b. rhodesiense* survival. In response, human *APOL1* polymorphisms (G1 and G2) were selected for: G1 and G2 alter key SRA binding sites within the C terminus of APOL1, disallowing binding of SRA to APOL1. This effectively reinstates APOL1 lytic activity against *T. b. rhodesiense*. This has occurred in areas of Africa where *T. b. rhodesiense* is endemic, and has resulted in a relatively strong positive pressure for the selection of the G1 and G2 alleles. Compared to areas other than Africa, the

APOL1 G1 and G2 alleles have reached relatively high frequencies in Africa (7.3-18.0% and 11.1-15.0%, respectively) (Genovese et al., 2010, Kasembeli et al., 2015).

While the positive effect of resistance to *T. b. rhodesiense* infection is conferred if just one G1 or G2 allele is present, patients who have two risk alleles (G1/G1, G2/G2 or more commonly G1/G2) are at increased risk for developing CKD and ultimately ESRD (Genovese et al., 2010). The risk for CKD should exert a negative selective pressure against the *APOL1* alleles, effectively driving the G1/G2 allele frequency down. However, as CKD develops over years, the negative selective pressure of the G1/G2 alleles for CKD is likely to become apparent after the positive selective pressure of trypanosomal resistance has been appreciated by the individual (Additionally, only one G1 or G2 allele is necessary for resistance to trypanosomal infection). Thus, the most likely pattern seen then is early resistance to HSS and later onset of CKD, resulting in the propagation of the G1/G2 alleles to the next generation before the onset of ESRD, allowing the G1/G2 alleles to remain at relatively high frequencies in African, but not other populations (Genovese et al., 2010, Pays et al., 2014, Kasembeli et al., 2015).

As *APOL1* G1 and G2 alleles are known to increase risk for ESRD in FSGS, HIVAN and H-ESKD, they could be considered as risk alleles for LN. Interestingly, different studies of these *APOL1* polymorphisms have resulted in contradictory results, showing both an association and no association with LN (Freedman et al., 2014, Lin et al., 2012, Kasembeli et al., 2015). These contradictory results led us to investigate a cohort of Black African patients with SLE from the Rheumatology clinic in the Chris Hani Baragwanath Academic Hospital, Johannesburg, South Africa, for the G1 and G2 *APOL1* polymorphisms and an association with renal disease in SLE.

8.3 Materials and methods

8.3.1 Ethics

Ethical approval for this study was obtained from the University of the Witwatersrand Ethics committee (Certificates M10707 and M130727).

8.3.2 Patients

One hundred and seventy eight Black South African patients with SLE were enrolled into the study. All patients fulfilled the Systemic Lupus International Collaborating Clinics (SLICC) 2012 criteria. The patients were recruited from the Chris Hani Baragwanath Academic Hospital Rheumatology clinic. Informed consent was obtained. Patients were excluded if they did not fulfil the SLICC criteria. Basic demographics, average creatinine since diagnosis, urine protein:creatinine ratio, serological data, SLICC score, previous renal biopsy results and previous treatment for tuberculosis were recorded. Average estimated glomerular filtration rate (eGFR) was calculated using the MDRD formula and each patient was staged according to the KDIGO guidelines (Levey and Bosch, 1999, Eknoyan, 2013). Chronic kidney disease was defined as an average eGFR < 60ml/min/1.73m^2 (stage 3 or worse renal failure) or biochemical, histological or radiological evidence of renal damage over three or more months. Data for each patient was anonymized and entered into a database. One hundred and eight Black South African individuals without any known disease were used as controls. The control samples were sourced from the Division of Human Genetics, National Health Laboratory Service, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, and were reported by Kasembeli et al., 2015.

8.3.3 *APOL1* Genotypes

Approximately 10ml of whole venous blood was taken from each patient and DNA was extracted using a salting out procedure (Miller et al., 1988). The *APOL1* G1 genotype was identified using an in-house polymerase chain reaction (PCR). The restriction endonucleases *HindIII* and *NspI* were used to digest the PCR product, and subsequently electrophoresed on a 3% agarose gel. Allelic data for rs73885319 and rs60910145 for each sample was scored and entered into a database. G1 was defined as both the presence of a guanine (G) residue at rs73885319 and a G residue at rs60910145. The *APOL1* G2 allele was identified by utilizing an in-house PCR with a fluorescently labelled primer. The resultant amplicon was sized on an ABI 3130xl sequencer and G2 was defined as the presence of a deletion at rs71785313. Allelic data for each sample was scored and entered into a database. The *APOL1* G0 genotype was defined as being present if neither the G1 nor the G2 allele were present.

8.3.4 Statistical analysis

Allelic frequencies were calculated by allele counting for G0, G1 and G2 for all cases and controls. Odds ratios with 95% confidence intervals were calculated and tested for significance using Fisher's Exact Probability test and Pearsons Chi squared test. Association was sought for CKD, LN, and SLE nephropathy. All data derived from this study were analysed using either the Stata/IC 13.1, Statistica version 12, or Epi info^{tm} 7.1.4.0 statistical analysis programs. A P value of <0.05 was considered significant.

8.4 Results

In total, 178 Black patients with SLE were recruited to this study. Control data was obtained entirely from Kasembeli et al. (N = 108) (Kasembeli et al., 2015). Patient

and control characteristics are shown in Table 8-1. Females comprised the majority of the patients studied (92.7%), with a female:male ratio of 13:1. Lupus nephritis was common, occurring in 72 patients (40.4%). Lupus nephritis grade 3 and 5 were the most frequent LN seen (30.6 and 48.6% respectively). Dual grade LN with was seen in six patients, again with LN grades 3 and 5 being commonest.

APOL1 G0, G1 and G2 genotypes were successfully determined in 92.6% (165) of the SLE subjects, and are summarised in Table 8-2. Subsequently, the G0, G1 and G2 genotypes were grouped into haplotypes, which are summarised in Table 8-3.

Odd ratios were calculated for the presence of 0, 1 or 2 risk haplotypes (G1 or G2) for SLE, CKD, stage 3 renal disease (eGFR < 60) and LN. These were calculated compared to the control samples, and are summarised in Table 8-4. Associations were seen between the presence of two risk alleles and SLE, CKD, stage 3 renal disease and LN, while the strongest association was with stage 3 renal disease and two risk alleles (19.44 (3.71 – 101.91), $P = 6.88 \times 10^{-4}$). Interestingly, no association was found between an eGFR< 60 and 1 risk allele.

Table 8-1: Characteristics of study and control populations

Characteristic	**SLE** (N = 178)	**CKD** (N = 107)	**eGFR< 60** (N = 20)	**LN** (N = 72)	**Control**(N = 108)
Female N (%)	165 (92.7)	99 (92.5)	16 (80.0)	66 (91.7)	69 (63.9)
Mean age (±SD)	39.2 (13.7)	37.0 (13.0)	36.5 (12.2)	33.6 (10.7)	38.7 (7.9)
Mean age at diagnosis (±SD)	32.4 (11.3)	31.2 (10.9)	31.4 (11.1)	28.1 (8.8)	N/A
Mean follow up period (±SD)	6.8 (6.5)	5.8 (6.3)	5.2 (6.5)	5.4 (6.0)	N/A
Mean SLICC criteria (±SD)	8.1 (2.5)	8.9 (2.5)	9.8 (2.4)	9.0 (2.5)	N/A
Acute Cutaneous Lupus (%)	94 (52.8)	55 (51.4)	12 (60.0)	37 (51.4)	N/A
Chronic Cutaneous Lupus (%)	92 (51.7)	62 (57.9)	13 (65.0)	37 (51.4)	N/A
Oral or Nasal Ulcers (%)	43 (24.2)	26 (24.3)	6 (30.0)	15 (20.8)	N/A
Non-Scarring Alopecia (%)	42 (23.6)	28 (26.2)	6 (30.0)	17 (23.6)	N/A
Synovitis (%)	116 (65.2)	62 (57.9)	12 (60.0)	42 (58.3)	N/A
Serositis (%)	44 (24.7)	31 (29)	9 (45.0)	26 (36.1)	N/A
Renal Disease (%)	107 (60.1)	107 (100)	20 (100.0)	72 (100.0)	N/A
Neurologic Disease (%)	28 (15.7)	22 (20.6)	4 (20.0)	12 (16.7)	N/A
Hemolyticanemia (%)	9 (5.1)	7 (6.5)	4 (20.0)	3 (4.2)	N/A
Leukopenia (%)	131 (73.6)	84 (78.5)	14 (70.0)	54 (75.0)	N/A
Thrombocytopenia (%)	40 (22.5)	25 (23.4)	7 (35.0)	15 (20.8)	N/A
ANA Positive (%)	173 (97.2)	104 (97.2)	19 (95.0)	69 (95.8)	N/A
Anti-dsDNA Positive (%)	75 (42.1)	53 (49.5)	14 (70.0)	40 (55.6)	N/A
aSm Positive (%)	129 (72.5)	85 (79.4)	15 (75.0)	56 (77.8)	N/A
aPL Positive (%)	117 (65.1)	76 (71.0)	14 (70.0)	53 (73.6)	N/A
Low Serum C3/C4 complement (%)	128 (71.9)	85 (79.4)	18 (90.0)	60 (83.3)	N/A
Direct Coombs positive	98 (55.1)	65 (60.7)	13 (65.0)	43 (59.7)	N/A
eGFR (ml/min/1.73m^2) (±SD)	110.00 (48.1)	103.9 (54.3)	36.7 (15.5)	103.20 (53.7)	N/A
Average UPCR (g/mmolcreat) (±SD)	0.150 (0.415)	0.241 (0.52)	0.221 (0.222)	0.290 (0.612)	N/A

Characteristics of the study and control populations. Systemic Lupus Erythematosus (SLE) is the main group studied, and comprises other subgroups, namely Chronic Kidney Disease (CKD),estimated Glomerular filtration rate less than 60ml/min/1.73m2(eGFR < 60) or Lupus nephritis (LN, Either biopsy proven (N = 67) or with clinical suspicion

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and awaiting biopsy (N = 5)). CKD, eGFR < 60 and LN are not mutually exclusive. The control data is taken from Kasembeli et al. 2015 and was not matched for age or sex, but was matched for race. . SD: Standard deviation. SLICC: SLE international collaborating clinic. ANA: Anti-nuclear antibody. Anti-dsDNA: Anti Double Stranded DNA antibody. aSm: Anti Smith Antibody. aPL: Antiphospholipid antibodies . eGFR: Estimated Glomerular Filtration Rate. N/A: Not Available.

Table 8-2: G0, G1 and G2 allele frequencies in the SLE, CKD, eGFR < 60 and LN groups compared to controls

	SLE	P	CKD	P	eGFR< 60	P	LN	P	Control
G0 (%)	210 (63.6)	**$< 1.0 \times 10^{-5}$**	122 (61.6)	**$< 1.0 \times 10^{-5}$**	18 (52.9)	**1.4×10^{-4}**	87 (65.9)	**7.6×10^{-4}**	175 (81.0)
G1 (%)	36 (10.9)	**8.9×10^{-4}**	22 (11.1)	**1.0×10^{-2}**	8 (23.5)	**8.0×10^{-5}**	12 (9.1)	0.0722	11 (5.1)
G2 (%)	84 (25.5)	**5.8×10^{-4}**	50 (25.3)	**1.2×10^{-3}**	8 (23.5)	0.0722	33 (25.0)	**4.5×10^{-3}**	30 (13.9)
Total	330		194		34		132		216

Testing for significant difference between the G0, G1 and G2 allele frequencies in the SLE group, and the SLE subgroups of CKD, eGFR < 60 and LN, relative to the control group. P values calculated using the Z test. A P value of < 0.05 was considered significant. Greyed out blocks indicate P < 0.05.

Table 8-3: *APOL1* genotype distribution in the control and study groups

Genotype	SLE (%)	CKD (%)	eGFR< 60 (%)	LN (%)	Control (%)
0 risk alleles					
G0/G0	66 (40.0)	39 (40.2)	6 (35.3)	28 (42.4)	70 (64.8)
1 risk alleles					
G0/G1	17 (10.3)	8 (8.2)	2 (11.8)	5 (7.6)	11 (10.2)
G0/G2	61 (37.0)	36 (37.1)	4 (23.5)	26 (39.4)	24 (22.2)
2 risk alleles					
G1/G1	1 (0.6)	1 (1.0)	1 (5.9)	1 (1.0)	0 (0.0)
G1/G2	17 (10.3)	12 (12.4)	4 (23.5)	6 (6.1)	0 (0.0)
G2/G2	3 (1.8)	1 (1.0)	0 (0.0)	0 (0.0)	3 (2.8)
Total	165	97	17	66	108

Summary of APOL1 genotyping results for the entire SLE population studied (N = 165) and various subgroups of the SLE population: those with CKD (N = 97), those with an eGFR < 60 (N = 17) and those with LN (N = 66). The subgroups are not mutually exclusive. Risk alleles are defined as the presence of either the G1 or G2 allele. The number of risk alleles is totalled for each patient, and should be 0, 1 or 2, with the presence of 2 risk alleles theoretically representing the highest risk for renal impairment, 1 an intermediate risk and 0 for the lowest risk. Control data from Kasembeli et al., 2015.

Table 8-4:*APOL1* risk alleles and association with renal disease in SLE

	Risk alleles (%)			OR 2 vs. 0	P Value	OR 1 vs. 0	P Value
	0	1	2	(95% CI)		(95% CI)	
Controls (N = 108)	70 (64.8)	35 (32.4)	3 (2.8)	–	–	–	–
SLE (N = 165)	66 (40.0)	78 (47.3)	21 (12.7)	7.42 (2.11 - 26.06)	**$P = 4.09 \times 10^{-4}$**	2.36 (1.40 – 3.98)	**$P = 1.11 \times 10^{-3}$**
CKD (N = 97)	39 (39.4)	44 (44.4)	14 (14.1)	8.38 (2.67 – 30.95)	**$P = 2.97 \times 10^{-4}$**	2.26 (1.25 – 4.08)	**$P = 6.63 \times 10^{-3}$**
eGFR < 60 (N = 17)	6 (35.3)	6 (35.3)	5 (29.4)	19.44 (3.71 – 101.91)	**$P = 6.88 \times 10^{-4}$**	2.00 (0.60 – 6.65)	P = 0.20
LN (N = 66)	28 (42.4)	31 (47.0)	7 (10.6)	5.83 (1.41 – 24.18)	**$P = 1.3 \times 10^{-2}$**	2.21 (1.15 - 4.25)	**$P = 1.60 \times 10^{-2}$**
LN 1 (N = 4)	0 (0.0)	4 (100.0)	0 (0.0)	N/A	N/A	N/A	N/A
LN 2 (N = 7)	5 (71.4)	1 (14.3)	1 (14.3)	4.67 (0.41 - 53.45)	P = 0.28	0.40 (0.05 - 3.56)	P = 0.36
LN 3 (N = 20)	5 (25.0)	12 (60.0)	3 (15.0)	14.0 (2.22 – 88.12)	**$P = 1.13 \times 10^{-2}$**	4.80 (1.57 – 14.70)	**$P = 3.41 \times 10^{-3}$**
LN 4 (N = 8)	3 (37.5)	4 (50.0)	1 (12.5)	7.78 (0.61 - 98.74)	P = 0.20	2.67 (0.57-12.58)	P = 0.19
LN 5 (N = 33)	16 (48.5)	14 (42.4)	3 (9.1)	4.38 (0.81-23.71)	P = 0.10	1.75 (0.77-3.99)	P = 0.18

To determine if the risk alleles (G1 or G2) were risk factors for the groups studied (SLE, CKD, eGFR <60 or LN (LN here is subdivided into grades 1 – 5)), the number of risk alleles were totalled for each group and odds ratios (OR) were calculated. Odds ratios of two against no risk alleles (OR 2 vs. 0) and one against no risk allele (OR 1 vs. 0) were calculated. Significance was tested for using using Pearsons Chi squared test or Fishers exact test if all expected cell frequencies are < 5. A P value of < 0.05 was considered significant, and are greyed out if below this value. N/A: Not applicable, data points are too few for meaningful calculation. LN1 – LN5: Lupus Nephritis grades one to five. Sum of LN1 to LN5 (72) is greater than total LN (66) due to dual LN seen in six biopsies.

8.5 Discussion

Lupus nephritis is a dreaded complication of SLE, and as LN can be clinically silent until ESRD is established, active screening at every visit is needed for early detection. Furthermore, as patients of African ancestry have a poorer outcome with SLE compared to other population groups, and ESRD develops earlier and more rapidly in this population; early identification and aggressive treatment is needed (Tikly and Navarra, 2008, Wadee et al., 2007, Freedman et al., 2014, Genovese et al., 2010, Lin et al., 2012). The association of the *APOL1* polymorphisms with FSGS, HIVAN and H-ESKD in patients of African ancestry (African Americans and Black South Africans) makes G1 and G2 attractive candidates as risk alleles for renal disease in SLE (Genovese et al., 2010, Kasembeli et al., 2015). This assumption is corroborated by Freedman et al., who describe the presence of two *APOL1* risk alleles as being a significant prognosticator for ESRD in SLE patients of African ancestry, accelerating progression to ESRD by as much as two years (Freedman et al., 2014). In addition to this, Freedman et al. found that the presence of two G1/G2 alleles allowed for a weak trend towards an earlier onset of SLE (Freedman et al., 2014). These findings are offset by Lin et al., who find no association with LN and the *APOL1* gene in patients of African ancestry (Lin et al., 2012). In a sub-analysis, Lin et al. note a marginally significant association ($p = 0.0418$) between rs71785313 (allele G2) and LN. They argue that as they were powered to detect a p-value of 0.01 in 85% of cases, a p value of 0.0418 suggests there is no effect of APOL1 in LN, ESRD patients (Lin et al., 2012).

Our results clearly show an association between SLE, LN, CKD and the presence of two risk alleles, corroborating Lin et al.'s findings. However, our strongest association is between the two risk alleles and an eGFR< 60 in SLE, in keeping with

Freedman et al.'s findings (Freedman et al., 2014). Our sample size for eGFR < 60 was modest in our cohort (N = 17), and as such caution must be used when interpreting these results. Patient ascertainment bias is likely to be the cause of the low number of patients with stage 3 renal failure seen in our cohort as SLE patients with early renal disease are referred for renal assessment and possible renal replacement therapy. Due to large patient loads and extended waiting times in our setting, patients are often only able to follow up at a single clinic. Therefore, recruiting patients from only the Rheumatology clinic is likely to have caused us to select patients without CKD, possibly biasing our sample against these patients and underestimating the G1/G2 genotype frequencies. In addition to this, the higher mortality seen in CKD in developing countries such as South Africa, may also partially explain the low number of patients in our cohort with an eGFR< 60 (Tikly and Navarra, 2008, Wadee et al., 2007, Bernatsky et al., 2006).

Systemic lupus erythematosus and LN are heterogeneous disease processes, allowing for a wide range of clinical and biochemical presentations (Seshan and Jennette, 2009, Bertsias et al., 2012). The initiating event for LN is immune mediated, but the exact mechanism is unclear. The proposed mechanisms range from preformed immune complexes deposited within the glomerulus, de novo antibody-antigen complex formation in the glomerular apparatus from antigens deposited previously and lastly due to antibodies binding to intrinsic glomerular antigens. These mechanisms are not mutually exclusive (Oates and Gilkeson, 2002). While the initiating event in LN is an inflammatory response within the glomerulus, this response does not necessarily lead to CKD (Oates and Gilkeson, 2002). A "second hit" may be needed, either to allow progression to CKD, or expedite it. The *APOL1* G1/G2 genotypes may function as such, requiring an initial insult such as

HIV, hypertension, LN or other, and subsequently sanctioning the progression to CKD. Evidence for this was scanty, until recently. Previous understanding of APOL1 protein function was that it was a constituent of HDL, and conveys resistance to HSS (Pays et al., 2014, 2015). Recently, Lan et al. have shown that expression of *APOL1* G1 or G2 genotypes in healthy podocytes leads to apoptosis via increased lysomal permeability (Lan et al., 2014). This may be analogous to the trypanosomal toxicity due to pore formation within the trypanosomal endosome (Pays et al., 2014). The APOL1 G0 protein can also induce podocyte injury, but only at supra-physiological concentrations. Lan et al. were able to induce podocyte apoptosis due to raised APOL1 G0 concentrations, by incubating the podocytes with interferon γ, mimicking an inflammatory response within the glomerulus (Lan et al., 2014). This suggests that inflammation within the glomerulus, as seen in LN, may lead to upregulation of APOL1, resulting in increased podocyte apoptosis. In the presence of a single G1 or G2 allele, the podocyte destruction is likely increased, while with two alleles, the destruction is likely to be relatively catastrophic, resulting in rapid establishment of renal dysfunction, and rapid progression to CKD. This is corroborated by Anderson et al., who have shown that the zebra fish protein *apol1*, which is orthologous to human APOL1, is needed for normal kidney function in zebra fish embryos. In *apol1* knockdown zebra fish embryo's, kidney function is rescued by introduction of wild type human APOL1 (G0) into the embryo, but not by APOL1 G1 or G2 protein (Anderson et al., 2015).

In summary, this study provides evidence that the *APOL1* genotypes G1 and G2 are risk alleles for SLE and LN. In addition, the G1 and G2 are also likely to be associated with progression to CKD, in that these two genotypes are most likely the "second hit" that allows progression to CKD. This is in keeping with Freedman et al.,

who have demonstrated a strong association with ESRD and the *APOL1* G1/G2 compound heterozygous state in LN (Freedman et al., 2014). As Anderson et al. have shown rescue of *apol1* knockdown zebra fish embryo's with human APOL1 G0, it would be interesting to assess the effect of APOL1 G0 protein on injured podocytes, such as FSGS, HIVAN, hypertensive nephropathy or even LN (Anderson et al., 2015, 2015).

Chapter 9: Appendix 1 – Clinical and immunologic criteria used in the SLICC classification criteria.

Clinical criteria

1. Acute cutaneous lupus, including lupus malar rash (do not count if malar discoid)
    a. bullous lupus
    b. toxic epidermal necrolysis variant of SLE
    c. maculopapular lupus rash
    d. photosensitive lupus rash in the absence of dermatomyositis or subacute cutaneous lupus (nonindurated psoriaform and/or annular polycyclic lesions that resolve without scarring, although occasionally with post inflammatory dyspigmentation or telangiectasias)
2. Chronic cutaneous lupus, including classical discoid rash
    a. localized (above the neck)
    b. generalized (above and below the neck)
    c. hypertrophic (verrucous) lupus
    d. lupus panniculitis (profundus)
    e. mucosal lupus
    f. lupus erythematosus tumidus
    g. chillblains lupus
    h. discoid lupus/lichen planus overlap
3. Ulcers (In the absence of other causes, such as vasculitis, Behcets, infection (herpes), inflammatory bowel disease, reactive arthritis, and acidic foods)
    a. Oral (Palate, buccal or tongue)
    b. Nasal
4. Nonscarring alopecia (diffuse thinning or hair fragility with visible broken hairs) in the absence of other causes such as alopecia areata, drugs, iron deficiency and androgenic alopecia
5. Synovitis involving two or more joints, characterized by swelling or effusion OR tenderness in 2 or more joints and thirty minutes or more of morning stiffness.
6. Serositis(in the absence of other causes, such as infection, uremia, and Dressler's pericarditis)
    a. Typical pleurisy for more than 1 day
        i. Or pleural effusions
        ii. Or pleural rub
    b. Typical pericardial pain (pain with recumbency improved by sitting forward) for more than 1 day
        i. Or pericardial effusion
        ii. Or pericardial rub
        iii. Or pericarditis by ECG
7. Renal
    a. Urine protein/creatinine (or 24 hr urine protein) representing 500 mg of protein/24 hr
    b. Or red blood cell casts
8. Neurologic
    a. Seizures
    b. Psychosis

    c. Mononeuritis multiplex (in the absence of other known causes such as primary vasculitis)
    d. Myelitis
    e. Peripheral or cranial neuropathy (in the absence of other known causes such as primary vasculitis, infection, and diabetes mellitus)
    f. Acute confusional state (in the absence of other causes, including toxic-metabolic, uremia and drugs)
9. Hemolytic anemia
10. Leukopenia/Lymphopenia
    a. Leukopenia (< 4000/mm^3 at least once) (in the absence of other known causes such as Felty's, drugs, and portal hypertension)
    b. Lymphopenia (< 1000/mm3 at least once) (in the absence of other known causes such as corticosteroids, drugs and infection)
11. Thrombocytopenia (<100,000/mm3) at least once (in the absence of other known causes such as drugs, portal hypertension, and TTP)

Immunological criteria

1. ANA above laboratory reference range
2. Anti-dsDNA above laboratory reference range, except ELISA: twice above laboratoryreference range
3. Anti-Sm
4. Antiphospholipid antibody: any of the following
    a. Lupus anticoagulant
    b. False-positive RPR
    c. Medium or high titer anticardiolipin (IgA, IgG or IgM)
    d. Anti-β2 glycoprotein I (IgA, IgG or IgM)
5. Low complement
    a. Low C_3
    b. Low C_4
    c. Low CH50
6. Direct Coombs test in the absence of hemolytic anemia

Criteria are cumulative and need not be present concurrently. A total of at least 4 criteria with a minimum of one in each of the clinical and serological criteria, or biopsy proven lupus nephritis with a positive ANA or anti DNA antibody are needed to diagnose SLE.

Chapter 10: Appendix 2 - The 2003 International Society of Nephrology/Renal Pathology Society classification of lupus nephritis

Class I	**Minimal mesangial lupus nephritis** Normal glomeruli by light microscopy, but mesangial immune deposits are seen by immunofluorescence.
Class II	**Mesangial proliferative lupus nephritis** Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits. Maybe a few isolated subepithelial or subendothelial deposits visible by immunofluorescence or electron microscopy, but not by light microscopy.
Class III	**Focal lupus nephritis** Active or inactive focal, segmental or global endo- or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations.
Class IV	**Diffuse lupus nephritis** Active or inactive diffuse, segmental or global endo- or extracapillary glomerulonephritis involving ≥ 50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations.
Class V	**Membranous lupus nephritis** Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations. May occur in combination with class III or IV.
Class VI	Advanced sclerosis lupus nephritis ≥ 90% of glomeruli globally sclerosed without residual activity.

Taken from Markowitz and D'Agati, 2007.

Chapter 11: Appendix 3 – Ethics clearance certificate M10707

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Nimmisha H Govind

CLEARANCE CERTIFICATE	M10707
PROJECT	The Genetics of Rheumatoid Arthritis in Black South Africans (revised title)
INVESTIGATORS	Dr Nimmisha H Govind
DEPARTMENT	Department of Medicine/Rheumatology
DATE CONSIDERED	30/07/2010
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 18/01/2013 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof M Tikly

DECLARATION OF INVESTIGATOR(S)

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

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Chapter 12: Appendix 4 – Ethics clearance certificate M130727

M130727M130727

R14/49 Dr Wesley van Hougenhouck-Tulleken et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M130727

NAME: (Principal Investigator)	Dr Wesley van Hougenhouck-Tulleken et al
DEPARTMENT:	Internal Medicine School of Health Sciences
PROJECT TITLE:	Systemic Lupus Erythematosus in a Black South African Population - Screeening for Candidate Susceptibility Loci
DATE CONSIDERED:	26/07/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Mohammed Tikly
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 13/08/2013

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

Chapter 13: Appendix 5 – Study Patient Information Sheet

INVITATION TO PARTICIPATE IN A STUDY ON SYSTEMIC LUPUS ERYTHEMATOSUS GENETICS

Patient Information Sheet

Hi,

My name is Wesley Tulleken. You have been diagnosed with systemic lupus eryhthematosis (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 refuse. 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. The blood sample will be stored for ten (10) years and may be used for further genetic studies on SLE in the future. 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

The University of the Witwatersrand

Prof. M Tikly

(011) 933-9577

Chris Hani Baragwanath Hospital

The University of the Witwatersrand

Professor Peter Cleaton-Jones

Chairman of the Human Research Ethics Committee

University of the Witwatersrand

(011) 717-2301

Chapter 14: Appendix 6 – Study Patient Consent Form

INFORMED CONSENT TO 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 eryhthematosis (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

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: _____

Chapter 15: Appendix 7 – Certificate of submission for examination of masters research report signed by higher degrees candidates

Full name	Wesley van Hougenhouck-Tulleken		
Student number	9809684w		
Title of submitted Research Project: Systemic Lupus Erythematosus in a Black South African population – Screening for Candidate Susceptibility Loci *Please note that if this title is different to your previously approved title, no further action can be taken by the Faculty Office until a change of title has been approved.*			
Contact no	083 311 5076	E-mail	westulleken@gmail.com

1. If you are likely to move in the next 6-12 months please give the anticipated date of move: Not Applicable
2. I hereby submit my **Masters (research report)**
3. I have checked all copies of my research report and declare that no pages are missing or poorly reproduced.
4. I have submitted two bound copies and one copies on CD

5. I confirm that I have:

a. A signed declaration indicating my understanding of the concept of plagiarism and a denial of plagiarism in my research document
b. A report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document included as an appendix.

6. I confirm that I have:

a. Not used either human or animal tissue or records No
b. If yes: I have included the ethics waiver letter pertinent to my research as an appendix Yes
c. Done research using animals No
   If yes: I have included a copy of the animal ethics committee clearance certificate as an appendix in this document Not applicable
d. Done research using human subjects, human tissue or patient records Yes
   If yes: I have included a copy of the human ethics clearance certificate as an appendix to the research document Yes

7. I understand that I may not graduate unless my University fees have been paid in full
8. My supervisors names, departments, telephone numbers and email addresses are as follows:

Name	Prof. M. Tikly		
Department	Internal Medicine		
Telephone	011 933 8940	E-mail	tikly.mohammed01@gmail.com
Name	Prof. M. Ramsay		
Department	Human Genetics		
Telephone	011 717 6630	E-mail	Michele.Ramsay@wits.ac.za
Name	Dr. N. Govind		
Department	Internal Medicine		
Telephone	011 933 8940	E-mail	nimmisha.govind@gmail.com

List all publications, which you have published in peer-reviewed journals from your postgraduate research report/dissertation/thesis during the course of your studies in the Faculty of Health Sciences (Include authors, year, title of paper, name of journal, volume number and page numbers). This information is mandatory.

Signature of candidate:  Date: 15 December 2015

Chapter 16: Appendix 8 – Certificate of submission for examination signed by supervisors of higher degrees candidates

Full name	Wesley van Hougenhouck-Tulleken
Student number	9809684w

Candidate for the degree of: MMed (Internal Medicine) has submitted his research report entitled: Systemic Lupus Erythematosus in a Black South African population – Screening for Candidate Susceptibility Loci

Contact no	083 311 5076	E-mail	westulleken@gmail.com

Mark with an X on appropriate box	**Yes**	**No**
Has this thesis/dissertation/research report been submitted with the acquiescence of the supervisor?	X	
To the best of your knowledge are you able to verify that: This is the candidate's work except as otherwise stated by the candidate?	X	
The substance (nor any part of it) has not been submitted in the past nor is being submitted for a degree in any other university	X	
The candidate has acknowledged wherever any information used in the thesis, dissertation or other work has been obtained by him/her while employed by, or working under the aegis of, any person or organization other than the University or its associated institutions	X	
Have examiners been nominated and approved?	X	

I certify that this thesis/dissertation/research report has the approval of the Animal Ethics Committee/Committee for Research on Human Subjects and the Number of the Certificate of Approval is: M10707 and M130727

List all publications, which your student have published in peer-reviewed journals from his/her postgraduate research report/dissertation/thesis during the course of his/her studies in the Faculty of Health Sciences (Include authors, year, title of paper, name of journal, volume number and page numbers). This information is mandatory.

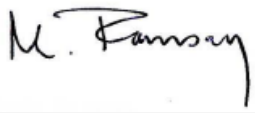
_____
_____
_____
_____
_____

Name of Supervisor: Prof. M. Tikly Telephone: (011) 933 9377

Signature:  E-mail: tikly.mohammed01@gmail.com

Date: 15/12/2015

Name of Supervisor: Prof. M. Ramsay Telephone: (011) 717 6635

Signature:  E-mail: Michele.Ramsay@wits.ac.za

Date: 15/12/2015

Name of Supervisor: Dr. N. Govind Telephone: (011) 933 9377

Signature:  E-mail: nimmisha.govind@gmail.com

Date: 16/12/2015

IMPORTANT NOTICE WITH REGARD TO THE SENATE STANDING ORDERS:

A.22 Submission against advice of Supervisor

If the Supervisor is not prepared to agree to the submission of a thesis, the candidate shall still be entitled, if he or she wishes, to submit it for examination. When a thesis is submitted against the advice of the Supervisor, this should be recorded in the minutes of the Faculty Graduate Studies Committee. In such a case, no internal examiners are appointed but a Supervisor's report will still be required. After the examination process, the external examiner(s) will be advised by the Chairperson of the Faculty Graduate Studies Committee that the thesis was submitted against the advice of the Supervisor.

A.24 *Nomination of Examiners*:

Nomination of examiners should take place at least six weeks before submission of the thesis or dissertation. *(The Postgraduate Office will not accept any submission for examination without the confirmed appointment of the nominated examiners*.)

A.25 Confidentiality of names of examiners (both external and internal)

The names of the examiners should be confidential during the examination process and may only be revealed to the candidate with the acquiescence of the examiner once the final version of the thesis has been submitted to the Faculty and the process has been completed.

Chapter 17: Appendix 9 – Turnitin report

Turnitin Originality Report

MMedWesleyStLucia.docx by Wesley Van Hougenhouck-tulleken

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