GENETIC ASSOCIATION AND INTERACTIONS STUDIES OF HB S AND C GENOTYPES AND *APOL1* AND *MYH9* VARIANTS IN PATIENTS WITH CHRONIC KIDNEY DISEASE

BY

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Declaration

I ERNESTINE KUBI, do hereby declare that with the exception of references to other people's work, which have been duly acknowledged, this thesis is the outcome of my own research conducted at the Noguchi Memorial Institute for Medical Research, University of Ghana, under the supervision of Dr. Anita Ghansah and Prof. Gordon Awandare. Neither all nor parts of this project have been presented for another degree elsewhere.

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Dedication

This work is dedicated to my mother. And it is also dedicated to my cousin Priscilla Kafui Lamptey who is a sickle cell patient and all other sickle cell patients.

Acknowledgement

I thank the Almighty God, for kindness, Grace and for making it possible for me to produce this dissertation. My heartfelt gratitude goes to my supervisor, Dr Anita Ghansah whose constructive criticisms, support, painstaking corrections and guidance helped me immensely during this study. I am also grateful for to my co-supervisor Prof Gordon Awandare for his support throughout the project.

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List of Abbreviations

AKI	Acute Kidney Injury
APOL1	Apolipoprotein 1
APS	Ammonium Per Sulphate
ATP	Adenosine Tri Phosphate
BCL2	B Cell Lymphoma 2
BH3	Bcl-2 Homology region 3
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney disease Epidemiology Collaboration
CKDRN	Chronic Kidney Disease Research Network
CVD	Cardiovascular Diseases
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ESRD	End-Stage Renal Failure
eGFR	Estimated Glomerular Filtration Rate
FSGS	Focal Segmental Glomerular Sclerosis
GWAS	Genome Wide Association Study
H3A	Human Hereditary and Health in Africa
HDL	High Density Lipoprotein
HIV-AN	HIV-Associated Nephropathy
KDOQI	Kidney Disease Outcome Quality Initiative
LD	Linkage disequilibrium
LDR	Ligation Detection Reaction
MAD	Membrane Addressing Domain

MDRD	Modification of Diet in Renal Disease
MYH9	Human non-muscle Myosin Heavy Chain 9 gene
NIH	National Institute of Health
NMIMR	Noguchi Memorial Institute for Medical Research
NSAIDS	Non-Steroidal Anti-Inflammatory Drugs
OPD	Out Patients Department
PTH	Parathyroid Hormone
PCR	Polymerase Chain Reaction
PFD	Pore Forming Domain
SCD	Sickle Cell Disease
SCT	Sickle Cell Trait
SSA	sub-Saharan Africa
SRA	Serum Resistance Associated binding domain
SNP	Single Nucleotide Polymorphism
TBE	Tris-borate-EDTA
TEMED	Tetramethylethylenediamine
W.H.O	World Health Organization

Abstract

Chronic Kidney disease (CKD) is a global public health threat with higher incidence in African populations that is partially associated with SNP variants in *MYH9* and *APOL1* genes. Studies suggests that haemoglobin variants including Hb S and C play a role in CKD pathogenesis. The study aimed to investigate the associations between Hb S, C genotypes, *APOL1* and *MYH9* variants in CKD and their possible interactions to modifying the risk of CKD. Four SNPs, each in *MYH9* and *APOL1* (representing G1 and G2) Hb S and C were genotyped in 537 samples (260 cases-CKD of unknown aetiology and sickle cell nephropathy, 270 controls - healthy individuals and SCD without nephropathy) selected from archived H3Africa Kidney disease Research Network samples. Using chi squared test and logistic regression, the association and strength of association between each SNP and CKD was estimated, adjusting for gender, age and clinical site. Hb S was associated with about 2-9 folds increased risk of developing nephropathy (p-value < 0.02). APOL1 G1 variant rs73885319 conferred between 30-70% increased risk of developing CKD of unknown aetiology (p-value ≤ 0.01). In a recessive and heterozygote model, *MYH9* variant rs4821481 increased the risk of developing nephropathy in SCD by 2-4 folds (OR=1.99 and 4.44 respectively, p-value < 0.01). There was a synergistic epistatic interaction between Hb AS genotype and *APOL1* rs73885319 resulting in a further increased risk (130%) of CKD for heterozygote *APOL1* rs73885319 individuals who are Hb S carriers. Results of the study though preliminary, will inform public health decisions on screening of new-borns for SCD, SCT and other common genetic predispositions of CKD.

CHAPTER ONE

INTRODUCTION

1.1 Background

Chronic kidney disease is an important non-communicable disease of public health menace with an estimation of 200 million people affected worldwide (Ojo, 2014). It involves a range of different pathophysiologic processes that includes all degrees of reduced renal function, from injured through mild, moderate, to severe irreversible kidney disease (Arora, 2017). Untimely death is only part of the problem associated with CKD because some CKD patients eventually progress to End-Stage Renal Disease (ESRD), a condition resulting in disability, poor quality of life and financial cost (De Nicola & Zoccali, 2016). At ESRD, CKD patients require Renal Replacement Therapy (RRT); dialysis or organ transplant to survive.

Over the years, occurrence of CKD has increased in both developed and developing countries at approximately 6% annually with a clear burden on sub-Saharan African (SSA) (Sumaili *et al.*, 2009), affecting mortality, morbidity, and livelihood of individuals (Schoolwerth *et al.*, 2006). Global deaths due to CKD is also rising showing a rise from 27th in 1990 to 13th in 2013 cause of death annually (Jager & Fraser, 2017). The annual incident rate of patients starting RRT is also significantly high, ranging from 150 to 400 per million people (pmp) in developed countries and 50 pmp in low-income countries (Jha *et. al*, 2013).

There are racial, regional and economic disparities in the occurrence and incidence rates of CKD and the rate of progression to ESRD (Nzerue *et al*., 2002). In the US for example, research has shown that African Americans have a disproportionate risk for developing ESRD compared with European Americans and Caucasians (Ashley-Koch *et al*., 2011). African Americans have 4–5 fold higher rates of developing progressive renal disorders including focal segmental glomerulosclerosis (FSGS), hypertensive CKD and HIV associated Nephropathy (HIVAN) as compared to European Americans (Kopp *et al*., 2011). The two leading aetiologies of CKD; Diabetes and hypertension, as well as differences in demographic characteristics, socioeconomic status, lifestyle, clinical factors and access to health care are not enough to account adequately for the excess risk of CKD and ESRD in African Americans (McClellan, 2006). Suggesting that genetics play a role in high risk of kidney disease in Africans.

Genetic studies of CKD has revealed the contribution of 2 genes; *MYH9* and *APOL1* on chromosome 22 have strongly been associated with the risk, incidence and progression of non-diabetic CKD (Freedman *et al*., 2011). Polymorphisms previously identified in *MYH9* gene account for 70% cases of non-diabetic chronic kidney disease and between 40-50% of HIVAN cases (Bostrom & Freedman, 2010). More recent studies have attributed genetic association of kidney disease in Africans to *APOL1* based on stronger statistical association of *APOL1* (G1 and G2) risk variants to CKD compared to the *MYH9* (Genovese *et al*., 2010). Mutations in *APOL1* have been related to the pathogenesis of non-diabetic forms of CKD including FSGS, Nephritic Syndrome, Nephrotic syndrome and HIVAN (Hu *et al*., 2012) all of which can lead to ESRD.

Remarkably not all individuals who have been identified to be either homozygous or heterozygous for the *APOL1* risk variants or with *MYH9* SNPs have been found to develop kidney disease (Freedman & Skorecki, 2014). In individuals with *APOL1* high-risk variants, some additional genetic and/or non-genetic factors have been suspected to be contributing to kidney disease progression (Freedman & Skorecki, 2014).

Individuals homozygous for haemoglobin variant Hb S, develop sickle cell disease (SCD), manifested by anaemia, episodes of vascular occlusion, pulmonary disease, organ damage, infectious complications, and premature mortality (Prabhakar *et al*., 2010). Kidney disease in Sickle cell anaemia has been reported to affect a few patients with SCD and has been considered a key risk cause of early death (Platt *et al*., 1994; Guasch, 2006). Sickle cell trait has also been recognized as an important risk factor for CKD even though there are some conflicting reports (Mukendi *et al*., 2015)

1.2 Rationale

African countries like other developing countries are undergoing an epidemiological transition of disease burden from infectious disease to chronic complex diseases (such as diabetes, obesity, hypertension, and CKD) (Boutayeb & Boutayeb, 2005). Among these diseases, CKD is one of the leading causes of early mortality in SSA. More than 500,000 individuals in SSA develop ESRD (Ojo, 2014) every year and about 70% of CKD patients with ESRD are projected to be from low-income countries of SSA (Stanifer *et al*., 2014). The cost for RRT is high and less than 2% of ESRD patients in SSA have access to it, making ESRD a death penalty for majority of patients (Ojo,

2014). The age onset of CKD in Africans is earlier than in people of other racial origins (Ojo, 2014). Each year there is about 6% -8% increase in ESRD rate in Africa and at current projections, none of the 54 SSA countries will be able to afford the cost of pre-dialysis CKD medical care estimated at $2500 to $20,000 per patient annually (Ojo, 2014).

These disturbing factors raise an imperative need for research to identify the various predisposing factors that may be influencing the disease burden. This will help alleviate the incidence and severity of kidney disease on the African continent. As the knowledge will be employed in developing diagnostics, therapy, and management plan to reduce early disease onset and progression, which will increase the life expectancy of individuals at risk.

APOL1 and *MYH9* do not completely explain the genetic predisposition of Africans to CKD and ESRD. Undeniably, the influence of genetic factors on non-communicable diseases, such as CKD, is complex and certainly involves multiple genes with several gene-gene interactions (Freedman *et al.*, 2011). This, however, calls for further investigations to identify other genetic loci, which could be contributing to development of CKD, and also in understanding the underpinnings of the disease in the African population. In identifying genetic risk factors for the disease in Africa, it may be suitable to revisit some of the most common genetic disorders of African history, such as haemoglobinopathies (Cavanaugh & Lanzkron, 2010).

Haemoglobinopathy associated nephropathy has focused primarily on homozygous Hb SS (Drawz *et al.*, 2016). There are also conflicting reports on the contribution of sickle cell trait to development of nephropathy (Mukendi *et al.*, 2015). In addition

other haemoglobin variants such as Hb C have been associated with CKD (Naik *et al.*, 2015) but have not been fully studied. Further research on the association between haemoglobinopathy S/C genotypes and CKD and their interactions with commonly known *MYH9* and *APOL1* risk variant to the developing renal damage is therefore timely and very significant.

1.3 Hypothesis:

Haemoglobin genotypes S and C are associated with development of CKD and they interact with *APOL1*, and *MYH9* SNPs to modify the risk of Chronic Kidney disease.

1.4 Aim

To determine the associations between Haemoglobin genotypes (S and C), *APOL1* risk variants, and *MYH9* and the risk of developing Chronic Kidney Disease and to characterise any possible gene-gene interactions between these genes and the occurrence of CKD.

1.5 Objectives

The study objectives were to,

1. Genotype Hb S and C, variants in Hb S and C, *APOL1* and *MYH9* variants of the study participant
2. Determine genotype and allele frequencies of Hb S and C, *APOL1* and *MYH9* variants in the study population
3. Determine the association of Hb S and C genotypes, *APOL1* and *MYH9* variants to kidney disease phenotypes.
4. Determine the interactions between Hb S/C genotypes with *APOL1*/*MYH9* variants to the risk of CKD.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Kidney Structure

The kidney is a vital systemic organ which helps in maintaining the body's homeostasis. The human kidneys are a pair of reddish brown, bean-shaped organs located in the high back of the abdominal cavity. Each part of the kidney is well structured such that they are able to meet their functional roles. They have the renal artery which supplies unfiltered blood to the kidney and the renal vein through which filtered blood is delivered back to the body (Guyton *et al.*, 2014). The functional units of the kidney is the nephron. There are about eight thousand to one million nephrons in a normal functioning human kidney (Guyton *et al.*, 2014). Each nephron is divided into two major parts: the renal corpuscle and the renal tubule as shown in fig. 2.1 below. These parts of the nephron filter blood and are consequential to the body's survival.

The renal corpuscle is the beginning of the nephron. It is comprised of a cluster of capillaries called the glomerulus and a surrounding sac-like structure called the Bowman's capsule. The glomerular capillaries consist of endothelial cells, the glomerular basement membrane, and visceral epithelial cells (podocytes) necessary for controlling filtration of molecules based on size and charge. Extending from the glomerular capsule is the renal tubule which is partitioned into; the proximal tubule, loop of Henle, and distal convoluted tubule. The proximal convoluted tubule, has

cells with microvilli extending into the lumen which allows the efficient removal of useful substances from the filtrate, which are eventually reabsorbed into the blood.

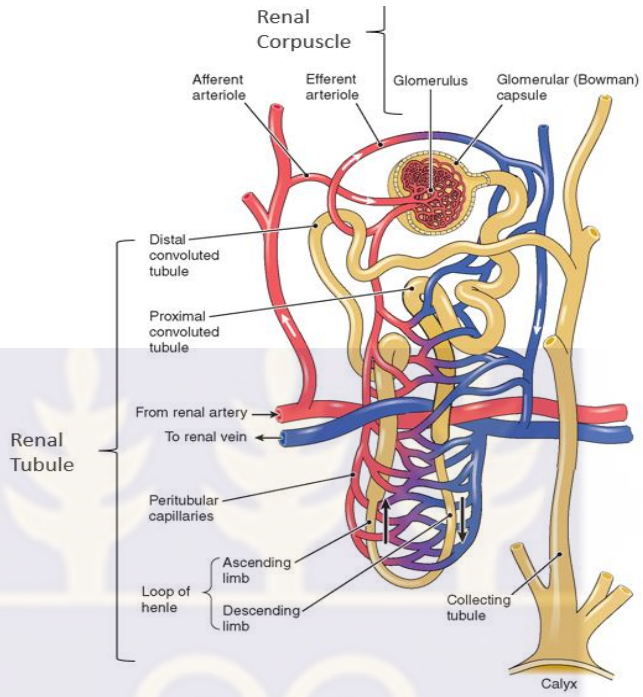

Figure 2. 1 Structure of the nephron (http://what-when-how.com/nursing/the-urinary-system-structure-and-function-nursing-part-1/).

2.2 Functions of the Kidney

The kidney plays several roles in a mammalian system but its core function is to maintain and regulate the body's homeostasis. The regulatory function of the kidney is pivoted by its ability to excrete substance (waste of metabolic processes from the blood, drugs and chemicals) and excess water out of the body. Metabolic waste products like urea, uric acid, metabolites of hormones and creatinine that can accumulate over time and damage the body are filtered from the blood by a cleaning process through filtration and purification using its functional unit, the nephron

(Robson, 2014). Balanced fluid, electrolytes and ions in the cells is vital for sustaining regular cell volume and creating steady environment for physciological functioning of the body. As a complex organ linked with circulatory and excretory systems, it orders the systemic maintenance of the pH of body fluids (Robson, 2014). In addition, the kidneys also serve as important endocrine organs. They secrete hormones necessary for bone formation (calcitriol), red blood cell production (erythropoietin), and regulation of blood pressure (renin) (Guyton *et al.*, 2014).

2.3 Chronic Kidney Diseases (CKD)

The major impact of kidney associated diseases is the loss and reduction of functioning nephrons and the rate of glomerular filtration which becomes lethal by the inability of the kidney to maintain its homeostatic function over a period of time (Hogg *et al.*, 2007). Kidney disease is a multi-aetiologic syndrome which may lead to temporary acute kidney injury (AKI) or persistent enduring chronic kidney disease (Perkins *et al*., 2016; Schwartz *et al.*, 2003).

Chronic kidney disease unlike AKI is progressive, irreversible and occurs over a long period of time with a gradual decline in kidney function (Freedman *et al*., 2012). It is associated with pathological abnormalities that affect the glomerular or tubular structures of the nephron, which eventually damage the kidney (Levey *et al*., 2005). The National Kidney Foundation Kidney Disease Outcome Quality Initiative (NFK-KDOQI) has defined CKD as the presence of kidney damage and estimated glomerular filtration rate (eGFR) less than 60ml/min/1.73m2 persisting for at least three months irrespective of aetiological origin (Hogg *et al*., 2003).

In 2002 CKD was classified into five stages based on the estimated GFR and the severity of damage which is determined by the measure estimated GFR (Table 2.1). This classification serves as guidelines for observing the progression and severity of CKD with associated adverse health effects and possible treatment stage considering the underlying causes (National Kidney Foundation, 2002). As kidney deterioration progresses from the early stages, it reaches a stage (stage 5) referred to as end stage renal disease (ESRD). At this advanced stage of kidney damage, there is almost a complete loss in the ability of the kidney to get rid of the body's waste effectively. This leads to a build-up of electrolytes, metabolic waste and fluids in the body which is fatal without any renal replacement therapy (Mcclellan & Flanders, 2003). The rate of CKD progression to ESRD is dependent on coexisting pathologies and associating risk factors (Nugent *et al.*, 2011).

Table 2. 1 Classifications of Chronic Kidney Disease (Levey *et al.* , 2003; Hogg *et al.*, 2003).

Stage	GFR*	Description	Actions/Interventions
1	90+	Normal kidney function but urine findings, structural abnormalities and genetic traits point to kidney disease	Treat comorbid conditions, reduce cardiovascular disease risk, slow the progression
2	60-89	Mildly reduced kidney function and other findings (stage 1) point to kidney disease	Estimate progression, treat underlying cause to slow the progression
3A 3B	45-59 30-44	Moderately reduced kidney function	Evaluate and treat complications
4	15-29	Severely reduced kidney function	Prepare for kidney replacement therapy
5	<15/ on dialysis	Very severe or **end stage kidney disease** (sometimes established as **kidney failure**	Kidney replacement (dialysis/transplant)

2.3.1 Symptoms of CKD

The initial stages of CKD are mostly undetected (asymptomatic) until after stage three when about 50% of kidney functions are lost (Wachukwu *et al.*, 2016). Nonetheless, early detection can help in early treatment and management to delay progression and keep the disease from getting worse (Wing *et al.*, 2014). The early symptoms that are usually associated with CKD are similar for some other common diseases and may be symptoms of the underlying cause (National Kidney Foundation, 2010). Some of the common symptoms associated with CKD, include insomnia, muscle constrictions, swollen feet and ankles due to fluid retention, restless leg syndrome, fatigue, poor concentration, a poor appetite, dry itchy skin, puffy eyes, paleness and excessive urination (National Kidney Foundation, 2010; Rahman *et al.*, 2012). Abnormal functioning of the kidney may also lead to accumulation of toxins and fluid which may express different signs that include Oedema in lower legs, feet and face and hands swelling (Vassalotti *et al*., 2007).

2.3.2 Diagnosis of chronic kidney disease

The asymptomatic nature of CKD poses as a challenge towards early diagnosis and clinical management. Most times, CKD is detected by chance, especially when the patient presents clinical manifestations defining another infection or disease or when routine laboratory tests are performed to detect other underlying aliments (National Kidney Foundation, 2010).

Generally, kidney function is measured by its ability to clear toxins from and metabolic waste from the blood. The gold standard for measuring the filtering capacity of the kidney has been by measuring the renal clearance of Inulin; a plant polysaccharide molecule which is easily filtered by the glomerulus (Khurana, 2008). Though very accurate, the procedure is expensive, invasive and cumbersome.

Largely, common signs and symptoms pointing to kidney defects form the basis and background for detecting chronic kidney disease. The significant rise in blood urea and levels of creatinine in the blood, high blood pressure and proteinuria are often markers for diagnosis. An alternative to inulin clearance 24-hours creatinine clearance test has been recommended for routine clinical testing (Khurana, 2008). The accurate measure of creatinine by renal clearance is used to estimate the eGFR. The blood test analysis is also sometimes complemented by urinalysis (Iseki, 2011). Universal urinalysis screening has also been considered as a standard diagnostic procedure that measures blood and urine urea levels for prompt and early detection of chronic kidney disease (Iseki, 2011).

A good understanding of the anatomy of the kidney has been established to be a useful tool in the diagnosis of chronic kidney disease. Radiography is helpful in identifying and evaluating patients with rise in creatinine level with realistic precision and accuracy (Neill, 2014). Tomography and 3D-Imaging of the kidney tissue provide information on the stage of the chronic kidney disease, and the extent of damage done to the kidney by different risk factors. Nephrologists commonly use ultrasound for kidneys diagnostic studies (Neill, 2014); this achieved by kidney tissue biopsy, guided by renal sonographic appearances, to detect, and diagnose CKD (Neill, 2014).

2.3.4 Complications Associated with Chronic Kidney Disease

The complications associated with CKD affects the quality of life and ultimately lead to death in due course or at the end (Levey *et al*., 2005). The common complications CKD include cardiovascular disease, high blood pressure, anaemia, malnutrition, bone disorders, neuropathy, gastrointestinal symptoms, electrolyte imbalance, abnormal calcium and phosphorous metabolism, immune disorder, low quality of life, progression to kidney failure and premature death (Levey *et al*, 2003; Levey *et al*, 2005; Snyder & Pendergraph, 2005).

2.3.4.1 Anaemia

Anaemia is one of the major complications of chronic kidney disease. It is defined as a shortage in one of the major indicators of red blood cell, such as RBC count or concentration of haemoglobin (Lau *et al*., 2015). Anaemia in CKD occurs primarily due to reduced production of erythropoietin by reduced total kidney mass (McClellan *et al*., 2004). It has been reported that a decrease in the erythrocytes indices accompanies rate of progression of CKD (McClellan *et al*., 2004). Chronic kidney disease associated anaemia increases morbidity and death from cardiovascular complications which may lead to further decline in renal function and the formation of a vicious cycle termed the ‘‘cardiorenal anaemia syndrome’’ (Thomas *et al.*, 2008).

2.3.4.2 Mineral and bone disorders

Moe *et al.* (2006) found out that complications associated with CKD can also be attributed to mineral and bone disorders (Thomas *et al*., 2008). These disorders were defined as bone formation or mineral metabolism abnormalities with secondary

effects on CKD (Gal-Moscovici & Sprague, 2007). Bone and mineral disorders occur when there is an imbalance in calcium and phosphorus in the blood. It is mostly manifested by irregularities of calcium, phosphorus, parathyroid hormone (PTH), and vitamin D metabolism.

The kidneys excrete excess phosphorus from the blood to balance calcium and phosphate levels which helps maintain proper and bone structure (Thomas *et al*., 2008). Calcitriol is produced in the kidney to help the kidneys maintain blood calcium level and promotes the formation of bone. Reduced renal function reduces calcitriol and blood calcium levels increasing phosphate levels. This causes an imbalance of blood calcium phosphate levels with a resultant increase in PTH secretion (Thomas *et al.*, 2008). Increases PTH secretion stimulates osteoclasts, which break down bone to release calcium into the bloodstream. The breakdown of bone tissue by osteoclasts causes weaker bone and distorts bone architecture. Some of the calcium released into the blood may get deposited in the heart and blood vessels increasing the risk of cardiovascular disease in CKD (Thomas *et al.*, 2008). In effect, mineral and bone disorders associated with CKD increase the rate of mortality and morbidity of the CKD patients (Gal-Moscovici & Sprague, 2007).

2.3.4.3 Cardiovascular Diseases (CVD)

The rate of mortality of cardiovascular disease associated with renal disease at the final stage is higher in patients on dialysis as compared to the general population of CKD patients (Sarnak *et al*., 2003). As the progression in kidney diseases increases, the risk of cardiovascular disease increases; from mild to moderate, and to maximum

as the condition gets worse. Studies have shown mortality due to cardiovascular disease to be about twice as high in patients with stage 3 CKD and three times higher at stage 4 than that in individuals with normal kidney function (Ardhanari, Alpert, & Aggarwal, 2014).

Chronic Kidney Disease patients are most likely to die of CVD than to develop kidney failure (Schulman *et al*., 2014). A report by the U.S. Renal Data System published in 2013, also showed 43% of patients with CKD patients with CVD had heart failure (HF), and 15% had a history of acute myocardial infarction; with corresponding non-CKD patients with CVD showing 18.5% and 6.4% respectively (Ardhanari *et al.*, 2014).

The association of CVD with CKD is relevant because CVD in CKD is treatable, potentially preventable, and CKD is also a risk factor for CVD (Ardhanari *et al.*, 2014). The NFK task force proposed that patients with CKD be considered in the "highest risk group" for future CVD episodes and that treatment recommendations based on CVD risk stratification should consider the highest-risk status of patients with CKD (Sarnak *et al*., 2003).

2.3.5 Treatment of chronic kidney disease

Like other diseases, infectious or otherwise, early detection of CKD and treatment provides proper handling procedures of the complications that are involved (Qaseem *et al*, 2013). Appropriate clinical management will perhaps prevent and delay the complicated stages of CKD and advancement to ESRD (Bastos & Kirsztajn, 2011).

Treatment of CKD is dependent on the different stages of the disease and the progressive state of the complications and damage associated (Hogg *et al.*, 2005). The stage by stage treatment guideline provided by the NFK is shown in table 2.1 above.

Interventions through the inhibition of angiotensin converting enzyme or angiotensin receptor blockers (to reduce proteinuria), control of blood glucose levels, and control of blood pressure have been established to delay the progression of chronic kidney disease (De Jong & Gansevoort, 2008; Mayer, 2014). In advanced and severe cases of CKD when the GFR declines to a point lower than 15ml/min/1.73m^2, RRT is required (Levey *et al.* , 2003; Hogg *et al.*, 2003).

To circumvent total kidney failure, dialysis is the considerable treatment and most common approach used to assume the role of filtering and cleaning of the blood. Though dialysis cannot completely play the role of a healthy kidney, but still, helps preserve the lives of CKD patients; though, time-consuming and effort demanding (Just *et al*., 2008). Kidney transplant has been established as the best form of treatment for patients with total kidney failure even though it's much more expensive and requires a willing donor (Karam *et al*., 2014). Generally, the cost of renal RRT is high and imposes patients, health care systems and the society with an enormous economic burden (Schoolwerth *et al*., 2006).

2.3.6 Epidemiology of Chronic Kidney Disease

Aging of the world's population, diet and other lifestyle-related adjustable risk factors, accompanied by a drop in early-life infectious diseases, have stemmed the emergence of chronic diseases as a major global health menace (WHO, 2005). Chronic Kidney Disease is of particular significance and has been listed as one of the non-communicable chronic diseases that are becoming more and more acknowledged in developing countries (Stanifer *et al*., 2014). Chronic Kidney Disease is a global public health problem with a huge burden on the public health system, and a risk factor for cardiovascular disease and increase in premature mortality (Hill *et al.*, 2016). It was reported as the 27th cause of total global deaths in 1990 rising to the 18th by 2010 according to a global disease study (Lozano, 2010); it has currently been ranked as the 12th highest cause of death and 17th highest cause of disability worldwide (Nugent *et al*, 2011).

The estimation of CKD prevalence is associated with age, gender, race, and several other clinical and demographic factors (Coresh *et al*., 2007). The rapid surge in the incidence of diabetes and hypertension, as well as obesity, is projected to impose an even greater and more overwhelming burden of CKD that developing countries are not equipped to manage (Naicker, 2009). The racial disparities in incidence of CKD present a strikingly high prevalence in non-Hispanic black and Mexican-American and Africans than other racial groups (Coresh *et al.*, 2007).

In a small population survey, an estimation of 200-300 per million people are known to be affected by CKD (Naicker, 2010). In SSA CKD affects largely young adults aged 20–50 years, with hypertension and glomerular diseases as the predominant

cause, unlike developed countries, where CKD presents in middle-aged and elderly people (Jha *et al*., 2013). Hypertension associated CKD, the leading aetiology of CKD in SSA estimated prevalence across Africa ranges from 25% in Senegal to 29.8% in Nigeria, 45.6% in South Africa, and 48.7% in Ghana (Naicker, 2013). The overall prevalence of CKD in Africa is estimated at 14% with both Ghana and Nigeria (the target population for this study) recording about 17% prevalence (Jha *et al*., 2013).

The exact prevalence data on chronic kidney disease is however lacking, mostly because the disease is asymptomatic and not detectable at the earlier stage (Hill *et al*., 2016). Most of the available statistics and prevalence data are estimated from those on renal replacement therapies. In developing countries, especially majority of the African countries, information on the occurrence and frequency of CKD is scarce and limited due to lack of documentation and renal record and database (Naicker, 2009). Nonetheless, the incidence rate of CKD in developing countries is projected to be about 3-4 times higher than in high-income countries (Naicker, 2013). In developed countries, ESRD patients on RRT are monitored, allowing for a reasonably reliable data on the prevalence of ESRD, though the proportion of patients with ESRD is just a small fraction of the entire CKD prevalence (Collins *et al*., 2012).

The global incidence estimate of CKD stages made in a systemic review and meta-analysis of CKD prevalence was; Stage 1; 3-5%, stage 2; 3-9%, Stage 3; 7-6%, Stage 4; 0-4% and Stage-5; 0-1% (Hill *et al*., 2016). According to a global dimension perspective made on CKD in 2013 (Jha *et. al*, 2013), 150 to 400 per million people (pmp) in developed countries to 50 pmp or maybe less in low-income countries where access to health care is limited are estimated to be on RRT. By the end 2013, an

estimation of 3.2 million people were on renal replacement therapy, with approximately 2,522,000 people undergoing dialysis treatment and 678,000 people to be living with renal transplants ("ESRD Patients in 2013," 2013).

Despite the high global menace of CKD, more than 100 countries have no provisions for long-term maintenance of renal replacement therapies and thus, more than 1 million people die annually from ESRD (Lozano *et al.*, 2012). This presents a significant public health challenge, especially in developing countries and therefore, demands special attention and preventive interventions.

2.3.7 Aetiologies of Chronic Kidney disease

Many factors contribute to the incidence of CKD. Among the most widely spread causes of CKD, hypertension and diabetes are the most predominant, accounting for up to about 60% of CKD ("National Kidney Foundation," 2010). Other factors that lead to CKD include chronic glomerulonephritis, autoimmune disorders, systemic infections, urinary tract infections, bladder stones and genetic diseases.

2.3.7.1 Diabetes

Diabetes is progressively recognized as a public health menace associated with increased mortality and co-morbid conditions and has been established as the number one leading cause of kidney failure and ESRD (Naicker, 2013). Diabetic kidney disease is known to be one of the very severe complications of type 2 diabetes with an estimation of 40% diabetic patients recorded to develop CKD annually (Evans, 2011) and is the most common cause of ESRD worldwide.

In the course of diabetes, blood vessels in the body including the filters of the kidney get damaged due to a build-up of sugar in the blood. This causes a pathological and functional effect on the kidney which eventually leads to kidney failure. The earliest indicator of kidney failure in diabetes is the presence of albuminuria. The presence of albuminuria is known to increase prevalence and rate of initiation of diabetic nephropathy by 80% (Dronavalli *et al*., 2008). Diabetic nephropathy is more common in diabetic type II patients and this has been attributed to genetic susceptibility (Bergrem & Leivestad, 2001). Aside the genetic factors, behavioural and environmental factors are considered very relevant to the development of diabetic nephropathy (Bergrem & Leivestad, 2001).

2.3.7.2 Hypertension

Hypertension is the second leading aetiology of CKD that plays a major role in the onset, progression of CKD and ESRD (Bidani & Griffen, 2004). It is an initiation factor that causes renal injury and is also a complication of kidney damage, contributing to the complexity of hypertensive nephropathy (Staples, 2010). During hypertension, a point at which the force of blood flow is high, veins expand so blood can flow easily and effectively. Inevitably, this expansion scars and debilitates veins throughout the body, including those in the kidneys. Long-term uncontrolled hypertension leads to high intra glomerular pressure, causing damage to the glomerulus and ultimately impairing glomerular filtration (Hering *et al.*, 2016). The kidney ultimately becomes unable to regulate blood pressure due to a build-up of extra fluid in blood raising the blood pressure even the more. Regardless of the underlying cause of hypertension, once hypertension develops, a cycle of kidney

injury, nephrosclerosis, worsening of hypertension and further renal damage arises (Fervenza, 2012). Thus, early detection and proper control procedures are important for managing hypertension and prevention or slowing down progression of kidney disease.

2.3.7.3 Glomerulonephritis

Glomerulonephritis also known as glomerular disease is used to refer to conditions that cause inflammation and damage to the glomerulus of the kidney. Even though the exact cause of glomerulonephritis is not really known it may occur on its own or as part of other illness such as lupus, repeated episodes of acute glomerular inflammation, infection and diabetes (Vos *et al*., 2015). Chronic glomerulonephritis is the third leading cause of CKD globally and is known to have accounted for about 10% of all kidney patients on dialysis (Barsoum, 2006). Mostly, proteinuria develops as a result of damaged glomerular membrane, and sometimes it may be due to weakening of the kidney glomerular re-absorption (Aitken *et al*., 2014). Proteinuria in effect facilitates activation of complement leading to inflammatory responses in tubules of the epithelial which is responsible for progression of CKD (Abbate *et al.*, 2006).

2.3.8 Risk factors of CKD

In addition to the major underlying causes of kidney failure, the age of disease onset and the rate of disease progression may be influenced by the presence of certain risk factors. Studies have shown kidney diseases to have been associated with certain risk

factors; modifiable (can be adjusted) and non-modifiable (cannot be adjusted or changed) supported by clinical and epidemiological data (Levin, 2001). Depending on how these factors contribute to CKD occurrence, they are also grouped as, initiating factors, susceptibility factors, progression factors, and end stage factor (Levey *et al*., 2003) as shown in table 2.2 below.

Table 2. 2 Categorized risk factors for chronic kidney disease (Levey et al., 2003)

Risk Factor	Definition	Examples
Susceptibility factors	Increase susceptibility to kidney damage	Older age, family history of chronic kidney disease, reduction in kidney mass, low birthweight, U.S. racial or ethnic minority status, low income or education
Initiation factors	Directly initiate kidney damage	Diabetes, high blood pressure, autoimmune diseases, systemic infections, urinary tract infections, urinary stones, lower urinary tract obstruction, drug toxicity
Progression factors	Cause worsening kidney damage and faster decline in kidney function after initiation of kidney damage	Higher level of proteinuria, higher blood pressure, poor glycaemic control in diabetes, smoking
End-stage factors	Increase morbidity and mortality in kidney failure	Lower dialysis dose (Kt/V), temporary vascular access, anaemia, low serum albumin level, late referral

2.3.8.1 Lifestyle

While a number of factors such as diabetes, hypertension and infections have been strongly associated with CKD, a number of modifiable lifestyle factors such as excessive alcohol intake, smoking, and obesity are also considered to pose risk for developing CKD (Stengel *et al.*, 2003). These factors are known to be associated with certain types of CKD eg. Diabetic CKD (Stengel *et al.*, 2003).

Obesity is a global challenge, and it has been linked with increased incidence of ESRD and contributes to the burden of CKD, especially in developing countries, with a higher incidence in Africa (Brown *et al*., 2000). High body mass index can be responsible for high risk of kidney disease and also contribute to the faster progression of CKD (Brown *et al*., 2000). According to the NFK, obesity, excessive smoking, poor diet, and physical inactivity increases the risk of kidney disease to about 300% irrespective of age, gender and race (Levey *et al*., 2011). In diabetic patients, physical inactivity and chronic smoking have been associated with increased rate of nephropathy and renal damage (Stengel *et al.*, 2003). Smoking and alcoholism which increases the risk of cardiovascular diseases also potentially increases the risk of CKD in hypertension patients (Ghonemy *et al*., 2015).

2.3.8.2 Use of Analgesics

Analgesics are mostly used to relieve pain and causes no harm when taken in the right dosage and not abused. Prolonged use of analgesics, especially over-the-counter (OTC) drugs that contain phenacetin or acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin or ibuprofen increase the risk of kidney damage (Nderitu *et al*., 2013). Analgesic nephropathy involves damage within the internal structures of the kidney causing renal papillary necrosis and interstitial nephritis (Turgut *et al*., 2007). Research shows that the effects of analgesics such as acetaminophen, aspirin, and non-selective NSAIDs on CKD increase aggressively in a dose-dependent manner (Kuo *et al*., 2010). Though the minimal use of analgesics is considered harmless, it is considered a significant contributing factor on renal

function and can cause acute or chronic renal failure, especially in the elderly (Turgut *et al.*, 2007) hence its use should be monitored and managed.

2.3.8.3 Age

Aging is a normal, progressive and unavoidable biological process characterized by a steady decline of cellular function as well as progressive structural changes in many organs. Aging is a common non-modifiable factor that contributes to the incidence of CKD and disease progression in both males and females, health condition notwithstanding (Hare *et al.*, 2007). Age significantly contributes to the progression of CKD, although there is little information on whether the CKD in older patients should be differently defined as compared to the general concept of CKD (Hare et al., 2007; Nitta, Okada, Yanai, & Takahashi, 2014). Scarring of the glomerulus begins at about age 30, and by the age 70, the mesangial has increased by 12% (Nitta *et al.*, 2013). This is followed by the structural and physiological changes of the glomerulus and the nephron. The scarring of the glomerulus also results in sclerosis, causing about 30% decline in kidney function by age. The deterioration in glomerular filtration progression rate varies significantly amongst individuals, and faster in CKD patients as compared to the general population (Voormolen *et al.*, 2007).

2.3.8.4 Gender

Though there is some disparity, what has consistently been reported by most research finding is that the incidence of CKD and disease progression is higher in men than women (Denic *et al.*, 2016). A survey conducted by the Japanese Society for Dialysis Therapy recorded a lower mean age of disease onset of CKD in men compared to

women (Iseki, 2008). Men with diabetes have a higher risk of nephropathy than women with diabetes (Denic *et al.*, 2016). The gender disparities in the occurrence of CKD and progression to ESRD may be influenced by other risk factors such as such as hypertension, hyperglycemia, albuminuria, dyslipidemia, body mass index, lifestyle and renal structure and sex hormones (Chang *et al.*, 2016). The prevalence of kidney failure is known to be higher in women than men, however, there seems to be a higher progression rate and mortality risk of CKD in men compared with women, except in post-menopausal women and diabetic patients (Goldberg & Krause, 2016). Higher estrogen levels in women give women a protective effect against CKD which declines gradually as one approaches menopausal age as estrogen level drops (Weinstein & Anderson, 2010).

2.3.8.5 Ethnicity

Ethnicity is difficult to qualify as a risk factor in seclusion, due to its association with a number of confounding factors related to socioeconomic status (Evans, 2011). This notwithstanding, epidemiological data shows that ethnicity can still be considered as an important risk factor even after ruling out economic factors (Evans, 2011). The 23rd annual US Renal Data Report System reports that the incidence of CKD in African Americans and Native Americans is about 4 and 2 times respectively higher than in European Americans (Collins *et al*., 2012). However, higher prevalence of CKD has been observed in European Americans (13.8%) as compared to African Americans (11.7%) (Coresh *et al*., 2007). This indicates that CKD is more prevalent in white populations but less likely to progress to severe stages as compared to African populations (Evans, 2011). More recent studies have also shown that African

Americans and indigenous Africans especially those with sub-Saharan African Ancestry have a higher risk of CKD and earlier age onset of disease as compared to other racial groups.

2.3.8.6 Genetic Factors

i. Myosin Heavy Chain 9 (MYH9)

The identification of the *MYH9* gene to be associated with non-diabetic kidney disease came as a breakthrough in kidney disease genomics (Bostrom & Freedman, 2010). The 110kb MYH9 gene encodes a 1960 amino acid residue protein, a non-muscle myosin made of two heavy chains and four light chains with the heavy chain having a head region and a tail region (Kopp *et al*., 2008). In the kidney MYH9 is synthesised in the glomerulus, precisely the podocyte, peritubular capillaries and tubules (Bostrom & Freedman, 2010).

About 45 mutations in the *MYH9* gene, have associated with a number of rare disorders (Giant Platelet Syndromes) such as deafness autosomal dominant type 17, Epstein syndrome, Alport syndrome and Fechtner syndrome, with progressive sensorineural deafness (Tzur *et al*., 2010). Renal biopsies have revealed ultrastructural evidence of podocyte injury in individuals with such *MYH9* related disorders (Seaghdha & Fox, 2011). Genetic studies have led to the identification of haplotype SNPs in *MYH9* to be associated with the increased risk of certain kidney diseases such as glomerulonephritis and ESRD (Bostrom *et al*., 2010).

The mechanism by which MYH9 induces kidney damage has not been well elucidated. MYH9 protein binds to actin at the head region and this aids in its role in cell motility, cell shape maintainance and cytokinesis. Considering its function, Johnstone *et al.* hypothesized that MYH9 contributes to renal diseases by disrupting the podocyte (Johnstone *et al*., 2011). This hypothesis was based on the knowledge that podocytes are sensitive to the discomposure of the cytoskeleton, and the interaction of the protein with actin to contract the cytoskeleton and maintain membrane tension and cell shape (Johnstone *et al*., 2011). It is therefore important to note that most of the mutations that tend to lead to more severe diseases are found in the head domain which drives the function of the protein (Nganga, 2015).

Association between MYH9 and CKD

The genetic association of *MYH9* to chronic kidney disease was first reported in 2008 (Kopp *et al.*, 2008). A Mapping by admixture linkage disequilibrium (MALD) study was carried out by Kopp *et al* in 2008 and they identified *MYH9* as a candidate gene related to and involved in the high racial disparities in HIVAN and FSGS. MALD is suitable for detecting genetic risk variants that have different frequencies between populations and contribute to diseases in admixed populations (Kao *et al*., 2008). The gene was identified when Kopp and his colleagues identified the apex of MALD to be occurring at SNP, rs735853, found on an intron close to the 3' end of the *MYH9* gene (Kopp *et al.*, 2008). Out of 17 known genes expressed in the kidney and found in the same region with LOD score of about 0.86% to be contributing to the high risk of CKD in African Americans, *MYH9* was the most expressed (Kao *et al*., 2008). And considering that it is specifically expressed in the podocytes which are significant to

glomerular filtration (Kopp *et al.*, 2008), Kopp and his colleagues considered it as the probable candidate gene for genetic association of CKD.

Twenty *MYH9* SNPs from the Yoruba (YRI), reference West Africa Ethnic group and European (CEU) populations of the International HapMap Project data were selected and grouped into haplogroups by Kopp and his colleagues for their study in 2008. The *MYH9* alleles haplotypes contributing to CKD were found to have a higher frequency of approximately 69% in individuals with African ancestry as compared to Europeans with almost less than 4% (Kopp *et al*., 2008). After SNP genotyping a four SNP haplotype (G/C/C/T; rs4821480, rs2032487, rs4821481, and rs3752462) in *MYH9* was identified to be significantly associated with FSGS, HIVAN, and hypertensive nephropathy in African Americans (Bostrom *et al*., 2010;Kopp *et al*., 2008). Three of these SNPs, located on intron 23 (rs4821480, rs2032487 and rs4821481) were found to be in strong linkage disequilibrium and were referred to as the E-1 or Extended-1 haplotype. This haplotype recorded a frequency of nearly 60% in African Americans and 4% in European Americans and confers 4-5 fold increased disease risk in a recessive model (Kopp *et al.*, 2008). The *MYH9* E-1 haplotype was thought to clarify approximately all of the additional burden of CKD disease in individuals of African ancestry; conferring about 70% increased risk (Oleksyk *et al*., 2010).

Other studies identified new haplotypes that also significantly showed some association with the major forms of CKD and ESRD. The L-1 haplotypes showed a significant relatedness with hypertensive ESRD, with SNPs rs7078, rs12107, rs735853 and rs5756129 in a recessive model (Freedman *et al*., 2009). The study showed that rs5756152 was independently associated with hypertensive associated

ESRD even after adjusting for the E-1 and L-1 haplotypes. MALD analysis also displayed an association between *MYH9* SNPs including rs12107, rs4821480 and rs5756152 and non-diabetic ESRD (Kao *et al*., 2008). Further genome-wide association studies also supported the initial *MYH9* findings of an association with hypertension and albuminuria in African Americans and non-diabetic ESRD in Hispanic Americans (Behar *et al*, 2010). Behar *et al* (2010) identified the F-1 haplotype, comprising of SNPs rs16996674, rs16996677 and rs11912763, which were significantly associated with non-diabetic ESRD and O'Seaghdha *et al* (2011) identified the S-1 haplotype that spans intron 13 -15 of the *MYH9* gene, which also showed association with a much higher risk of CKD.

Other *MYH9* SNP haplotypes were identified to have been associated with kidney disease and occur at different frequencies worldwide (Oleksyk *et al*., 2010). These are the E-2 haplogroups which are known to be protective and the neutral haplotypes; E-3, E-4, and E-5. The E-2 haplotype has the highest frequency of 62% among the European populace and the neutral haplotypes being the highest among the Oceania population with 98% frequency.

ii. Apolipoprotein L1 (APOL1)

The Apolipoprotein L1 (*APOL1*) gene belongs to a family of genes called the Apolipoprotein (APOL) which is composed of six genes;

, *APOL2, APOL3, APOL4, APOL5 and APOL6*. It is a 14,522 base pairs long gene encoding a 398 amino acids residue protein. It is located on chromosome 22 position on the long arm at position 12.3 and is comprised of seven exons and six introns (fig

2.2). The encoded APOL1 protein is a minor apolipoprotein component of HDL (High-density lipoprotein) class 3.

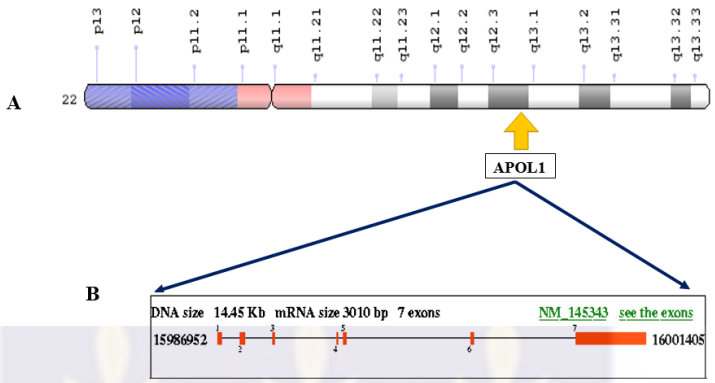

Figure 2. 2 Chromosome 22 showing the position of APOL1 (A), (Genetics Home Reference, 2017) *and* ***APOL1*** **gene structure showing introns and exons *(B).*** (Fu *et al*., 2017) *APOL1 is located 14kb 3' downstream MYH9 on the long (q) arm of chromosome 22 at position 13.1.*

APOL1 is synthesized in the liver and some other tissues, such as the pancreas, kidney, and brain. Expressed APOL1 is found in vascular endothelium, liver, heart, lung, placenta, podocytes, proximal tubules, and arterial *(Limou* et al., *2014)*. The APOL1 protein is comprised of the following domains from the N terminal, a Signal peptide domain (SD), pore forming domain (PFD), B-cell lymphoma 2 homology domain 3 (BH3), membrane addressing domain (MAD), and serum resistant associated interacting domain (SRA) (Limou *et al*., 2014) (Fig 2.3).

APOL1 belongs to the B-cell lymphoma 2 (BCl2) class of proteins which are known for their roles in autophagy and induction of immune response (Wan *et al*., 2008). Circulating APOL1 is involved in innate immunity by possessing a trypanolytic factor giving protection against Trypanosoma infection, a parasite transmitted by tsetse fly.

APOL1 complexed to high density lipoprotein class 3 which are known as trypanolytic factors (TFL), binds to the lysosomal wall of the parasite in an infected person creating a conformational change in the protein. The ionic PFD then creates an anionic pore which allows an influx of chloride ion creating an anion imbalance. This creates an osmotic pressure eventually disrupting the parasite lysosomal wall leading to its rapture and death.

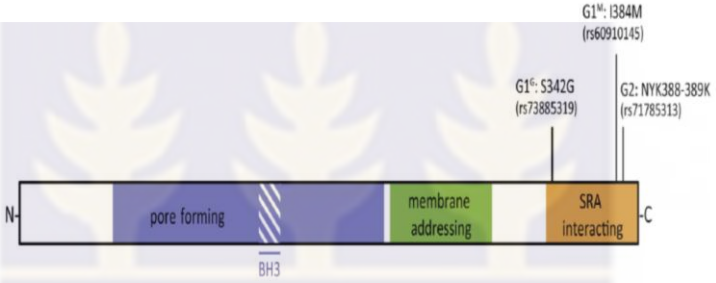

Figure 2. 3 Protein structure showing the domains of Apol1 protein with the location of the G1 allele and G2 allele (Limou *et al*., 2014). *The coding region of APOL1 is comprised of seven exons and six introns. APOL1 protein is comprised of four domains: Signal peptide domain, pore forming domain, B-cell lymphoma 2 homology domain 3, membrane addressing domain, and serum resistant associated interacting domain. The mutations associated with risk of CKD occurs in the serum resistant associated interacting domain.*

Evolution of APOL1 Risk Variants

Despite this trypanolytic ability of circulating APOL1, two evolving subspecies of Trapanosoma (*Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*) still showed some level of virulence (Wasser *et al*., 2012). When a human is infected by *T. brucei rhodesiense* which drove the evolution of two kidney disease risk variants in Africa, the virulence SRA protein binds to the SRA domain of APOL1 protein and inactivates the lysosomal and endosomal components (Hadjuk 2011). This allows for proliferation and survival of the parasite even in the presence of serum

circulating APOL1. Two *APOL1* coding variants (G1 and G2) however evolved, modifying the C-terminus of the protein to inhibit the binding of the SRA protein of Trypanosoma parasite at the C-terminal domain of the APOL1 protein (Wasser *et al*., 2012) as shown in figure 2.6 above. The non-binding of parasite SRA to APOL1, restore the lytic ability of APOL1 as an adaptive advantage to confer resistance against *T. brucei rhodesiense* infection (Kasembeli, 2015).

The G1 allele include two variants (Ser342G-rs73885319 and Ile384Met - rs60910145) which are non-synonymous missense mutations occurring in the last exon of *APOL1* (Thomson *et al*., 2014). These two variants are a haplotype in an almost perfect linkage disequilibrium (LD) (Genovese *et al*., 2010). The G2 variant is an in-frame deletion of 6 nucleotide bases resulting in the deletion of two amino acids (p.N388del/Y389del). The Ser342G missense variant is located within the SRA-binding domain but outside the SRA-binding epitope whereas the Ile384Met and the G2 alleles are occurring within the SRA-binding epitope covering amino acids 370 to 392 (Limou *et al*., 2014). The G1 and G2 variants do not occur together on the same chromosome and are known to be casually related (Kopp *et al*., 2011).

These two alleles are have been found in people with recent African ancestry; African Americans and indigenous Africans particularly sub-Saharan Africa (Limou *et al*., 2014) where Trypanosoma infection was endemic. The selective advantage of natural selection to gain resistance against *Trypanosoma rhodesiense* led to high frequencies of these variants in sub-Saharan Africa and African American population (Palmer *et al*., 2015). The distribution of these alleles shows high frequencies in SSA (Fig 2.4) where most African Americans are known to originate; high prevalence in West

Africa, low in the Northeastern and absent in the Ethiopian population (Tzur *et al*., 2010). The frequency of the G1 and G2 variants in West Africa is mostly observed in Ghana and Nigeria with an estimation of >40% frequency for G1 and between 6%-24% for G2 (Limou *et al*., 2014).

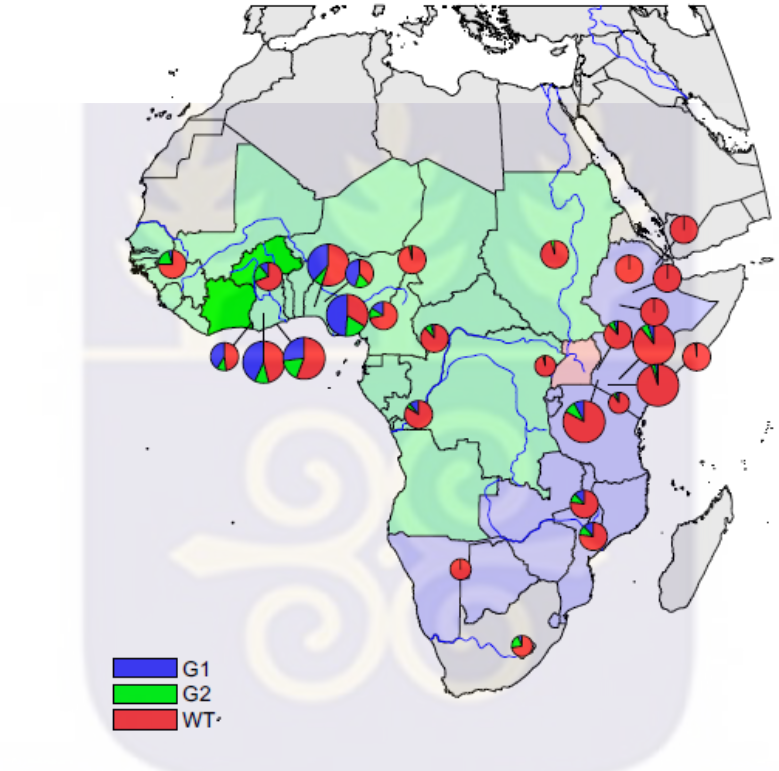

Figure 2. 4 Distribution of the G1 and G2 APOL1 variants across Africa. (Thomson *et al*., 2014) . *Allele frequencies of the G1 and G2 variants, and the wild-type are differentiated by blue, green and red segments, respectively. The different circle sizes indicates the approximate number of genotyped individuals: small, <10 individuals/20 chromosomes; medium, 10–100 individuals/20–200 chromosomes; big, >100 individuals/200 chromosomes.*

Association between APOL1 and CKD

After the discovery of *MYH9* as a candidate gene for the observed disparities in the occurrence of CKD amongst African descents and European Americans, there have been the speculations of uncertainty of *MYH9* association (Genovese *et al*., 2010). This led to further studies such as the human 1000 genome project which led to the discovery of *APOL1* mapped near *MYH9* to be more strongly associated with the observed risk of CKD in individuals of recent African ancestry than all previously associated *MYH9* SNPs (Wasser *et al*., 2012). This conclusion was made due to a stronger statistical association of *APOL1* risk variants compared to the *MYH9* gene (Genovese *et al*., 2010) and lack of independent association of *MYH9* with earlier stages of related kidney disease phenotypes (Tzur *et al.,* 2010). Nonetheless Linkage disequilibrium patterns have shown that *APOL1* G1 and G2 variants are in strong LD with *MYH9* E-1 haplotype (89% in G1haplotypes and in 76% in G2 haplotypes) that was previously reported to be strongly linked to CKD (Genovese *et al.*, 2014). This, however, explains the initial association of *MYH9* haplotypes with kidney disease in the African population.

The two genetic variants; G1and G2 have been linked with the pathogenesis of non-diabetic CKD including hypertensive associated nephropathy, FSGS, HIVAN, ESRD (Tzur *et al*., 2010) (Fig 2.5), severe progressive sickle cell associated nephropathy and lupus nephritis (Ashley-Koch *et al*., 2011). In HIVAN, *APOL1* has been shown to modify the pathology and progression to ESRD in HIV and non-HIV patients. In a large kidney biopsy studies of both HIV and non-HIVAN, 86% of patients with two

APOL1 risk alleles recorded presence of focal glomerulosclerosis (FSGS) and hypertensive nephropathy (Fine *et al*., 2012).

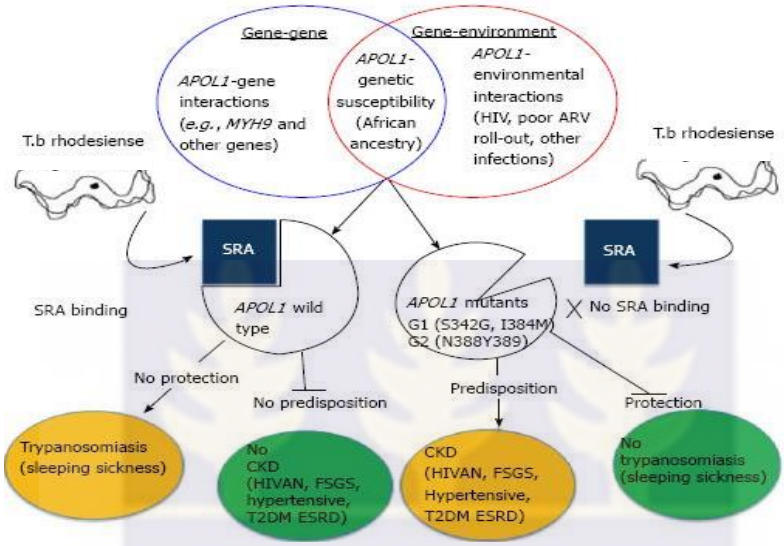

Figure 2. 5 Association between APOL1 variants and CKD and the positive selection of APOL1 risk variants as a result of Trypanosomiasis (Kasembeli, 2015)**.** *The mutant alleles G1 and G2 variants encode forms of APOL1 that does not bind to and evade SRA protein of Trypanosoma and remain active against T. b. rhodesiense. The mutant alleles predisposes an individual to different forms of kidney HIVAN, FSGS and ESRD.*

In an African American cohort study, having two risk variants contribute about 50% of CKD in HIV patients without antiretroviral drugs (Kopp *et al*., 2011.). Conversely, the relative scarcity of the G1 and G2 alleles in the Ethiopian population has been linked to protection from HIVAN in the Ethiopian HIV patient population (Baher *et al*, 2011; (Wasser *et al*., 2012)) even though there was an observed high frequency of *MYH9* haplotype in this population.The *APOL1* kidney disease risk variants also account for early onset of CKD and younger age of ESRD and haemodialysis initiation (Kanji *et al*., 2011). Work done by Revees-Daniel et al shows that the graft

survival of kidney donated by *APOL1* risk variants carries was significantly shorter irrespective of the recipients status (Reeves-daniel *et al.*, 2011). This finding may imply that the presence of *APOL1* risk variants in donors may account for the poor outcomes of kidney allografts. The association of the two risk variants to renal failure could be either in a homozygous or heterozygous state.

iii. Haemoglobin (Hb)

Haemoglobin is an iron-rich globular protein responsible for transporting oxygen from the lungs to other parts of the body and maintaining the shape of the red blood cells. The haemoglobin molecule also functions in transporting some carbon dioxide from tissues to the lungs and acts as a very important buffer in the blood. It consists of two pairs of polypeptide globin subunit (two alpha-globin chains and two beta-globin), each folded around a haem molecule that contains iron. There are more than one haemoglobin genes that encodes the protein. In an adult human, the main form of haemoglobin present is haemoglobin A (Hb A) and its encoded by *HbA1*, *HbA2*, and *HbB* (Hardison *et al.*, 2014). The gene for α globin is located on the short arm of chromosome 16 and the β, γ, and δ genes are positioned on the short arm of chromosome 11.

Haemoglobinopathies

Haemoglobinopathies are a group of blood disorders characterised by an abnormal structure of the globin molecules (Hb variants) or a reduced or no synthesis of one or two of the globulin subunits (Thalassaemia syndrome) (Trent, 2006). With a Worldwide prevalence of about 7% (Kohne, 2011), haemoglobinopathies are known

as the most common inherited genetic disorder (Trent, 2006) with high prevalence in malaria endemic regions such as Africa (Cappellini *et al.*, 2008). The high prevalence of haemoglobinopathies malaria endemic regions is attributed to positive selective advantage provided against malaria. Both thalassaemia and abnormal Hb variants are inherited in an autosomal recessive manner and can both lead to mild to severe anaemia in affected patients (Trent, 2006) with homozy gous individuals having more severe complications (Kohne, 2011)

Mutations leading to thalassaemia occurs in either the α-globin or β-globin of the haemoglobin molecule of an adult human (Kohne, 2011). Depending on which subunit affected, the condition is referred to as α or β thalassaemia. It is caused by either a genetic substitution or deletion of certain nucleotides from the genes that code for these subunits.

Haemoglobin variants are mostly caused by missense mutations that lead to amino acids substitutions of the either or both globin proteins (Thom *et al*., 2013). The most common and clinically significant Hb variants worldwide are Hb S (Sickle cell), Hb C and Hb E (Kohne, 2011). Sickle cell disease/Aneamia is the most severe form of haemoglobinopathies. Sickle cell disease occurs as a single nucleotide mutation in the β-globin chain of adult haemoglonin (Hb A) (Chukwuemeka *et al*., 2015). It is characterized either homozygous Hb SS or as compound heterozygous Hb SC, Hb SD, Hb S-β0-thalassaemia and Hb S-β+-thalassaemia (Chukwuemeka *et al*., 2015). Haemoglobin C (Hb C) is also another structural Hb variant which is associated with sickling of the red blood cell but results in a milder form of haemolytic anaemia and

other clinical complications (Trent, 2006) but have a similar spectrum of organ involvement (Guasch *et al*., 2006).

There are many complications that occur in individuals with haemoglobinopathies especially and they vary from person to person. These complications include a wide range of physiological, neurocognitive, and psychological comorbidities (McClellan *et al*., 2008; Midence & Shand, 1992; Schatz *et al*., 1993). Symptoms of SCD include vaso-occlusive pain crises, anaemia, eye damage, splenic sequestration, immunosuppression, acute chest pain, slowed growth and puberty, leg ulcers, stroke gallstones, priapism and organ damage (Centers for Disease Control and Prevention (CDC), 2011).

Haemoglobinopathy Associated Nephropathy

Kidney damage is one of the major target organ related complications associated with SCD. Sickle cell nephropathy affects a minority of SCD patients and is associated with early mortality in affected individuals (Ashley-Koch *et al*., 2011; Platt *et al*, 1994). Sickle cell nephropathy has been well characterised and consists of a variety of renal abnormalities, ranging from tubular changes and glomerulopathy, to gross alterations of the kidney (Hicks *et al*., 2011). The manifestations associated with SCD nephropathy include papillary necrosis, hyposthenuria, impaired renal acidification, proteinuria, haematuria, supranormal proximal tubular function, urinary concentrating defect and renal failure (Stuart & Nagel, 2004). Clinically significant renal involvement occurs more frequently in sickle cell disease than in sickle cell trait or in other haemoglobinopathies (Nath & Hebbel, 2015).

The general mechanism underlying sickle cell nephropathy is mainly hypoxia and ischemia caused by blockage of sickled red blood cells in the renal medulla (Alhwiesh, 2014). Sickle cell nephropathy may also be consequence effects of an underlying functional vascular abnormalities resulting in haemodynamic changes (Nath & Hebbel, 2015). This creates a perfusion paradox with hypoperfusion occurring concurrently with cortical hyperperfusion (Guasch *et al*., 2006). The renal vasculopathy also leads to unusual renal vascular responses to stress that occur systemically or in distant organs and tissues. This response is characterized by enhanced renal vasoconstriction and resultant vaso-occlusion. Persistent sequences of ischaemia and ischaemia–reperfusion injury occur, leading to interstitial diseases, glomerular disease or repeated episodes of AKI which eventually may lead to progressive renal damage (Nath & Hebbel, 2015).

The prevalence of renal failure in sickle cell disease alone is estimated to be between 5 and 18% of the total populace of SCD patients (Guasch *et al*., 2006). Usually, the manifestations of renal disease in sickle cell patients comes with ageing with nephropathy occurring between the third and fifth decade of life (Nolan *et al*., 2005). Younger people with SCD tend to have normal renal function and as they grow older, the kidneys begin to deteriorate gradually progressing to ESRD (Ham *et al*., 2000). However, some manifestations of renal damage including hyperfiltration, hypertrophy, and impaired urinary concentration may be present as early as early as in infancy in SCD (Nath & Hebbel, 2015).

It seems that renal manifestations are generally less common or less severe in SCT and other haemoglobinopathies but may involve painless microscopic or gross

haematuria, impaired urinary concentration, asymptomatic hematuria, and papillary necrosis (Abbud-Filho, 2013). Unlike SCD, the long term impairment of kidney has not been fully established in individuals with sickle cell trait and other haemoglobinopathy variants such as Hb C. There are also conflicting reports on the contribution of inheritance of one HbS to the initiation and progression of CKD since sickle cell traits are normally known to be benign (Hicks *et al*., 2011). Nonetheless, the fact that some renal abnormalities have been characterised in less severe forms of haemoglobinopathy genotypes, they may be important risk factors for initiating the progression of renal damage in the African population where haemoglobinopathies are predominant. Thus, with the uncertainty in SCT being an independent risk factor to kidney disease, it will be thought-provoking to examine the interaction of SCT with other recently identified genetic risk variants for nephropathy in Africans, such as *APOL1* and *MYH9* (Mukendi *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research Design and Study Population

This study was a case-control study with CKD as cases and patients without any form of nephropathy as controls. The study used archived samples selected from the H3Africa Kidney disease Research network (H3A KDRN) study population. The H3Africa kidney genomics project is aimed at conducting genomics research that will aid in identifying new genetic and interacting environmental factors that contribute to the incidence of kidney disease in Africa. The participants used in this study were patients recruited between May 2013 and December 2016. The samples were randomly selected from Ghana and Nigeria within the H3A CKDRN study population. Ghana and Nigeria form the study population because both countries have recorded almost similar prevalence of CKD.

3.2 Sample size

To avoid false positive associations, is it necessary to have a large enough sample size that will give enough statistical power for a successful association analysis. The minimum sample size that was required to make the research findings significant was calculated using case-control sample size calculation as described by Schlesselman and Stolley, 1982. The calculated sample size was based on an estimated 16% prevalence of CKD in sub-Saharan Africa (Osafo *et al*., 2015) and an odds of CKD

occurring in African population at 3 times higher than in other populations. To display a 95% confidence interval and a statistical power of 80%, minimum total sample size was calculated as 150 (75 control and 75 cases). For this study, 260 cases and 277 controls were selected to make a total of 537 study population. The sample size calculation is shown in appendix 3.

3.3 Inclusion Criteria

Only individuals who had consented to participate and gave their biological specimen to be included in any genetic study within the interest of the Human Hereditary and Health in Africa (H3A) Kidney disease research (KDR) under IRB approval from both Ghana and Nigeria in addition to their medical histories were included in this study. The H3A KDR studies included 4000 kidney disease cases and 4000 controls however only a total of 537 non-related participants were selected to be part of this study.

Patients within an age range of 1-74 years were selected for this study. This age range was selected to include paediatric patients who are likely to have kidney disease from genetic factors and older patients who have the highest prevalence of chronic kidney disease. Participants who had been clinically diagnosed with CKD stage 3, 4 and 5 based on KDOQI criteria with or without clinically diagnosed SCD were included as cases.

Healthy individuals and patients attending hospitals or have been hospitalized for other medical reasons with no evidence of kidney damage and dysfunction made up

the control group. Healthy individuals were also selected from the communities as part of the control group to have a true representative of the general population. Glomerular Filtration Rate (MDRD) greater than 60 ml/min/1.73m2 and random albumin/creatinine ratio mg/mmol of (<3.5 mg/mmol (female); <2.5 mg/mmol (male)) were also considered as an inclusion criteria for the control group.

3.4 Exclusion Criteria

Any patient with hypertension, diabetes or any systemic disease that could explain the diagnosis of CKD except for sickle cell disease were not included in the study. Pregnant women, prisoners, patients suffering from cancer of the kidney, multiple myeloma and polycystic kidney disease were also excluded from this study.

3.5 Laboratory Procedures

3.5.1 DNA Isolation and Purification

DNA was isolated from archived frozen venous blood samples collected from study participants by professional laboratory technicians in Ethylenediamine Tetra-acetic Acid (EDTA) tubes. Before DNA isolation, the frozen blood samples were allowed to thaw completely. Qiagen (QIAamp) midi kit spin column DNA isolation protocol for blood DNA isolation and purification with some adjustments was used to isolate genomic DNA from all samples. All buffers used were provided by Qiagen in addition to the DNA isolation column.

Qiagen Protease (200 µl) was pipetted into the bottom of a 15 ml falcon tube and 2 ml buffy coat was added and mixed briefly by rocking up and down. To this, was added 2.4 ml lysis buffer and mixed thoroughly by inverting the tube 15 times, followed by additional vortexing for 1 minute. The mixture was incubated at 70°C for 10 minutes to ensure complete lysis of the cell and breakdown of unwanted cellular proteins whiles DNA is kept intact. After the incubation, the following procedures were carried out on ice and centrifuging was done using a refrigerated centrifuge at 4°C. This is to ensure the integrity of the DNA is preserved. To the incubated samples, 2 ml molecular grade absolute ethanol (96–100%) was added and mixed by inverting the tube 10 times, followed by brief vortexing. The mixture was transferred carefully onto the QIAamp midi column placed in a 15 ml centrifuge tube and centrifuged at 3000 rpm for 3 minutes. This was repeated till all the sample mix was exhausted and filtrate discarded. At this point, the DNA is bound to a silica gel in the spin column. The DNA was washed to get rid of all cell components and debris. This was done by first adding 2 ml washing Buffer 1 to the QIAamp Midi column and centrifuging at 3500 rpm for 3 minutes. A second wash was done with 2 ml washing Buffer 2 at 3500 rpm for 25 minutes to ensure all buffer residue was removed. To separate the DNA from the column, 300 µl of DNA elution buffer was added to the column placed in a clean 15 ml centrifuge tube and incubated for 5 minutes. The incubation allows for maximum elution of isolated DNA. The DNA was finally eluted by centrifuging at 3500 rpm for 5 minutes. The eluted DNA was pulled out of the centrifuge tube into a clean 2 ml screw cap tube and stored for all downstream assays.

3.5.2 DNA Concentration and Quality

DNA purity and concentration was tested using the Qubit 2.0 fluorometer. High sensitive double-stranded DNA assay was used to determine the concentration of intact DNA. The Qubit 2.0 Fluorometer is a benchtop instrument for the quantitation of DNA, RNA, and protein, using the highly sensitive and accurate fluorescence-based quantitation assays. Low fluorescent dyes bind to the molecules and become highly fluorescent when bound to the nucleic acid molecules. There is a wide difference between the fluorescence intensity of bound and unbound such that, no significant fluorescence is given off unless the dye is bound to a molecule. The fluorescence is measured as a property of the molecule which determines the concentration. The qubit fluorometric assay is very specific and selective for dsDNA, RNA, and protein minimizing the effects of contaminants in the sample that may affect the quantitation and the measurement is very distinguished based on the nucleic acid of interest.

To quantify isolated genomic DNA, highly sensitive DNA specific fluorescent dye (pico red) was mixed with the Qubit double-stranded high specific buffer in a ratio of 1:199 (µl). The isolated DNA was then added to the assay mixture at 1:199 (µl) and incubated in the dark for 2 minutes. The concentration was read using the fluorometer and recorded as nanograms per microliter (ng/µl). Preferred working concentration ranged between 20 ng/µl and 60 ng/µl.

Quality of DNA was analysed by agarose gel electrophoresis. Using a 1% agarose gel (pre-stained with ethidium bromide), 4 µl of the genomic DNA was run with 1X SB buffer at 200V for 30 minutes. UV spectrophotometer was used to view the gel in the

dark. For a good quality, intact and non-degraded DNA, a single tight band is expected. The presence of a smeary streak was an indication of degraded DNA and poor DNA quality.

3.5.3 Genotyping

In all 10 SNPs were selected for this study. For Haemoglobinopathy HB S (rs334) and C (rs33930) were selected since they are most frequently reported in Sickle Cell Disease cases. SNPs found in Haplotypes which are in high LD and have a significantly high frequency in African populations ((Behar *et al*., 2010) were selected for *MYH9*. The selected SNPs are rs4821481 and rs2032487 for the E-1 haplotype and for the S-1 haplotype rs5750248 and rs5750250. Reported *APOL1* variants G1 (rs60910145 and rs73885319) and G2 (rs71785313) which have been reported as the true genetic association of chromosome 22 to CKD were selected along with the variants rs9622363, a tagging SNP.

3.5.3.2 Polymerase Chain Reaction (PCR)

A multiplex PCR assay was employed in amplifying the flanking regions in the genes of interest (*APOL1, MYH9* and Hb) where the SNPs are located. Multiplex PCR reaction is a PCR technique used to amplify more than one target sequence in a single PCR reaction. Triplex PCR was used to amplify the coding sequences of *APOL1, MYH9* and HB. Each triplex assay has two different SNPs with Amelogenin XY (AM XY) as an internal control (as shown in table 3.1). The primers used in each triplex

assay were designed to be specific to each target sequence with melting temperature within the same range (Primer sequences and product size shown in Appendix 1).

Preparation of the PCR mix was done in a clean UV sterilised PCR workstation. The total master mix for each reaction was prepared in a sterile 1.5 ml Eppendorf tube, mixed well by vortexing gently and centrifuged to settle the components in the tube. The volume and concentration of each reagent needed per reaction is shown in Table 3.2. Each final reaction mix comprised of 5 µl of the final master mix added to 5 µl DNA template for each sample.

The amplification was done with the aid of a Techne Prime thermal cycler using the following cycling conditions; initial denaturation at 94°C for 3 minutes, 8 cycles of denaturation at 94°C for 30seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute, 8 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 1 minute, and extension at 72°C for 1 minute, denaturation at 94°C for 30seconds, annealing at 57°C for 1 min, and extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The PCR products were run on a 1% agarose gel and visualized under UV light to detect successful amplification. These reactions conditions were for only triplexes 1, 2, 4, and 5. For triplex 3 the annealing temperature was 57°C with all other condition the same as has been described.

3.5.3.3 Universal PCR (UNI PCR)

Universal PCR reaction was carried out using a universal primer to further amplify the amplicons from the multiplex PCR. This is to ensure there is maximum DNA template for downstream assays. The volume and concentration of each reagent

needed per sample and reaction conditions are shown Tables 3.3 and 3.4 respectively. To the reaction product from the initial triplex reaction 10 µl of Uniplex master mix was added for the Uniplex reaction.

Table 3. 1. Llist of Triplexes

TRIPLEX	SNPS
1	Hb C, *APOL1* rs60910145, AmXY
2	Hb S, *APOL1* rs73885319, AmXY
3	*MYH9* rs4821481, *MYH9* rs2032487,AmXY
4	*MYH9* rs5750250, *MYH9* rs5750248, AmXY
5	*APOL1* rs9622363, *APOL1* rs71785313, AmXY

Table 3. 2. Volume and concentration of each reagent used in Triplex PCR assay

REAGENTS	Working Concentration	Volume (µL)	Final concentration in PCR master mix
DNAse free Water		1.58	
PCR Buffer	10X	1	2X
$MgCl_2$	50mM	1	10mM
DNTP	2.5mM	0.8	0.4mM
Triton X	2.50%	0.2	0.10%
Primer set for marker one	20µM	0.1	0.4 µM
Primer set for marker two	20µM	0.1	0.4 µM
Primer set for marker Amelogenin	20µM	0.1	0.4 µM
Taq Polymerase	5units/µl	o.12	0.12units/ µl

Marker set one and two are representative of the target sequences in each reaction

Table 3. 3. Volume and concentration of each reagent used in Universal PCR assay

Reagents	Working Concentration	Volume (µL)	Final concentration in PCR master mix
DNAse free Water		6.78	
PCR Buffer	10X	1	2X
$MgCl_2$	50mM	1	10mM
DNTP	2.5mM	0.8	0.4mM
Universal Primer Set	20µM	0.1	0.4 µM
Taq Polymerase	5units/µl	o.12	0.12units/ µl

Table 3. 4. PCR cycling conditions for universal PCR assay.

Steps	Temperature(°C)	Reaction Time	Number Of Cycles
Initial Denaturation	94	3mins	1
Denaturation	94	30secs	
Annealing	55	1min	
Extension	72	1min	35
Final Extension	72	10mins	1

3.5.3.4 Protease Reaction

Protease treatment was done to digest and stop the activity of the Taq polymerase enzyme which could interfere with the ligation reaction in the next step. To deactivate the taq enzyme, 10 µl of 12.5 mg/ml protease was added to total volume of PCR product (about 20 µl). The reaction was run in a Techne Primer thermal cycler at 50°C for 20min and 75°C for 15min.

3.5.3.5 Ligation Detection Reaction (LDR)

Ligation detection reaction is an assay developed for genotyping multiple SNPs, micro-deletions and insertions (Fitness, 2001). This technique has been employed in polymorphism typing to detect mutations in disease genes (Zhang *et al*., 2009). Ligation detection reaction technique is such that oligonucleotide probes are designed to be complementary to the point where polymorphism is occurring. For loci that contain two alleles, i.e. the wildtype allele and the mutant allele, three different probes are designed. One common probe(L2) that anneals to the 5' end of the template DNA and two allelic probes (L1) with one having a 3' nucleotide that complementary to the wildtype and the other to the variant allele (Fitness, 2001). Once the common probe and the allelic probe bind to the template DNA adjacent to each other, a nick is formed at the point where the polymorphism is occurring (fig 3.1). Ligation detection reaction uses ligase enzyme to ligate the adjacent probes. The allelic probe which is complementary to the test sample DNA is ligated to the common probe to form a double stranded DNA. Ligation products are only formed when the allele-specific and common probes are perfectly paired and complementary to target sequence.

One usefulness of LDR is that more than one SNP occurring at different genes and loci can be typed simultaneously at the same time (Fitness, 2001). However, one thing to consider is to ensure the Probes designed to be complementary to each polymorphic site are not complementary to each other and can anneal to their complementary stands at comparable temperatures.

To SNP genotype *APOL1*, *MYH9*, and HB S and C SNPs, probes were designed to have varying product length and also tagged with different fluorescent labels

(Appendix 2). The unique length and fluorescent labels allow each specific allele to be differentiated by both size and/or fluorescence following acrylamide gel electrophoresis. The cycling ligation reaction was set at 94°C initial denaturation for 3 minutes, followed by followed by 40 cycles of final denaturation at 94°C for 15secs, and annealing and ligation at 61°C for 1mins. For triplex 3 all conditions remained the same as described above but for annealing and ligation temperature which was set at 57°C.

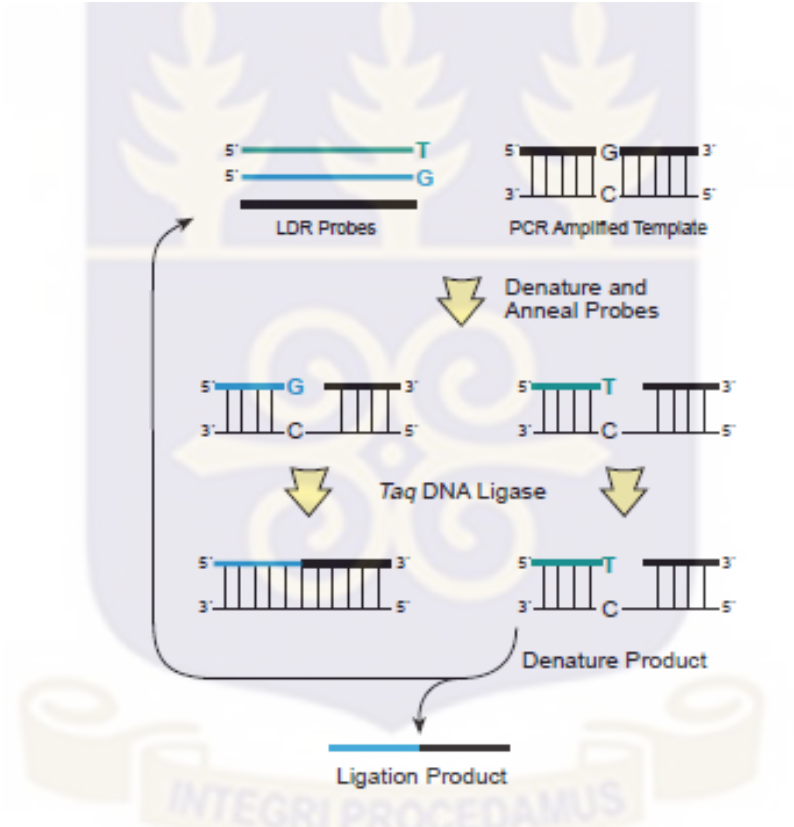

Figure 3. 1 Schematic Presentation of LDR assay (Zhang *et al*, 2009). *Genotyping of each polymorphism requires three LDR probes, one common (shown in black), and two discriminating (green and blue, one for each allele of the polymorphism). The common probe is composed of target-specific sequence complementary to the region adjacent to the polymorphism. In this example, the template has been amplified from an individual homozygous for the wildtype G allele. Hence only the wildtype allelic probe is ligated to the common probe, generating a single type of ligation product. The thermal cycling is repeated a few times in order to generate sufficient ligation product for detection*.

Table 3. 5. Volume and concentration of each reagent used in LDR assay.

Reagents	Working Concentration	Volume (µL)	Final concentration in PCR master mix
Ligase Buffer	10X	2	1X
AmX L1	5mM	1	0.5µM
AmX L2	100mM	1	100µM
AmY L1	5mM	1	0.5µM
AmY L2	100mM	1	100µM
Marker 1 L1 mix	5mM	1	0.5µM
Marker 1 L2 mix	100mM	1	100µM
Marker 2 L1 mix	5mM	1	0.5µM
Marker 2 L2 mix	100mM	1	100µM
Taq ligase	80units/µl	0.12	16units/ µl

Marker 1 and 2 are representative of the target sequences in each reaction

3.5.3.6 Polyacrylamide Gel Electrophoresis

To analyse the LDR genotyping results, the product was run on 10% polyacrylamide gel. The gel was prepared as follows; 12.5 ml of 40% Accugel (19:1) was added to 5 ml 10X Tris-Borate-EDTA (TBE) buffer, 500 µl of Ammonium persulfate (APS) (freshly prepared), 32.5 ml distilled water and 50 µl of TEMED in a clean sterile beaker and mixed by swirling gently. The mixture was quickly then poured in between already assembled glass plate with spacers (1.5 mm). The glass plates were clamped together at the sides to avoid leakage of the mixture. The gel comb was also inserted carefully making sure bubbles are not trapped under the teeth. The polyacrylamide was left for about 15 minutes at room temperature and allowed to polymerize. The gel still in the glass plate was then placed in the acrylamide gel tank. 1X TBE buffer was added to the top and bottom avoiding the formation of bubbles at the bottom of the gel. The wells were flushed out using Pasteur pipette with 1X TBE

to get rid of any bubbles. The LDR product for each sample was mixed with 5 µl bromophenol and loaded into the wells. The gel was run at 250V for 60 minutes. The gel viewed using UV trans-illuminator and the observed genotypes were scored and recorded accordingly.

3.6 Quality Control

In an attempt to ensure quality of laboratory procedures and avoid false positives, certain quality measures were put in place. As part of the PCR-LDR multiplex assay, each triplex reaction included two target SNPs and Amelogenin as a means of confirming gender of the study participants. Amelogenin is a gene found on chromosomes X and Y and encodes a protein found in the enamel matrix. There is a distinct difference between the Amelogenin X and Amelogenin Y in nucleotide sequence and size. They also do not undergo recombination and so has been the most preferred marker for sex typing (Bansal *et al.*, 2012). It was used in the PCR-LDR assay as an internal control for determining a successful reaction. Also negative controls (i.e reaction with no template DNA) were included as part of each set of reaction, to ensure there were no contamination during laboratory procedures.

Hardy-Weinberg test of random mating in the study population was also calculated to assess the quality of genotype data generated. The principles of Hardy-Weinberg is that, genotype frequencies in a population will remain constant from generation to generation without any evolutionary forces. Hardy-Weinberg Equilibrium is achieved when the population is large population, there is random mating and from generation to

generation there are no evolutionary forces such as mutation, migration and natural selection (Crow and Dove, 1998).

3.7 Statistical Analysis

Prevalence of the various *APOL1* and *MYH9* SNPs, Hb S and C in the study population was calculated as a proportion of the frequency of occurrence of each SNP to the total number of participants. The allele frequency and Genotype frequency calculated as observed counts expressed in percentage.

Chi-square test was used to estimate the association between the individual *APOL1*, *MHY9* variants and Hb S and C and CKD. To determine the strength of associations observed above, odds ratio was calculated with a confidence interval at a significance level of 5% using four different models of genetic association. The additive model; which is the increase in the risk/protection conferred by an allele is increased by x-fold for heterozygotes and 2x-fold for homozygote, dominant model; indicates that either one or two copies of allele *B* are required for a x-fold increase in disease risk, recessive model; shows that two copies of allele *B* are required for an x-fold increase in disease risk and heterozygotes advantage; where the heterozygous genotype has a higher relative fitness than homozygotes (Clarke *et al.*, 2011). The Logistic regression model was used to calculate the association and gene-gene interaction between cases and controls, adjusting for age, gender and clinical site. Age, gender and clinical site are also potential risk factors of CKD but not dependent on the genotypes under study and also their distribution not equal between cases and controls. In order not miss the

true representations of the genetic association they had to be adjusted for in the regression model.

The analysis was done using Stata (version 13.0). All Chi-square values with their P-values of the association analysis are shown in the appendix 4-13 and odds ratios with their confidence intervals and P-values are shown in the results section.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristics

This study was composed of 537 unrelated individuals from seven different clinical sites from Ghana and Nigeria made up of 260 cases and 277 controls. The characteristics of study participants are summarised in table 4.1 by CKD status expressed as frequency count and percentages. Generally, there were more females than males in the study, 285 and 252 respectively (χ^2 = 4.125, p-value = 0.05). The median age was not significantly different between the cases and controls, 38.95 vs 38.55, respectively. The overall prevalence of CKD (eGFR ≤ 60 ml/min/1.73 m2) in the study population was 48.42% with males having the highest prevalence (54.37% at p-value = 0.01). As illustrated in table 4.1, there was a significant difference in distribution of participants by country with those from Nigeria having high prevalence in CKD (p-value = 0.02). Out of a total of 260 CKD patients (cases), 202 (77.69%), were without sickle cell with the remaining 58 (22.31%) having sickle cell related nephropathy. The CKD cases without sickle cell disease were classified as CKD of unknown aetiology based on the exclusion of diabetes, hypertension and any other factor that could explain nephropathy. The control group also comprised of 110 (39.71%) sickle cell patients without CKD and 167 (42.26%) healthy controls. The participants without sickle cell disease and CKD comprised the general population control and were considered as healthy controls since they had no symptoms of kidney damage.

Table 4. 1. Demographic characteristics of Study Population stratified by CKD status

CHARACTERISTICS	CASES	CONTROL	χ^2 (P-VALUE)	TOTAL
GENDER :				
MALE	137(57.34)	115(45.63)	**6.72 (0.010)**	252
FEMALE	123(43.16)	162(56.84)		285
MEAN AGE	38.95(37.11-40.79)	38.56 (36.79-40.33)	0.76(5.76)	38.83
SICKLE CELL (YES)	58(22.31)	110(39.71)	**18.89 (0.000)**	168
SICKLE CELL (NO)	202(77.69)	167(60.29)		369
GHANA	92(35.38)	125(45.12)	**5.29 (0.022)**	217
NIGERIA	168(64.62)	152(54.87)		320

Significantly association if P-value < 0.05

4.2 Genotype scoring

To detect the genotypes of each individuals, the PCR-LDR product was run on 10X polyacrylamide gel and gel images captured and saved for SNP scoring. The representative gel image as shown in figure 4.1 display 3 possible genotypes. The gel images were analysed for homozygosity or heterozygosity of each SNP. Heterozygosity showed 2 bands for each SNP. Homozygosity, on the other hand, was indicated by one band of either of alleles differentiated by size and colour.

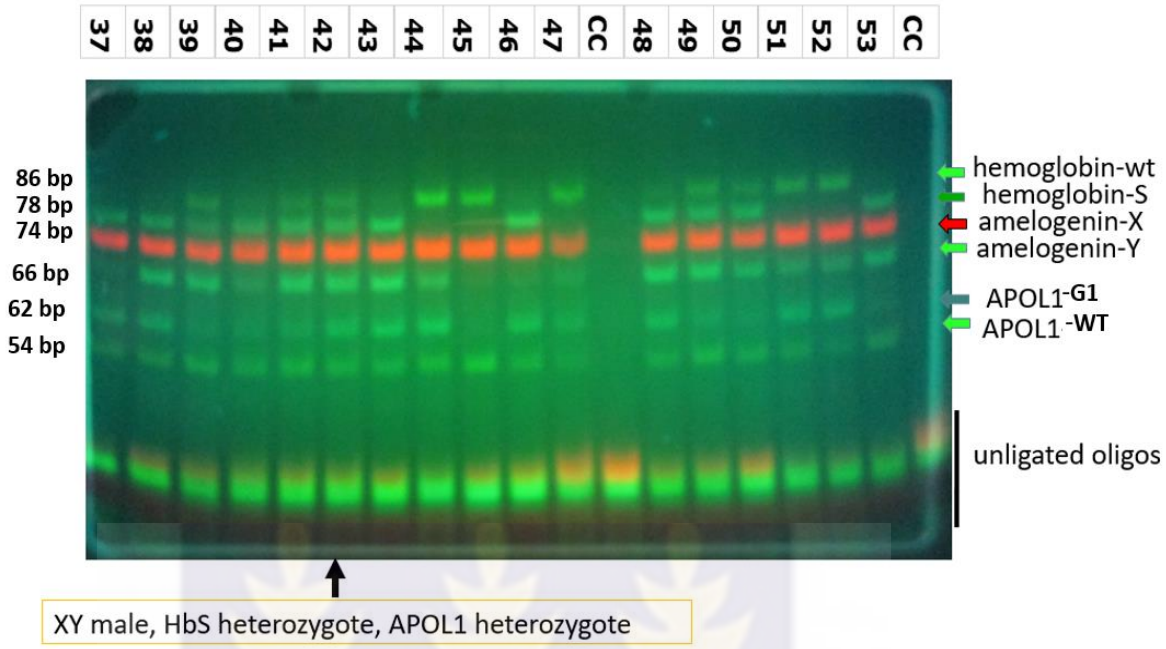

Figure 4. 1 A representative gel showing a genotype assay using triplex Ligation detection reaction. *Allele specific LDR product are assigned by colour and size (represent at far left of the gel image) following 10% polyacrylamide gel electrophoresis. Each band as shown in the image represents a specific genotype of each allele. At the bottom of the gel are unused LDR probes from the reaction mix. Each lane (37-58) represents LDR product of study samples and the lane labelled as CC represents LDR product of non-template DNA sample (Negative Control).*

4.3 Allele and Genotype Frequencies of Hb S, Hb C, APOL1 SNPs and MYH9 SNPs Variants in Study Population

Tables 4.2a-4.2c show the allele and genotype frequencies and distribution of each SNP between cases and controls, and Ghana and Nigeria. The frequency distribution of all SNPs did not different significantly between the two countries except for Hb C that had higher frequency in Ghana than Nigeria (p-value = 0.02). Hb C, Hb S and rs9622363 recorded significant difference in frequency between cases and controls (p-value < 0.01, p-value = 0.02 and 0.015 respectively). However for rs9622363 (table 4.2b), the allele frequency did not differ between the two groups.

Table 4. 2a. Allele and Genotype frequency of Hb S and C in study participants

SNP	CKD	NON CKD	P-VALUE	GHANA	NIGERIA	P-VALUE
Hb C						
G/G	250(97.00)	244(88.00)	12.853(0.02)	187(86.57)	306(96.22)	12.853(0.02)
G/A	9(3.00)	30(11)		29(13.43)	10(3.16)	
A/A	0(0.00)	2(1.00)		0(0.00)	2(0.63)	
G	0.98	0.94		0.93	0.97	
A	0.02	0.06	5.474(0.02)	0.07	0.03	5.474(0.02)
Hb S						
A/A	161(62.16)	132(47.65)	19.114(0.00)	120(55.30)	173(54.23)	0.561(0.75)
A/T	66(25.45)	71(25.63)		52(23.96)	85(26.66)	
T/T	32(12.35)	74(26.71)		45(20.74)	61(19.12)	
A	0.68	0.61		0.67	0.68	
T	0.32	0.39	2.855(0.09)	0.33	0.32	0.300(0.54)

Table 4. 2b. Allele and Genotype frequency of *APOL1* SNPs in Study Participants

SNP	CKD	NON-CKD	χ^2 (P-VALUE)	GHANA	NIGERIA	χ^2 (P-VALUE)
rs60910145						
T/T	99(38.22)	119(43.12)	1.766(0.41)	92(42.60)	126(39.50)	0.532(0.76)
T/G	130(50.1)	132(47.82)		102(47.20)	160(50.16)	
G/G	30(11.58)	25(9.06)		22(10.19)	33(10.34)	
T	0.63	0.67		0.66	0.65	
G	0.37	0.33	0.939(0.33)	0.34	0.35	0.256(0.80)
rs73885319						
A/A	79(30.50)	110(39.7)	4.989(0.08)	80(36.87)	109(34.17)	0.59(0.75)
A/G	155(59.84)	143(51.62)		119(54.84)	179(56.11)	
G/G	25(9.65)	24(8.66)		18(8.29)	31(9.72)	
A	0.60	0.66		0.64	0.62	
G	0.40	0.34	2.066(0.15)	0.36	0.38	0.221(0.64)
rs71785313						
Ins/Ins	207(80.50)	187(78.20)	0.402(0.82)	158(79.40)	236(79.46)	0.0(1.00)
Ins/Del	50(19.50)	52(21.80)		41(20.60)	61(20.54)	
Del/Del	0(0.000)	0(0.000)		0(0.00)	0(0.00)	
Ins	0.90	0.89		0.90	0.90	
Del	0.10	0.11	0.681(0.41)	0.10	0.10	0.0(1.00)
rs9622363						
A/A	10(3.88)	1(4.00)	8.447(0.015)	3(1.51)	8(2.68)	1.147(0.54)
A/G	169(65.5)	174(73.00)		141(71.21)	202(67.79)	
G/G	79(30.6)	63(26)		54(27.27)	88(29.53)	
A	0.37	0.37		0.37	0.37	
G	0.63	0.63	1(0.000)	0.63	0.63	0.00(1)

Table 4. 2c Allele and Genotype frequency of *MYH9* SNPs in Study Participants

SNP	CKD	NON-CKD	χ^2 (P-VALUE)	GHANA	NIGERIA	χ^2 (P-VALUE)
rs5750248						
C/C	20(7.72)	23(8.30	0.531(0.37)	22(10.14)	20(6.27)	2.919(23)
C/T	125(48.26)	125(44.16)		96(44.24)	154(48.28)	
T/T	114(44)	129(57.04)		99(45.62)	145(45.45)	
C	0.32	0.37		0.32	0.30	
T	0.68	0.63	1.476(0.22)	0.68	0.70	0.242(0.62)
rs5750250						
A/A	20(7.72)	25(9.03)	4.375(0.11)	22(10.14)	23(7.21)	1.484(0.47)
A/G	135(52.12)	124(44.77)		104(47.93)	155(45.59)	
G/G	104(40.15)	128(46.21)		91(41.94)	141(44.20)	
A	0.34	0.31		0.34	0.32	
G	0.66	0.69	0.546(0.46)	0.66	0.68	0.239(0.63)
rs4821481						
T/T	12(4.72)	14(5.07)	4.23(0.12)	10(4.69)	16(5.08)	1.484)
T/C	118(45.46)	104(37.68)		88(41.31)	134(42.27)	
C/C	124(48.82)	158(57.25)		115(54.00)	167(52.68)	
T	0.28	0.24		25	0.26	
C	0.72	0.76	2.54(0.11)	0.75	0.74	0.067(-.77)
rs2032487						
T/T	12(4.72)	11(5.07)	0.221(0.87)	9(4.22)	14(4.42)	0.131(0.94)
T/C	90(35.43)	101(46)		75(35.21)	116(36.59)	
C/C	152(59.84)	164(59.57)		129(60.56)	187(59.00)	
T	0.22	0.22		0.22	0.23	
C	0.78	0.78	0.00(1)	0.78	0.77	0.073(0.79)

4.4 Hardy-Weinberg Equilibrium (HWE) to Assess quality of Genotype Data

The control group was used to calculate Hardy-Weinberg equilibrium test of random mating in the study population. Table 4.3 shows that all the SNPs tested were in HWE except for Hb S, rs73885319 and rs9622363 which were significantly out of HWE at p-value ≤ 0.02.

Table 4.3. Hardy-Weinberg Distribution of SNP in Study Population.

SNP	Allele	HWE (χ^2)	P-Value
rs 334 (HBS)	A/T	56.60	0.00
rs 33930165(HBC)	G/A	0.98	0.32
rs 60910145	T/G	0.88	0.17
rs 73885319	A/G	5.64	0.02
rs 9622363	A/G	76.96	0.00
rs 71785313	Ins/Del	3.56	0.06
rs 5750248	C/T	0.91	0.34
rs 5750250	A/G	0.42	0.52
rs 4821481	T/C	0.35	0.56
rs 2032487	T/C	0.88	0.35

Significantly out of Hardy Weinberg Equilibrium (HWE) if P-value < 0.05

4.5 Association between Genetic Variants and Disease Phenotypes

Association between each genotyped SNP and disease outcome was tested for under four models of association; dominant, additive, recessive and heterozygote using logistic regression adjusting for age, gender and clinical sites after a crude (unadjusted) chi-square test of association and odds ratio analysis. For testing the association between Hb S, Hb C, *APOL1* and *MYH9* variants and disease outcome, all CKD cases were compared with the general population control group. The association of *APOL1* and *MYH9* were tested for by disease phenotype comparing Sickle cell nephropathy to Sickle cell control and CKDU was compared to the general population.

Before testing for association between selected SNPs and CKD, potential risk factors of CKD were tested to determine their effect size on having chronic kidney disease in the study participants (table 4.4). After controlling for age and clinical site, results indicate that males were on the average at 59% more at risk of having the kidney disease as compared to females. Study participants from Nigeria had about 54% (p-value = 0.0076) risk of developing CKD as compared to participants from Ghana. Age did not show any significant effect in risk of developing CKD. Because sex and clinical site had an association with CKD they were later included in the regression analysis as confounders and age was included in the model as a covariate.

Table 4. 4. Association between potential confounders and CKD

Covariate	OR (95% CI)	P-value
Age	1.00 (0.99 – 1.01)	0.66454
Sex (Male)	1.59 (1.13 – 2.25)	0.00765
Site (Nigeria)	1.54 (1.09 – 2.19)	0.01564

Significantly association if P-value < 0.05

4.5.1 Association between Hb S and C variants and Chronic Kidney Disease

All CKD cases were compared with the general population control to test for association between Hb S and C, and disease outcome. Significant increased risk of developing CKD was observed for having at least a copy or having an increasing number of the allele. Having double copy of Hb S showed a very high increased risk in relation to the other models of association even (OR=9.5, p-value < 0.001) (table 4.5). Hb S heterozygotes were not found to be at significant risk of developing CKD. Individuals who were heterozygous, having at least one copy or increasing number of Hb C were having about 30-57% significant protection against kidney disease even though no significant association was observed for individuals with two copies of the variant.

Table 4. 5. Association between Hb Variants (S and C) and CKD Cases tested under 4 genetic models In the Study Population

SNP	Risk Allele	Association Model	Unadjusted		Adjusted	
			OR(95% CI)	P-value	OR(95% CI)	P-value
HBS	T	ADDITIVE	1.83(1.35-2.46)	0.001	1.73(2.3-43.9)	0.000
		DOMINANT	1.87(1.21-2.88)	0.005	1.72(1.10-2.68)	0.016
		RECESSIVE	11.62(2.74-49.2)	0.001	9.5(2.22-40.98)	0.000
		HETEROZYGOTE	1.12(0.71-1.76)	0.619	1.11(0.70-1.77)	0.648
HBC	A	ADDITIVE	0.57(0.37-0.88)	0.011	0.54(0.34-0.83)	0.006
		DOMINANT	0.31(0.14-0.73)	0.007	0.28(0.12-0.66)	0.004
		RECESSIVE	1	-	1	-
		HETEROZYGOTE	0.36(0.15-0.85)	0.02	0.32(0.14-0.73)	0.011

Significantly association if P-value < 0.05, marginal trend of association if P-value ≥ 0.05 ≤ 0.09

4.5.2 Association between *APOL1* SNPs Variants and CKD Phenotypes

All CKD cases were compared with general population control to determine the association between the *APOL1* SNPs and CKD. *APOL1* rs71785313 (G2) did not show any significant association with developing CKD under either of inheritance patterns even after adjusting for confounders. Generally significant association was observed with carrying at least a copy of rs73885319 variant and an increased risk of developing CKD (30-80% (P < 0.05)), even though no significant association was observed for double copies of the allele (table 4.6a). There was also an increased risk

of developing CKD given that an individual had the rs60910145 variants (49%, P=0.05). On the other hand, a trend of protection against disease was observed for individuals who were heterozygote for the rs9622363 variant.

Further association analysis was performed by subgrouping the participants into disease phenotypes and this is summarised in table 4.6b (CKD of unknown aetiology) and table 4.6c (Sickle cell Nephropathy). This was done in order to determine whether the observe association as described in the general case group will be different after stratifying by disease phenotype group. Similar to the association that was observed, in the total CKD population, significant associations was observed with rs73885319 general genotype and developing CKD of unknown aetiology (table 4.6b) under additive and recessive models with significance of 0.006 and 0.005 respectively. There was also marginal increased risk of developing CKD of unknown aetiology for heterozygote rs73885319 individuals (OR = 1.47, p-value = 0.73). There was about 50% significant increased risk of developing CKD of unknown aetiology given that one had the *APOL1* rs60910145 variant at 0.05 significant level. There was however no significant association between any of the APOL1 SNPs and developing sickle cell nephropathy.

Table 4. 6a. Association between *APOL1* Variants and CKD Cases tested under 4 genetic models In the Study Population

SNP	Risk Allele	Association Model	Unadjusted		Adjusted	
			OR(95% CI)	P-value	OR(95% CI)	P-value
rs73885319		ADDITIVE	1.28(1.06-1.54)	0.009	1.30(1.08-1.57)	0.006
	G	DOMINANT	1.72(1.15-2.58)	0.008	1.81(1.20-2.74)	0.005
		RECESSIVE	1.37(0.67-2.82)	0.379	1.35(0.65-2.82)	0.408
		HETEROZYGOTE	1.501.01-2.23)	0.040	1.59(1.06-2.37)	0.024
rs60910145						
	G	ADDITIVE	1.15(0.93-1.41)	0.181	1.81(0.95-1.46)	0.119
		DOMINANT	1.41(0.95-2.10)	0.083	1.49(0.99-2.24)	0.050
		RECESSIVE	1.6(0.84-3.4)	0.141	1.73(0.85-3.52)	0.129
		HETEROZYGOTE	1.17(0.79-1.73)	0.410	1.23(0.82-1.83)	0.309
rs71785313	DEL	GENOTYPE	0.93(0.55-1.57)	0.787	0.92(0.53-1.58)	0.775
rs9622363						
	G	ADDITIVE	1.13(0.74-.173)	0.787	1.11(0.72-1.75)	0.611
		DOMINANT	0.19(0.02-1.50)	0.116	0.18(0.02-1.53)	0.118
		RECESSIVE	1.36(0.85-2.20)	0.202	1.34(0.82-2.19)	0.236
		HETEROZYGOTE	0.63(0.39-1.02)	0.067	0.64(0.40-1.04)	0.07

Significantly association if P-value < 0.05, marginal trend of association if P-value ≥ 0.05 ≤ 0.09

Table 4. 6b. Association between *APOL1* Variants and CKD of Unknown aetiology tested under 4 genetic models

SNP	Risk Allele	Association Model	Unadjusted		Adjusted	
			OR(95% CI)	P-Value	OR(95% CI)	P-Value
rs73885319	G	ADDITIVE	1.28(1.05-1.56)	0.011	1.30(1.06-1.57)	0.008
		DOMINANT	1.69(1.10-2.60)	0.015	1.77(1.14-2.74)	0.010
		RECESSIVE	1.58(0.76-3.31)	0.218	1.58(0.75-3.33)	0.230
		HETEROZYGOTE	1.40(0.93-2.13)	0.103	1.47(0.96-2.24)	0.073
rs60910145	G	ADDITIVE	1.38(0.94-2.45)	0.145	1.08(0.82-1.40)	0.58
		DOMINANT	1.40(0.95-2.19)	0.086	1.51 (0.99-2.32)	0.055
		RECESSIVE	1.51(0.71-3.16)	0.278	1.54(0.73-3.27)	0.256
		HETEROZYGOTE	1.25(0.83-1.89)	0.230	1.34(0.86-1.99)	0.214
rs71785313	DEL	GENOTYPE	0.97(0.56-1.67)	0.914	1.00(0.57-1.74)	0.982
rs9622363	G	ADDITIVE	1.05(0.67-1.67)	0.813	1.03(0.65-1.65)	0.875
		DOMINANT	0.14(0.01-1.15)	0.068	0.14(0.02-1.15)	0.069
		RECESSIVE	1.35(0.80-2.18)	0.269	1.35(0.80-2.24)	0.254
		HETEROZYGOTE	0.62(0.38-1.02)	0.060	0.61(0.37-1.01)	0.056

Significantly association if P-value < 0.05, marginal trend of association if P-value ≥ 0.05 ≤ 0.09

Table 4. 6c. Association between *APOL1* Variants and Sickle Cell Nephropathy tested under 4 genetic models

SNP	Risk Allele	Association Model	Unadjusted		Adjusted	
			OR(95% CI)	P-value	Or(95% CI)	P-value
rs73885319		ADDITIVE	1.05(0.76-1.42)	0.779	1.07(0.78-1.47)	0.660
	G	DOMINANT	1.270.63-2.53)	0.492	1.36(0.67-2.77)	0.388
		RECESSIVE	0.44(0.12-1.64)	0.225	0.44(0.11-1.66)	0.227
		HETEROZYGOTE	1.58(0.81-3.05)	0.172	1.69(0.86-3.33)	0.126
rs60910145						
	G	ADDITIVE	0.91(0.64-1.28)	0.610	0.88(0.62-1.25)	0.484
		DOMINANT	0.91(0.47-1.76)	0.796	0.88(0.45-1.72	0.717
		RECESSIVE	1.35(0.54-3.39)	0.515	1.56(0.59-4.09)	0.366
		HETEROZYGOTE	0.79(0.42-1.51)	0.488	0.73(0.37- 1.40)	0.624
rs71785313	DEL	GENOTYPE	0.97(0.56-1.67)	0.914	1.00(0.57-1.74)	0.982
rs9622363						
	G	ADDITIVE	1.19(0.59-2.37)	0.614	1.37(0.67-2.82)	0.385
		DOMINANT	1	-	1	-
		RECESSIVE	0.84(0.42-1.66)	0.614	0.73(0.35-1.49)	0.385
		HETEROZYGOTE	0.84(0.42-1.66)	0.614	0.73(0.35-1.49)	0.385

Significantly association if P-value < 0.05, marginal trend of association if P-value ≥ 0.05 ≤ 0.9

4.5.3 Association between *MYH9* SNP Variants and CKD Phenotypes

Table 4.7a summarises the association between the selected *MYH9* SNP variants and development of kidney disease. There was no evidence of significant association between any of the *MYH9* SNPs and renal damage in the general study population.

Stratifying by kidney disease phenotype (table 4.7b and 4.7c), there was a trend towards an increased risk of CKDU given that an individual was heterozygote for rs5750250 (OR = 1.43, p-value 0.09). There was an evidence of protection against development of CKDU for heterozygous rs2032487 (OR = 0.82, p-value =0.03). There was a significant increased risk of having sickle cell nephropathy under the additive and heterozygote models (OR= 1.99 and 4.40 respectively at p-value < 0.001) for rs4821481. Paradoxically, there was a rather significant protection against sickle cell nephropathy in the individuals with double copy of rs4821481 (OR = 0.26, p-value < 0.001).

Table 4. 7a. Association between *MYH9* Variants and CKD Cases tested under 4 genetic models In the Study Population

SNP	Risk Allele	Association Model	Unadjusted		Adjusted	
			OR(95% CI)	P-Value	Or(95% CI)	P-Value
rs5750248		ADDITIVE	0.94(0.78-1.12)	0.512	0.91(0.76-1.09)	0.385
	T	DOMINANT	0.92(0.44-1.95)	0.838	0.75(0.35-1.63)	0.453
		RECESSIVE	0.87(0.59-.26)	0.565	0.84(0.56-1.25)	0.403
		HETEROZYGOTE	1.12(0.75-1.64)	0.47	1.09(0.73-1.63)	0.65
rs5750250						
	G	ADDITIVE	0.86(0.63-1.19)	0.388	0.81(0.58-1.12)	0.206
		DOMINANT	1.00(0.48-2.08)	0.981	0.88(0.41-1.85)	0.793
		RECESSIVE	0.80(0.54-1.19)	0.275	0.75(0.49-1.12)	0.162
		HETEROZYGOTE	1.24(0.84-1.83)	0.275	1.27(0.85-1.90)	0.288
rs4821481						
	C	ADDITIVE	1.09(0.91-1.32)	0.323	1.08(0.90-1.31)	0.375
		DOMINANT	0.75(0.27-2.04)	0.576	0.71(0.25-2.00)	0.527
		RECESSIVE	0.75(0.51-1.12)	0.168	0.76(0.51-1.14)	0.189
		HETEROZYGOTE	1.26(0.85-1.87)	0.246	1.24(0.83-1.86)	0.286
rs2032487						
	C	ADDITIVE	0.97(0.85-1.10)	0.675	0.97(0.84-1.10)	0.660
		DOMINANT	0.75(0.27-2.04)	0.576	0.71(0.25-2.00)	0.527
		RECESSIVE	1.02(0.68-1.52)	0.909	1.02(0.67-1.53)	0.922
		HETEROZYGOTE	0.92(0.62-1.39)	0.724	0.93(0.61-1.40)	0.715

Significantly association if P-value < 0.05, marginal trend of association if P-value ≥ 0.05 ≤ 0.09

Table 4. 7b. Association between *MYH9* Variants and CKD of Unknown Aetiology tested under 4 genetic models

SNP	Risk Allele	Association Model	Unadjusted		Adjusted	
			OR(95% CI)	P-Value	Or(95% CI)	P-Value
rs5750248		ADDITIVE	0.91(0.76-1.11)	0.366	0.89(0.73-1.08)	0.231
	T	DOMINANT	0.96(0.43-2.11)	0.919	0.77(0.34-1.74)	0.521
		RECESSIVE	0.82(0.55-.23)	0.355	0.78(0.51-1.19)	0.258
		HETEROZYGOTE	1.12(0.80-1.82)	0.36	1.18(0.78-1.80)	0.421
rs5750250						
	G	ADDITIVE	0.83(0.59-1.15)	0.251	0.77(0.55-1.70)	0.128
		DOMINANT	1.05(0.48-2.26)	0.908	0.91(0.41-2.00)	0.813
		RECESSIVE	0.72(0.47-1.10)	0.136	0.67(0.43-1.03)	0.071
		HETEROZYGOTE	1.34(0.91-2.08)	0.131	1.43(0.93-2.18)	0.098
rs4821481						
	C	ADDITIVE	1.03(1.0-1.23)	0.759	0.63(0.90-1.31)	0.634
		DOMINANT	0.69(0.24-1.94)	0.487	0.69(0.23-2.01)	0.502
		RECESSIVE	0.97(0.64-1.47)	0.912	0.98(0.64-1.50)	0.947
		HETEROZYGOTE	0.96(0.63-1.46)	0.861	0.95(0.62-1.47)	0.832
rs2032487						
	C	ADDITIVE	0.96(0.84-1.11)	0.337	0.97(0.85-1.11)	0.727
		DOMINANT	0.69(0.24-1.94)	0.487	0.69(0.23-2.01)	0.502
		RECESSIVE	1.13(0.74-1.73)	0.585	1.14(0.74-1.75)	0.551
		HETEROZYGOTE	0.82(0.53-1.33)	0.375	0.82(0.52-1.27)	0.037

Significantly association if P-value < 0.05, marginal trend of association if P-value ≥ 0.05 ≤ 0.09

Table 4. 7c. Association between *MYH9* Variants and Sickle Cell Nephropathy tested under 4 genetic models

SNP	Risk Allele	Association Model	Unadjusted		Adjusted	
			OR(95% CI)	P-Value	Or(95% CI)	P-Value
rs5750248		ADDITIVE	1.08(0.81-1.44)	0.566	1.11(0.83-1.59)	0.471
	T	DOMINANT	1.18(0.38-3.57)	0.772	1.21(0.39-3.74)	0.736
		RECESSIVE	1.2(0.63-2.26)	0.575	1.2(0.66-2.43)	0.477
		HETEROZYGOTE	0.88(0.46-1.67)	0.697	0.84(0.43-1.62)	0.607
rs5750250						
	G	ADDITIVE	1.08(0.67-1.74)	0.759	1.11(0.83-1.49)	0.471
		DOMINANT	1.29(0.43-3.8)	0.641	1.30(0.43-3.98)	0.635
		RECESSIVE	0.04(0.55-1.96)	0.901	1.09(0.56-2.10)	0.788
		HETEROZYGOTE	1.05(0.55-2.05)	0.989	1.007(0.52-1.9)	0.983
rs4821481						
	C	ADDITIVE	2.01(1.45-2.79)	0.00	1.99(1.43-2.75)	0.000
		DOMINANT	2.19(0.45-10.6)	0.332	2.27(0.44-11.5)	0.323
		RECESSIVE	0.25(0.12-0.50)	0.00	0.26(0.13-0.52)	0.000
		HETEROZYGOTE	4.56(2.30-9.05)	0.00	4.40(2.20-9.80)	0.000
rs2032487						
	C	ADDITIVE	1.14(0.92-1.40)	0.233	1.11(0.89-1.38)	0.326
		DOMINANT	1.33(0.25-7.09)	0.736	1.28(0.22-7.19)	0.779
		RECESSIVE	0.71(0.37-1.35)	0.303	0.75(0.39-1.45)	0.394
		HETEROZYGOTE	1.47(0.77-2.82)	0.237	1.38(0.72-2.68)	0.329

Significantly association if P-value < 0.05, marginal trend of association if P-value ≥ 0.05 ≤ 0.09

4.6 Epistatic Interaction

Table 4.8a and 4.8b shows the interaction between Hb S genotypes with *APOL1* and *MYH9* variants in the general case-control and also by disease type and the effect on developing CKD. There was an increased risk of having CKD (OR=2.37, p-value= 0.009) for individuals who were both heterozygote for *APOL1* rs73885319 and are Hb S carries. There was no significant interaction between Hb S and any of the *MYH9* variants even after adjusting for possible confounding factors.

Table 4. 8a. Epistatic Interactions between Hb S and *APOL1* variants in CKD

SNP Combination		Sickle Cell CKD		CKD of Unknown aetiology		TOTAL CKD CASES	
		OR(CI)	P-Value	OR(CI)	P-value	OR(CI)	P-value
Hb S and rs60910145	SS* G/G	1.16(0.093-14.65)	0.94	-	-	1	-
	SS*T/G	0.93(0.088-10.0)	0.9	-	-	1	
	AS*G/G	9.37(0.4-239)	0.17	1.76(0.3-10.2)	0.52	3.36(0.65-17.2)	0.145
	AS*T/G	1.37(0.12-15.68)	0.78	1.12(0.5-2.2)	0.7	1.59(0.80-3.14)	0.182
Hb S and rs73885319	SS* G/G	0.55(0.0358.64)	0.65	-	-	1	-
	SS*A/G	0.76(0.06-9.32)	0.83	-	-	1	
	AS* G/G	0.007(0-NA)	1	1.31(0.2-8.3)	0.78	1.46(0.23-9.2)	0.687
	AS* A/G	1.58(0.13-19.2)	0.72	1.4(0.69-2.8)	0.34	2.37(1.23-4.54)	0.009
Hb S and rs9622363	SS* G/G	0.76(1.000 -NA)	0.9	1.28(0-NA)	1	1	-
	SS*A/G	0.54(0-NA)	1	0.03(0-NA)	1	1.93(0.32-11.4)	0.467
	AS* G/G	0.81 1.000)	1	0.003(0-NA)	0.98	1	
	AS* A/G	0.8(0-NA)	1	0.0001(0-NA)	0.98	0.38(0.13-1.18)	0.095
Hb S and rs71785313	SS* I/D	0.21(0.038-1.14)	0.07	0.589-NA)	1	1	-
	AS* I/D	0.09(0.007-1.12)	0.06	0.85(0.28-2.56)	0.78	0.93(0.32-2.73)	0.899

Significant interaction if P-value < 0.05

Table 4. 8b Epistatic Interactions between Hb S and *MYH9* variants in CKD

SNP Combination		Sickle Cell CKD		CKD of Unknown aetiology		TOTAL CKD CASES	
		OR(CI)	P-Value	OR(CI)	P-Value	OR(CI)	P-Value
Hb S and rs5750248	SS* T/T	527421.8(0-NA)	0.98	0.74(0-NA)	-	1	-
	SS*C/T	319414.6(0-NA)	0.98	0.5e-7(0-NA)	0.98	3.8(0.7-22.06)	0.137
	AS*T/T	373641(0-NA)	0.98	0.94(0.31-2.8)	0.92	1.32(0.5-3.83)	0.607
	AS*C/T	601533.2(0-NA)	0.98	0.75(0.25-2.2)	0.63	1.15(0.4-3.29)	0.788
Hb S and rs570250	SS* G/G	647043.2(0-NA)	0.98	0.72(0-NA)	1	1	-
	SS*A/G	491067(0-NA)	0.98	0.05e-7(0-N)	0.98	3.79(0.7-21.4)	0.13
	AS* G/G	619810.9(0-NA)	0.98	0.96(0.3-2.7)	0.98	1.31(0.46-3.7)	0.606
	AS* A/G	1181703(0-NA)	0.98	0.80 (0.3-2.23)	0.67	1.20(0.45-3.2)	0.701
Hb S and rs4821481	SS* C/C	1.05(0.10-10.24)	0.96	-	-	1	-
	SS*T/C	4.6(0.48-44.4)	0.18	-	-	5.6(0.88-36.3)	0.67
	AS* C/C	1.34(0.12-14.4)	0.8	0.91(0.5-1.75)	0.78	0.88(0.25-3.1)	0.845
	AS* T/C	4.25(0.43-41.7)	0.12	0.88(0.4-1.96)	0.77	1.330.36-4.95)	0.644
Hb S and rs2032487	SS* C/C	0.69(0.14-3.2)	0.6	-	-	1	-
	SS*T/C	0.69(0.13-3.5)	0.65	-	-	2.77(0.4-18.6)	0.295
	AS* C/C	0.61(0.11-3.34)	0.57	1.02(0.54-1.92)	0.94	1.05(0.29-3.7)	0.933
	AS* T/C	1.22(0.23-6.3)	0.81	0.64(0.3-1.5)	0.3	1.06(0.3-3.9)	0.931

Significant interaction if P-value < 0.05

CHAPTER 5

DISCUSSION

5.1 Hb S and C Association with CKD

Several studies have suggested a genetic component to the occurrence of CKD and ESRD especially in the African population (Cavanaugh & Lanzkron, 2010). The genetic predispositions have been attributed to *APOL1/MYH9* locus found on chromosome 22 but does not fully explain the susceptibility of Africans to a higher risk of developing CKD. While these findings have great consequences for CKD and ESRD, the identification of other risk factors for CKD, both environmental and genetic in association with eGFR, will help improve the understanding of the fundamentals of CKD in African populations (Parsa *et al*., 2013).

Chronic kidney disease is known to occur throughout the life of Sickle cell patients and reported as an early cause of death in sickle cell patients (Nath & Hebbel, 2015). Most reports on CKD in sickle cell patients have been fixated on Hb SS patients (Saraf *et al*., 2014). Hb S has however been reported to have a dominant effect on risk of incidence of CKD, with SCT being associated with ESRD (Hicks *et al*., 2011). Nonetheless, with limited literature on sickle cell trait in the development of renal complications (Hicks *et al*., 2011), there are conflicting reports as to whether SCT is a risk factor for nephropathy (Cavanaugh & Lanzkron, 2010). The haemodynamic changes in the arteries of the kidney that occur in sickle cell anaemia due to hypoxia

causes structural changes in the glomerulus which is marked as a hallmark of renal damage.

The results of this study revealed that Hb S has a dominant effect on the incidence of CKD, increasing the risk of developing nephropathy to about 70% and about 9 fold increased risk for individuals homozygote for Hb S (table 4.5). Out of a total number of 202 CKD patients, 22.3% of them had sickle cell disease which makes 34% of the sickle cell patients selected for the study (table 4.1). The observed prevalence of CKD in sickle cell patients in this study is similar to what have been reported by other studies separately in Ghana (39%) (Ephraim *et al*., 2015) and in Nigeria (~40%) (Aneke *et al*., 2014). The prevalence of sickle cell nephropathy recorded in this study and the observed 2-9 fold increases risk of developing CKD adds to knowledge that kidney damage is a common comorbidity in Sickle cell disease marked by the presence of sickled red blood cells in the renal vessels (Platt *et al*., 1994; Guasch *et al*.,, 2006; Nath & Hebbel, 2015).

However, there was no significant change in risk of disease observed in the heterozygotes (Hb AS) individuals. This finding however is in agreement with a study in Congo which reports no independent association of SCT with CKD (Mukendi *et al.*, 2015) but conflicting with studies with a larger population size in African American populations that show a significant association between SCT and CKD incidence and ESRD in individuals with non-diabetic and non-hypertensive CKD (Naik *et al.*, 2017). The inconclusive role of Hb AS in disease association with CKD observed in this study as compared to other studies could be due to small sample sizes, the category of renal disease being studied and the different study population.

Interestingly our study agrees with what is reported by Mukendi and his colleagues who used an indigenous African population as represented in our study but did not discriminate against diabetes and hypertension, which was a major exclusion criterion for this study (Mukendi *et al.*, 2015). This however points out some of the challenges faced in identifying genetic predispositions to complex disease phenotypes (Ashley-Koch *et al*., 2011).

Sickle cell trait is known as a benign condition and generally associated with less severe complication as compared to sickle cell disease. Nonetheless, under certain conditions such as urinary tract infection, severe pneumonia, altitude hypoxia, gross haematuria, and complications associated with exercise, serious morbidity or mortality can occur (Kark, 2000.). Therefore, the association of Hb AS with renal abnormalities may be attributed to other comorbid conditions of CKD such as diabetes and hypertension (Cavanaugh & Lanzkron, 2010). Some renal abnormalities that have been associated with sickle cell trait include microscopic haematuria, mild urinary concentrating defect, renal medulla carcinoma and renal papillary necrosis even though the relationship between SCT and long-term kidney impairment has not been well established (Naik *et al.*, 2014). These conflicting findings therefore highlight the need to further investigate the role of SCT in CKD, where it could possibly be interacting with other risk factors both genetic and environmental to confer susceptibility to CKD rather than being an independent risk factor.

Contrast to Hb S, Hb C was not associated with increased risk of developing CKD but rather showed a protection against developing renal damage in the study population (table 4.5). Unlike Hb S, Hb C does not cause linear intracellular polymerization in

intravascular areas of low oxygen tension and there is also reduced red cell deformability associated with the Hb C variant. The protection of Hb C individuals from developing renal damage can be attributed to less severity of Hb C and the rare occurrence of renal damage in individuals with Hb C genotypes (Okafor & Aneke, 2013). Naik and colleagues in 2017, recorded 25.9% of 250 haemoglobin C trait individuals with CKD in their study. However, after adjusting for age, sex, smoking status, diabetes, hypertension and *APOL1* high-risk genotype status, no association of increased risk was found in these individuals. Also, other reported associations of Hb C with increased risk developing kidney disease were found in Hb SC which presents with sickling disorder and severe pathologies similar to Hb SS (Bachir & Galacteros, 2004). The above among other reasons implies that being positive for Hb C may not be an independent risk factor for developing renal abnormalities. However possible association with CKD may occur when inherited with other severe forms of haemoglobinopathy variants (eg. Hb S), and with other comorbidities and risk factors. This may only be a speculation and requires further studies to ascertain the role and contribution of Hb C in pathogenesis and risk of CKD.

5.2 *MYH9* Association with CKD

The genetic predisposition of the chromosome 22 locus in previous studies have mostly focused on Diabetes, hypertension, FSGS, and HIV associated nephropathy in African American and Hispanic American population (Kao *et al.*, 2008; Genovese *et al.*, 2010; Tzur et al., 2010; Kasembeli *et al.*, 2015) . The purpose of this study was to focus on association between known SNPs in this locus in SCD CKD and CKD of

unknown aetiologies in a representative sample of Africans who are reportedly at a much higher risk of developing CKD.

Generally, the study recorded a weak association between *MYH9* and CKD (table 4.7a). A further analysis, after stratifying by disease phenotype sub-groups, showed an evidence of association between *MYH9* SNPs and risk of developing CKD. A marginal trend towards reduced risk and increased risk of developing CKD of unknown aetiology given that an individual inherited the rs5750250 risk allele homozygotes or heterozygotes, respectively. Heterozygotes for rs2032487 variant showed about 20% protection from developing CKD of unknown aetiology (table 4.7b). The study also showed that inheriting the *MYH9* variant rs482148 may increase the risk of sickle cell nephropathy by between 1- 4 folds under additive and heterozygote models of genetics association (table 4.7c).

MYH9 has been previously associated with excess risk of non-diabetic CKD and ESRD in African Americans and Hispanic Americans (Freedman *et al*., 2009). This high risk observed in hypertension-associated ESRD, FSGS and HIV-associated nephropathy (HIVAN) in African Americans appears to be due to a comparatively lower frequency of *MYH9* risk alleles in European Americans (4%), compared to African Americans (60%) (Kao *et al*., 2008). Most of *MYH9* associated nephropathy has been attributed to the SNPs in the E-1 (rs4821480, rs2032487, and rs4821481), S-1(rs5750250, rs2413396, rs5750248) and F-1 (rs16996674, rs16996677, rs11912763) haplotypes. Even though S-1 and F-1 haplotype are known to have stronger disease association as compared to the E-1, the former have been shown to be in a linkage disequilibrium with the latter (Behar *et al*., 2010). In a genetic association study in

SCD in African American population, an increased risk of disease conferred by individual *MYH9* SNPs and after repeating analysis in SNP haplotypes was observed (Ashley-Koch *et al*., 2011).

The genetic models of association between *MHY9* SNPs and developing CKD identified by Behar *et al* (2010) and other studies showed an increased risk of developing nephropathy under a recessive and additive inheritance mode. In this study, for rs4821481 under both heterozygote and additive mode there was a significant increased risk of association between the SNP and sickle cell nephropathy. It is intriguing to however note that the under the recessive model both rs5750250 and rs4821481 rather showed a reduced risk of developing CKDU and sickle cell nephropathy respectively. Though the model of genetic association observed between *MHY9* SNPs and the disease phenotypes included in this study is does not reflect what have generally been observed in previous studies, it is evident the *MYH9* is also possibly associated with sickle cell nephropathy and CKD of unknown aetiology in an indigenous African population. This association would therefore be better understood with a larger sample size and in a haplotype analysis.

5.3 *APOL1* Association with CKD

APOL1 has mostly been associated with high risk of CKD in HIV, hypertension and FSGS among African Americans (Genovese *et al*., 2010; Tzur et al., 2010). The purpose of this study was to investigate the evidence of association between *APOL1* and both sickle cell nephropathy and CKD of unknown aetiology in a sample of population of West Africans (Nigeria and Ghana). The results represented in this

study (4.6b) provide evidence that *APOL1* is similarly associated with CKD of unknown aetiology as have been previously been reported in African Americans, Hispanic Americans and an indigenous Yoruba population (Tayo *et al*., 2013) with other forms of nephropathy (hypertensive, HIVAN, FSGS). The disease association found in this study with the two *APOL1* G1 variants (rs73885319 and rs60910145).

Association between *APOL1* renal risk variants and CKDU has not really been reported elsewhere and this may be the first observed association. The association found between *APOL1* and CKDU in this study may not be entirely surprising because, even though CKD of unknown aetiology is less mentioned in studies that have associated *APOL1* with nondiabetic nephropathy (Tayo *et al*., 2013; Genovese *et al*., 2010; Tzur et al., 2010), CKD of unknown aetiology could have been captured in these studies that did not discriminate against type of non-diabetic nephropathy being investigated. This study also reported about 2 fold increased risk of association between *APOL1* G1 variants and CKDU (table 4.6b). This association may not be as strong as the 7 fold increased odds of CKD in hypertensive (Freedman *et al*., 2010) and a stronger even much higher odds of about 30 folds increased association in HIV nephropathy (Tzur *et al.*, 2010). This notwithstanding this study was able to show that, the *APOL1* kidney disease risk may also associated with the risk of developing CKD of unknown aetiology.

The direct effect of *APOL1* on podocyte and glomerular function has not been known and there have been controversies on whether *APOL1* is a direct causal factor for renal abnormalities (Beckerman *et al*., 2017). Some in-vitro studies have demonstrated that APOL1 could induces cytotoxicity which results in cellular damage

(lan and olabisi). Studies by Beckerman and his colleagues showed that podocyte specific expression of APOL1 either of the *APOL1* risk variants in mice resulted in functional (albuminuria and azotemia), structural (foot-process effacement and glomerulosclerosis) and molecular (gene-expression) changes that almost resembled kidney disease in humans. Linking the above mentioned studies to the association between *APOL1* and CKD of unknown aetiology observed in this study, it may be that *APOL1* is causal for podocyte and glomerular injury and can potentiate long term kidney damage (Beckerman *et al*., 2017).

In addition, the results did show find any significant association between *APOL1* and sickle cell nephropathy (table 4.6c). The result of the study as indicated earlier stand in contrast to a study with a population of 521 African Americans sickle cell patients (Ashley-Koch *et al*., 2011). They, reported an association between *APOL1* was and proteinuria, an indication for kidney damage in sickle cell patients. Another study conducted in Europe with a predominantly (78%) SSA origin study cohort, also recorded a significant association of *APOL1* with age-dependent decrease in GFR and ESRD in sickle cell patients (Kormann *et al.*, 2017). The inconsistency of our results with the findings from these two studies could also be attributed partly to smaller size of our sickle cell group and differences in the genetic backgrounds of the population in these different studies. Their study was conducted in an African American population unlike the indigenous African population used for our study. Current generation of African Americans have mixed genetic background due to random mating with other races and exposure to different environmental factors such as

climate changes which could lead to evolution of certain genetic information (Tishkoff *et al*., 2009) from their ancestral Africans.

Previous studies have reported strong independent disease associations with both *APOL1* risk alleles G1 (rs73885319 and rs60910145) and G2 (rs71785313) in an autosomal recessive manner (Tayo *et al.*, 2013). In this study, associations were observed between the G1 risk allele and CKD in a dominant and additive models with no significant associations in the recessive model. The G2 allele had no significant association with either of the CKD phenotypes; CKD of unknown aetiology of Sickle cell Nephropathy. This discrepancy may be partly due to the effect of sample size and the observed low minor allele frequency for G2 (10%) compared to 24% prevalence recorded in Africa (Thomson *et al*., 2014).

5.4 Epistatic Gene-Gene Interaction

Since not all genetically predisposed individuals develop CKD, modifying factors contribute to the incidence and the percentage risk of developing CKD (Bostrom *et al*., 2012). The influence of genetic factors on non-communicable diseases is complex and certainly involves multiple genes with several gene-gene interactions. Thus based on the anticipation that Hb S, *APOL1* and *MYH9* may be interacting with each other to modify the genetic risk of developing CKD, gene-gene interaction analysis was performed to determine possible association between Hb S genotypes and the selected SNPs from the *APOL1/MYH9* locus.

From the individual association analysis, individuals who were heterozygote for *APOL1* variant rs73885319 were recorded to have about 50% increased risk of developing CKD (table 4.6a), whereas no significant association was observed for Hb AS carriers (table 4.5). However, upon conducting gene-gene interaction analysis, there was a further increased risk of having nephropathy (130%) (table 4.8a) as compared to what was observed in only heterozygote rs73885319. This, indicates a possible synergistic epistatic interaction between the two SNPs. In contrast to prior study by Hicks *et al*., 2011, this study was able to show that Hb AS is also associated with an increased risk of developing CKD as was independently reported by Naik *et al*., even though the association found in this study is in the presence of *APOL1* G1 variant. This finding is rather intriguing, as it presents us with a possibility that Hb S carriers may also be at risk of renal damage as they are likely to be exposed to other common genetic variants associated with Kidney disease in African populations to potentiate their risk of developing CKD.

Hicks *et al*., (2011) after finding lack of independent association of Hb AS with CKD, also performed a gene-gene interaction analysis but again did not find any significant interactions between any *APOL1* genotype and Hb AS in conferring increased risk of developing CKD. They argued that lack of genetic interaction between these two genes is backed by the fact that *APOL1* and Hb S are associated with susceptibility to renal disease in an autosomal recessive patterns and the absence of evidence of risk of nephropathy in individuals with sickle cell trait. However, the interaction found in this study cannot be doubted on the bases of their arguments since their study recorded

relatively small Hb S frequency (Naik *et al*., 2017) and there is evidence of similar interaction of *APOL1* variants with some other genes (Divers *et al.*, 2014).

Sickle cell trait individuals are normally unaffected till they in low oxygen tension environments (Kark John, 2000.). Injection radiographs of the renal medulla have confirmed vascular disruption in individuals with Hb SS disease and SCT compared with those with Hb AA, suggesting a common mechanism of renal injury in both genotypes (Naik *et al.*, 2017). In SCD, hypoxia in the renal medulla leads to ischemic-reperfusion injuries and vaso-oclusion resulting in progressive glomerulosclerosis. A similar mechanism with mild consequences may underlie the risk of developing nephropathy in SCT (Ashley-Koch *et al.*, 2011). Some studies have suggested that the expression of these risk variants induces cytotoxicity (Olabisi *et al*., 2016) resulting in cellular injury (Lan *et al*., 2015) but the mechanism of inducing toxicity has not been fully clarified. It is however clear that, additional functional study is necessary to confirm and understand how these two gene-gene interaction may be occurring to cause kidney disease.

CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This study was a unique study in the sense that it was done using indigenous African population and also excluded hypertension and diabetes, the two known leading causes of CKD which have been represented in most genetic predisposition to CKD studies.

People with Hb S in this study were found to have about 2-9 fold increased risk of having nephropathy although Hb AS did not show any significant association with CKD. Hb C on the other hand rather showed protection against disease development in both homozygous and heterozygous individuals.

APOL 1 G1 variants (rs73885319 and rs60910145) are associated with increased risk of developing CKD of unknown aetiology with rs73885319 presenting 70-100% risk of developing nephropathy. There was a trend towards increasing the risk of having nephropathies, given that an individual had the *APOL1* variant rs60910145. In contrast to the G1 variants, there was an observed trend of being protected against developing CKD given that one had he *APOL1* variant rs9622363 which is a tagging SNP in the G1 locus. There was no association of *APOL1* with sickle cell nephropathy as reported in other populations. However, even though Hb AS and heterozygotes for *APOL1* variant rs73885319 were not individually found to have an increased risk of developing CKD, there is an increased risk of disease (130%) in individuals who are heterozygous for both Hb S and *APOL1* rs73885319. The risk

conferred by the presence of Hb AS and heterozygous *APOL1* rs73885319 indicates a possible synergistic epistatic interaction between both SNPs which need to be further investigated.

MYH9 showed an evidence of association with CKD with variant rs4821481 associated with sickle nephropathy increasing the risk (99%) of renal damage in sickle cell patients and rs5750250 presenting a trend of increasing risk of CKD of unknown aetiology.

Some associations reported in other literature may not have been replicated here due to, rather small sample sizes, different case inclusion criteria and different genetic backgrounds (haplotypes) surrounding the SNPs being analyzed. However, in view of the above findings, this study has potential extensive public health implications. Public information on the genetic predispositions to CKD imposed by these SNPs would allow for screening especially at an early age to identify those at risk. Screening at birth to identify SCD patients and Hb S carriers will help in prevention of certain lifestyle modifying factors such as smoking, chronic alcohol intake and NSAID which could potentiate CKD.

6.2 Recommendations

Based on the observed associations and interactions, it is recommended that,

1. Future studies using a larger sample size would allow for confirmation of the observed associations and detect new associations.

2. Further studies with same case definitions across study sites studies will also be required to replicate these observed associations.
3. Genome-Wide Association Studies is also recommended in order to be able to cover other SNPs that may have been missed in this study and other studies.
4. In studying risk factors of CKD of unknown aetiology in the future, other factors such as lifestyle (e.g. Smoking, eating habit, alcohol intake) of study participants and environmental factors; (eg. Infectious diseases) should be considered in further studies.
5. Also, other common Genetic factors which are known to be associated with *APOL1* induced non-diabetic forms of CKD; NPHS2, and Bone Morphogenic Protein 4 (Divers *et al.*, 2014) should also be considered in the pattern of genetic susceptibility to renal damage in sickle cell patients and people with CKD of unknown aetiologies.
6. Further studies to understand the mechanisms by which these SNPs individually and together cause renal abnormalities will help in developing therapies as interventions for early onset of disease, delay progression and possibly prevent the occurrence of CKD in individuals who are genetically at risk.

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Appendix

Appendix 1: Primer sequences of SNP used for PCR

Gene	SNP	Forward Primer	Reverse Primer
Beta Globin	Hb S and C	GGAGCACGCTATCCCGTTAGACCTCACCACCAACTTCATCCA	CGCTGCCAACTACCGCACATGGCAACCTCAAACAGACACCA
APOL1	rs60910145	GGAGCACGCTATCCCGTTAGACAGAGACAGCTGAGGAGCTGAA	CGCTGCCAACTACCGCACATGGCCCTGTGGTCACAGTTCTT
APOL1	rs73885319	GGAGCACGCTATCCCGTTAGACCCTGGAAATGAGCAGAGGAG	CGCTGCCAACTACCGCACATGGACTTTGCCCCCTCATGTAA
APOL1	rs9622363	GGAGCACGCTATCCCGTTAGACAAGTCAGCTGCCACCAAAAC	CGCTGCCAACTACCGCACATGGCCTCAGTGCACACACAAAT
APOL1	rs71785313	GGAGCACGCTATCCCGTTAGACTGAAGAAGGTGGCTCAGGAG	CGCTGCCAACTACCGCACATGATATCTCTCCTGGTGGCTGC
MYH9	rs5750248	GGAGCACGCTATCCCGTTAGACAAGGTCATCCCCAAACACAG	CGCTGCCAACTACCGCACATGAGCTTCGAGAGTGGCTGAAG
MYH9	rs5750250	GGAGCACGCTATCCCGTTAGACGTCAGCGGATGTGCACTTTT	CGCTGCCAACTACCGCACATGGGCTGACACCTTTACCCAAG
MYH9	rs4821481	GGAGCACGCTATCCCGTTAGACTTTACACAGGCTCGGGTCTC	CGCTGCCAACTACCGCACATGGGGCCTTAGTTGAGGGATTT
MYH9	rs2032487	GGAGCACGCTATCCCGTTAGACAGAGAGGGGCACGGAGAAG	CGCTGCCAACTACCGCACATGTGGGTGAGAAAGGATTCCAG
Amelogenin X and Y		GGAGCACGCTATCCCGTTAGACCCCTGGGCTCTGTAAAGAATAGTG	CGCTGCCAACTACCGCACATGATCAGAGCTTAAACTGGGAAGCTG
Uni Primer		GGAGCACGCTATCCCGTTAGAC	CGCTGCCAACTACCGCACATG

Appendix 2: LDR probe sequences showing attached fluorescent labels and product size for each allele

SNP	L1 -Reference Allele/ Product Size	L1-Risk Allele/ Product size	L2
HB S	/5TET/CAATTAGCACCATGGTGCATCTGACTCCTGA/ 90bp	/56-FAM/ACCATGGTGCATCTGACTCCTGT/ 82bp	/5Phos/GGAGAAGTCTGCCGTTACTGCCCATGCTCAGACACAATTAGCGCGACCCTTAATCCTTA
HB C	/56-FAM/ACCATGGTGCATCTGACTCCTG/ 78bp	/5TET/ATTAGCACACCATGGTGCATCTGACTCCTA/ 86bp	/5Phos/AGGAGAAGTCTGCCGTTACTGCCATGCTCAGACACAATTAGCGCGACCCTTAATCCACGT
rs60910145	/5TET/AGGAGCTGGAGGAGAAGCTAAACATT/ 54bp	/56-FAM/ACGTACGTAGGAGCTGGAGGAGAAGCTAAACATG/ 62bp	/5Phos/CTCAACAATAATTATAAGATTCTGCAGG
rs73885319	/5TET/TCACGGATGTGGCCCCTGTAA/ 50bp	/56-FAM/ACGTACGTTCACGGATGTGGCCCCTGTAG/ 58bp	/5Phos/GCTTCTTTCTTGTGCTGGATGTAGTCTAC
rs9622363	/56-FAM/CCCCAGTTCCTTATCTGCAATTCCATA/ 54bp	/5TET/ACGTACGTCCCCAGTTCCTTATCTGCAATTCCATG/ 62bp	/5Phos/ATTCCCAGATCCCTGCAAACCAAACTG
rs71785313	/56-FAM/ACTGGAGGAGAAGCTAAACATTCTCAACAATAATTATAA/ 90bp	/5TET/TGGAGGAGAAGCTAAACATTCTCAACAATAA/ 82bp	/5Phos/GATTCTGCAGGCGGACCAAGAATGCTCAGACACAATTAGCGCGACCCTTAA
rs5750248	/5TET/ACGTACGTGGGAGCCATGCATCTGCCAC/ 58bp	/56-FAM/CGGAGCCATGCATCTGCCAT/ 50bp	/5Phos/GGCCACCTCACCTTCCTGTCAATGCTCAGA

rs5750250	/5TET/TGACACCTTTACCCAAGGATCCATCA/ 81bp	/56-FAM/ACGTACGTTGACACCTTTACCCAAGGATCCATCG/ 89bp	/5Phos/CGGTGGTTCTCCAGCATTAGTGAAAATGCTCAGACACAATTAGCGCGACCCTTAA
rs4821481	/5TET/ACGGCTGGCAAAGAAGAGCTTTCT/ 82bp	/56-FAM/ACGTACGTACGGCTGGCAAAGAAGAGCTTTCC/ 90bp	/5Phos/AGAGGGGAAAGGCAAACCCTTATGCTCAGACACAATTAGCGCGACCCTTAATCCTTAG
rs2032487	/5TET/ACGTACGTCCACACGGCGCTCACCTGT/ 58bp	/56-FAM/CCACACGGCGCTCACCTGC/ 50bp	/5Phos/GCCACCAGGCCACCTTCTCCAACTGACTAAA
Ame X	/56-ROXN/TGTTGATTCTTTATCCCAGATGTTTCTCAAG/ 74	N/A	/5Phos/TGGTCCTGATTTTACAGTTCCTACCACCATGCTCAGACACAAT
Ame Y	/56-FAM/CGATTCTTCATCCCAAATAAAGTGGTTTC/ 66	N/A	/5Phos/TCAAGTGGTCCCAATTTTACAGTTCCATGCTCAGACA

Appendix 3: Sample size calculation

$$n = \left(\frac{r+1}{r}\right)\frac{(\bar{p})(1-\bar{p})(Z_{\beta}+Z_{\alpha/2})^2}{(p_1-p_2)^2}$$

N is Sample size for case

P_1 is the prevalence of case

P_2 is the prevalence of the control

r is the ratio of case to control

$Z_{\alpha/2}$ is the type II error at a desired power

Z_{β} is the significant level

Therefore,

For 80% power, $Z_{\beta} = 0.84$

For 0.05 significance level, $Z_{\alpha} = 1.96$

For and equal ratio of case to control, r = 1

To calculate for prevalence of control group,

$$OR = \frac{P_2(1-P_1)}{P_1(1-P_2)}, \qquad 3 = \frac{P_2(1-0.16)}{0.16(1-P_2)}$$

$$0.48(1-P_2) = 0.84P_2, \qquad 0.48-0.48P_2 = 0.84P_2,$$

$$0.48 = 0.84P_2 + 0.48P_2$$

$$P_2 = 0.36$$

$$\bar{P} = \frac{(P_2 + rP_1)}{1+r}, \qquad \bar{P} = \frac{(0.36+0.16)}{2}$$

$$\bar{P} = 0.26$$

n is therefore, $= \frac{1+1}{1}\frac{(0.26)(1-0.26)(0.84+1.96)^2}{(0.16-0.36)^2}$

n = 75.42

The least estimated sample size for cases and controls is 75 each.

Appendix 4: SNP Association in individuals with CKD Compared to Population Control Testes under heterozygote Model

SNP	RISK ALLELE	TEST	CASE	CONTROL	χ^2 (P-value)
HB					
rs334	T	A/A*T/T	193(74.52)	128(76.65)	
		A/T	66(25.48)	39(23.35	0.247(0.619)
rs33930165	A	G/G*A/A	250(96.53)	152(91.02)	
		G/A	9(3.47)	15(8.98)	5.79(0.016)
APOL1					
rs73885319	G	A/A*G/G	104(59.85)	84(50.30)	
		A/G	155(59.85)	83(49.70)	4.2(0.040)
rs60910145	G	G/G*T/T	129(49.81)	90(53.89)	
		G/T	130(50.19)	77(46.11)	0.67(0.410)
rs9622363	G	A/A*G/G	89(34.50)	33(25.19)	
		A/G	169(65.50)	98(74.81)	3.49(0.062)
MYH9					
rs5750248	T	C/C*T/T	134(51.74)	91(54.49)	
		C/T	125(48.26)	76(45.51)	0.30(0.578)
rs5750250	G	A/A*G/G	124(47.88)	89(53.29)	
		A/G	135(52.12)	78(46.71)	1.19(0.275)
rs4821481	T	C/C*T/T	136(53.54)	99(59.28)	
		C/T	118(46.46)	68(40.72)	1.34(0.246)
rS2032487	T	C/C*T/T	164(64.57)	105(62.85)	
		T/C	90(35.43)	62(37.13)	0.12(0.724)

Appendix 5: SNP Association in individuals with CKD Compared to Population Control under Dominant Model

SNP	RISK ALLELE	TEST	CASE	CONTROL	χ^2 (P-value))
HB					
rs334	T	A/A	161(62.16)	126(75.45)	
		A/T*T/T	98(37.84)	41(24.55)	8.15(0.004)
rs33930165	A	G/G	250(96.53)	150(89.92)	
		G/A*A/A	9(3.47)	17(10.18)	7.96(0.005)
APOL1					
rs73885319	G	A/A	79(35.50)	72(43.11)	
		A/G*G/G	180(69.50)	95(56.89)	7.05(0.008)
rs60910145	G	T/T	99(38.22)	78(46.71)	
		T/G*G/G	160(61.78)	89(53.29)	3.00(0.083)
rs9622363	G	A/A	10(3.88)	1(0.76)	
		A/G*G/G	248(96.12)	130(99.24)	3.063(0.080)
MYH9					
rs5750248	T	C/C	20(7.72)	12(7.19)	
		C/T*T/T	239(92.28)	155(92.81)	0.0420(0.838)
rs5750250	G	A/A	20(7.72)	13(7.78)	
		A/G*G/G	239(92.28)	154(92.22)	0.0006(0.981)
rs4821481	C	T/T	12(4.72)	6(3.59)	
		C/T*C/C	242(95.28)	161(96.41)	0.31(0.574)
rs2032487	C	T/T	12(4.72)	6(3.50)	
		T/C*C/C	242(95.28)	161(96.41)	0.004(0.95)

Appendix 6: SNP Association in individuals with CKD Compared to Population Control under Dominant Model

SNP	RISK ALLELE	TEST	CASE	GPC	χ^2 (P-value)
HB					
RS 334	T	A/A*A/T	227(87.64)	165(98.80)	
		T/T	32(12.36)	2(1.20)	17.21(0.00)
RS 33930165	A	G/G*G/A	259(100)	165(98.80)	
		A/A	0(0.00)	1.20(2.00)	1.21(0.39-3.74)
APOL1					
rs73885319	G	A/A*A*G	234(90.35)	155(92.81)	
		G/G	25(9.65)	12(7.19)	0.77(0.377)
rs60910145	G	T/T*T/G	229(88.42)	155(92.81)	
		G/G	30(11.58)	12(7.19)	2.21(0.137)
Rs9622363	G	A/A*A/G	175(73.53)	99(75.57)	
		G/G	63(26.47)	32(24.43)	1.63(0.201)
MYH9					
Rs5750248	T	C/C*C/T	145(55.98)	88(52.69)	
		T/T	114(44.02)	79(47.31)	0.44(0.505)
Rs5750250	A	A/A*A/G	155(59.85)	91(54.49)	
		G/G	104(40.15)	76(45.51)	1.19(0.275)
Rs4821481	C	T/T*C/T	130(51.18)	74(44.31)	
		C/C	124(48.82)	93(55.69)	1.9(0.168)
RS2032487	C	T/T*T/C	102(40.16)	68(40.72)	
		C/C	152(59.84)	99(59.28)	0.0132(0.909)

Appendix 7: SNP Association in individuals with Sickle Cell Nephropathy Compared to Sickle Cell Control under Additive Model

SNP	RISK ALLELE	TEST	SCN	SCC	χ^2 (P-value)
APOL1					
rs73885319	G	A/A	17(29.31)	38(34.55)	2.50(0.286)
		A/G	38(65.52)	60(54.55)	
		G/G	3(5.17)	12(10.91)	
rs60910145	G	TT	41(37.61)	41(37.61)	0.659(0.719)
		T/G	55(50.46)	55(50.46)	
		G/G	13(11.93)	13(11.93)	
rs71785313	"_"	Ins/Ins	50(86.21)	83(76.85)	0.2550.613)
		Ins/Del	8(13.79)	25(23.15)	
		Del/Del	0	0	
rs9622363	G	A/A	0(0.00)	0(0.00)	0.255(0.613)
		A/G	39(67.24)	76(71.3)	
		G/G	19(32.76)	31(28.97)	
MYH9					
rs5750248	T	C/C	5(8.62)	11(10.00)	0.330(0.848)
		C/T	24(41.38)	49(44.55)	
		T/T	29(50.00)	50(45.45)	
rs5750250	G	A/A	5(8.62)	12(10.91)	0.219(0.896)
		A/G	25(43.10)	46(41.82)	
		G/G	28(48.28)	52(47.27)	
rs4821481	T	T/T	2(3.45)	8(7.27)	20.13(0.000)
		C/T	40(68.97)	36(32.73)	
		C/C	16(27.59)	66(60.00)	
rs2032487	T	T/T	5(4.55)	5(4.55)	1.427(0.490)
		C/T	26(44.83)	39(35.45)	
		C/C	30(51.72)	66(60.00)	

Appendix 8: SNP Association in individuals with Sickle Cell Nephropathy Compared to Sickle Cell Control under Heterozygous Model

SNP	RISK ALLELE	TEST	SCN	SCC	χ^2 (P-Value)
APOL1					
rs73885319	G	A/A*G/G	20(38.48)	50(45.45)	
		A/G	38(65.52)	60(54.55)	1.88(0.170)
rs60910145	G	G/G*T/T	32(55.17)	54(49.54)	
		G/T	26(44.83)	55(50.46)	
Rs9622363	G	A/A*G/G	19(32.76	31(28.97)	
		A/G	39(67.24)	76(71.03)	0.25(0.613)
MYH9					
Rs5750248	T	C/C*T/T	34(58.62)	61(55.45)	
		C/T	24(41.38)	49(44.55)	0.15(0.694)
Rs5750250	G	A/A*G/G	33(56.90)	64(58.18)	
		A/G	25(43.10)	46(41.82)	0.025(0.873)
Rs4821481	T	C/C*T/T	18(31.03)	74(67.27)	
		C/T	40(68.97)	36(32.73)	20.13(0.000)
RS2032487	T	C/C*T/T	32(55.17)	71(64.55)	
		T/C	26(44.83)	39(35.45)	1.40(0.236)

Appendix 9: SNP Association in individuals with Sickle Cell Nephropathy Compared to Sickle Cell Control under Dominant Model

SNP	RISK ALLELE	TEST	SCN	SCC	χ^2 (P-Value)
APOL1					
rs73885319	G	A/A	17(29.31)	38(34.55)	
		A/G*G/G	41(70.69)	72(65.45)	0.472(0.492)
rs60910145	G	T/T	23(39.66)	41(37.61)	
		T/G*G/G	35(60.34)	68(62.39)	0.066(0.796)
rs9622363	G	A/A	58(100.0)	0(0.00)	
		A/G*G/G		107(100.0)	4.26(0.03)
MYH9					
rs5750248	T	C/C	5(8.62)	20(7.72)	
		C/T*T/T	53(91.38)	239(92.28)	0.08(0.772)
rs5750250	G	A/A	5(8.62)	12(10.91)	
		A/G*G/G	53(91.38)	98(89.09)	0.21(0.640)
rs4821481	C	T/T	2(3.45)	8(7.27)	
		C/T*C/C	56(96.55)	102(92.73)	0.99(0.319)
rs2032487	C	T/T	2(3.45)	5(4.55)	
		T/C*C/C	56(96.55)	105(95.45)	0.1140.735)

Appendix 10: SNP Association in individuals with Sickle Cell Nephropathy Compared to Sickle Cell Control under Recessive Model

SNP	RISK ALLELE	TEST	SCN	SCC	χ^2 (P-value)
APOL1					
rs73885319	G	A/A*A*G	55(94.83)	98(89.09)	
		G/G	3(5.17)	12(10.91)	0.53(0.215)
rs60910145	G	T/T*T/G	49(84.48)	96(88.07)	
		G/G	9(84.48)	13(11.93)	0.43(0.514)
rs9622363	G	A/A*A/G	39(67.24)	76(71.03)	
		G/G	19(32.76)	31(28.97	0.25(0.613)
MYH9					
rs5750248	T	C/C*C/T	29(50.00)	60(54.55)	
		T/T	29(50.00)	50(45.45)	0.31(0.575)
rs5750250	A	A/A*A/G	30(51.72)	58(52.73)	
		G/G	28(48.28)	52(47.27)	0.015(0.901)
rs4821481	C	T/T*C/T	42(72.41)	44(40.00)	
		C/C	16(27.59)	66(60.00)	15.96(0.000)
rs2032487	C	T/T*T/C	28(48.28)	44(40.00)	
		C/C	30(51.72)	66(60.00)	1.06(0.303)

Appendix 11: SNP Association in individuals with CKD of Unknown aetiology Compared to Population Control under Additive Model

SNP	RISK ALLELE	TEST	CKDU	GPC	χ^2 (P-value)
APOL1					
rs73885319	G	A/A	62(30.85)	72(43.11)	
		A/G	117(58.21)	83(49.70)	
		G/G	22(10.95)	12(7.19)	6.38(0.041)
rs60910145	G	TT	76(37.81)	78(46.71)	
		T/G	21(10.45)	77(46.11)	
		G/G	104(51.74)	12(7.19)	3.39(0.181)
rs71785313	Deletion	Ins/Ins	157(78.89)	104(79.39)	
		Ins/Del	42(21.11)	27(20.61)	
		Del/Del	0	0	1.03(0.60-1.77)
rs9622363	G	A/A	10(5)	1(0.76)	
		A/G	130(65)	74(98.81)	
		G/G	60(30)	32(24.43)	6.27(0.044)
MYH9					
rs5750248	T	C/C	15(7.46)	12(7.19)	
		C/T	101(50.25)	76(45.51)	
		T/T	85(42.29)	79(47.31)	0.95(0.622)
rs5750250	G	A/A	15(7.46)	13(7.78)	
		A/G	110(54.73)	78(46.71)	
		G/G	76(37.81)	76(45.51)	2.47(0.29)
rs4821481	T	C/C	108(55.10)	93(55.69)	
		C/T	78(39.80)	68(40.72)	
		T/T	10(5.10)	6(3.59)	0.49(0.782)
rs2032487	T	C/C	122(62.24)	99(59.28)	
		C/T	64(32.65)	62(37.13)	
		T/T	10(5.10)	6(3.59)	1.12(0.572)

Appendix 12: SNP Association in individuals with CKD of Unknown aetiology Compared to Population Control under Heterozygote Model

SNP	RISK ALLELE	TEST	CKDU	GPC	χ^2 (P-value)
APOL1					
rs73885319	G	A/A*G/G	84(41.79)	84(50.30	
		A/G	117(58.21	83(49.70)	2.66(0.10)
rs60910145	T	G/G*T/T	97(48.26)	90(53.89)	
		G/T	104(51.74)	77(46.11)	1.16(0.282)
rs9622363	G	A/A*G/G	70(35)	33(24.19)	
		A/G	130(65)	98(74.81	3.55(0.05)
MYH9					
rs5750248	T	C/C*T/T	100(49.72)	91(54.49)	
		C/T	10(50.25)	76(45.51)	0.82(0.37)
rs5750250	G	A/A*G/G	91(45.27)	89(53.29)	
		A/G	110(54.73)	78(46.71)	2.34(0.13)
rs4821481	C	C/C*T/T	118(60.20)	99(59.28)	
		C/T	78.3980)	68(40.72)	0.0319(0.858)
rs2032487	C	C/C*T/T	132(67.35)	105(62.87)	
		T/C	64(32.65)	62(37.13)	0.79(0.37)

Appendix 13: SNP Association in individuals with CKD of Unknown aetiology Compared to Population Control under Dominant Model

SNP	RISK ALLELE	TEST	CKDU	GPC	X^2(P-value)
APOL1					
rs73885319	G	A/A	62(30.85)	72(43.11)	
		A/G*G/G	139(69.15)	95(56.89)	5.9(0.015)
rs60910145	G	T/T	76(37.81)	78(46.71)	
		T/G*G/G	125(62.19)	89(53.29)	2.96(0.085)
rs9622363	G	A/A	10(5.00)	1(0.76)	
		A/G*G/G	190(95.00)	130(99.24)	4.42(0.035)
MYH9					
rs5750248	T	C/C	15(7.46)	12(7.19)	
		C/T*T/T	186(92.54)	155(92.81)	0.010(0.919)
rs5750250	A	A/A	15(7.46)	13(7.78)	
		A/G*G/G	186(92.54)	154(92.22)	0.0134(0.908)
rs4821481	C	T/T	10(5.10)	6(3.59)	
		C/T*C/C	186(94.90)	161(96.41)	0.48(0.485)
rs2032487	C	T/T	10(5.10)	6(3.50)	
		T/C*C/C	186(94.90)	161(96.41)	0.48(0.485)

Appendix 14: SNP Association in individuals with CKD of Unknown aetiology Compared to Population Control under Recessive Model

SNP	RISK ALLELE	TEST	CKDU	GPC	X^2(P-value)
APOL1					
rs73885319	G	A/A*A*G	179(89.05)	155(92.81)	
		G/G	22(10.95)	12(7.19)	1.53(0.215)
rs60910145	G	T/T*T/G	180(89.55)	155(92.81)	
		G/G	21(10.45)	12(7.19)	1.189(0.276)
rs9622363	G	A/A*A/G	140(70.00)	99(75.57)	
		G/G	60(30.00)	32(24.43)	1.22(0.268)
MYH9					
rs5750248	T	C/C*C/T	116(57.71)	88(52.69)	
		T/T	85.45.29)	79(47.31)	0.44(0.505)
rs5750250	G	A/A*A/G	125(62.19)	91(54.49)	
		G/G	76(37.81)	76(45.51)	2.22(0.135)
rs4821481	C	T/T*C/T	88(44.90)	74(44.31)	
		C/C	108(55.10)	93(55.69)	0.0126(0.911)
rs2032487	C	T/T*T/C	74(37.76)	68(40.72)	
		C/C	122(62.24)	99(59.28)	0.33(0.564)