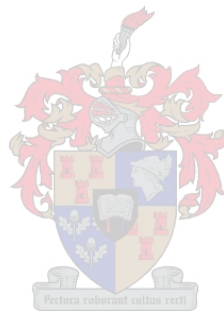# The occurrence of genetic variations in the MYH9 gene and their association with CKD in a mixed South African population

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Medical Science, at Stellenbosch University

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December 2012

i

DECLARATION:

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ii

ABSTRACT:

The purpose of this study was to investigate the association of the selected *MYH9* single nucleotide polymorphisms (SNPs) with chronic kidney disease (CKD) and its related co-morbidities in the South African mixed ancestry population residing in Bellville South, Cape Town. In 2008, two landmark studies identified SNPs in the *MYH9* gene which explained most of the increased risk for non-diabetic CKD in African Americans. These polymorphisms were later found to be weakly associated with diabetic nephropathy.

Three SNPs that exhibited independent evidence for association with CKD were selected (rs5756152, rs4821480 and rs12107). These were genotyped using a Taqman genotyping assay on a BioRad MiniOpticon and confirmed by sequencing in 724 subjects from Bellville South, Cape Town, South Africa. Prevalent CKD was defined based on the estimated glomerular filtration rate calculated using the modification of diet in renal disease (MDRD) formula.

Chronic kidney disease was present in 214 subjects (29.6%), 96.3% were stage 3 and only 8 subjects were stage 4. In additive allelic models, adjusted for age and gender, rs5756152 demonstrated an association with kidney function whereby each G allele of rs5756152 increased eGFR by 3.67 ml/min/1.73, reduced serum creatinine by 4.5% and increased fasting plasma glucose by 0.51 mmol/L. When an interaction model was used, the effect of rs5756152 on serum creatinine, eGFR and blood glucose levels was retained, and enhanced, but only in diabetic subjects. In addition, rs4821480 T allele increased eGFR while rs12107 A allele decreased glucose levels in diabetic subjects.

In contrast to reports that *MYH9* SNPs are strongly associated with non-diabetic end stage renal disease, our study demonstrated that rs5756152 and rs4821480 are associated with early kidney function derangements in type 2 diabetes whilst rs12107 is associated with glucose metabolism. Our findings, along with previous reports, suggest that the MYH9 gene may have a broader genetic risk effect on different types of kidney diseases than previously thought.

iii

OPSOMMING:

Hierdie studie het ondersoek ingestel na die verband tussen drie gekose MYH9-enkelnukleotied-polimorfismes (SNP's) en chroniese niersiekte (hierna 'niersiekte'), wat verwante ko-morbiditeite insluit, onder 'n Suid-Afrikaanse populasie van gemengde afkoms in Bellville-Suid, Kaapstad. Twee rigpuntstudies het in 2008 op SNP's in die MYH9-geen afgekom wat verklaar het waarom Afro-Amerikaners 'n hoër risiko vir niediabetiese niersiekte toon. Later is bevind dat hierdie polimorfismes ook 'n swak verband met diabetiese nefropatie het.

Drie SNP's wat elk onafhanklik bewys gelewer het van 'n verband met niersiekte is vervolgens gekies (rs5756152, rs4821480 en rs12107). Die SNP's is daarná met behulp van die Taqman-toets op 'n BioRad MiniOpticon aan genotipering onderwerp, en is toe deur middel van reeksbepaling by 724 proefpersone van Bellville-Suid, Kaapstad, Suid-Afrika, bevestig. Die voorkoms van niersiekte is bepaal op grond van die geraamde glomerulêre filtrasietempo (eGFR), wat aan die hand van die 'niersiekte-dieetveranderings'- (MDRD-)formule bereken is.

Daar is bevind dat 214 proefpersone (29,6%) aan chroniese niersiekte ly – 96,3% was in fase 3 en slegs agt proefpersone in fase 4. In toegevoegde alleliese modelle wat vir ouderdom en geslag aangepas is, het rs5756152 'n verband met nierfunksie getoon: Elke G-allel van rs5756152 het eGFR met 3,67 ml/min/1,73 verhoog, serumkreatinien met 4,5% verlaag en vastende plasmaglukose met 0,51 mmol/L verhoog. Toe 'n interaksiemodel gebruik is, is die effek van rs5756152 op serumkreatinien, eGFR en bloedglukosevlakke behou en versterk, hoewel slegs by diabetiese proefpersone. Daarbenewens het die T-allel van rs4821480 eGFR verhoog, terwyl die A-allel van rs12107 ook glukosevlakke by diabetiese proefpersone verlaag het.

In teenstelling met bewerings dat MYH9-SNP's 'n sterk verband met niediabetiese eindstadiumniersiekte toon, het hierdie studie bewys dat rs5756152 en rs4821480 met vroeë nierfunksieversteurings by tipe 2-diabetes verband hou, terwyl rs12107

iv

weer met glukosemetabolisme verbind word. Tesame met vorige studies, doen hierdie navorsingsbevindinge dus aan die hand dat die MYH9-geen dalk 'n groter genetiese risiko-effek op verskillende tipes niersiekte het as wat voorheen vermoed is.

v

ACKNOWLEDGEMENTS:

I wish to thank my supervisors, Professor TE Matsha and Professor RT Erasmus, for their guidance and direction during this learning experience.

To my mom, I thank you for your continuous support.

A portion of this work has been presented at the Annual Stellenbosch Academic Day 2012 and will be presented at the International PathPoint conference on 29th September 2012, at Crystal Towers, Cape Town

DEDICATIONS:

For Papa ♥

vi

TABLE OF CONTENTS

viii

ix

LIST OF FIGURES:

LIST OF TABLES:

LIST OF ABBREVIATIONS:

1.25(OH)2D – 1,25-dihydroxyvitamin D

25(OH)D – 25-hydroxyvitamin D

A – Adenine

ACE – Angiotensin-Converting Enzyme

ACR – Albumin;Creatinine Ratio

ACTN4 – Actinin Alpha 4

ADMA – Asymmetric Dimethylarginine

AKI – Acute Kidney Injury

ANP – Atrial Natriuetic Peptide

Apo A-IV – Apolipoprotein A-IV

APOL – Apolipoprotein

ARPKD – Autosomal recessive polycystic kidney disease

BLAST – Basic Alignment Search Tool

BMI – Body Mass Index

BNP – Brain Natriuetic Peptide

C – Cytosine

CEU – Europeans

CI – Confidence Interval

CKD – Chronic Kidney Disease

CKD-EPI – Chronic Kidney Disease – Epidemiology Collaboration

CUBN – Cubulin

CVD – Cardiovascular Disease

DBP – Diastolic Blood Pressure

DM – Diabetes Mellitus

DN – Diabetic Nephropathy

DNA – Deoxyribose Nucleic Acid

EDTA – Ethylenediamine Tetra Acetic Acid

eGFR – Estimated Glomerular Filtration Rate

ELMO1 – Engulfment and Cell Motility 1

EMEA – European Medicines Agency

ESRD – End-Stage Renal Disease

FBG – Fasting Blood Glucose

FDA – Food and Drug Administration

FGF-23 – Fibroblast Growth Factor 23

FSGS – Focal Segmental Glomerulosclerosis

G – Guanine

GCKD – Glomerulocystic Disease

GFR – Glomerular Filtration Rate

GLDH – L-Glutamate Dehydrogenase

GWAS – Genome – Wide Association Studies

HBA1C – Haemoglobin A1c – glycated haemoglobin

HDL – High Dense Lipoprotein

HDL-c – High Density Lipoprotein - Cholesterol

H-ESRD – Hypertensive End Stage Renal Disease

HIV – Human Immunodeficiency Virus

HIVAN – Human Immunodeficency Virus Associated Nephropathy

HN – Hypertensive Nepherosclerosis

HPT – Hypertension

HWE – Hardy-Weinberg Equilibrium

IDT – Integrated DNA Technology

IFG – Impaired Fasting Glucose

IGT – Impaired Glucose Tolerance

IL -1 – Interleukin 1

IL-1Ra – Inter-Leukin-1 Receptor Antagonist

INF2 – Inverted Forming 2

IQR – Interquartile range

KDIGO – Kidney Disease: Improving Global Outcomes

KIM-1 – Kidney Injury Molecule 1

LCAT – Lecithin-Cholesterol Acyltransferase

LD – Linkage Disequilibrium

LDL-c – Low Density Lipoprotein - Cholesterol

LOD – Logarithm of Odds

LPL – Lipoprotein Lipase

MALD – Mapping of Admixture Linkage Disequilibrium

MDRD – Modification of Diet in Renal Disease

MTHFR – Methenyltetrahydrofolate Reductase

MTHFS – Methenyltetrahydrofolate Synthase

MYH9 – Myosin Heavy Chain 9

NCBI – National Centre for Bioinformatics Institute

NGAL – Neutrophil Gellatinase-Associated Lipocalin

NGSP – National Glycohameoglobin Standarisation Program

NHANES – National Health and Nutrition Examination Survey

NO – Nitric Oxide

NPHS2 – Nephrosis 2

NT-proBNP – N-Terminal prohormone Brain Natriuetic Peptide

OGTT – Oral Glucose Tolerance Test

OR – Odds Ratio

PCR – Polymerase Chain Reaction

PTH – Parathyroid Hormone

q-PCR – Real-Time Polymerase Chain Reaction

RAS – Renin-Angiotensin System

RFU – Relative Fluorescence Units

SADTR – South African Dialysis and Transplant Registry

SBP – Systolic Blood Pressure

SGA – Small Gestational Age

SLE – Systemic Lupus Erythematosus

SNP – Single Nucleotide Polymorphism

T – Thymine

TB – Tuberculosis

TCF7L2 – Transcription Factor 7 Like-2

TE – Tris EDTA

TG – Triglycerides

TGF-β – Transforming Growth Factor

T_m – Melting Temperature

TRPC6 – Transient Receptor Potential Cation Subfamily 6

UAE – Urinary Albumin Excretion

UMOD – Uromodulin

USRDS – United States Renal Data System

UV/VIS – Ultraviolet Visible

WHO – World Health Organization

YRI – Yorubas

CHAPTER 1

[Literature Review]

1.1 INTRODUCTION

Background:

Chromosome 22 is a widely researched chromosome in regard to gene mutation and disease association. Two genes mapped on chromosome 22q12 have been linked to the risk and progression of chronic kidney disease (CKD), the myosin nonmuscle heavy chain 9 (*MYH9*) gene and more recently, apolipoprotein 1 (*APOL1).* More than 45 mutations have been found in the *MYH9* gene and implicated in a wide range of disorders that have subsequently been grouped and termed the *MYH9*-related disorder, however the mechanisms underlying the cause of CKD has not yet been discovered. Chronic kidney disease is a condition marked by the gradual decline of kidney function over time.

Many studies have found an association of *MYH9* variants with an increased CKD risk, including focal segmented glomerulosclerosis (FSGS), hypertensive end stage renal disease (H-ESRD), non-diabetic end-stage renal disease (ESRD), HIV associated nephropathy (HIVAN) and a weak association with diabetic nephropathy (DN). Single nucleotide polymorphisms (SNPs) increasing the risk for these kidney diseases were identified in different regions of the *MYH9* gene, clustered into groups according to linkage disequilibrium (LD) blocks, E-1, S-1, F-1 and L1 haplotypes, with three SNPs (rs5756152, rs4821480 and rs12107) showing independent association with CKD.

The most compelling part about the *MYH9* locus is the significant and increased risk allele frequency in African Americans. This population is an inter-mating product of Africans with European Caucasians. The mixed ancestry (Coloured) ethnic group in South Africa is also heterogeneous with its origin, with predominantly African, European, and a proportion of Asian ancestry. This has prompted the speculation that the *MYH9* gene may play a role in the development of CKD in the mixed ancestry population group in South Africa, and therefore, investigation as a predisposing factor of this disease is warranted. Although the magnitude of CKD in this population in South Africa is currently unknown due to the lack of reliable national data systems in place to collect, analyze and report this information; several studies have reported a high

prevalence of diabetes and hypertension in the mixed ancestry ethnic group. Diabetes, hypertension and glomerulonephritis are the three leading causes of CKD, and the incidence of ESRD has been observed to mirror that of these three disorders. Thus the research question of this thesis is: Is there an association between *MYH9* gene polymorphisms and CKD and its related co-morbidities within the mixed ancestry ethnic group of South Africa?

Problem statement:

No detailed genetic analyses of kidney diseases have been done in the mixed ancestry population of South Africa, and it is not known if there is an association between *MYH9* SNPs rs4821480, rs5756152 and rs12107; and CKD and its related re-comorbidities.

Research aim:

The aim of this study is to determine whether or not *MYH9* polymorphisms have an association with CKD and its related co-morbidities in the South African mixed ancestry population residing in Bellville South, Cape Town.

Research objectives:

The objectives of this study are to:

I.   Genotype both CKD and non-CKD subjects' extracted deoxyribose nucleic acid (DNA) samples for *MYH9* polymorphisms, namely, rs12107, rs4821480, rs5756152, that were shown in other populations to be independently associated with CKD

II. Determine the allele and genotype frequencies of the three *MYH9* polymorphisms in the selected study population.

III. Determine the association between *MYH9* alleles and CKD and its related co-morbidities - obesity, type 2 diabetes mellitus and hypertension.

Chapter overview:

This chapter will cover CKD definition, diagnosis, classification and treatment; epidemiology, aetiology and research into CKD initiation and progression markers. Details into the genetics of CKD and expansion into *MYH9* gene research will be discussed.

4

1.2 CHRONIC KIDNEY DISEASE

1.2.1 CKD Definition

Chronic kidney disease (CKD) is a condition marked by the gradual decline of kidney function over time. The 'Kidney Disease: Improving Global Outcomes (KDIGO)' defines CKD as kidney damage for greater than 3 months with or without a decreased glomerular filtration rate, which can lead to a lowered glomerular filtration rate (GFR) (Levey *et al*, 2005). Chronic kidney disease is manifested by either a pathogenic abnormality or kidney damage markers, or a glomerular filtration rate of less than 60 ml/min/1.73 m² for more than 3 months with or without kidney damage; or abnormal imaging tests (Hogg *et al*, 2003).

1.2.2 Symptoms of CKD

Symptoms may not appear until the kidney function is severely decreased but they include fatigue, poor concentration, a poor appetite, insomnia, muscle cramping at night, swollen feet and ankles, dry itchy skin, nocturia, restless legs, dyspnoea as well as puffiness around the eyes, especially in the morning. (National Kidney Foundation, 2010). The clinical presentation of CKD is generally non-specific but presentation trends have been seen with various types of kidney disorders that have resulted in the loss of kidney function. Diabetic kidney damage and glomerular diseases, namely proliferative glomerulonephritis and non-inflammatory diseases present with asymptomatic urinalysis abnormalities such as proteinuria, hematuria, casts and pyuria; whereas vascular diseases manifest as high blood pressure due to the urine abnormalities. Likewise, tubulointerstitial and cystic diseases have asymptomatic urinalysis abnormalities but include urinary tract symptoms such as dysuria, incontinence, flank pain and an increase in urine frequency and urgency (Levey *et al*, 2003).

1.2.3 Diagnosis of CKD

Chronic kidney disease is often silent, preventing clinical presentation from being used for early diagnosis. It is often detected by chance, when routine blood and urine tests are run in search of another underlying health problem. Increased urea and creatinine levels in the blood, elevated blood pressure as well as proteinuria are the common abnormalities that are found and result in further testing.

The methods for the analysis of the urea and creatinine levels, in both blood and urine, differ between laboratories, depending on the available resources. High performance liquid chromatography is considered the gold standard of creatinine determination due to its high specificity (Bishop, Fody and Schueff, 2005). This is a highly specialized test but is expensive both to purchase, set up and to run. Most laboratories use the kinetic-Jaffe reaction, which is inexpensive, rapid and easy to perform. In rural areas where laboratories are basic or non-existent, this kinetic-Jaffe reaction is done directly on a sample aliquot and the change in absorbance determined using spectrophotometry (Bishop, Fody and Scheuff, 2005). This absorbance result is used to calculate the creatinine level. The typical human reference ranges for serum creatinine are as follows: 45-90 µmol/l for women and 60-110 µmol/L for men (Bishop, Fody and Scheuff, 2005). If filtering in the kidney is deficient, creatinine blood levels rise. A rise in blood creatinine level is observed only with marked damage to functioning nephrons rather than an indication of early-stage kidney disease. Low creatinine can also be a sign of certain diseases which cause decreased muscle mass, such as myasthenia gravis and muscular dystrophy (Marks and Mesko, 2002). These patients present with weakness, muscle wasting, and other symptoms; in addition to the abnormal blood levels. However these levels may not always be associated with disease states, with vegetarians showing lower creatinine levels due to the non-existence or decrease in protein intake and elderly people also having a lower creatinine level due to the decrease of muscle mass with age. A blood creatinine level of 110 µmol/l can indicate significant renal disease in an elderly female while 150 µmol/l is a normal range for a male body builder (Mandal, 2004).

The analysis of urea in blood or urine samples uses an enzymatic reaction, where urea is hydrolyzed and the ammonium ions that are produced are measured by the electrode of the analyzer (Bishop, Fody and Scheuff, 2005). As with creatinine, this method may be altered in rural areas and the ammonium from the reaction can be measured by the color change with a pH indicator. The gold standard for urea analysis is an enzymatic method involving both urease and L-glutamate dehydrogenase (GLDH) (Bishop, Fody and Scheuff, 2005). Urine is analyzed further with the use of dipsticks and microscopy analysis. Depending on the type of dipstick used, urine can be tested for the following: pH, protein, hemoglobin, glucose, ketones, specific gravity, urobilinogen and leukocyte esterases (Provan, 2010). The coincidental detection of proteins in a urine sample using the dipstick method provides important information as persistent proteinuria, specifically albuminuria, is a principal marker of kidney damage. Unfortunately, results obtained from a dipstick can be fairly unreliable as they are affected by the dilution of the urine and the specificity of the reaction (Levey *et al*, 2003). A positive protein result should be confirmed or investigated further with urine albumin determination. The urinary albumin excretion (UAE) is considered the gold standard for determining albumin levels in urine samples but is inconvenient and sometimes unavailable, as it requires a 24 hour urine sample. For this reason, the albumin:creatinine ratio (ACR) is recommended (Bishop, Fody and Scheuff, 2005). As renal function declines due to the loss of nephrons in the kidney, the glomerular capillary permeability increases. This allows small volumes of albumin to pass into the urine. These albumin levels are detected in the laboratory setting using albumin-specific immunoassays. The term microalbuminuria is used when describing low levels of albumin in the urine (Bishop, Fody and Scheuff, 2005). An ACR of less than 30 mg/mmol is considered normal in a healthy individual (National institute for health and clinical excellence, 2008). A result of >30 mg/mmol should be repeated with an early morning urine sample to confirm the presence of proteinuria. An initial result of greater than 70 mg/mmol, need not be repeated. In diabetic patients, microalbuminuria is clinically significant with a reference range of > 2.5 mg/mmol in men and > 3.5 mg/mmol in women.

Renal function is determined by the clearance of a specific substance from the body via the kidneys. This assesses the glomerular filtration rate (GFR) and is accurately measured by the renal clearance of inulin, the gold standard for the determination of renal function (Khurana, 2008). Inulin is a dye that does not exist naturally in the body and is therefore freely filtered by the glomeruli, neither reabsorbed nor secreted in the nephron or metabolized in the body. Once a constant level of plasma inulin concentration is established, the GFR may be calculated using the following variable, where V is the urine flow rate in ml/min, and the urine and plasma inulin concentrations are measured in mg/ml (Khurana, 2008):

$$GFR = \frac{U_{Inulin} \times V}{P_{inulin}}$$

Due to the invasive and expensive nature of the inulin clearance test, the clearance of creatinine is a more routinely used method for the determination of renal function and GFR calculation (Khurana, 2008). Creatinine has a fairly constant plasma concentration, is fully filtered by the glomeruli and is only secreted marginally in the tubules within the nephron. The samples required are a urine sample collected for a full 24 hour period and a random serum creatinine sample. A normalization factor is used to correct for body surface area and the formula becomes (Daniel, 2008):

24 hour creatinine clearance (ml/min) $= \dfrac{U \times V}{P} \times \dfrac{1.73}{A}$

U = Urine creatinine concentration (mg/dl)

V = Urine volume excreted in 24 hours (ml)

P = Serum creatinine concentration (mg/dl)

A = The patient's body surface area (m^2)

(Perry, 2008)

A limitation of this test is the collection of a 24 hour urine sample. This can be challenging for patients and as a result it is generally not accurately collected (Puri, 2005). An equation that provided an estimate was proposed to replace the 24-hour creatinine clearance using easily obtainable serum biochemistry, and as an easier method to determine renal function. This calculated the estimated GFR (eGFR), with GFR defined as the volume of fluid that is filtered by the glomerulus per minute (Levey *et al*, 2003). This equation became the preferred method of renal function estimation (Daniel, 2008). It does not require a urine sample and is corrected for age and weight, allowing an easier determination of GFR through calculation.

$$\text{Creatinine clearance (ml/min)} = \frac{(140 - \text{age}) \times \text{weight} \times \underline{0.85 \text{ (if female)}}}{72 \times \text{serum creatinine}}$$

The 'Modification of Diet in Renal Disease (MDRD)' study equation is the most recent, commonly used formula for the estimation of GFR. The initial equation included serum albumin and urea nitrogen, however these were later dropped. The ethnicity factor usually used in the MDRD formula has been proven to be irrelevant in the black population of South Africa (Van Deventer *et al*, 2008). The result of this calculation is used for grading, treatment and dosing of renally excreted medication (Snyder and Pendergraph, 2005; Perry, 2008).

$$\text{GFR (mL/min/1.73m}^2) = 175 \times (\text{Standardized serum creatinine})^{-1.154} \times (\text{Age})^{-0.203} \times (0.742 \text{ if female})$$

(Stevens & Levey, 2009)

Both formulas used to estimate GFR were developed based predominantly on young, Caucasian patients (Daniel, 2008). However, these are not the individuals that are most at-risk for the development of CKD. The first at-risk group is the elderly. As the body ages, there is a natural loss of glomeruli due to a decline in the number of nephrons, glomerular capillary atrophy and overall kidney mass decline. Metabolic waste, specifically creatinine, is not excreted fully and the concentration builds up in the serum. This makes both the Cockcroft-Gault and the MDRD formulae somewhat inaccurate for estimating the GFR as the natural

decline in creatinine excretion by the kidneys is not accounted for. Many studies (Botev *et al*, 2009; Gouin-Thibault *et al*, 2007) have compared the use of both formulas, and observed that the Cockcroft-Gault formula underestimates the GFR slightly as it underestimates the creatinine clearance. This formula, therefore, is more applicable and accurate in elderly people for the estimation of GFR where creatinine excretion is less pronounced (Daniel, 2008). Other clinical situations where the eGFR may be unreliable or misleading include extremes in body size, high intake of dietary protein, vegetarian diets, or creatine supplements, severe liver disease, diseases of skeletal muscle, amputees or paraplegics due to the disparity in creatinine levels (Johnson *et al*, 2009).

In 2009, Levey and co-workers developed another equation, the Chronic Kidney Disease – Epidemiology Collaboration (CKD-EPI) equation, based on serum creatinine levels that would be as accurate as the MDRD equation at a GFR of less than 60mL/min/1.73m² but more accurate at a higher GFR. Like the MDRD equation, the CKD-EPI includes race, age and sex and takes serum creatinine into account. This study showed that the CKD-EPI is more accurate than the widely used MDRD equation as it has a lower bias, preventing over-estimation of the GFR (Levey *et al*, 2009). The few studies that have been carried out since 2009 (Michels *et al*, 2010; Madero and Samack, 2011) are all in agreement with the accuracy of the CKD-EPI equation.

CKD-EPI equation:

eGFR = 141 x minimum(Serum Creatinine/k,1)^{a} x maximum(Serum Creatinine/k,1)$^{-1.209}$ x 0.993^{age} x [1.018 if female] x [1.159 if black]

k = 0.7 for females and 0.9 for males
a = -0.329 for females and -0.411 for males

Kidney damage may be detected by other markers, including abnormalities in urine sediment such as casts and epithelial cells, renal tubular acidosis in blood and urine chemistry measurements; and abnormal findings on imaging studies including polycystic kidneys, hydronephrosis and small kidneys (Levey *et al*,

2005; Levey *et al*, 2003). Cystatin C is a more recently discovered marker of renal function (Hojs *et al*, 2006). It is an endogenous, low molecular weight protein that is produced by all nucleated cells, and levels in the blood are not affected by age, gender, race or lean muscle mass. This protein is filtered by the glomeruli and reabsorbed and catabolized by the tubular epithelial cells, with only small amounts excreted in the urine. Since Cystatin C is not produced by the muscle, the problem of low creatinine in elderly patients due to low muscle mass or high creatinine levels due to high muscle mass is eliminated. Cystatin C is however affected by inflammation and immunotherapy (Daniel, 2008). In a study done by Stevens *et al* in 2008, the weak association between Cystatin C and age, sex and race was confirmed. Cystatin C alone was found to be more accurate in estimating the GFR than serum creatinine alone, but was slightly erroneous when compared to serum creatinine adjusted for age, sex and race. However, the main relevant outcome of the study was that an equation that used adjusted serum creatinine from the CKD-EPI equation, as discussed previously, as well as Cystatin C was the most accurate measure of the true GFR (Stevens *et al*, 2008). The CKD-EPI cystatin and creatinine equation adjusted for age, sex and race was defined as follows:

$$\text{eGFR:} \quad 177.6 \times \text{SCr}^{-0.65} \times \text{CysC}^{-0.57} \times \text{age}^{-0.20} \times 0.80 \text{ (if female)} \times 1.11 \text{ (if African American)}$$

Imaging studies are done once CKD has been diagnosed and helps with its prognosis by documenting the size of the kidneys, with a normal size indicating CKD that is amenable to treatment and smaller kidneys indicative of more irreversible damage. Imaging studies are also useful in identifying the cause of the kidney disease (Snyder and Pendergraph, 2005). When the cause cannot be determined using the patient's history, medical conditions, laboratory results and imaging studies, a biopsy is suggested. This yields a definitive diagnosis but involves a risk. Kidney damage cannot be seen with the naked eye, and patients will only be diagnosed once symptoms are visible, when the glomerular filtration rate is already severely decreased. Target screening is therefore very important. With a known genetic variation in a specific race, this target screening will be made more specific.

1.2.4 Classification of CKD

The results of many studies provide a strong foundation for using the GFR for classification purposes. The GFR is estimated using specific equations and based on a level less than 90 ml/min/1.73m^2, as discussed in 1.2.3. The results are used to determine staging and treatment information for CKD, creating a standard for physicians and nephrologists worldwide by KDIGO in 2002 (Table 1.1) (Levey *et al*, 2003). Many issues have been raised about stages 3, 4 and 5 being defined solely on eGFR with no requirement of kidney damage or adjustment for age and gender (Glassock and Winearls, 2008). The KDIGO acknowledged the challenge against the 2002 CKD definition, a point that was already raised in 2004. Their response was to conduct a worldwide survey with nephrologists with the major concerns being documented and seriously discussed in 2008. Many suggestions of amendment were put forward but as of yet, no studies have been done to compare how they would perform against the 2002 KDIGO system. No common solution has been reached so far but it is likely to be revised in the near future (Hallan and Orth, 2010).

Table 1.1: Classification of Chronic Kidney Disease. Taken from (Levey *et al*, 2003; Levey *et al*, 2005; Hogg *et al*, 2003)

STAGES	DESCRIPTION	eGFR (ml/min/1.73m²)	ACTION
-	At increased risk	≥ 90 with CKD risk factors	Screening, CKD risk reduction
1	Kidney damage with normal or increased GFR	≥ 90	Treat comorbid conditions, reduce cardiovascular disease risk, slow the progression
2	Kidney damage with mild decrease in GFR	60-89	Estimate progression
3	Moderately decreased GFR	30-59	Evaluate and treat complications
4	Severely decreased GFR	15-29	Prepare for kidney replacement therapy
5	Kidney failure (End stage renal disease)	<15	Kidney replacement

1.2.5   Complications associated with CKD

The major complications of chronic kidney disease include progression to kidney failure, cardiovascular disease, hypertension, anemia, malnutrition, bone diseases, neuropathy, gastrointestinal symptoms, electrolyte abnormalities, disordered calcium, phosphorous metabolism, immune dysfunction, decreased quality of life and premature death (Levey *et al*, 2005; Levey *et al*, 2003; Snyder and Pendergraph, 2005).

1.2.6   Treatment of CKD

As with most diseases, starting treatment at the earliest stage of CKD allows for the best possible outcome. The diagnosis of CKD, along with its stage or severity, complications or risk factors as well as comorbid conditions first need to be determined. This will allow for early intervention, with proper and possibly personalized treatment or therapy, based on lifestyle and specific genetic risk factors present in affected individuals. Identification of risk factors and availability of proper treatment and disease management strategies may postpone the loss of kidney function and prevent the development of accompanying cardiovascular disease (CVD) (reviewed by He and Whelton, 1999). Currently, affected individuals who have progressed to ESRD undergo dialysis, which is costly; or transplantation, which depends on the availability of compatible donors (Hogg *et al*, 2003).

Table 1.1 details actions to be taken at specific CKD stages. Treatment of any comorbid conditions, interventions to slow progression of kidney disease, in addition to reducing the risk of cardiovascular disease, should all be initiated during stages 1 and 2. This stage of treatment is vital in increasing the length of time before ESRD. During stage 3, any complications that arise should be evaluated and treated. The prevalence of complications increases with a decreasing GFR of below 60ml/min/1.73m². Preparation for kidney replacement therapy should be done in stage 4, with preparation for dialysis and kidney transplantation beginning when the GFR drops below 15ml/min/1.73m², stage 5

(Levey *et al*, 2003; Hogg *et al*, 2003). Three interventions have been proven to slow the progression of CKD; the control of blood pressure, glucose level control in diabetic patients and the reduction of proteinuria with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (de Jong *et al*, 2008).

1.3   EPIDEMIOLOGY OF CKD

Chronic kidney disease is currently the 12th highest cause of death and 17th highest cause of disability worldwide (Nugenta *et al*, 2011). Factors contributing to the increase in the global prevalence of CKD include greater referral and acceptance due to a greater awareness in developed countries, and the rapid increase in the prevalence of diabetes and hypertension, which are both significant risk factors in CKD (Ronco, Brendolan and Levin, 2005). It is expected that the number of individuals with hypertension will increase from 972 million to 1.56 billion people by 2025 and the diabetes incidence from 171 million in 2000 to 366 million by 2030, of which 298 million of these will be in developing countries (Nugenta *et al*, 2011). Worldwide, more than a million patients are undergoing ESRD therapy, but the true prevalence of CKD is lacking (Molony and Craig, 2008). In developed countries, the ESRD patients taking treatment are monitored, allowing for a fairly accurate burden of ESRD to be determined, however the number of patients with ESRD is just a small proportion of the entire burden of CKD (Goldsmith, Tayawardene and Ackland, 2007). The number of patients with stage 1 to 4 CKD is not as easy to determine as statistics only show those being treated, not those who do annual follow-ups for basic comorbid condition control or treatment. Information from developing countries in Asia, Africa and South America is scarce due to the lack of renal registries and databases, but it can be estimated that the CKD prevalence and burden will be significantly higher than developed countries. There are multiple potential explanations for the variability between developed and developing countries; however the most apparent factors include access to health care, the effectiveness of detection and treatment of kidney disease and population specific risk factors (Molony and Craig, 2008).

This rapid rise of diabetes and hypertension, as well as obesity, will result in an even greater and more profound burden that developing countries are not equipped to handle. With a 10 - 16% prevalence of diabetes in South Africa and 1 in 5 adult South Africans with hypertension, CKD is a scary reality in this country (Katz, 2005; Southern African Hypertension Society, 2011). At the same time, many developing countries are experiencing significantly high rates of infectious diseases which initiate and increase progression of CKD such as schistosomiasis, human immunodeficiency virus (HIV), tuberculosis (TB), amyloidosis and hepatitis B and C (White, 2008). Poverty and socio-economic status are highly correlated with the common risk factors of CKD, including hypertension, diabetes, obesity, smoking and dyslipidaemia, making low to middle income countries, such as South Africa, particularly vulnerable to the CKD burden (Nugenta *et al*, 2011).

A review done in Cape Town (South Africa) of renal biopsy results over a 10 year period showed that primary or secondary glomerulonephritis was the main cause of CKD. 53.7% of the study population consisted of patients of the mixed ancestry ethnic group (Okpechi *et al*, 2010). Glomerulonephritis is caused, by among others, autoimmune diseases such as systemic lupus erythematosus (SLE), HIV, viral hepatitis, drug abuse and infections such as TB and malaria. HIVAN increased from 6.6% in 2000 to 25.7% in 2009. In 2007, South Africa had 17% of the global burden of HIV and one of the world's worst TB epidemics (Karin *et al*, 2009). These statistics, coupled with the diabetes and hypertensive prevalence, make CKD a grave burden in South Africa.

Chronic kidney disease has not been given enough attention globally, largely due to the global health community's focus on infectious diseases, importantly HIV, as well as the lack of awareness about CKD. There is thus a critical need for funding in developing countries in order to implement more comprehensive, cost-effective, and preventative interventions against CKD. Providing care for patients who require dialysis or transplantation is a major and growing healthcare problem in both developed and developing nations in terms of cost, premature mortality and economic impact (Goldsmith, Tayawardene and Ackland, 2007).

1.4   MARKERS FOR CKD PROGRESSION

Markers for CKD initiation and progression are important in the prediction of patients at risk for progression of CKD and its consequences. The identification of reliable and accurate markers will allow for a better understanding of the pathogenesis and progression of CKD. Proteinuria, along with other kidney abnormalities, such as urine sediment and imaging irregularities, has already been identified as kidney damage markers. However, new markers are needed to detect kidney damage prior to the reduction of the GFR. Novel low molecular weight proteins are being increasingly studied, with many showing great promise.

1.4.1 Asymmetric Dimethylarginine (ADMA): The reduced availability of nitric oxide (NO) was hypothesized to play a role in the progression of kidney disease (Fliser *et al,* 2005). Nitric Oxide is a vasodilator that is found in the endothelium of the kidney and is important in the regulation of blood flow. Decreased levels of NO results in decreased renal plasma flow, increased blood pressure, and increased renovascular resistance (reviewed by Kronenberg *et al*, 2009). ADMA is an endogenous inhibitor of NO synthase that has a long duration of action, with the kidney being the main site of ADMA excretion. The role of increased plasma - -ADMA has been studied in diabetes, pre-eclampsia, strokes and vascular and coronary heart diseases (Vallance, 2001; Cooke, 2004; Achan *et al*, 2003). Individuals with ESRD undergoing hemodialysis have high plasma ADMA levels and this is a strong and independent predictor of overall mortality and cardiovascular outcome (Zoccali *et al*, 2001). Though data showing elevated ADMA levels in CKD patients (Cooke, 2004) and experimental data demonstrating the association between increased ADMA levels and the progression of CKD had been published, a study done by Fliser *et al* (2005) was the first to demonstrate the role of ADMA and CKD progression in humans, namely non-diabetic CKD patients. Remarkably, plasma ADMA was the only independent predictor of progression apart from serum creatinine. A subsequent study (Ravani *et al*, 2005) was done on CKD patients with either diabetes or cardiovascular complications, and a 20% increased risk of mortality was identified for every 0.1 µmol/L increase of plasma ADMA, independent of

hemoglobin, GFR and proteinuria. To further prove the association between increased ADMA and DN, Hanai *et al* (2009) did a cohort study on type 2 diabetic patients of Japanese descent. They demonstrated that higher levels of ADMA was associated with faster progression of nephropathy in diabetic individuals, based on increased albuminuria and decreased eGFR. ADMA shows immense promise as a CKD marker but has as yet, not been submitted to the Food and Drug Administration (FDA) and European Medicines Agency (EMEA) for recognition as a biomarker associated with nephrotoxicity (reviewed by Fassett *et al*, 2011).

1.4.2 Fibroblast growth factor 23 (FGF-23): FGF-23 has been identified as a phosphatonin, decreasing phosphate reabsorption in the kidney, and is associated with increased phosphate excretion and decreased plasma phosphate concentrations (reviewed by Bernt, Schiavi and Kumar, 2005). A disturbed calcium-phosphate metabolism and CKD progression have been linked (reviewed by Block *et al*, 2004), and increased levels of FGF-23 have been noted in CKD patients and those already in ESRD (Imanishi *et al*, 2004; Larsson *et al*, 2003) However, the assays used in these studies detected both the intact FGF-23 molecule and its COOH-terminal fragments. This made it difficult to assess whether there was a decrease in the degradation of full-length FGF-23 or if the FGF-23 fragments are biologically inactive. A study by Flisher *et al* (2007) examined the association of CKD progression with both intact FGF-23 as well as the c-terminal level. The findings showed a significantly faster CKD progression time when levels of c-terminal FGF-23 were above the optimal cut-off level of 104rU/mol (46.9 months) when compared to patients who had intact FGF-23 level above the median concentration of 35pg/ml (54.6 months). Additionally, CKD patients with c-terminal levels within the normal limits had a longer progression time (72.5 months) than patients with intact FGF-23 levels below the threshold (69.8 months). After adjustment for GFR, the CKD progression estimates by FGF-23 were still significant, suggesting that FGF-23 is an independent prediction marker and not simply a surrogate marker of GFR. Finally, should the FGF-23 c-terminal increase to 250rU/ml, the risk for CKD progression increases to 35% ($p < 0.001$), adjusted for age, sex, GFR and

proteinuria (as reviewed by Kronenberg *et al*, 2009). The c-terminal of FGF-23 is easier to measure than the intact FGF-23 molecule, and given this, subsequent studies have focused on the c-terminal levels in CKD progression association studies.

1.4.3 Vitamin D: Vitamin D deficiency has long been linked to traditional cardiovascular risk factors such as hypertension, insulin resistance, diabetes, and dyslipidemia (Forman *et al*, 2007; Pittas *et al*, 2006); and is also associated with albuminuria and a higher prevalence of mortality in the Third National Health and Nutrition Examination Survey (NHANES) cohort (Mehrotra, Kermah and Salusky, 2009; De Boer *et al,* 2007). The adequacy of body vitamin D stores is best assessed by the measurement of the serum level of 25-hydroxyvitamin D (25(OH)D) (reviewed by Kandula *et al*, 2011). However, 25(OH)D needs to undergo 1-α hydroxylation for it to be converted into the active form, 1,25-dihydroxyvitamin D (1,25(OH)2D). The kidneys are the primary site for 1-α hydroxylation of vitamin D, due to the presence of 1α hydroxylase in the proximal and distal tubules, as well as the ascending Loop of Henlé (Zehnder *et al*, 1999). The renal synthesis of 1,25(OH)2D is tightly regulated by complex interactions between the parathyroid hormone (PTH), calcium and phosphate. An association between mortality and vitamin D deficiency has been shown in both dialysis and non-dialysis dependent CKD (Wolf*,* 2008; Mehrotra, Kermah and Salusky, 2009). Intervention studies have also been done using an active Vitamin D analog, showing a reduction in proteinuria among CKD patients (Fishbane *et al*, 2009; Agarwal, 2009). A definitive study done by Ravani *et al* (2009), revealed that for a 10ng/ml increase in 25(OH)D levels, the associated CKD progression had a 40% decrease in the hazard ratio. This study proved that 25(OH)D is an independent inverse predictor of disease progression and death in patients with stage 2-5 CKD.

18

1.4.4 Adiponectin: Adiponectin is a cytokine specific to, and produced in the adipocytes. It has a central role in glucose and lipid metabolism as an insulin sensitizer, an anti-inflammatory, anti-atherosclerotic and vasculo-protective cytokine (reviewed by Kronenberg *et al*, 2009). Adiponectin is abundant in plasma but a large number of studies have shown an association between low adiponectin levels and negative outcomes, such as obesity and type 2 diabetes mellitus (Arita *et al*, 1999; Hotta *et al*, 2001), in addition to coronary artery disease (Kumada *et al*, 2003; Pishon *et al*, 2004; Schulze *et al*, 2005). However, adiponectin is elevated in patients with kidney impairment, with kidney function being an important determinant of circulating levels of cytokines (Becker *et al*, 2005). When kidney function decreases, the proposed mechanisms for adiponectin level disturbances are changes in the ligand or receptor reactivity as shown for other hormone or receptor systems in renal failure, reduced adiponectin clearance by the kidney, or a counter-regulatory response to metabolic derangements in renal failure (reviewed by Shen, Peake and Kelly, 2005; Isobe *et al,* 2005; Zoccali *et al*, 2003). Adiponectin levels are also increased in type 1 diabetes patients and DN (Imagawa *et al*, 2002; Saraheimo *et al*, 2005). A study done by Kollerits *et al* (2007), found that increased adiponectin levels were an independent predictor of CKD progression, however only in men. Conflicting results on the cause and effect of high adiponectin levels in CKD patients are rife, with some studies suggesting that the high levels are a marker of poor prognosis (Menon *et al*, 2006), and others suggesting that the high levels play a protective role by reducing albuminuria through a direct effect on podocyte function and modulation of inflammation and oxidative stress (Sharma *et al*, 2008; Komura *et al*, 2010). Supportive of this protective view are studies showing that high adiponectin levels have been associated with a better prognosis in ESRD and CVD patients, with a lower risk for cardiovascular events in ESRD patients (Zoccali *et al*, 2002). Conversely, Saraheimo *et al* (2008) demonstrated that patients with normoalbuminuria or microalbuminuria had no differences in the baseline adiponectin concentrations between those who had CKD progression or those with no CKD progression. However, in the macroalbuminuria group, progressors had significantly higher serum adiponectin concentrations compared with non-progressors. A review by Fassett *et al* (2011),

is against the view that adiponectin cannot be considered as a biomarker for early detection of CKD progression due to the association between albuminuria and adiponection with CKD progression. Because of its association with other lifestyle diseases, adiponectin cannot be disregarded and further studies should be done to determine the effect of elevated adiponectin levels in CKD.

1.4.5 Apolipoprotein A-IV (Apo A-IV): Apolipoprotein A-IV is a plasma protein synthesized in the intestines and excreted into the circulation on chylomicron particles (Weinberg and Spector, 1985). Apo A-IV activates lecithin-cholesterol acyltransferase (LCAT) enhancing the formation of small high dense lipoprotein (HDL) particles, modulates the activation of lipoprotein lipase (LPL), has anti-oxidant and anti-atherogenic properties, stimulates cholesterol efflux from peripheral cells, as well as having involvement in several steps of the reverse cholesterol transport pathway. Although the liver is the main site of degradation of apo A-IV, the kidney contributes significantly (Haiman *et al*, 2005). The initial localization of apo A-IV in the kidney identified it in the brush border cells of the proximal tubules and in the granules of the epithelial cells in the proximal tubule, where the apo A-IV was degraded within the lysosomes. Localization of apo A-IV was also done in the distal tubules, concluding that apo A-IV is not filtered by the glomerulus but rather reaches the tubules to be absorbed. There has been little doubt that CKD is associated with abnormalities in lipoprotein metabolism, however a study done by Boes *et al* (2006) was the first to investigate the association between the apo A-IV concentration and the progression of CKD. The findings showed that plasma apo A-IV concentrations above the baseline level was one of the best predictors for the progression of kidney disease, apart from baseline GRF. This association was independent of other lipoproteins, ADMA, proteinuria, blood pressure and inflammatory status. Additionally, an increase of apo A-IV to 10mg/dL results in a 60% increase in risk of CKD progression. These findings, along with the reports on increased apo A-IV concentration in hemodialysis patients (Seishima and Muyo, 1987; Dieplinger *et al,* 1992; Kronenberg *et al*, 1995) and the demonstration that patients with kidney disease have significantly increased apo A-IV concentrations even when the GFR is still within normal range (Kronenberg *et al*, 2002), allows for the conclusion that apo A-IV is an early marker for renal insufficiency. This is

supported by the recent findings of apo A-IV immunoreactivity in kidney tubular cells, suggesting a direct role of the human kidney in apo A-IV metabolism

1.4.6 Natriuretic peptides: A natriuretic peptide refers to proteins that induce the loss of sodium in the urine, and includes the atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) (Shils *et al*, 2005). ANP is a powerful vasodilator, involved in maintaining cardiovascular and renal homeostasis, and is secreted by the atrial monocytes of the heart. A study done by Dieplinger *et al* (2009) showed by using multiple Cox-proportional hazard regression analyses, that an elevated plasma concentration of ANP was strongly predictive of the progression of CKD after adjustments for age, gender, GFR, proteinuria and pro-B-type natriuretic peptide, suggesting it to be a useful new marker. BNP is secreted by the ventricles of the heart, with similar physiological actions as ANP (Shils *et al*, 2005). The N-terminal fragment (NT-proBNP) is an inactive molecule and results from the cleavage of the prohormone pro-BNP, and is solely reliable on renal excretion. Both the BNP and NT-proBNP levels increase as the GFR declines (Austin *et al*, 2006). The increasing ratio of NT-proBNP/BNP with decreasing GFR was seen in the same study, indicating that NT-proBNP is influenced to a greater degree by renal function. These results were consistent with other studies (DeFilippi *et al*, 2005; Richards *et al*, 2006). The independent influence of renal function on BNP was taken into account due to the presence of heart disease among participants, and the cases were separated into CKD with evidence of heart disease, and CKD without evidence of heart disease. As found by Takami *et al* (2004), without fluid overload, distinctive of heart disease, there was no elevation of BNP even with the presence of severe renal dysfunction. The BNP and NT-proBNP levels were all normal for the group without heart disease. BNP and NT-proBNP was then deemed an indicator for mortality due to cardiac complications or cardiac hospitalization in patients with renal disease, and a marker for early detection of cardiac complications arising from CKD. The precise influence of CKD on BNP and NT-proBNP levels continues to be debated and studies show plasma BNP dependence on the GFR among patients with and without heart failure. Additional prospective studies will be required to validate and better define the relationship between BNP, NT-proBNP and CKD

progression, independently of myocardial dysfunctions. Nevertheless, should these validations never arise, the screening of a sensitive marker for cardiac complications of CKD is still of importance.

1.4.7 Neutrophil gelatinase-associated lipocalin (NGAL): Neutrophil gelatinase-associated lipocalin is an iron-carrying protein that is expressed and released in large amounts from the tubular epithelium of the distal nephron following acute kidney injury (AKI), ischemia or toxicity (Bolignano *et al*, 2009). Increased NGAL levels in the urine have been associated with an increased CKD progression to ESRD, with the levels increasing as the CKD increases in severity (reviewed by Fassett *et al*, 2011). Serum and urine NGAL levels are increased in diabetic patients, suggesting a role in the development of DN. Additionally; significant correlations have been found between serum and urine NGAL levels and eGFR, and urinary NGAL levels and the degree of proteinuria. There is now strong evidence that increased urinary and serum NGAL reflect damage across a spectrum of kidney diseases, as well as AKI, and may predict progression of CKD.

1.4.8 Kidney injury molecule-1 (KIM-1): Kidney injury molecule-1 is a transmembrane tubular protein with uncertain function, however it is believed to play a role in tubulo-interstitial damage (Ichimura *et al*, 1998). This homologue is predominant in the kidney, predominantly expressed in tubular cells, and contains a highly conserved tyrosine kinase phosphorylation motif at position 350 (reviewed by Waanders *et al*, 2010). KIM-1 is not detectable in the urine from individuals with normally functioning kidneys. Elevated urine KIM-1 levels have been described in AKI and CKD. A study done by Van Timmeren *et al* (2007) found significantly higher KIM-1 levels in patients with tubular necrosis as opposed to the still significant but substantially lower levels in various chronic renal diseases. Urinary KIM-1 levels in renal disease were associated with tubular KIM-1 and renal inflammation, but not to proteinuria. There was no relation found between urinary KIM-1 with glomerular damage and interstitial fibrosis. Urinary KIM-1 levels might therefore represent only the degree of ongoing tubular damage, and might be a promising biomarker for assessing the

progressive nature of renal disease. However, the almost complete absence of KIM-1 in healthy kidneys resulted in some studies concluding that KIM-1 plays a role in the renal regeneration process (Ichimura *et al*, 1998). This is yet to be determined and controversy exists as to whether KIM-1 is involved in actively regulating the inflammation process, or if the expression of KIM-1 is purely a response to kidney damage, does KIM-1 attempt to repair damage or is it part of a recovery process (reviewed by Waanders *et al*, 2010). Long term observational studies in large populations are required to assess urinary KIM-1 as a CKD biomarker.

It is unlikely that one single marker will satisfy the need to predict CKD progression, morbidity and mortality. Chronic kidney disease is a complex disease with multiple pathophysiological processes involved. A panel of confirmed biomarkers would be the ideal conclusion, with a review of all the markers done together as they may have additive effects on CKD progression, morbidity and/or mortality. Large studies are required urgently for the more established markers and more intense investigation into the 'newer' biomarkers, such as pentraxin-3, urinary interleukin 8 and liver-type fatty acid binding protein. Several novel urinary markers show promise of non-invasive demonstration of kidney damage or prediction of disease progression. None appear to be ready at this time for widespread application in clinical practice.

1.5   AETIOLOGY OF CKD

1.5.1 Non-modifiable factors

Non-modifiable factors refer to factors that cannot be changed or adjusted. These are termed susceptibility factors, increasing the risk for adverse outcomes of CKD. Susceptibility factors increase the vulnerability to kidney damage such as old age, gender, a family history of CKD, ethnicity, a low birth weight or small kidneys, as well as genetic variants proven to be associated with CKD or any of the known conditions causing CKD (Hogg *et al*, 2003) These genetic mutations will be discussed in further detail in 1.5.4.

1.5.1.1 Age: The NHANES is a group of studies that assess the health and nutritional status of the population in America. Due to the lack of CKD prevalence studies in South Africa, we have to rely on external statistics and extrapolate the results. According to the NHANES Annual Data Report, 85% of individuals with an eGFR of less than 60ml/min/1.73m^2 are 60 years or older (Bethesda, 2011). This value confirms the increasing risk of CKD with age. A significant observation that must be noted is the natural aging of the kidney, with deterioration of both structural and physiological features (reviewed by Silva, 2005). The scarring of the glomerulus (glomerulosclerosis) starts from the age of 30, and by the age of 70 the mesangium has increased by 12%. This is followed by the formation of a direct channel between the afferent and efferent arterioles. The arterioles show the deposition of hyaline and collagen fibers while the smaller arteries are thickened due to the proliferation of the elastic tissue. The renal tubules undergo atrophy and fibrosis (reviewed by Silva, 2005). These anatomical changes cause a decrease in the GFR as the plasma flow is affected. The diminished response to stimuli with the reduced sodium reabsorption and plasma concentrations of renin and aldosterone causes a greatly increased fractional excretion of sodium and overall urine sodium excretion. The ability to concentrate and dilute ones urine is also slowly lost along with decreased potassium secretion and urea absorption (Lindeman, Tobin and Shock, 1985). These changes can easily be mistaken for CKD and need to be investigated (these normal changes in healthy patients can be determined by a normal hemoglobin concentration, normal erythropoietin levels and a normal urinalysis result); as these natural changes can themselves initiate CKD or cause cardiovascular disease, which in turn leads to CKD (Musso and Oreopoulos, 2011). The monitoring of patients is important.

1.5.1.2 Gender: Information on the gender differences in GFR and therefore CKD prevalence is conflicting. The Third NHANES noted prevalence of a GFR of less than 80ml/min/1.73m^2 in 54% of men and 68% of women when using the MDRD formula and a 25% and 53% prevalence respectively using the Cockcroft-Gault formula. When breaking down the prevalence by age group, men and women in the age group 30-59 years and 60-79 years, had prevalence's of 9%:17.5% and 45.2%:50.2% respectively. However, the most significant GFR difference between men and women is for those over the age of 80 with 45.8% of

men and only 32% of women falling in this category (Clase, Garg and Kiberd, 2002). Other studies have confirmed the statement that women have a lower GFR than men (Rule *et al*, 2004). However, there are studies showing that it is the rate of progression of renal disease that differs between genders, rather than the initial prevalence in men and women, which is more rapid in men than in women (Silbiger and Neugarten, 1995). There are genetically determined differences between the sexes in renal structure and function as well as the influence of sex hormones, all of which will have a bearing on the susceptibility and progression of CKD (Sabolić *et al*, 2007). These observations are said to give women a gender-related advantage, which is lost after menopause as estrogen levels drop. Whether these differences favor males, females or neither is controversial and debatable. Latest KDOGI guidelines state that 18 studies have addressed the impact of gender on GFR. Results suggest either an association of faster progression with males, association of faster progression with females or finally no association between either gender or progression. Though these results are indecisive, the impression is that progression is faster among men. Until conclusive findings are obtained through research, this decision is taken as the gold standard of guidelines (National Kidney Foundation, 2006).

1.5.1.3 Ethnicity: Racial risk studies conducted, initially in the United States of America and subsequently globally, have noted that racial minorities and indigenous populations are mostly at risk, specifically, African Americans, Native Americans, the Asian population and Aboriginal people (Mehrotra *et al*, 2008). According to the United States Renal Data System (USRDS) 2011 report, African Americans had the highest prevalence of CKD in America with 5284 patients per million followed by Native Americans with 2735 patients per million. This entirely overshadows the 1279 patients per million in the Caucasian American population. Due to this obvious increase in risk, MDRD, CKD-EPI and CKD-EPI Cystatin C equations each have an African American factor included to equate for this increase in risk. Higher rates of modifiable metabolic-related disorders such as obesity, diabetes, and hypertension are becoming more predominant among indigenous populations (Mehrotra *et al*, 2008). This can be explained by the changing environment, adoption of a Western diet with less physical activity

25

and poor lifestyle factors associated with low socio-economic status. It is these environmental changes and socio-economic conditions that exert a great influence on the health and disease among the subpopulations (Nicholas *et al*, 2005). The disproportionately high rate of CKD among the racial minorities worldwide, emphasizes the need to re-evaluate the identification, diagnosis and treatment of CKD in countries with diverse communities, such as South Africa.

1.5.1.4 Low birth weight: Low birth weight has been identified as a risk factor not only for obesity, but for the development of CKD as well (Griffen, Kramer and Bidani, 2008). The relationship was first described by Dr Barker in the 1980's, when he showed that the lower the weight of the baby at birth, the higher the risk of developing coronary heart disease in adulthood (Brenner, Garcia and Anderson, 1988). Further studies showed that the low birth weight was also associated with hypertension, stroke and type 2 diabetes, and was deemed the 'Fetal Origins Hypothesis', and later the Barker's Hypothesis. These manifestations were due to under nutrition during fetal life, leading to a low birth weight, which permanently changed the body's structure, physiology and metabolism (Brenner, Garcia and Anderson, 1988). Barker's hypothesis has been modified into the Brenner Hypothesis, which states that a congenital reduction in nephrons due to a low birth weight or intrauterine growth retardation results in a greater likelihood of the onset of adult hypertension and subsequent renal failure (Wadsworth *et al*, 1985). A low birth weight has been associated with a decreased number of nephrons, and should there be delayed fetal growth, even in a full-term pregnancy, there can be a decrease of up to 20% of the number of nephrons. This becomes evident in adulthood with the manifestation of hypertension and a decreasing GFR (Mañalich *et al*, 2000). The decreased number of nephrons (termed oligonephronia) results in an increased pressure on the remaining nephrons' capillaries, contributing to glomerular damage. This increases renal vulnerability and exacerbates the progression of renal disease (Reyes and Mañalich, 2005). Congenital, hereditary and acquired CKD has also been associated with children born prematurely, with a study in 2010 by Franke *et al* having statistics as high as 39.3%, 24.7% and 15.5%, respectively. These results suggest that children who are born prematurely and at a lower than normal birth weight are more predisposed to high-grade CKD.

1.5.1.5 Small kidney size: As with low birth weight, small kidneys have also been associated with a reduced number of nephrons (Franke *et al*, 2010). With a decrease in glomerular number and size, there is an unmistakable correlation with decrease in kidney weight. This results in glomerular hypertension in the few nephrons available, which, in turn, triggers a cycle of progressive loss of renal function (Nvengaard and Bendtsen, 1992).

1.5.1.6 Family history of CKD: Family history of CKD can be mainly attributed to the genetic factors associated with CKD, which will be discussed later in this review (1.5.4). A study completed between 1995 and 2003 demonstrated a 23% incident of dialysis in patients with first or second degree relatives who had been diagnosed with ESRD, and far more are likely to have family members with silent proteinuria or CKD (Freedman *et al*, 2005). This percentage did not include cases of ESRD with Mendelian inheritance. This high prevalence could partially be explained by the presence of CKD susceptibility genetic factors and common lifestyle factors.

1.5.2 Lifestyle factors

Lifestyle factors contributing to CKD, also referred to as modifiable factors, describe causes of CKD that are controllable and can be prevented or avoided. The two main modifiable causes of chronic kidney disease are diabetes and high blood pressure, which are responsible for up to two-thirds of the cases of CKD (National Kidney Foundation, 2010). These medical conditions are referred to as initiation factors as they directly initiate kidney damage (Hogg *et al*, 2003). Additional modifiable factors include smoking, excess alcohol intake (Stengel *et al*, 2003), excessive sun exposure, pollutants such as toxic waste, diesel particles, and organic pollutants (Delfino *et al*, 2009; Orth and Hallan, 2008; Gee and Payne-Sturges, 2004).

Type 1 diabetes is characterized by the autoimmune destruction of the insulin producing β-cells of the pancreas, whereas in type 2 diabetes, cells with insulin receptors are resistant to the action of insulin (Gillespie, 2006; Stumvoll,

Goldstein and van Haeften, 2005). Current WHO diagnostic criteria for diabetes mellitus is a fasting plasma glucose ≥ 7.0 mmol/l or 2–h plasma glucose ≥ 11.1 mmol/l (WHO, 2006). Between 20%-40% of patients with diabetes will ultimately develop DN (Dronavalli, Duka and Bakrus, 2008). Initiation and progression of DN differs according to the type of diabetes present as well as the presence or absence of microalbuminuria. The presence of albuminuria increases the prevalence of initiation of DN to 80% (Dronavalli, Duka and Bakrus, 2008). There are many distinct stages of the development of DN. Initially, functional changes will occur in the nephron before any biochemistry results show a problem. This hyperperfusion and hyperfiltration is caused by the decrease in the resistance to blood flow in both afferent and efferent arterioles in the glomerulus (Dronavalli, Duka and Bakrus, 2008). This results in increased blood flow into the glomerulus, thereby increasing glomerular pressure. Decreased resistant action has been attributed to many factors including prostaglandins, vascular endothelial growth factor A, repeated stretching and relaxation of the mesangial cells and transforming growth factor β (TGF-β). TGF-β is an extensively researched mediator in DN (Zihadeh, 2004). It is stimulated by, to mention a few, a high glucose concentration, products of the gylcation of proteins, oxidative stress, glucoseamine overproduction and high levels of angiotension II, endothelin and thromboxane. This cytokine promotes the synthesis of extracellular matrix and decreases the matrix degradation by inhibiting proteases.

Cytokines and growth factors are involved in the cascade leading to kidney damage by causing oxidative stress, abnormal glycosylation, lipid peroxidation, and the production of additional inflammatory elements (Dronavalli, Duka and Bakrus, 2008). Ultimately, albumin leakage from the glomerular capillaries ensues, accounting for the microalbuminuria. Initial hemodynamic changes due to growth factors, cytokine and vasoactive system disturbances associated with diabetes are further exacerbated by the hyperglycemia. Hyperglycemia is associated with an increase in mesangial cell proliferation and hypertrophy, resulting in glomerular hypertrophy, mesangial expansion and glomerular basement membrane thickening (Dronavalli, Duka and Bakrus, 2008). Mesangial expansion due to an increase in matrix production further stimulates TGF-β

secretion and reduces the capillary area for filtration, further decreasing the renal function (Ichnose, Kawasaki and Eguchi, 2007). The development and outcomes of DN, as well as the decline in the rate of kidney function, occur via multiple mechanisms. It is greatly variable between patients and is influenced by additional factors including blood pressure, glycaemic control that may lead to metabolic and hemodynamic changes, as well as genetic predisposition.

Hypertension is the second leading cause of ESRD and plays a predominant role in the progression of CKD (Bidani and Griffen, 2004). Systolic blood pressure of above 140 mmHg is considered hypertensive (WHO, 2004). Hypertensive nephrosclerosis (HN) is either benign or malignant, with the benign nephrosclerosis pattern showing non-specific hyaline arteriosclerotic lesions developing slowly over time with no obvious proteinuria and only severely compromised GFR in susceptible individuals; whereas malignant nephrosclerosis is characterized by severe hypertension and disruptive vascular and glomerular injury accompanied by fibrosis, necrosis, myointimal hyperplasia and thrombosis (Bidani and Griffen, 2004). Arteries and arterioles are narrowed, resulting in glomerular ischemia. Blood pressure load can be controlled by maintaining the blood pressure within a certain limit, this is characteristic of benign nephrosclerosis. Regulatory vasoconstriction of the afferent arteriole supplying blood to the glomerulus attempts to maintain a relatively constant renal blood flow, protecting against renal damage. Should blood pressure exceed the maintainable limits, malignant nephrosclerosis ensues, causing vascular injury (Bidani and Griffen, 2004). Hypertension is an initiation factor causing renal damage and is also a consequence of renal disease, contributing to the complexity of HN. When renal function is compromised due to other factors, the sodium-potassium pump within the kidney is disrupted, affecting the blood pressure regulation, resulting in secondary HN from primary renal disease. Conversely, malignant hypertension has been associated with renal damage, with increased blood pressure a strong predictor of a decline in renal function resulting in CKD (Fervenza, 2012). The difficulty in determining whether it was the renal damage or the hypertension which manifested first, leads to difficulty in CKD typing. HN may be diagnosed on a long-standing history of hypertension

and moderate proteinuria, without any evidence indicating an alternative diagnosis (Fervenza, 2012). However, regardless of the aetiology of the HN, once hypertension develops, a cycle of renal injury, nephrosclerosis, aggravation of hypertension and further renal damage ensues and main focus on prevention, early detection and proper control procedures is important.

Susceptibility to hypertensive vascular injury with a reduced blood pressure load limit and lower threshold for damage is seen in already existent CKD, due to diabetes or other factors such as genetic susceptibility. Collectively, hyperinsulinemia in diabetes plays a role in hypertension initiation and aggravation by increasing platelet adhesion and aggregation, causing an imbalance between coagulation and fibrolytic activity and ultimately leading to a pro-coagulant state, endothelial dysfunction, lipoprotein abnormality, and vascular smooth muscle alteration (Acharya, 2001). This links the first major risk factor in CKD, diabetes, to the second, hypertension. Each condition promotes and aggravates the other, as one is uncontrolled, so the next is initiated or enhanced.

Obesity is another chronic disease risk factor that plays a significant role in the initiation and progression of CKD (Schmitz *et al*, 1992; Wolf, 2003; Chagnac *et al*, 2003). There are many factors that contribute to the obesity epidemic and these include high intake of refined carbohydrates and fats, urbanization, sedentary lifestyle that results in reduced physical activity due to technological advances and the use of transportation (World Health Organization, 2006). Obesity is the main driving force for both the diabetes and hypertension epidemics (Coldits *et al*, 1990; Ascherio *et al*, 1998; Field *et al*, 2001). Adipose tissue secretes many hormones that regulate insulin sensitivity such as cytokines, leptin, resistin and adiponectin. (Silha *et al*, 2003). Adiponectin is the main focus of type 2 diabetes-related insulin resistance. It regulates insulin by promoting glucose uptake into the myocytes, suppressing gluconeogenesis in the hepatic cells and increasing fatty acid oxidation in both muscle and hepatic tissue. With the increase in adipose tissue, the adiponectin receptors become

resistant to adiponectin and there is a decrease in fatty acid oxidation, reduced insulin signaling and an eventual insulin resistance (reviewed by LeRoith, 2011).

The exact mechanism for obesity causing hypertension is not fully understood, but it is postulated that the link is through insulin resistance. Hyperinsulinemia enhances sodium resorption through its effect on the distal renal tubules, resulting in hypertension. Another mechanism involves increased secretion of aldosterone due to elevated insulin, causing hypervolemia and ultimately contributing to hypertension (Barnett, Barnett and Kumat, 2009). Countless studies have shown the association between obesity and CKD through diabetes and hypertension, and other components of the metabolic syndrome (Stengel *et al*, 2003; Kramer *et al*, 2005; Locatelli, Pozzini and Vecchio, 2006). A number of studies have attempted to find a direct association between metabolic syndrome, which is characterized by abdominal obesity, high triglycerides, low HLDs and high blood pressure; and CKD, however it is difficult to differentiate the effect caused by each of the factors, the metabolic syndrome, hypertension and impaired glucose metabolism (El Nahas, 2005). Kurella, Lo and Chertow (2005) investigated the effect of type 2 diabetes and components of the metabolic syndrome on the development of CKD, and after adjusting for hypertension and diabetes there was still an increased risk of CKD among the study participants, suggesting that other conditions of the metabolic syndrome may be involved in the mechanism initiating CKD, however this is yet to be proven (Kurella, Lo and Chertow, 2005).

1.5.3 Folk medicine

Folk remedy usage is prevalent throughout Africa and Asia, and the effect of its intake on renal damage requires investigation in African populations as the medication is widely used in Africa. The use of folk medication is most popular amongst the poorer sections of society in the developing world. Over three-quarters of the population in sub-Saharan Africa depend on traditional medicine as their primary source of health care, more than 60% of the Chinese use herbal therapy and a large part of the rural population in India relies on indigenous

medical systems that use herbs, ash and heavy metals (reviewed by Jha, 2009). The source and composition of the herbal remedies varies around the world but the origin of botanical ingredients is alike. The prescription and distribution of these remedies is done by community trained herbalists and the ingredients of the given mixtures are never tested for safety or toxicity. Aristolochic acid nephropathy is the most frequent herb-induced form of CKD. The toxic ingredient is aristolochic acid, which is a component of a Chinese herb, Aristolochia fangchi (reviewed by Jha, 2009). Common African plants associated with toxicity and acute and chronic renal failure, include the *Cape Aloes, Callilepis laureola* and the *Securidacea Longepedunculata* (Luyckx, Steenkamp and Stewart, 2005). The reasons suggested for consultation with traditional healers include the protection of a child, increased sexual potency, infertility treatment, removal of evil spirits, getting rich and protection against witchcraft. Many patients only resort to Western medicine after the use of folk treatment has failed, due to tradition and culture. The use of herbal remedies is often withheld by patients due to the fear of judgment, as there is much stigma attached to the use of folk medicine (Luyckx, Steenkamp and Stewart, 2005). True incidence of CKD due to nephrotoxic herbs remains uncertain.

1.5.4 Genetic factors associated with CKD

The role of genetics in the development of CKD has been an immense breakthrough that has partly elucidated the aggregation of kidney disease in families and populations. Many years of positional cloning studies have led to the identification and characterization of numerous genes and mutations in kidney diseases. It is clear that kidney diseases have a strong genetic component, and can therefore be classified into monogenic and polygenic CKD (reviewed by Hildebrandt, 2010). Rare single mutations causing monogenic kidney disorders have been identified and almost all demonstrate Medelian Inheritance patterns. The degree in which these genes cause the kidney disease depends on the mode of inheritance (reviewed by Hildebrandt, 2010). Monogenic recessive disorders have a tight genotype-phenotype correlation due to the full or high penetrance and usually manifest by adolescence, including autosomal recessive

polycystic kidney disease (ARPKD) and pediatric nephrotic syndrome caused by mutations in the nephrosis 2 (*NPHS2*) gene (Boute *et al*, 2000). An important factor of monogenic diseases is the fact that the mutation itself represents the primary cause of the disease. Dominant monogenic diseases develop in adulthood with a reduced genotype-phenotype correlation because of incomplete penetrance and varying expressivity such as glomerulocystic kidney disease (*GCKD*) and FSGS due to actinin alpha 4 (*ACTN4*), transient receptor potential cation subfamily 6 (*TRPC6*) and inverted forming 2 (*INF2*) mutations (Kaplan *et al*, 2000; Winn *et al*, 2005; Brown *et al*, 2010; reviewed by Hildebrandt, 2010). At the other end of the spectrum of causality, with very weak genotype-phenotype correlation, are polygenetic disorder mutations. Multiple mutated alleles in different genes have to act in concord to cause disease (reviewed by Hildebrandt, 2010). These diseases manifest in adulthood and are more frequent than monogenic diseases, with less heritability; there is more room for environmental changes.

Linkage analysis has been successful in identifying renal disease-causing mutations with a mendelian mode of inheritance (reviewed by Friedman and Pollak, 2011). Linkage analysis is informative when investigating genes for rare, Mendelian, monogenic disorders for which there is strong familial risk, and for identifying loci that are strongly associated with complex diseases (reviewed by Hirschhorn and Daly, 2005). Linkage analysis makes use of the shorter distance between genetic loci, assuming that the closer they are positioned to each other, the more likely the variants are co-inherited, first described by A Sturtevant in 1913. However, linkage analysis has failed to detect most of the polymorphisms associated with common CKD phenotypes (ESRD and DN, GFR, and albuminuria) (reviewed by Friedman and Pollak, 2011). Genome wide association studies (GWAS) were subsequently introduced, which allowed for the analyzing of the entire genome and identification of polymorphisms with small effects that might increase predisposition to common complex diseases (McCarthy and Hirschhorn, 2008). The drawback of this approach is the large amount of genotyping that is required. The number of polymorphisms that can be screened and the cost of the analyses can be reduced by genotyping one variant within a

haplotype (Lunetta, 2008). Another disadvantage is the marked difference in allele frequency between mixed populations, which results in the overestimated effect of polymorphisms within complex disease. This was resolved by introducing admixture mapping, also known as mapping of admixture linkage disequilibrium (MALD). This strategy is cost effective as it required studying 2000-3000 markers, which is 200-500 times fewer than is needed for genome-wide association analysis (Briscoe, Stephens and O'Brien, 1994; McKeigue, 1998; reviewed by Darvasi and Shifman, 2005). The ability to detect haplotypes that contain a disease-associated polymorphism for a specific complex disease using MALD is maximised by analysing markers that are most divergent between the parent populations of the mixed ethnic group (for example, African and American in the case of the African-American group) (Briscoe, Stephens and O'Brien, 1994; Dean and Santis, 1994; McKeigue, 1998; reviewed by Smith and O'Brien, 2005; Darvasi and Shifman, 2005). Since the commencement of this genetic research, several genes have been implicated in CKD susceptibility, initiation and progression and its many risk factors, including *UMOD*, *SHROOM3*, *TCF7L2*, *CUBN*, *ELMO1*, Angiotensin gene*, MTHFS, MTHFR, IL-1RA* and *APOL1.* Additionally, it was through GWAS and MALD that specific haplotypes in the *MYH9* locus were found to be associated with an increased risk for FSGS and CKD in African Americans and later hypertensive-, non-diabetic and diabetic-ESRD (reviewed by Hildebrandt, 2010).

The uromodulin (*UMOD)* gene is located on chromosome 16q12.3 and exclusively expressed in the renal tubular cells of the thick ascending Loop of Henle in the kidney (Hart *et al*, 2002). *UMOD* encodes for the Tamm-Horsfall protein (also called uromodulin), and although isolated almost 6 decades ago, the physiologic role of this protein remains unclear. The increased concentration of uromodulin in urine has been associated with the development of CKD (Köttgen *et al*, 2010) and medullary cystic kidney disease 2 (Hart *et al*, 2002). The minor T allele at rs12917707 on the *UMOD* gene has been associated with a 20% reduced risk of CKD (Köttgen *et al*, 2009). These studies were all conducted in people of European decent, and it has not shown any reproducible results in

other populations. However in the Japanese population, *UMOD* polymorphisms have been associated withhypertension (Iwai *et al*, 2006).

The *SHROOM3* gene is found on chromosome 4q21.1 and codes for the actin-binding protein, shroom3, which is important in apical epithelial constriction, and therefore regulation of the shape of epithelial cells (reviewed by Divers and Freedman, 2010). The rs17319721 A allele in the *SHROOM3* gene has been associated with a lower GFR and lower urine albumin:creatinine ratio in the European and African populations (Ellis *et al*, 2010)

The transcription factor 7-like 2 gene (*TCF7L2*) encodes the transcription factor 4, which acts as a downstream effector in the canonical Wnt signaling pathway and has been strongly associated with type 2 diabetes risk and impaired glucose tolerance in the European, Asian and African populations (Köttgen *et al*, 2008). In all populations studied, the T allele of rs7903146 has been strongly linked with type 2 diabetes. Few other SNPs in *TCF7L2* have shown reproducible association with type 2 diabetes (Cauchi *et al*, 2007). This gene is also significantly associated with CKD progression and function in individuals without diabetes in both American Caucasians and Africans. Kottengen *et al* (2008) showed that the rs7906195 C and rs7903146 T alleles have been associated with a 20% increase in CKD progression risk in Caucasians without diabetes, but only the rs7906195 has shown similar significant association in African Americans (Köttgen, 2008).

Cubulin, product of the *CUBN* gene, is found in the apical brush border area of the proximal tubules in the kidney (Birn *et al*, 1997). Together with megalin and amnionless, cubulin regulates receptor-mediated endocytosis of low-molecular-weight proteins (Christensen, Verroust and Nielsen, 2009). When cubulin is lacking or mutated due to variations in the *CUBN* gene, proximal tubular protein reabsorption is ineffective, resulting in proteinuria (reviewed by Böger and Heid, 2011). This explains the association between the rs1801239 C allele and consistently higher urine albumin:creatinine ratio and microalbuminuria. The

35

significant association was observed in European and African individuals with or without diabetes and hypertension (Böger *et al*, 2011).

The engulfment and cell motility 1 (*ELMO1)* gene is found on chromosome 7p14 and is ubiquitously expressed but predominantly in the spleen and placenta (Shimazaki *et al*, 2005). The ELMO1 protein interacts with other proteins to promote phagocytosis of dying cells and cell shape changes (Gumienny *et al*, 2001). Its association with type 2 diabetes was first reported in the Japanese population (Shimazaki *et al*, 2005), and later similar findings were obtained in African Americans in addition to having an effect on ESRD. An association with type 1 diabetes was also observed in the Caucasian population (Garrett, Pezzolesi and Korstanje 2010). Different regions of the *ELMO1* gene are associated with the significant findings in different ethnic groups, with intron 18 in the Japanese population, intron 13 in the African American population and typically intron 16 in European Americans, with different polymorphisms producing the common DN phenotype (Divers & Freedman, 2010)

The angiotensin-converting enzyme (ACE) is encoded by the angiotensin gene, which is localized on chromosome 17. ACE is a metalloenzyme which converts angiotensin I to angiotensin II, is a vasoconstrictor, and inactivates bradykinin, a vasodilator (Sayin *et al*, 2009). ACE is part of the renin-angiotensin system (RAS) that is involved in regulating blood pressure and fluid balance. Upregulation of RAS has been reported in CVD and also suggested to contribute to the development of CKD (Reveiwed by Wong, Kanetsky and Raj, 2008). RAS has also been implicated in the induction of cell proliferation and fibrosis, which affects development of CKD (Kagami *et al*, 1994). Polymorphisms in the angiotensin gene have been associated with an increased susceptibility or initiation of CKD in diseases such as Henoch-Schonlein purpura, diabetic nephrology and hypertension, as well as rapid progression of CKD (El Nahas and Levin, 2009). A deletion/insertion (I/D) polymorphism has been identified in intron 16, which is believed to be responsible for about 50% of the variability in plasma ACE levels among individuals (Gumprecht *et al*, 2000). Particularly, a homozygous deletion (D/D) was associated with higher ACE levels, which in turn

was implicated in ventricular hypertrophy and increased carotid intima-media thickness in ESRD (Balkestein *et al*, 2001). The D allele has also been linked with a rapid decline of renal function (Gumprecht *et al,* 2000), and homozygous D carriers had a worse disease prognosis and developed CKD at a younger age (Baboolal *et al*, 1997; Luttropp *et al*, 2009). The role of the I/D polymorphisms was also reported in relation to ACE inhibitor treatment, in which I/I carriers with immunoglobulin A nephritis receiving this medication had a reduced progression to ESRD (Yoshida *et al*, 1995).

SNP rs6495446 in the methenyltetrahydrofolate synthetase gene (*MTHFS*) has been associated with CKD in Caucasians, but the mechanism by which this leads to susceptibility, initiation, or the progression of CKD is not yet fully understood (El Nahas and Levin, 2009; Kottgen, *et al,* 2008). The *MTHFS* gene has been mapped on chromosome 15q25.1 and codes for the methenyltetrahydrofolate synthetase, which is responsible for tetrohydrofolate metabolism and helps in the biosynthesis of purines, thymidine and amino acids (El Nahas and Levin, 2009). Also involved in folate metabolism pathway is methenyltetrahydrofolate reductase (MTHFR), which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for remethylation of homocysteine to methionine (reviewed by Födinger, Höri and Sunder-Plassmann, 2000). A review by Rao, Reddy and Kanagasabapathy (2008) reported higher levels of homocysteine in CKD patients, and speculated that this may accelerate the atherosclerotic process by inducing lipoprotein oxidation and endothelial injury. The 677C>T polymorphism in the *MTHFR* gene has been associated with diabetic nephropathy, with the T allele predominantly found in affected individuals with early onset DN who progressed to ESRD (Ksiazek, Bednarek-Skublewska and Buraczynska, 2004). The *MTHFR* gene has been mapped to 1p36.3 (Goyette *et al*, 1994).

Interleukin-1 receptor antagonist (IL-1Ra), encoded by the *IL-1RN* gene, is a protein of the interleukin 1 (IL1) family (reviewed by Dinarello, 1994). It is a competitive inhibitor of IL-1. Intron 2 of *IL-1RN* contains an 86-base pair variable number tandem repeat consisting of 2-6 repeats. The genes coding for IL-1Ra

and IL 1 are located near to each other on the long arm of chromosome 2. The less common allele of *IL-1RN* containing 2 repeats has been associated with an increased production of IL-1Ra protein and a decreased production of IL-1 (Jaaskelainen *et al,* 2008). This 2-repeat allele has been associated with ESRD and CKD progression in patients with diabetes nephritis and glomerulonephritis (Luttropp *et al*, 2009). It has also been shown to play a role in the development of a severe clinical outcome of inflammatory diseases and increased risk for coronary artery diseases in patients with type 2 diabetes (Marculescu *et al*, 2002).

1.5.5 Nonmuscle myosin heavy chain (*MYH9*) and its association with CKD

The *MYH9* gene is located on the long arm of chromosome 22 at position 13.1, located from base pair 36 677 322 to base pair 36 784 062, approximately 110 kb with 41 exons (Kopp *et al*, 2008). This gene encodes for the myosin IIA heavy chain, a subunit of myosin IIA protein. Myosin IIA heavy chain consists of 1960 amino acids and is 227kDa in size, with a globular head in N-term and a coiled-coil tail in C-term (Schilwa, 2006). An actin binding site is found in the head region, interacting with actin, as well as a light chain binding site present in the long tail region. It is abundantly expressed in the kidney, platelets and liver and in smaller amounts in the thymus, spleen, intestine and cochlea. Within the kidney, it is expressed in the glomerulus, specifically the podocyte, peritubular capillaries and tubules. Myosin IIA heavy chain plays several physiological roles such as cytokinesis, cell motility and the maintenance of cell shape (Schliwa, 2006).

More than 45 mutations have been found in the *MYH9* gene, and implicated in a wide range of disorders that have subsequently been grouped and termed the *MYH9*-related disorder (Tzur *et al*, 2010). These disorders are characterized by large platelets and thrombocytopenia, both present from birth. The autosomal dominant giant-platelet disorders include May-Hegglin anomaly, Fechtner syndrome and Sebastian syndrome, all exhibiting dohle bodies within their leukocytes; and Epstein syndrome which is characterized by deafness and cataracts (Schliwa, 2006). Although the exact mechanism of the development

and progression of non-diabetic CKD by polymorphisms in the *MYH9* gene is unknown, aggregation of abnormal myosin and damage to the cytoskeleton of the podocyte and tubular cells, and the rearrangement of the actin cytoskeleton, ultimately disrupting the filtration barrier, have been hypothesized (Johnstone *et al*, 2011).

The initial discovery of the *MYH9* gene variation increasing CKD risk was made in 2008 by Kopp *et al*, a study that demonstrated a high risk (7.5%) of developing ESRD in African-Americans compared to European Americans (2.1%). According to Kopp *et al* (2008), African-Americans have a disproportionate risk for certain CKD types, namely DN, HN, lupus nephritis, FSGS and HIVAN. The authors suggested that this observation could be partly explained by lifestyle, social-economic status and clinical factors such as poor blood glucose or blood pressure control. Although diabetes and hypertension can be familial, with individuals from African decent having a greater risk of both, further making this ethnic group more susceptible to CKD, predisposing polymorphisms would better explain the large disproportion of the risk. The hypothesis of this 2008 study was that the fourfold increased risk for idiopathic FSGS and 18-50 fold increase in HIV-associated nephropathy would be associated with genetic variants that would be frequent and have strong effects in African Americans but would be infrequent in European Americans. The results of a mapping by admixture linkage disequilibrium (MALD) study done by this study group identified *MYH9* as a functional and possible candidate gene. Their scan yielded a single prominent peak located on chromosome 22 with a logarithm of odds (LOD) score of 12.4. *MYH9* was one of 35 genes that fell into the 95% interval of credibility of the MALD peak. The LOD score compares the likelihood of the genes in the chromosome 22 peak being linked; to the likelihood that the data obtained was purely by chance. A LOD score of higher than 3 is considered significant, signifying that the data is in fact linked (Morton, 1955). Twenty *MYH9* SNPs were selected as they demonstrated a pronounced frequency difference between Yorubas (YRI) (a West African ethnic group) and European (CEU) populations from the International HapMap Project data. Three *MYH9* SNPs located in intron 23 (rs4821480, rs2032487 and rs4821481), were in strong LD, and thus termed

extended or E-1 haplotype. The LD refers to the occurrence or frequency of the combinations of alleles in a population (Li and Stephens, 2003). This may happen more or less often in the given population when compared to the number of times the allele combinations would occur should a random formation of haplotype be formed based on their frequencies. LD is commonly described using D', with a range between -1 and 1 (Li and Stephens, 2003). The odds ratio (OR) ranged from 3.9 to 5.7 when comparing homozygous risk allele individuals and heterozygous risk allele individuals as opposed to an OR of close to 1 when comparing heterozygous and non-risk homozygous allele, clearly showing a recessive nature of these three intron 23 SNPs. Conversely, SNPs rs5756152, rs1005570 and rs16996677 had additive effects with higher ORs when comparing homozygous risk allele carriers to those harboring homozygous non-risk allele individuals. The OR provides an estimate of the relationship between the binary variables (Bland and Altman, 2000). It enables one to examine the effects of other variables on that relationship using logistic regression and is useful in case-control studies.

Kopp *et al* (2008) then did association studies with idiopathic and HIV-1-associated FSGS, revealing seven significantly associated SNP results, including rs4821480 ($p = 3 \times 10^{-5}$) and rs5756152 ($p = 1 \times 10^{-7}$). The susceptible allele in all seven SNP's was frequent in both African Americans (29 – 91%) and YRI (40 – 97%) but much less frequent in European Americans (3 – 52%). Haplotype association showed that the strongest risk association with FSGS was for the E-1 haplotype ($p = 4 \times 10^{-23}$). The E-1 haplotype had a frequency of 60% in African Americans but only 4% in European Americans. Consistent with this result, the E-2 haplotype was the most frequent in European Americans (69%) and showed the significant protective effect ($p = 0.008$). The four SNPs in the E-1 haplotype showed modestly significant associations between FSGS and European Americans, although the E-1 haplotype itself didn't boast any significance. Additionally, association studies were done regarding H-ESRD. Nine *MYH9* SNPs, including rs5756152 ($p = 0.0002$) as well as the E-1 haplotype (rs4821480, $p = 0.002$) showed significant associations with H-ESRD, consistent with the FSGS direction. Lastly, during survival analysis studies, haplotype E-1 showed no significant association with progression to ESRD.

Following this important finding, a further study was done by Freedman *et al* in 2009 in a larger group of unrelated African Americans with H-ESRD, which demonstrated an association between the E-1 haplotype and hypertensive African Americans (p = 4.52 x 10^{-12}). A novel second haplotype, termed L-1, consisting of rs7078, rs12107, rs735853 and rs5756129, also showed significant association with H-ESRD in a recessive model. The study demonstrated that rs5756152 was independently associated with H-ESRD even after adjusting for the E-1 and L-1 haplotypes. In addition, the study demonstrated rs5756152 (p = 2.92 x 10^{-5}) and rs12107 (p = 0.01) exhibited independent evidence for association. The evidence of association of rs12107, rs4821480, and rs5756152 with CKD was further replicated in other studies. Kao *et al* (2008) conducted a MALD analysis that revealed an association between *MYH9* SNPs that included rs12107 (p = 3.64 x 10^{-3}), rs4821480 (p = 1.72 x 10^{-11}) and rs5756152 (p = 4.49 x 10^{-9}) and non-diabetic ESRD. These authors also detected an association between the investigated *MYH9* SNPs and both hypertensive and FSGS ESRD. Additional genome-wide analysis has supported the initial *MYH9* findings of 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 has a statistically significant association 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 an even greater risk of CKD.

There has been much speculation following these initial findings, with a few articles concluding that the *MYH9* and renal disease association is non-existent. This uncertainty stemmed from the discovery of the second gene encoding apolipoprotein 1, which was also mapped in chromosome 22, near the *MYH9* locus. Genovese *et al* (2010) determined that the E-1 haplotype of the *MYH9* gene, the best predictor of renal disease in previous studies, was present in most haplotypes containing the G1 and G2 alleles of the *APOL1* gene (E-1 was present in 89% of haplotypes carrying G1 and 76% of haplotypes carrying G2), which were found to be stronger risk factors for CKD in African Americans than

polymorphisms within the *MYH9* gene. In contrast to the *MYH9*-CKD associated SNPs that seem to have no apparent functional defect, the three *APOL1* polymorphisms alter the amino acid sequence of the encoded protein, suggesting functionality: S342G and I384M referred to as G1, and a 6-base pair deletion that removes N388 and Y389 referred to as G2. The *APOL1* polymorphisms were initially reported to have a strong effect on FSGS risk, but were later found to be strongly associated with hypertensive-ESRD (Genovese *et al,* 2010). These polymorphisms also proved to be stronger genetic factors in HIVAN compared to FSGS and H-ESRD (Kopp *et al*, 2010). Studies done in African populations (Ethiopia, Cameroon, Congo, Ghana, Malawi, Mozambique and Sudan) found 0% occurrence of the *APOL1* G1 and G2 alleles in HIV affected individuals with no HIVAN from Ethiopia, further suggesting that *APOL1* may play a role in the development of HIVAN in this population group (Tzur *et al*, 2010). Importantly, *APOL1*-mediated kidney disease risk showed an autosomal-recessive inheritance rather than an additive pattern that is typical of most common complex disease. Although aspects of the *APOL1*-CKD association is compelling, there still remains no evidence that *APOL1* polymorphisms are strongly associated with CKD and data from the human protein atlas suggest that *APOL1* is not even expressed in the glomeruli (Johnstone *et al*, 2011). The results from a study done by Tzur *et al* (2010), suggest that *APOL1* polymorphisms may have arisen from positive selection due to Trypanosomiasis, which may explain the high allele frequency in African ancestries (Genovese *et al,* 2010). Humans are resistant to *Trypanosoma brucei* due to the lysis effect of the APOL1. However, a subspecies of this parasite that later emerged, is resistant to APOL1-mediated lysis and can only be destroyed by the presence of the G1 and G2 polymorphisms, which inactivates the virulence factor of the *T. brucei rhodesiense*.

This additional and post-*APOL1* genetic association discovery regarding the *MYH9* gene as well as the fact that this gene is already implicated in several rare inherited syndromes with glomerular involvement makes it impossible for CKD and *MYH9* gene variation association to be ruled out (Nelson *et al*, 2010). Indeed, the genetic involvement of chronic diseases, such as CKD, is complex

42

and undoubtedly involves many genes with multiple interactions, and it may be postulated that the *MYH9* and *APOL1* genes complement each other (Freedman *et al*, 2011). The compelling evidence of *MYH9* polymorphisms and their renal disease associations led to the motivation for this study, particularly in the mixed ancestry ethnic group of South Africa due to its admixture nature that may be similar to that of African Americans.

1.6 SIGNIFICANCE OF RESEARCH

It has been estimated that CKD affects approximately 1 in 10 adults or over 500 million people worldwide (Zhang and Rothenbacher, 2008). However, this prevalence only reflects the burden of the disease in developed countries. In low-income countries, including South Africa, the burden of chronic disease including CKD is also increasing but the prevalence of CKD to date is largely unknown due to no recent published data. The last reliable report of the South African Dialysis and Transplant Registry (SADTR) in 1994 indicated that 3399 patients were on treatment for ESRD, with 754 new patients having commenced therapy in that year (SADTR, 1994). It is likely that this number has risen since then and is therefore not a true representation of the actual prevalence. Diabetes, hypertension and glomerulonephritis are the three leading causes of CKD. With the increasing burden of obesity, type 2 diabetes and hypertension, it is expected that the incidence of ESRD will mirror that of these three diseases. This observation has dire implications for the developing world where the burden of diabetes is projected to increase by 250% by year 2030 (Wild *et al*, 2004). It is estimated that by 2030, more than 70 percent of patients with ESRD will be residents of developing countries (Barsoum, 2009).

The increasing prevalence of diabetes in most populations is partly due to the increase in the prevalence of obesity. Currently, more than one billion adults are overweight, with at least 300 million of them deemed clinically obese (WHO, 2006). Obesity is also associated with FSGS, a combination which has poor long term prognosis with one half of the patients ultimately developing advanced renal failure (Praga *et al*, 2001). In South Africa, where 29.2% of men and 56.6% of women are overweight or obese (Puone *et al*, 2002), diabetes is believed to

occur in 10% to 16% of adults although prevalence rates of up to 25% have been reported recently in some age groups (reviewed by Katz, 2005). Hypertension, on the other hand, is considered a cause of ESRD in 34.6% of Blacks, 20.9% of Mixed Ancestry, 13.9% of Indians, and 4.3% of Whites (reviewed by Katz, 2005). Several studies have also reported a high prevalence of diabetes in the mixed ancestry ethnic group, from 10.8% in 1996 (Levitt *et al*, 1999) to 25.6% in 2008 (Erasmus *et al*, 2012). South Africa has an additional burden of HIV, which has affected 19.9% of the adult population and contributes to 30% of deaths and numerous cases of HIVAN (reviewed by Katz, 2005). These risk factors, together with a high HIV/CKD prevalence result in a large burden of CKD in this country. Earlier interventions for CKD would be beneficial but the lack of knowledge on the number of affected people and those at risk of developing the disease hinders the adoption of appropriate preventive measures that may be useful. Establishing predisposing factors for CKD will enable early identification of individuals or groups of people who might benefit most from early screening, detection and intervention to prevent the initiation of CKD or delay progression to ESRD and associated cardiovascular risk.

1.7 CONCLUSION

Chronic kidney disease is a silent epidemic, the 12th highest cause of death worldwide. Despite adjustment for the many identified non-modifiable and lifestyle factors, certain populations still have a large disproportion of risk for CKD that could only be explained by predisposing polymorphisms. The role of genetics in the development of CKD has been an immense breakthrough that has partly elucidated the aggregation of kidney disease in families and populations. Polymorphisms in the *MYH9* gene have been associated with FSGS, H-ESRD, HIVAN and non-diabetic ESRD in the African American population with SNPs rs5756152, rs4821480 and rs12107 showing independent association. The mixed ancestry population in the Bellville South area in Cape Town, South Africa, is a population of interest due to the similar ancestry of both African and Caucasian. Establishing predisposing factors for CKD will allow for early identification of individuals of groups of individuals who would benefit from early screening, detection and intervention.

44

CHAPTER 2

[Research Methodology]

2.1 INTRODUCTION

As described in Chapter 1, research into the association between polymorphisms in the *MYH9* gene and CKD has been of great significance in the African American population. This has led to an increased interest in the mixed ancestry population of Bellville South, Cape Town, South Africa. The research design and methodology in this study is aimed at determining if there is an association between CKD and its related co-morbidities and *MYH9* polymorphisms in the mixed ancestry subjects residing in the urban township of Cape Town, through genotyping. This chapter will detail the research setting, study design; and population characteristics. The collection of data and participant medical information was obtained via questionnaires, physical examinations and biochemical analyses, with details given. Protocols of laboratory work required to extract DNA and genotype the samples are described and finally the statistical methods to be used will be detailed.

2.2 RESEARCH SETTING

In the Western Cape, the Bellville South community matched the requirements of the mixed ancestry population required for this study. Bellville South is located within the northern suburbs of Cape Town, South Africa. It is traditionally a community of a mixed ancestry ethnic population group formed in the late 1950s. The area, often referred to as a township, is an underdeveloped urban residential area that was previously reserved for non-Caucasians including the mixed ancestry group. The population has ancestry from Khoisan, African, European Caucasian and a small amount of Asian populations (Quintana-Murci *et al*, 2010). According to the 2001 population census (the latest census done in Bellville), its population stands at approximately 26 758 with the mixed ancestry group accounting for 80.48% (21 536). The target population for this study were subjects between the ages 35 – 65 years and their number was estimated to be 6 500 (City of Cape Town Census, 2001), however other age groups were also included due to the higher CKD prevalence observed across all age groups.

2.3 RESEARCH DESIGN AND STUDY POPULATION

The subjects in the study were selected from a main database from the Obesity and Chronic Diseases of Lifestyle Research Unit based at Cape Peninsula University of Technology, the main research project. This was a cross-sectional study aimed at establishing a cohort that can be followed up for insulin resistance and its sequel in randomly selected mixed ancestry. The data presented here was collected from mid-January 2008 to March 2009. Using a map of Bellville South, random sampling was conducted as follows: From a list of streets from each stratum, the streets were then classified as short, medium and long based on the number of houses within each street. Streets with houses ≤ 22 were classified as short, houses 23–40 as medium and long streets were those with > 40 houses. A total of 16 short streets representing approximately 190 houses, 15 medium streets representing approximately 410 houses and 12 long streets representing approximately 400 houses were randomly selected across the different strata. From the selected streets, all household members meeting the selection criteria were eligible to participate in the study. Community authorities requested that participants outside the random selection area benefit from the study; these were also included, but given a different code. Recruited subjects were visited by the recruitment team the evening before participation and reminded of all the study instructions. The instructions included overnight fasting, abstinence from drinking alcohol or consumption of any fluids in the morning of participation. Furthermore, participants were encouraged to bring along their medical/clinic cards and/or drugs they were currently using.

2.4 INCLUSION/EXCLUSION CRITERIA

The study population consisted of mixed ancestry participants residing in the Bellville South area, who consented to participate and gave blood for genetic analysis in addition to a medical history if necessary. The main research project consisted of 1254 participants however only a total of 724 subjects were included in this study, comprising of 526 random subjects between the ages 35 - 65 years and 198 voluntary subjects, age range 16 – 95. Ten subjects were from other

ethnic groups, whilst five did not consent to blood sampling and were excluded resulting in a total of 941 participants. Individuals with GFR of <60 ml/min and ACR of 17-250 mg/g for men or 25-355 mg/g for women were included as a case group. Those with GFR ≥ 60 ml/min were included as a control group. The control group was matched to the case according to age, gender, and ethnicity (mixed ancestry).

2.5 SAMPLE SIZE

The main research project consisted of 1254 participants from which the study population of the present project was selected. The study reported a CKD prevalence of 29.6% (unpublished data) according to the MDRD equation. To obtain statistically significant observations on allelic distributions and adequate power for association tests between diabetes, hypertension and obesity-related traits and genotypes given the reported prevalence, the number of a control group was chosen based on a 1:1 case-control ratio. In order to successfully analyze association studies, the sample size must be sufficiently large to create enough statistical power to reduce the probability of generating false-positive associations (Berry and Ho, 1988). The following guided our genotype sample size calculation: the number of people in the desired population, the estimated population variance, the desired precision, the confidence interval and the estimated response rate. The sample size was calculated as a minimum of 704 participants needed in order for this study to display a 95% confidence interval and a 3% margin of error.

2.6 DATA COLLECTION

To obtain the medical information and samples of the participants, a detailed protocol describing data collection procedures (questionnaires and physical examination) was developed. The team members, consisting of professional nurses and field workers, were trained, and a pilot study in a neighboring community with similar demographics was performed to validate the questionnaire and to synergize the workflow. A supervisor was allocated for each team to monitor the performance of the personnel and calibrate equipment

according to a standard protocol. In addition, weekly meetings were held to assess progress, solve problems and re-train the research team (if necessary). A questionnaire designed to retrospectively obtain information on lifestyle factors such as smoking and alcohol consumption, physical activity, diet, family history of CVD and diabetes mellitus (DM), demographics etc. was administered by trained personnel (appendix B). The questionnaire was adapted from several existing standards and recognized sources (Bradshaw *et al*, 1995; Ewing, 1984) and was also pre-tested in a neighboring community with similar demographics. Information about medication taken by participants was also obtained through clinic cards and record of drugs that participants brought to the study site. The more detailed the information retrieved, the more accurate and complete the database, allowing for more association tests during statistical analysis.

2.6.1 Anthropometric measurements

Clinical measurements obtained included: height, weight, hip and waist circumferences and blood pressure. Measurements were carried out by qualified healthcare professionals who underwent training to standardize all measurements prior to the commencement of the study. Blood pressure measurements were performed according to World Health Organization (WHO) guidelines (WHO, 1999). Measurements were performed using a semi-automatic digital blood pressure monitor (Rossmax MJ90, USA) on the right arm, in sitting and ambulatory position. After a 10 minute rest period, three readings were taken at 5 minute intervals and the lowest of the three readings was taken as the blood pressure. Weight was determined on a Sunbeam EB710 digital bathroom scale, which was calibrated and standardized using a weight of known mass. Weight measurements were recorded to the nearest 0.1 kilograms and taken with each subject in light clothing, without shoes and socks. Height was recorded in centimeters to one decimal place using a stadiometer, with subjects standing on a flat surface at a right angle to the vertical board of the stadiometer. Body Mass Index (BMI) was calculated as weight per square meter (kg/m^2). Waist circumference was measured using a non-elastic tape at the level of the narrowest part of the torso as seen from the anterior view. When difficult to observe the waist narrowing, especially in obese subjects, the waist

49

circumference was measured between the ribs and the iliac crest. All anthropometric measurements were performed three times and the average measurement used for analysis.

2.6.2 Biochemical analyses

All participants, except the self-reported type 2 diabetic subjects (confirmed by either medical card record or drugs in use) underwent a 75g oral glucose tolerance test (OGTT) as prescribed by the WHO, with fasting blood glucose in all participants. Categories of glucose tolerance were defined using the 1998 WHO criteria (WHO, 1999). Blood samples were transported daily in an ice-pack box for processing at the Metropolis Private Pathology Laboratory (Century City, Cape Town). Serum creatinine was determined using the kinetic-Jaffe reaction (Cobas 6000, Roche Diagnostics, USA) and the result used to determine the glomerular filtration rate using the MDRD and CKD-EPI formulae. Plasma glucose was measured by enzymatic hexokinase method (Cobas 6000, Roche Diagnostics, USA). Glycosylated hemoglobin (HbA1c) was assessed by turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics, USA). This method is National Glycohaemoglobin Standardization Programme (NGSP) certified according to Roche Diagnostics. High density lipoprotein cholesterol (HDL-c) and triglycerides (TG) were estimated by enzymatic colorimetric methods (Cobas 6000, Roche Diagnostics, USA). Low density lipoprotein cholesterol (LDL-c) was calculated using Friedwald's formula. Serum cotinine was measured by chemiluminescent assay (Immulite 1000, Siemens, Germany). This laboratory was accredited and performed all the necessary and required daily, weekly and monthly internal and external quality control.

2.6.3 Data management

To maintain patient confidentiality, all data captured sheets containing clinical and demographic info of each patient were coded by a study number and the specimen labeled accordingly, thus any info leading to the identity of the subjects

was kept separately. All consent forms and questionnaires were stored in confidential files and securely locked away.

2.7 GENETIC ANALYSIS

The genetic analysis of a set of samples requires DNA extracted from venous blood from each participant. The DNA quality and purity is determined to check viability of further testing. The genotyping of individuals for a specific polymorphism can be done using polymerase chain reaction (PCR) techniques. These methods require optimization for each specified polymorphism. In the present study, genotyping was conducted using real-time PCR (qPCR). Different amplification patterns obtained that represented each of the three genotypes were validated by conventional PCR, followed by automated sequencing.

2.7.1 DNA extraction

Venous samples were collected from consented subjects by professional medical nurses in Ethylenediamine Tetra acetic Acid (EDTA) tubes. These samples were transported to the laboratory at room temperature but were stored at $-20°C$ as the extraction could not be done immediately. The modified salting out procedure was used to extract the genomic DNA from the venous samples. Solution make-up can be found in Appendix D. A volume of 5-8 ml of whole blood was transferred from the EDTA tube into a 50 ml Falcon tube. Cold lysis buffer was adjusted according to the volume of blood used (according to the procedure, 30 ml of cold lysis buffer for every 10 ml of blood) and added to a final volume of 40 ml. All reagents used in subsequent steps were adjusted accordingly. This mixture was placed on ice for 15 minutes and inverted every 5 minutes to allow the contents to mix thoroughly. The mixture was then centrifuged at 1500rpm for 10 minutes at $4°C$ (J-6M/E centrifuge, Beckman, UK). The supernatant was carefully discarded and the pellet resuspended in 0.9% Phosphate Buffered Saline. This was followed by centrifugation at 1500 rpm for 10 minutes after which the supernatant was discard and the pellet resuspended in nuclear lysis buffer, 0.3 mg/ml Proteinase K and 1% Sodium Dodecyl Sulphate. The contents were mixed well and incubated at $55°C$ overnight. The following day, 6 M NaCl was

added to the solution and the tubes were shaken vigorously for 1 minute. The mixture was centrifuged at 2500 rpm for 30 minutes. The supernatant containing the DNA was transferred to a clean Falcon tube, and the pellet discarded. The supernatant was vortexed for 15 seconds, and centrifuged at 2500 rpm for 15 minutes. The subsequent supernatant was transferred to a clean Falcon tube, leaving behind the pellet and any foam. Cold 99.9% ethanol was added to each tube and agitated to precipitate the DNA. Using a sterile pipette, the DNA was pulled out of the Flacon tube, placed in a clean 1.5 ml eppendorf tube and washed with cold 70% ethanol. All tubes were centrifuged using a bench top microcentrifuge (Microcentrifuge® Lite, Beckman Coulter™, USA). This 'washing step' was repeated until the pellet of DNA was clear. The ethanol was discarded, tubes left at room temperature to dry and depending on the size of the pellet, 200 – 800 µl of 1X Tris Ethylenediamine tetra acetic acid (TE) buffer was added to dissolve the DNA. All tubes were shaken at room temperature using a rotator, to dissolve the DNA pellet.

2.7.2 DNA quality

DNA purity and concentration was tested using the NanoDrop® ND-100 spectrophotometer system. The NanoDrop® spectrophotometer used ultraviolet visible (UV/VIS) to accurately determine nucleic acid concentration in a sample, recorded in nanograms per microlitre (ng/µl). Those samples with high concentrations were diluted to obtain a final concentration of 200 ng/µl. The quality and purity of the DNA samples was also measured by measuring the ratio of absorbance at 260 nm and 280 nm. Purified DNA should give a result of ~1.8 from the 260/280 ratio measurement. A deviation of this ratio is normally indicative of contaminants.

2.7.3 Conventional polymerase reaction and sequencing

The aim of PCR is the continuous, exponential, semi-conservative replication of a well-defined DNA region present in the template DNA leading to the accumulation of large quantities of specific target DNA product (Van Pelt-Verkuil, van Belkum and Hays, 2008). PCR was first described in 1985 and two key improvements in

52

the PCR process have since improved the technique, run time and convenience (Van Pelt-Verkuil, van Belkum and Hays, 2008). The process initially relied on non-thermostable DNA dependent DNA polymerase which became heat inactivated after every PCR cycle. The addition of thermostable DNA polymerases meant the laborious task of adding fresh enzyme after each cycle was eliminated. This reduced the time required to perform the assay and the likelihood of cross-contamination. Secondly, machines, thermocyclers, which could heat and cool the sample automatically, eliminated the need for several separate water baths.

DNA to be amplified is initially removed from the nucleus of the cells via DNA extraction. The secondary and tertiary structures are then removed via heating the DNA, the denaturation step (Van Pelt-Verkuil, van Belkum and Hays, 2008). The double stranded DNA is separated by the heat into single stranded DNA strands, by the breaking of the hydrogen bonds between the complimentary base pairs on the opposite strands. The specific melting temperature (T_m) is dependent on the length of the DNA strand and the base composition, with Guanine-Cytosine rich DNA melting at a higher temperature than Adenine-Thymine rich DNA (Van Pelt-Verkuil, van Belkum and Hays, 2008). Following melting, the DNA binding sites for the subsequent annealing stage are exposed. Specifically designed and synthetically synthesized oligonucleotides act as primers for the DNA polymerase enzyme. Two primers are required, one complementary to the sense strand and the other complementary to the non-sense strand. The primers determine the specificity of the PCR and the length of the DNA region to be amplified, and initiate the DNA replication (Van Pelt-Verkuil, van Belkum and Hays, 2008). The extension step of the PCR process requires free deoxynucleotide triphosphate molecules, namely adenine, guanine, cytosine and thymine. These are incorporated into the growing strand by the DNA polymerase. Their energy rich triphosphate section is utilized by the DNA polymerase to catalyse a phosphodiester link between the 3'-hydroxy terminus of the primer or the previous added deoxynucleotide triphosphate molecule (Van Pelt-Verkuil, van Belkum and Hays, 2008). Hydrogen bonds between the complementary base pairs on the adjacent strands are formed, and complete an amplification cycle. To

yield a high concentration of the amplified product, this cycle is repeated many times. Successful amplification requires added chemicals, specifically magnesium, a cofactor of DNA polymerase, and sodium chloride to maintain a correct reaction pH. The reaction temperatures, incubation times and number of cycles depend on the specific primer and the kit used to make up the master mix.

This technique was used with automated sequencing as a quality control measure to validate the reliability of the real-time PCR technique. It was also used for identifying positive controls that could be incorporated in every qPCR. Thirteen samples were selected from the study population for sequencing.

Oligonucleotide primers (Table 2.1) for amplifying the regions containing the three SNPs were designed following primer design guideline using Primer3plus and Integrated DNA Technologies (IDT) programs. The three SNPs selected are located in the following *MYH9* regions: rs5756152 is found in intron 12 at bp65042418, rs4821480 in intron 23 at bp35025193 and rs12107 in exon 41 at bp35007928. Designed primers were checked for occurrence of dimers and hairpin loops using the IDT. Furthermore, primers were submitted to the National Centre for Bioinformatics Institute (NCBI) primer Basic Alignment Search Tool (BLAST) for possible non-specific binding. Primer sequences were analyzed for the presence or absence of polymorphisms that may inhibit amplification of corresponding gene regions.

Table 2.1 Oligonucleotide primers and their characteristics for amplifying *MYH9* regions containing SNPs of interest.

Primer name	Sequence (5'-3')	length in bases	Tm	GC content
rs4821480_For	AGC ACA GAA CAC TTG CTA GCC TCA	24	60.2 ºC	50.0 %
rs4821480_Rev	AAT CCT TTC TCA CCC AAG CCC ACA	24	60.5 ºC	50.0 %
rs5756152_For	CTC CTG ACC TCA AG CAA TCT G	21	60.0 °C	52.4 %
rs5756152_Rev	CAT CGG GAT CCT GGA CAT T	19	60.7 °C	52.6 %
rs12107_For	GAT TCC TCC CCA CCC TGT	18	59.7 °C	61.1 %
rs12107_Rev	CCT CTA ACG CTC TGG CTG TC	20	60.2 °C	60.0 %

The PCR protocol was prepared in a sterile environment using Bioline Taq PCR kit and nuclease-free tubes and tips as follows: a total volume of 50 µl reaction mixture contained 1x buffer, 1mM magnesium chloride, 5% DMSO, 0.3mM dNTP mix, 0.24 µM each of the forward and reverse primers, 0.1µl of 5U/µl Bio Taq, and nuclease-free water to obtain a final volume. The prepared PCR master mix was aliquoted to separate 0.2 ml tubes and 0.25 µg/µl of the DNA template was added to each tube. A non-template control was included in every PCR run to check the presence/absence of contamination. The gene regions were amplified in a Perkin Elmer 2720 thermal cycler (Applied Biosystems, USA) as follows: initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 2 minutes. All amplicons were electrophoresed on a 2% agarose gel and visualized in a GelDoc system (BioRad, GmbH, Germany).

All amplicons were purified with a mixture of exonuclease I and shrimp phosphatase, and then sequenced using the BigDye® Terminator version 3.1 Cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The sequence reaction mixture for each primer was prepared separately as follows: each primer to be used was diluted to 1.3 µM, and was mixed with 3 µl amplicon solution, and 4 µl BigDye® Terminator mix (2.5x), and BigDye® Terminator buffer (5x) or nuclease-free water to a final volume of 10 µl. The sequencing reaction was conducted according to the manufacturer's instructions, and resolved on an ABI 3130X ® Genetic Analyzer (Applied Biosystems, USA) at the Central Analytical Facility, University of Stellenbosch. The sequencing data was analysed using the Geospiza's FinchTV version 1.4 software programs.

2.7.4 Real-time polymerase chain reaction

PCR assays have undergone significant changes in the last decade. qPCR is a PCR-based technique used in DNA amplification and simultaneous quantification of the product (Mackay, 2007). It is sensitive, specific, fast and a closed system. The monitoring of the accumulation of the amplicon is made possible by the

addition of molecules with fluorescent potential that attach to the primer, oligoprobes or the amplicon (Mackay, 2007). The detection method may be non-specific fluorescent dyes that bind to any double stranded DNA, increasing in fluorescence as the number for double stranded DNA molecules increases; or sequence specific DNA probes that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target (Van Guilder, Vrana and Freeman, 2008). Real-time PCR allows for the quantification of the products during the PCR cycles, allowing for gene expression analysis (Applied Biosystems, 2005). The level of fluorescence as opposed to the amount of PCR product is measured. An amplification plot is used to display the fluorescence over the cycles. For allelic discrimination, the fluorescent results are viewed once the cycles are completed, hence end-point qPCR (Applied Biosystems, 2005). It is used to determine the genotype of a sample, allowing for the differentiation of a SNP. This was the required method for this study. Due to the determination of two alleles, a specific, fluorescent, dye-labeled probe for each allele is needed. The probes contain different fluorescent reporter dyes to differentiate between the amplification of each of the alleles. There are many dyes available, including VIC and FAM. VIC has an emission maximum of 554 nm, thus emitting a green-yellow color and in this study, measures the minor allele presence or absence (Life Technologies, USA). FAM, 6-carboxyfluorescein, is the most commonly used fluorescent dye and fluoresces blue in colour at 517 nm.

The study population was genotyped on the BioRad MiniOpticon Real time PCR system (Biorad, Germany) using TaqMan SNP genotyping assay (Applied Biosystems, USA) that was designed for each SNP, and data analyses conducted using a corresponding software (CFX Manager software). The initial step was to test the assay, and optimize it if necessary. The assay was bought ready-to-use and optimized from Applied Biosystems. However, a test run was recommended to make sure that the data generated was reliable for further analyses. For every run, a positive control (any of the samples with known genotypes according to sequencing results) was included plus at least 3 non-template controls. The real-time PCR master mix was prepared according to the manufacturer's instructions.

2.8 STATISTICAL ANALYSIS

General characteristics of the study group are summarized as count and percentage for dichotomous and as median and interquartile range (IQR) for quantitative traits, as some of the distributions were skewed. Traits were log-transformed to approximate normality, where necessary, prior to analysis. Linear models were used for the analysis of quantitative traits and logistic regression models for dichotomous traits. Using models enabled us to adjust all analyses for known confounders' age and gender (where possible).

Additive allelic association of each SNP was compared with each trait, between participants with and without DM (interaction between DM and SNP on trait), after adjusting for age and gender. The additive allelic association of each SNP was also tested with each trait in the combined group, while adjusting for age and gender. These models provided the effect sizes and p-values reported in the results section. Results corresponding to p-values below 5% are described as significant. Multiple testing was not adjusted for. Analyses were done in R, a language and environment for graphics and statistical computing, freely available from http://www.R-project.org. The R package genetics was used for genetic frequency distributions, Hardy-Weinberg Equilibrium (HWE) and LD testing. The HWE describes the equilibrium at a single locus in a randomly mating population (Gillespie, 2004). Hardy-Weinberg frequencies details unchanged genotype proportions in a large population from generation to generation should there be no evolutionary forces such as mutation, migration and selection (reviewed by Crow and Dove, 1998). It allows for the prediction of genotype frequencies from the knowledge of gene frequencies.

2.9 ETHICAL CONSIDERATIONS

The study was approved by the Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences (Project number: CPUT/HW-REC 2008/002 and CPUT/HW-REC 2010/H017) and the University of Stellenbosch Ethics Committee (N10/05/142) (Appendix A). The study was conducted

57

according to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed written informed consent forms after all the procedures had been fully explained in the language of their choice (Appendix C). The consent form included the agreement for obtained blood samples to be stored and used for biochemical tests and genetic analyses. In addition, permission was also sought from other relevant authorities such as the city and community authorities. These authorities granted permission to operate in the community and also to make use of designated places such as community halls or nearby schools for data and sample collection.

2.10 CONCLUSION

The area of Bellville South in Cape Town is an underdeveloped urban residential area that was previously reserved for non-Caucasians, including the mixed ancestry population. This population consists of African, Khoisan, European and some Asian ancestry. It is this mixed ancestry that prompted the selection of this population and research setting as it met the requirements of interest of research into the *MYH9* gene polymorphisms in the African American population. Following participant recruitment, anthropometric measurements and biochemical analyses were obtained. Samples were genetically analyzed from the initial DNA extraction, followed by genotyping and subsequently statistical analyses. The research objectives detail participant genotyping and analysis of the frequency and association results. This study design and methodology will achieve these objectives.

58

CHAPTER 3

[Results and Discussion]

Presented in the form of a full-length manuscript in preparation for future publication

in a scientific journal

59

3.1 INTRODUCTION

Myosin IIA heavy chain, a subunit of myosin IIA protein, consists of 1960 amino acids and is 227kDa in size, with a globular head in amino-terminus and a coiled-coil tail in carboxy-terminus (Schliwa, 2006). An actin binding site is found in the head region, interacting with actin, as well as a light chain binding site present in the long tail region (Figure 3.1). Myosin IIA heavy chain is encoded by the *MYH9* gene, which is approximately 110 kilobases long with 41 exons, and is located on the long arm of chromosome 22 at position 13.1 (Kopp *et al*, 2008). It is abundantly expressed in the kidney, platelets and liver and in smaller amounts in the thymus, spleen, intestine and cochlea. Within the kidney, it is expressed in the glomerulus, specifically the podocyte, peritubular capillaries and tubules. Myosin IIA heavy chain plays several physiological roles such as cytokinesis, cell motility and the maintenance of cell shape (Schliwa, 2006).
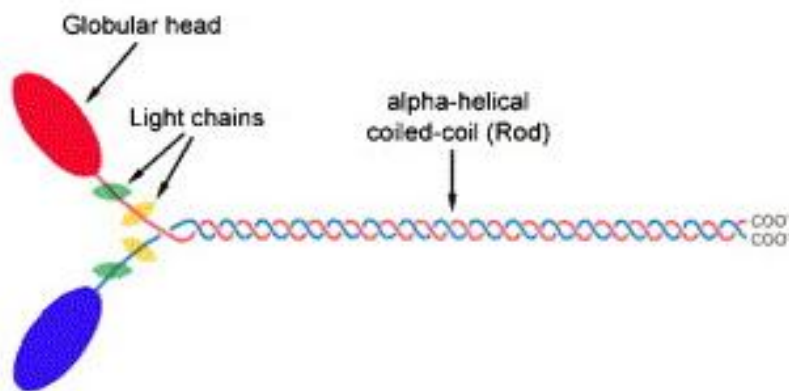
Figure 3.1: Schematic representation of the myosin non-muscle structure (Adapted from Straussman *et al*, 2007; permission in Addendum F)

More than 45 mutations have been found in the *MYH9* gene, and implicated in a wide range of disorders that have subsequently been grouped and termed the *MYH9*-related disorder. These disorders are characterized by large platelets and thrombocytopenia, both present from birth (Schliwa *et al*, 2006). The link between *MYH9* polymorphisms and CKD was first discovered in 2008 by Kopp *et* al in the African American population. The role of genes in the development of kidney diseases was an immense breakthrough, explaining the aggregation of kidney

disease in families and populations that could not be explained solely by other known risk factors. Although the exact mechanism by which *MYH9* polymorphisms contributes to the development and progression of non-diabetic CKD is unknown; it has been hypothesized that these sequence variants may cause aggregation of abnormal myosin and damage to the cytoskeleton of podocyte and tubular cells, and rearrangement of the actin cytoskeleton, ultimately disrupting the filtration barrier.

SNPs within *MYH9* were identified in different regions of the gene, and found to be in linkage disequilibrium. These SNPs were then clustered into groups according to linkage disequilibrium blocks (Figure 3.2): extended or E-1 haplotype (rs4821480, rs2032487, and rs4821481, rs3752462) (Kao *et al*, 2008), S-1 haplotype (rs5750248, rs2413396, rs5750250), F-1 haplotoype (rs16996674, rs16996677, rs11912763) (Behar *et al*, 2010) and L1 haplotype (rs12107, rs7078, rs735853, rs5756129) (Freedman *et al*, 2009). Furthermore, three SNP's (rs4821480, rs5756152, rs12107) exhibited independent evidence for association with hypertensive and non-diabetic CKD (Freedman *et al*, 2009). For this reason, the present study investigated the role of rs5756152, rs4821480, and rs12107 in CKD and its related clinical traits in a population of Mixed Ancestry origin.
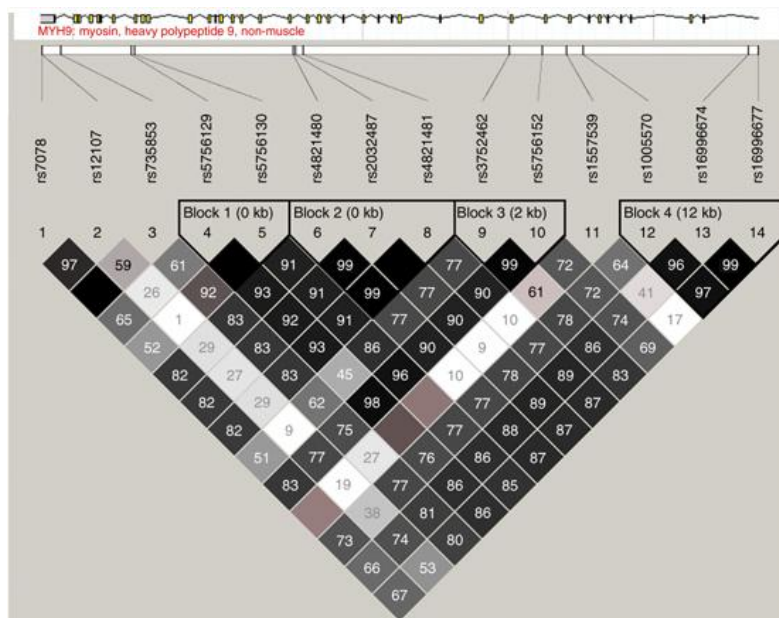
Figure 3.2: Gene structure and linkage disequilibrium plot of 49 kb of the *MYH9* gene. The three SNPs are shown located in different LD blocks (Taken from Freedman *et al*, 2009; permission in Addendum E)

3.2 MATERIALS AND METHODS

Data collection and study population: Participants were members of a cohort study conducted in Bellville South, Cape Town, a mixed ancestry Township formed in the late 1950s. Eligible participants, selected from 1000 households through a multistage random sampling, were invited to take part in a community based survey between January 2008 and March 2009, with data collection through standardized procedures. All consenting participants received a standardized interview and physical examination during which blood pressure was measured according to World Health Organisation (WHO) guidelines using a semi-automatic digital blood pressure monitor (Rossmax PA, USA) on the right arm in sitting position. Other clinical measurements included the body weight, height, waist and hip circumferences. All anthropometric measurements were performed three times and their average used for analysis. Participants with no history of medically diagnosed diabetes mellitus underwent a 75 g OGTT as prescribed by the WHO. Blood samples were collected and processed for further determinations. Plasma glucose was measured by enzymatic method (Cobas 6000, Roche Diagnostics), HbA1c was assessed by turbidimetric inhibition immunoassay (Cobas 6000, Roche Diagnostics) and urine albumin by immunoturbidimetric assay (Cobas 6000, Roche Diagnostics).

CKD definitions and estimation of GFR: Kidney function was approximated through eGFR for which both the 4-variable MDRD equation applicable to standardised serum creatinine values, and the CKD-EPI equation were used. An eGFR of <60 ml/min was used to define chronic kidney disease (or CKD stage 3-5). Urinary albumin excretion was quantified in terms of urinary ACR and participants ranked according to ACR levels as: normoalbuminuria (ACR<3.4 mg/mmol), microalbuminuria (3.4$\leq$ACR<33.9 mg/mmol) and macroalbuminuria (ACR$\geq$33.9 mg/mmol). Diabetes status was based on a history of doctor-diagnosis, a fasting plasma glucose ≥7.0 mmol/l and/or a 2-hour post-OGTT plasma glucose >11.1 mmol/l. Hypertension was based on a history of medically diagnosed hypertension and/or receiving medications for hypertension or average

systolic blood pressure ≥140 mmHg and average diastolic blood pressure ≥90 mmHg.

Genotyping of the study population: There is no published data on *MYH9* SNPs and haplotype variations in the mixed ancestry population from South Africa. We therefore selected the three SNPs that have been shown to exhibit independent evidence for association with CKD (rs4821480, rs575152 and rs12107) in prior studies. The SNPs were genotyped using a Taqman genotyping assay (Applied Biosystems, USA) on a BioRad Optica (Biorad, Germany) and confirmed by sequencing.

Statistical analysis: Statistical analyses were conducted as described in Chapter 2. Descriptive statistics were conducted and general characteristics of the study group summarized as count and percentage for dichotomous traits, mean and SD or median and 25th-75th percentiles for quantitative traits. Results corresponding to p-values below 5% were described as significant. Traits were log-transformed to approximate normality, where necessary, prior to analysis. SNPs were tested for departure from HWE expectation via a chi square goodness of fit test. LD was estimated using the D' statistic. Linear regression models were used for the analysis of quantitative traits and logistic regression models for dichotomous traits, always assuming additive models for the SNPs. Using linear and logistic models enabled us to adjust all analyses for known confounders as specified everywhere in the results. The additive allelic association of each SNP with each trait, overall and according to diabetes status was investigated, and tested for heterogeneity by adding the interaction term of diabetes and each SNP to a model that contained the main effects of diabetes and the relevant SNP. Results corresponding to p-values below 5% are described as significant. All analyses used the statistical package R (version 2.12.2 [2011-02-25], The R Foundation for statistical computing, Vienna, Austria).

63

3.3 RESULTS

3.3.1 Genetic analysis

Conventional PCR was used to run the initial samples of each SNP and the final products of the PCR were electrophoretically separated on 2% agarose gel. The gel electrophoresis images are available. Figure 3.3 shows the electrophoresed products of each of the three SNPs, rs5756152, rs4821480 and rs12107. Amplicons were sequenced and analyzed for the presence or absence of specified sequence variants. The sequencing results display the three possible nucleotide combinations (genotypes) present in that specific SNP. Heterozygous refers to the presence of two different nucleotides at a locus, one from each parent. Alternatively, two of the same nucleotides at a locus is denoted as homozygous. An individual may be homozygous for either of the two nucleotides present in the heterozygous state. Homozygosis may be for the allele with a lower frequency in a particular population, the minor allele and perhaps the risk allele if association is found; or for the allele with the highest frequency, the most common genotype in a population, the wild-type. Figure 3.4, 3.5 and 3.6 display chromatograms that represent the sequences around each SNP, and the corresponding genotypes there were identified in the study population. The rs5756152 SNP is an adenine guanine substitution (c.1380+90A>G) located in intron 12 of the *MYH9* gene (Figure 3.4). Figure 3.5 shows the guanine to thymine substitution (c.2977-159G>T) of the rs4821480 SNP, which is located in the intron 23 of *MYH9* gene. The rs12107 SNP is a cytosine to thymine substitution (c.*732C>T) located in exon 41 at the 3' untranslated region of the *MYH9* gene.

Subsequently all samples were genotyped using qPCR, using the BioRad MiniOpticon. Ready-to-use TaqMan SNP genotyping assay was used for detecting *MYH9* polymorphisms in individuals included in the study. The MiniOpticon was calibrated to use VIC and FAM fluorescent dyes. Figure 3.7, 3.8 and 3.9 exhibits the allele discrimination scatter plots showing VIC and FAM fluorescence results, the amplification of alleles. Each symbol represents an allele

64

that was amplified by qPCR in each sample. Amplified samples tend to cluster into groups corresponding to the three genotypes.
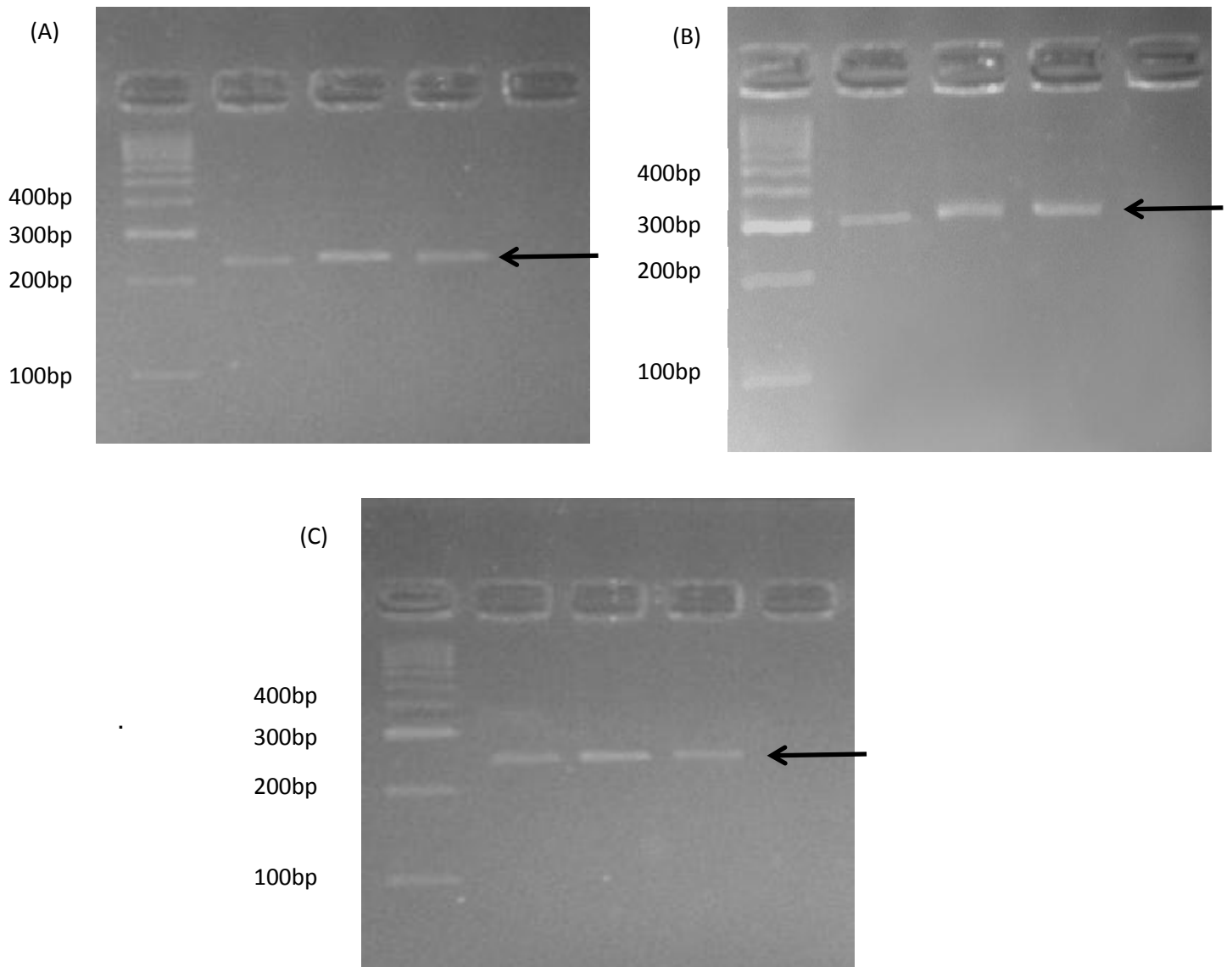
Figure 3.3: Conventional PCR products of the three SNPs genotyped on 2% agarose gel, stained by EtBr and visualised using ultraviolet light transillumination. **A:** The product of amplification of rs5756152, the arrow indicates the product, **B:** rs4821480 **C:** rs12107

Homozygous - GG

Heterozygous - AG

Homozygous - AA
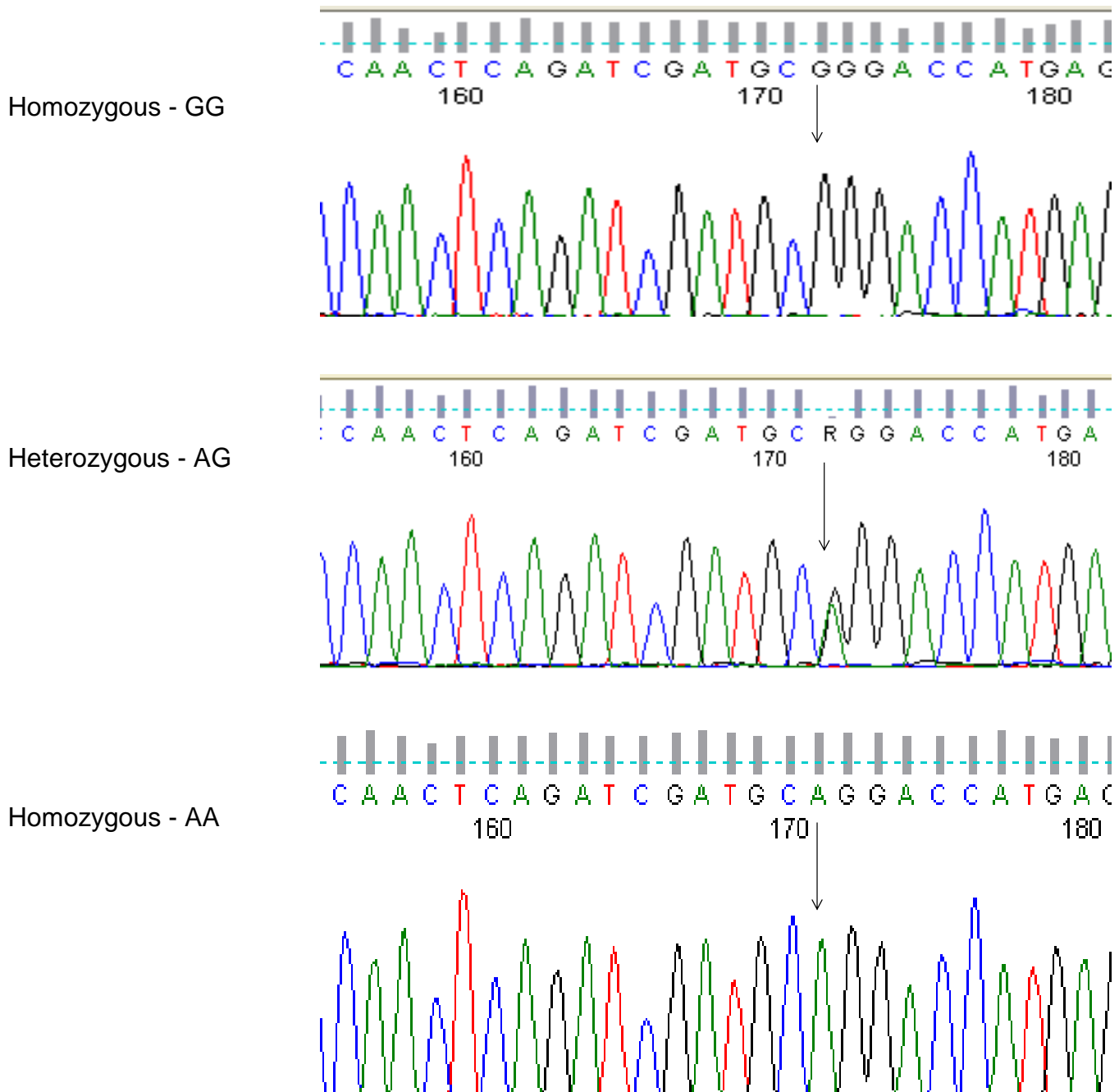
Figure 3.4: Sequencing chromatogram for rs5756152 showing the three genotypes obtained in the study population. The arrow indicates the affected nucleotide and R represents the presence of both nucleotides (A and G). Colour coding of the peaks: *green*, adenine (A); *blue*, cytosine (C); *black*, guanine (G); *red*, thymine (T).

66

Homozygous - GG

Heterozygous - GT

Homozygous - TT
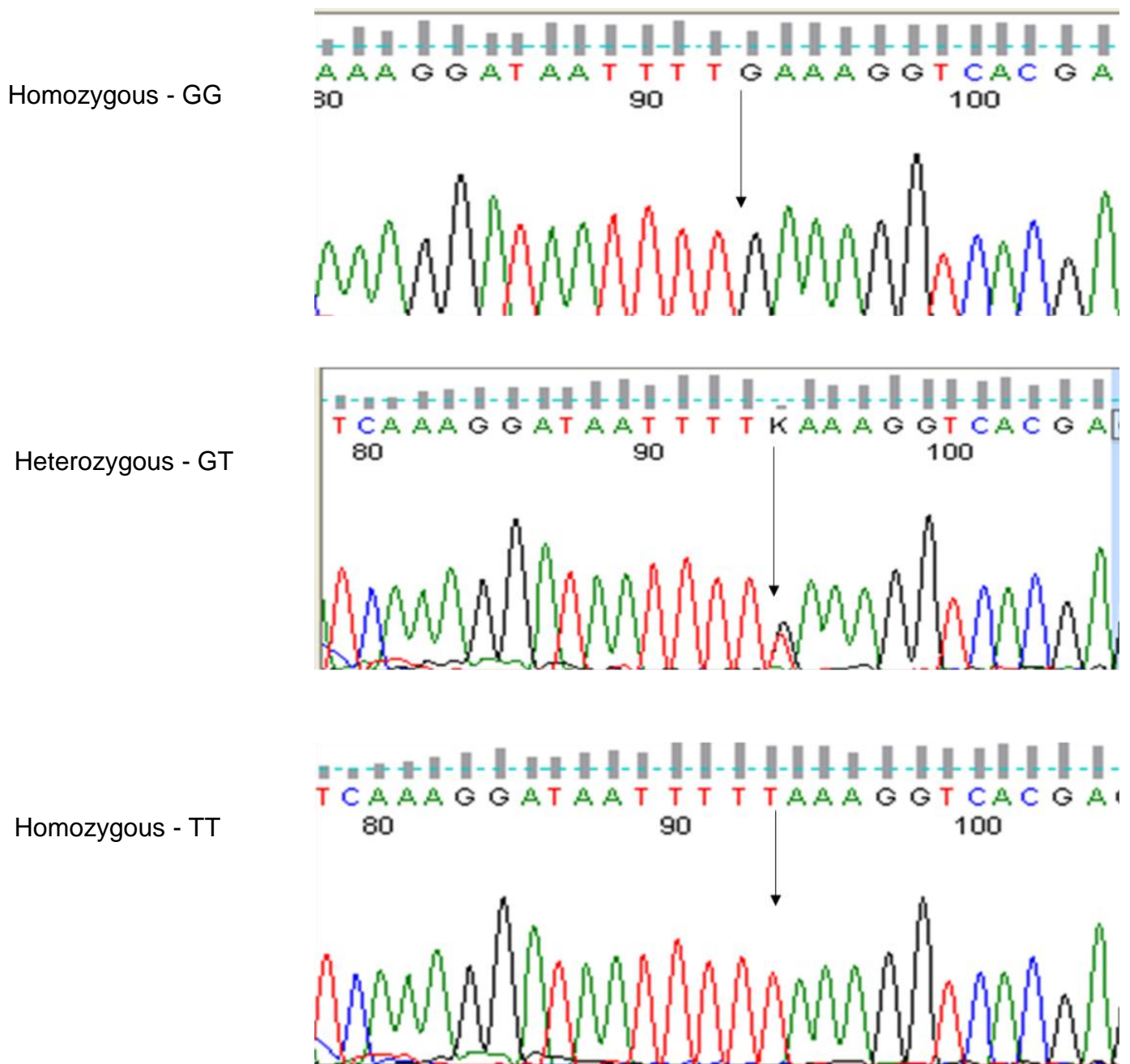
Figure 3.5: Sequence chromatogram for rs4821480. The arrow indicates the affected nucleotide and K represents the presence of both nucleotides (G and T). Colour coding of the peaks: *green*, adenine (A); *blue*, cytosine (C); *black*, guanine (G); *red*, thymine (T).

67

Homozygous - CC

Heterozygous - CT

Homozygous - TT
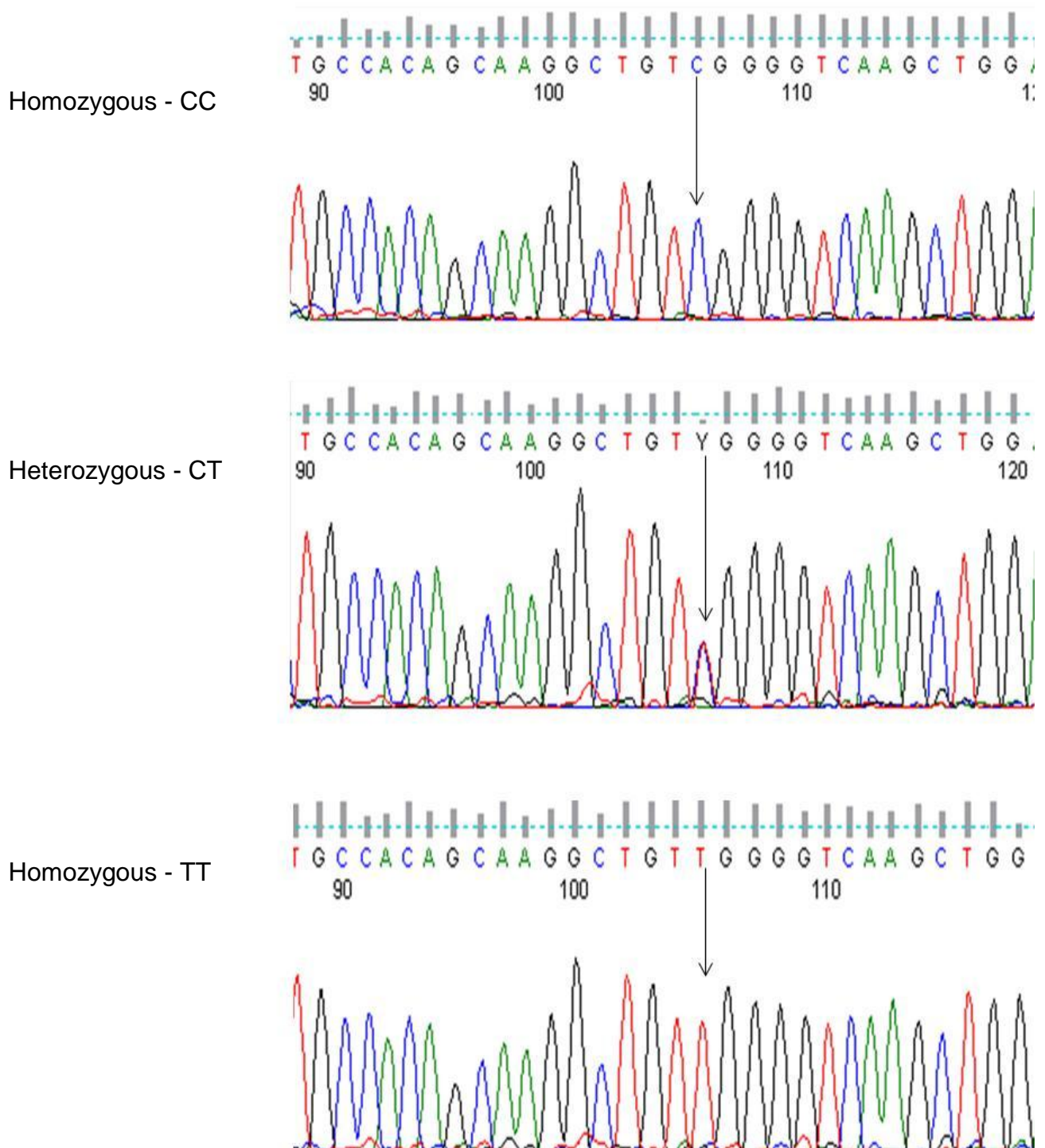
Figure 3.6: Sequence chromatogram for rs12107. Reverse primers were used. The arrow indicates the complementary base of the affected nucleotide and Y represents the presence of C and T, thus the G and A on the sense strand. Colour coding of the peaks: *green*, adenine (A); *blue*, cytosine (C); *black*, guanine (G); *red*, thymine (T).

68
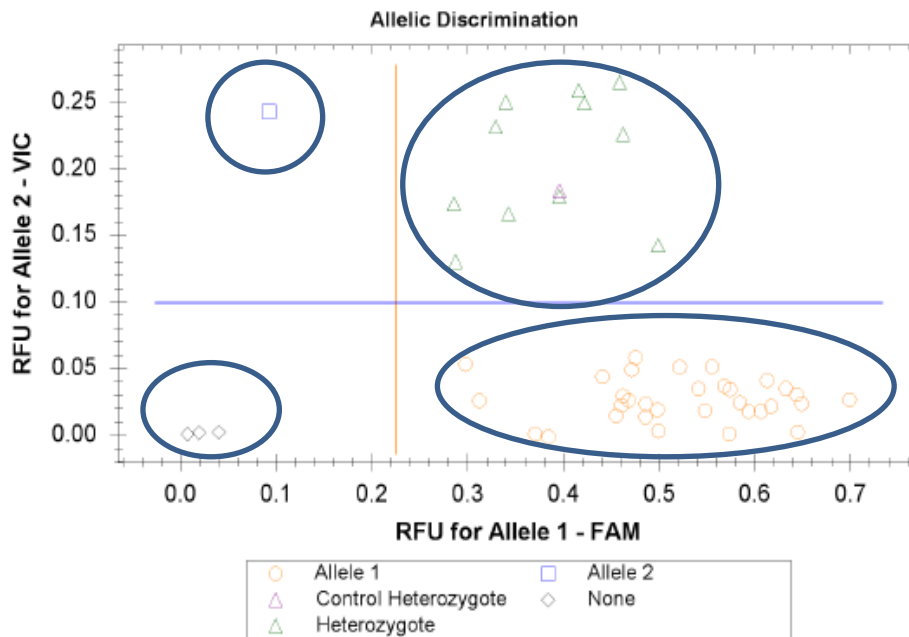
Figure 3.7: Allele discrimination scatter plot showing amplification of alleles of SNP rs5756152. Each symbol represents an allele that was amplified by qPCR in each sample. *Diamond* – no fluorescence, negative, *Square* - only VIC, minor allele (G/G), *Circle* – only FAM, wild-type (A/A), *Triangle* – VIC and FAM, heterozygous (G/A). *RFU – relative fluorescence units
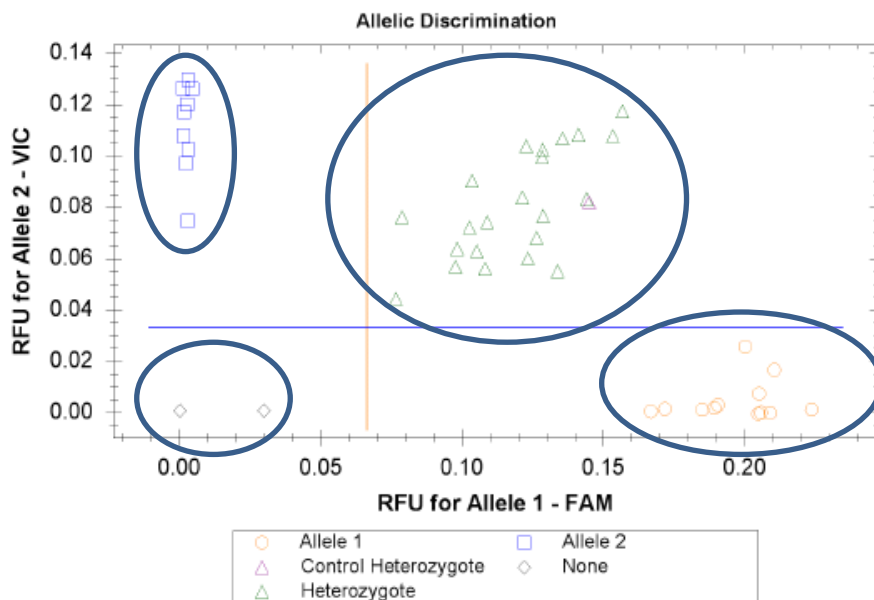
Figure 3.8: Allele discrimination scatter plot showing amplification of alleles of SNP rs4821480. Each symbol represents an allele that was amplified by qPCR in each sample. *Diamond* – no fluorescence, negative, *Square* - only VIC, minor allele (T/T), *Circle* – only FAM, wild-type (G/G), *Triangle* – VIC and FAM, heterozygous (T/G). *RFU – relative fluorescence units
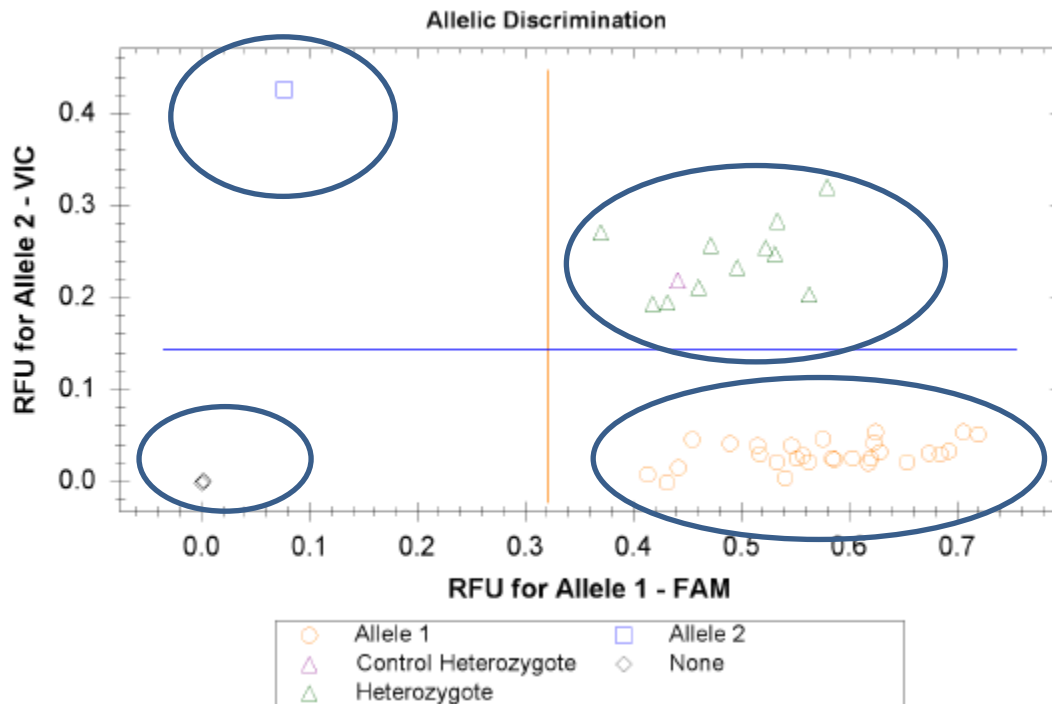
Figure 3.9: Allele discrimination scatter plot showing amplification of alleles of SNP rs12107. Each symbol represents an allele that was amplified by qPCR in each sample. *Diamond* – no fluorescence, negative, *Square* - only VIC, minor allele (A/A), *Circle* – only FAM, wild-type (G/G), *Triangle* – VIC and FAM, heterozygous (A/G). *RFU – relative fluorescence units

3.3.2 General Characteristics:

Baseline characteristics of participants are summarized in Table 3.1 and 3.2, by gender and CKD status respectively. Generally there were more females than men in the study, 566 and 162 respectively. The median age was not significantly different between the women and men, 53 vs 53, respectively. Significant differences were observed between males and females in serum creatinine (p <0.001), eGFR (p <0.001), systolic blood pressure (p = 0.003) and diastolic blood pressure (p = 0.021), with males demonstrating higher values than females (Table 3.1). The prevalence of CKD (eGFR ≤ 60 ml/min/1.73 m^2) was 29.6%, 206 participants were stage 3 and 8 were stage 4, while stage 2 had the

highest participant number (407). As illustrated in Table 3.2, the CKD prevalence increased with age, (p < 0.001). By selection, serum creatinine was significantly higher in CKD-affected participants (p <0.001), with a corresponding lower eGFR (p <0.001). Notably, was the considerably high prevalence of diabetes and hypertension with 58 CKD cases having both diabetes and hypertension; 78 having hypertension, but no diabetes; 20 having diabetes but no hypertension and 58 having neither diabetes nor hypertension.

Table 3.1: General characteristics of participants, stratified by gender. P-values are for the difference between groups. Significant p-values are highlighted.

Trait	Female	Male	P-value*
Number	**566**	**162**	
Age, median (IQR)	53 (43-65)	56 (44-68)	0.181
Serum Creatinine (umol/l)	78 (68-89)	93 (83-105)	< 0.001
eGFR (ml/min/1.73m^2)	68 (56-80)	75 (63-86)	< 0.001
GFR stage, n (%)			0.017
4	7 (1)	1 (1)	
3	175 (31)	31 (19)	
2	304 (54)	103 (64)	
1	76 (14)	27 (17)	
CKD.MDRD (GFRd)	182 (32)	32 (20)	0.002
FBG (mmol/l)	5.6 (5.0-6.5)	5.4 (5.0-6.6)	0.459
Post FBG (mmol/l)	6.9 (5.7-8.6)	6.3 (5.3-8.4)	0.057
WHO diagnosis			0.399
Normal	287 (51)	86 (53)	
DM	88 (16)	20 (12)	
IFG	21 (4)	3 (2)	
IFG+IGT	23 (4)	6 (4)	
IGT	76 (14)	19 (12)	
Known DM	67 (12)	28 (17)	
SBP (mmHg)	119 (108-132)	125 (115-135)	0.003
DBP (mmHg)	73 (66-82)	77 (69-84)	0.021
HPT, yes (%)	246 (44)	64 (40)	0.368

*Significant p-value < 0.005. Abbreviations: IQR, Inter Quartile Range; eGFR, Estimated Glomerular Filtration Rate; CKD, Chronic Kidney Disease; MDRD, Modification of Diet in Renal Disease; FBG, Fasting Blood Glucose; WHO, World Health Organization; DM, Diabetes Mellitus; IFG, Impaired Fasting Glucose; IGT, Impaired Glucose Tolerance; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; HPT, Hypertension.

Table 3.2: General characteristics of participants, stratified by CKD status. P-values are for the difference between groups, adjusted for age and gender, where possible. Significant p-values are highlighted.

Trait	CKD CASE	CONTROL	P-value*
Number	**214**	**510**	
Gender, male (%)	32 (15)	130 (25)	0.001
Age, median (IQR)	66 (58-74)	48 (39-58)	< 0.001
Urine creatinine (umol/day)	7.2 (4.8-11.0)	8.0 (5.3-12.1)	0.004
Urine microalbumin (µg/ml)	5.0 (3.0-15.2)	4.1 (3.0-10.4)	0.395
Serum creatinine (umol/l)	97 (90-112)	75 (66-83)	< 0.001
ACR (mg/mmol)	0.82 (0.47-1.97)	0.70 (0.39-1.50)	0.787
HbA1c (%)	6.0 (5.6-6.5)	5.8 (5.5-6.2)	0.014
FBG (mmol/l)	6.0 (5.0-7.0)	5.4 (5.0-6.3)	0.087
Post FBG (mmol/l)	7.5 (6.0-9.0)	6.5 (5.5-8.3)	0.166
SBP (mmHg)	126 (115-141)	118 (108-130)	0.150
DBP (mmHg)	74 (67-83)	74 (66-82)	0.059
eGFR (mL/min/1.73m^2)	53 (47-56)	76 (69-88)	< 0.001
DM, yes (%)	78 (36)	125 (25)	0.827
HPT, yes (%)	136 (64)	173 (34)	0.042
DM Yes, HPT Yes (%)	58 (27)	74 (15)	0.149

* Significant p-value < 0.005. Abbreviations: IQR, Inter Quartile Range; sACR, Albumin/Creatinine Ratio; HbA1c, Glycated Hemoglobin; FBG, Fasting Blood Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood pressure; eGFR, Estimated Glomerular Filtration Rate; DM, Diabetes Mellitus; HPT, Hypertension.

3.3.3 Genotype distribution:

Linkage disequilibrium analysis was performed in participants with and without CKD. SNP rs5756152 was in LD with both rs4821480 and rs12107, but LD was strongest but not complete with rs4821480 with D' = 0.71 and 0.66 in cases and controls respectively. On the other hand, rs4821480 and rs12107 were not in LD, with D' = 0.02 and 0.14 in cases and controls respectively (Table 3.3). Table 3.4 shows that all SNPs were in HWE in both cases and controls, except rs4821480 in control subjects (HWE = 0.007). The frequency distributions, both genotype and allele, did not differ significantly between the two groups. The A allele of rs575152, G allele of rs4821480 and G allele of rs12107 and were the most frequent in both cases and controls, deeming these the wild type alleles. The genotype distributions comparison between genders (Table 3.5) yielded slightly different results. Both genotype and allele frequency distribution did not differ significantly between rs5756152 and rs12107;

72

however a significant difference (p = 0.040) was seen for the G/G haplotype for rs4821480 between males and females, with a higher frequency in males (47%). The T allele frequency for rs4821480 between males and females was notably significant (p 0.010).
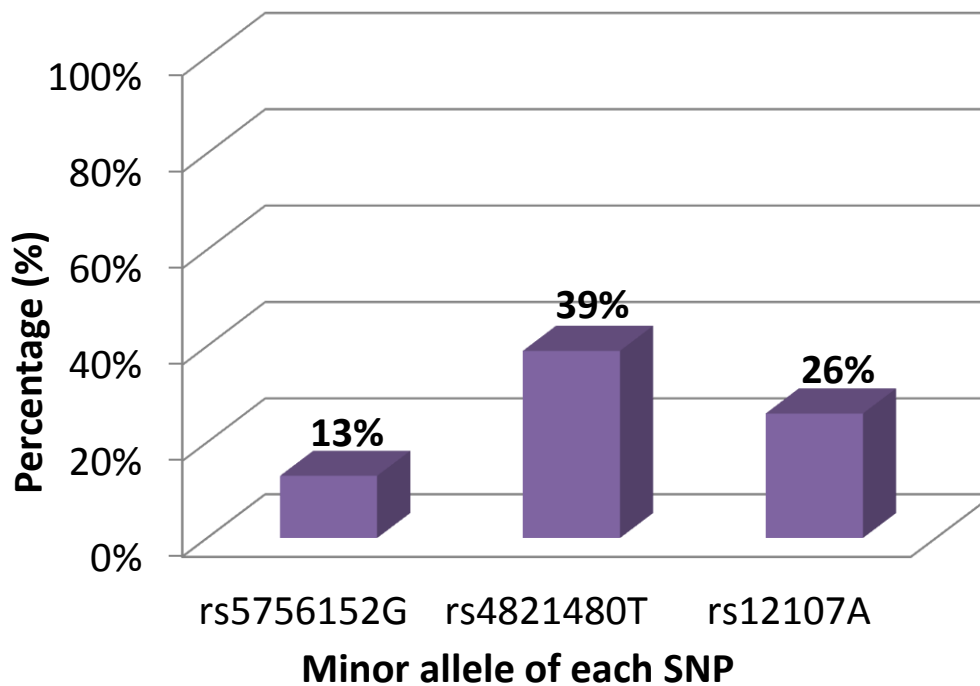
Figure 3.8: Minor allele frequencies of the three selected SNPs

Table 3.3: Linkage disequilibrium data between the three selected SNPs. **A:** rs5756152 compared to rs4821480 and rs12107 in participants with CKD. **B:** rs4821480 and rs12107 LD determination in CKD participants. **C:** rs5756152 compared to rs4821480 and rs12107 in participants without CKD. **D:** rs4821480 and rs12107 LD determination in non-CKD participants.

A: CKD: YES		rs4821480	rs12107
rs5756152	D	0.05	0.02
rs5756152	D'	0.71	0.23
rs5756152	Corr.	0.34	0.15
rs5756152	x^2	50.25	9.26
rs5756152	P-value	0.0000	0.0023
rs5756152	n	214	214

B: CKD: YES		rs12107
rs4821480	D	0.00
rs4821480	D'	0.02
rs4821480	Corr.	0.01
rs4821480	x^2	0.07
rs4821480	P-value	0.7921
rs4821480	n	214

C: CKD: NO		rs4821480	rs12107
rs5756152	D	0.06	0.02
rs5756152	D'	0.66	0.16
rs5756152	Corr.	0.33	0.11
rs5756152	x^2	110.62	12.15
rs5756152	P-value	<0.0001	0.0005
rs5756152	n	510	510

D: CKD: NO		rs12107
rs4821480	D	-0.01
rs4821480	D'	0.14
rs4821480	Corr.	-0.07
rs4821480	x^2	4.39
rs4821480	P-value	0.0361
rs4821480	n	510

*Significant p-values are highlighted.

74

Table 3.4: Genotype distributions, minor allele frequencies, and unadjusted p-values for comparing genotype distributions between CKD groups, additive allelic effects between CKD groups. HWE p-values are from exact tests.

	CKD CASE	CON	P-value
n	**214**	**510**	
rs5756152			
A/A	168 (0.79)	375 (0.74)	0.438
A/G	41 (0.19)	126 (0.25)	
G/G	5 (0.02)	9 (0.02)	
G	51 (0.12)	144 (0.14)	0.115
HWE	0.192	0.855	
rs4821480			
G/G	88 (0.41)	199 (0.39)	0.226
G/T	94 (0.44)	215 (0.42)	
T/T	32 (0.15)	96 (0.19)	
T	158 (0.37)	407 (0.40)	0.141
HWE	0.463	0.007	
rs12107			
G/G	117 (0.55)	283 (0.55)	0.303
G/A	87 (0.41)	191 (0.37)	
A/A	10 (0.05)	36 (0.07)	
A	107 (0.25)	263 (0.26)	0.808
HWE	0.275	0.644	

Abbreviation: HWE, Hardy Weinburg Equilibrium

Table 3.5: Genotype distributions, minor allele frequencies, and unadjusted p-values for comparing genotype distributions between genders.

	Female	Male	P-value
n	**566**	**162**	
rs5756152			
A/A	423 (0.75)	124 (0.77)	0.479
A/G	130 (0.23)	37 (0.23)	
G/G	13 (0.02)	1 (0.01)	
G	156 (0.14)	39 (0.12)	0.414
rs4821480			
G/G	211 (0.37)	76 (0.47)	0.040
G/T	247 (0.44)	66 (0.41)	
T/T	108 (0.19)	20 (0.12)	
T	463 (0.41)	106 (0.33)	0.010
rs12107			
G/G	304 (0.54)	97 (0.6)	0.372
G/A	224 (0.4)	57 (0.35)	
A/A	38 (0.07)	8 (0.05)	
A	300 (0.27)	73 (0.23)	0.142

*Significant p-values are highlighted.

3.3.4 Genotype associations:

In additive allelic models that were adjusted for age and gender, only rs5756152 demonstrated an association with CKD, whereby each G allele of rs5756152 increased eGFR by 3.67 ml/min/1.73 m^2 (95% CI: 1.29-6.05), p = 0.003, reduced serum creatinine by 4.5% (95%CI: 1.7-7.4%), p = 0.002 and added 0.51 mmol/L (95% CI: 0.06-0.96) to fasting blood glucose, p = 0.028 (Table 3.6). Interactive models were used to test additive allelic effect on the traits between those participants with diabetes and those with hypertension. It was the effect on the traits between the diabetic group and those without diabetes mellitus that showed significant results.   Seven SNP-trait pairs differed significantly between the diabetic group and those without diabetes. The results are summarized Table 3.7, starting with the interaction p-values. It was found that in each of the combinations

of trait and SNP showing a difference, the effect was significant in the DM group, but not in the normal group. The effect sizes in the DM group, with 95% confidence intervals, for the listed allele are also given. In the DM group, with each G allele of rs5756152, serum creatinine decreased by 10%, eGFR increased by 8.8 ml/min/1.73m^2, and FBG and post FBG increased by 1.3 mg/dl and 2.2 mg/dl respectively. Also in the DM group, each rs4821480 T allele increased eGFR with 4.9 ml/min/1.73m^2 whereas each rs12107 A allele reduced FBG and Post FBG by 0.9 mg/dl and 2.0 mg/dl respectively.

Table 3.6: Genotype association and additive allelic association p-values between traits and SNPs. Adjusted for age and gender

	Genotype			Additive allelic		
	rs5756152	rs4821480	rs12107	rs5756152	rs4821480	rs12107
Age	0.582	0.804	0.663	0.617	0.695	0.402
Serum Creatinine (mg/dl)	0.010	0.181	0.724	0.002	0.139	0.998
eGFR (ml/min/1.73m^2)	0.010	0.111	0.652	0.003	0.064	0.986
CKD.MDRD	0.226	0.303	0.438	0.115	0.141	0.808
FBG (mg/dl)	0.087	0.855	0.292	0.028	0.593	0.125
Post FBG (mg/dl)	0.502	0.422	0.217	0.247	0.642	0.114
SBP (mmHg)	0.528	0.637	0.935	0.613	0.389	0.717
DBP (mmHg)	0.784	0.159	0.176	0.493	0.097	0.918

*Significant p-value < 0.05. Abbreviations: eGFR, Estimated Glomerular Filtration Rate; CKD, Chronic Kidney Disease; MDRD, Modification of Diet in Renal Disease; FBG, Fasting Blood Glucose; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure.

Table 3.7: Significant SNP-trait pairs in additive allelic associations between DM and non DM groups. The interaction p-value, and the effect (95% CI) of each allele on that trait, in the DM group.

Interaction P-value	Trait	Allele	Effect (95% CI) in DM
0.0426	Serum Creatinine (%)	rs5756152 G	-10.0 (4.9-14.8)
0.0057	eGFR (ml/min/1.73m^2)	rs5756152 G	8.8 (4.5-13.2)
0.0013	FBG (mg/dl)	rs5756152 G	1.3 (0.7-2.0)
<0.0001	Post FBG (mg/dl)	rs5756152 G	2.2 (1.3-3.1)
0.0069	eGFR (ml/min/1.73m^2)	rs4821480 T	4.9 (2.0-7.9)
0.0050	FBG (mg/dl)	rs12107 A	-0.9 (0.4-1.4)
<0.0001	Post FBG (mg/dl)	rs12107 A	-2.0 (1.3-2.7)

*Significant p-value <0.05. Abbreviations: **CI: Confidence Interval. DM: Diabetes Mellitus. eGFR: Estimated Glomerular Filtration Rate. FBG: Fasting Blood Glucose.

3.4. DISCUSSION

The results of this study provide evidence that SNP's in the *MYH9* gene are associated with eGFR levels in type 2 diabetic mixed ancestry subjects from South Africa, with the reference alleles of rs5756152 and rs4821480 increasing the eGFR. Although the E-1 haplotype has previously been shown to confer all the excess burden of diabetes-unrelated ESRD in African Americans (Kopp *et al*, 2008; Nelson *et al,* 2010), the strongest association with eGFR was observed with rs5756152 and to a lesser extent by the E-1 rs4821480 after performing interaction model analysis. A serum creatinine effect was only seen with the G allele of rs5756152, decreasing creatinine levels by 10% (Table 3.7). This positive association has not been seen between this SNP and serum creatinine in previous research. An intriguing finding in this study was the association with the genotyped SNPs on blood glucose levels. The G allele of SNP rs5756152 G increased blood glucose levels while the A allele of rs12107 decreased blood glucose levels (Table 3.7).   Despite the identification of disease susceptible *MYH9* SNPs, the disease causal mechanism remains largely unknown. It is known that the non-muscle myosin protein is abundantly expressed in the kidney, platelets and liver and in smaller amounts in the thymus, spleen, intestine and cochlea. Within the kidney, it is expressed in the podocytes which are highly specialized cells, with the ability to ultra-filter blood and support glomerular capillary pressures (Perry *et al*, 2006; Saleem *et al*, 2008). The *MYH9* gene, specifically the E-1 haplotype, has been shown to modulate the genetic effect of FRMD3 for diabetes susceptibility (Freedman *et al*, 2011). In view of our results, it is possible that the *MYH9* exerts this effect through gene-gene interaction in the regulation of blood glucose levels. It is not clear why the two SNP's had opposing effects on blood glucose levels, we speculate that their different effects on glucose levels are likely cancelling each other if both are present in an individual, consequently the weak association with type 2 diabetes-associated ESRD.

Populations with an African ancestry, such as African Americans, have long been shown to be prone to the development of kidney diseases with a poorer prognosis compared to their European counterparts (Freedman *et al*, 1995).

These discrepancies have often been linked to lifestyle, social-economic status and clinical factors such as poor blood glucose or blood pressure control. In 2008, two studies took advantage of the differences in kidney disease prevalence and its cumulative lifetime risk (2.1% vs 7.5%, respectively) between Americans of European and African origin, and identified SNPs in the *MYH9* gene which conferred most or nearly all of the increased risk for non-diabetic kidney disease in African Americans (Kao *et al*, 2008; Kopp *et al*, 2008). The *MYH9* SNPs were clustered into groups according to linkage disequilibrium blocks: the E-1 haplotype (rs4821480) (Kao *et al*, 2008; Kopp *et al*, 2008), S-1 haplotype, F-1 haplotype (Behar *et al*, 2010) and L1 haplotype (rs12107) (Freedman *et al*, 2009). In this study, we genotyped three SNP's (rs5756152, rs4821480 and rs12107) that have been shown to exhibit independent evidence for association with non-diabetic ESRD (Freedman *et al*, 2009). This study is different from the previous reports as it demonstrates an early association between *MYH9* polymorphisms and renal phenotypes, and describes the effect of *MYH9* polymorphisms in a community based cohort as opposed to clinically diagnosed ESRD. These findings, and those previously published, suggest that the *MYH9* gene may have a broader genetic risk effect on different types of kidney diseases

Non-communicable diseases are the leading cause of death in the world, responsible for 59% of the 57 million deaths that occurred in 2008 (Aiwan *et al*, 2010). Thirty-six million of these deaths were attributed to cardiovascular disease, diabetes, cancers and chronic respiratory disease. Underlying all this is the much less appreciated epidemic of CKD, which in return brings with it a huge burden of CVD and ESRD (Davids, 2007). Our study showed that 29.6% of the population in Bellville South had CKD, of which 96.3% had an eGFR of <60 ml/min/1.73m^2 (Stage 3 CKD). This figure is significantly higher than the reported prevalence in developed countries like the USA (13.1%) (Huda, Alam and Rashid, 2012) and is likely explained by the significantly older age of participants with CKD. The lack of functioning registries in sub-Saharan Africa, including South Africa, has resulted in a lack of reliable statistics, but the impression is that the prevalence of CKD is at least 3-4 times more frequent than in developed

countries (Naicker, 2009; Ainah, 2010). The last report from the South African Dialysis and Transplant Registry was in 1994 and revealed a prevalence of 99 patients per million (3399 patients) on renal replacement therapy (reviewed by Davids, 2007). However, this only reports the incidence of patients with ESRD, on treatment and excludes the larger proportion of patients with CKD (Stages 3 and 4).

The development of CKD is strongly associated with hypertension and type 2 diabetes. Indeed, we observed a high prevalence of diabetes and hypertension in subjects with CKD. In Africa, hypertension is a major cause of CKD and conversely, secondary hypertension is most commonly initiated by the presence of CKD (Sinclair *et al*, 1987). In our population 64% of subjects with CKD also had hypertension compared to 34% in subjects without CKD. This figure is similar to those quoted in the USA, where hypertension is the number two cause of ESRD, following diabetes, and is a co-morbid condition in approximately 61-66% of those with eGFR <60 ml/min/1.73m^2 (Bethesda, 2011). Hypertension affects approximately 25% of the adult population and is a cause of CKD in 21% of patients on renal replacement therapy in the South African Registry (Variava *et al*, 1990; reviewed by Naicker, 2009). For these reasons, treatment of hypertension is a very important factor in CKD and CVD risk reduction strategy. Co-existence of hypertension is associated with more rapid progression of CKD and several studies have indicated that treating hypertension in patients with CKD and proteinuria may slow the decline in GFR and progression of CKD (Maki *et al*, 1995; reviewed by He and Whelton, 1999; Jafar *et al*, 2003; Casas *et al*, 2005).

Currently approximately 366 million individuals suffer from type 2 diabetes, and future predictions indicate that this number will surge to approximately 522 million by 2030 (International Diabetes Federation, 2011). The observed prevalence of diabetes in our population is 28.4%; higher than the world diabetes prevalence of 6.4% in 2010 among adults aged 20-79 years (Shaw, Sicree and Zimmet, 2010). Diabetes mellitus type 2 is becoming a global health problem due to several factors, including aging, urbanization, increasing prevalence of obesity and

physical inactivity (Wild *et al*, 2004). One third of diabetic patients will eventually develop CKD due to diabetic nephropathy (Bethesda, 2007). Diabetic nephropathy is characterized by proteinuria, and the earliest sign is considered to be microalbuminuria (Inomato *et* al, 1989). Traditional risk factors such as hypertension, hyperlipidaemia and smoking need to be managed in patients with co-existing diabetes mellitus as they contribute to the increased cardiovascular risk and worsening of renal failure. As highlighted before, our study cohort demonstrated that these factors strongly correlated with CKD.

To our knowledge, this is the first study in the mixed ancestry population of South Africa providing detailed genetic analysis of kidney diseases. A major limitation of this study pertains to the measurements of the general characteristics of the subjects. Blood glucose measurements were not repeated in asymptomatic subjects who were found to have diabetes, while according to the WHO type 2 diabetes diagnosis procedure requires at least one further test on a subsequent day (WHO, 2003). Urine samples to determine albumin levels were only collected and analysed once, with the KDIGO guidelines stating that albuminuria can only be diagnosed on two to three separate urine collections.

In conclusion, SNPs rs5756152 and rs4821480 are associated with early kidney diseases in the mixed ancestry population of South Africa with type 2 diabetes whilst rs12107 is associated with glucose metabolism. Our findings and other studies suggest that the *MYH9* gene may have a broader genetic risk effect on different types of kidney diseases. The findings of this study have important public health implications. Strong and specific genetic associations would allow for race specific screening, allowing for education on healthy living, delaying the onset of CKD as well as allowing for early implementation of appropriate treatment, with the hope of preventing disease progression. This would ease a large burden of dialysis patients of both state and private hospitals. This is the first genetic screening in the mixed race population of South Africa for CKD – a small step in a confidently long future of genetic screening in South Africa.

81

3.5. RECOMMENDATION FOR FUTURE STUDIES

This study was unique in the fact that it was a community based cohort describing MYH9 polymorphisms and their associations with early renal phenotypes, as opposed to clinically diagnosed ESRD. On the strength of these findings, the inclusion of ESRD-diagnosed patients in the same mixed ancestry population in a future study would allow for comparisons to be made between the significance and strength of the effect of the MYH9 polymorphisms on early stage CKD and ESRD. Between 20%-40% of patients with diabetes will ultimately develop DN (Dronavalli, Duka and Bakrus, 2008) and diabetes is the leading cause of ESRD (Bethesda, 2011). The DM prevalence in South Africa and the significant findings in this study of the *MYH9* polymorphisms' effect on diabetic individuals further substantiate the inclusion of ESRD individuals in future studies.

There is a lack of data mapping of the admixture ancestry markers of this mixed ancestry population. Although *MYH9* risk alleles found in the African American population are highly prevalent in populations with an African ancestry including the indigenous San and Bantu-South Africans (Oleksek *et* al, 2010), suggesting that the identified SNPs in the African American population will be prevalent in this population, it may be possible that there are other SNPs within the *MYH9* gene that may show a significance. In this study, rs5756152 showed the strongest association with renal phenotypes; however it must be established if other SNPs within the *MYH9* gene prove otherwise. In addition, it is recommended that these future studies include the *APOL1* gene. *APOL1* has recently been found to have stronger association with kidney diseases than polymorphisms within the *MYH9* gene in African Americans. The *APOL1* gene was mapped in chromosome 22, near the *MYH9* locus, with the two risk alleles, G1 and G2, overlapping the E-1 haplotype in the MYH9 gene by 89% and 76% respectively (Genovese *et al,* 2010). Suggesting functionality, the G1 and G2 polymorphisms alter amino acid sequence of the encoded protein, something the *MYH9*-CKD association lacks. *APOL1* polymorphisms have been found to be strongly associated with FSGS, H-ESRD and HIVAN (Genovese *et al,* 2010;

Kopp *et al*, 2010). Importantly, the *APOL1*-mediated kidney disease risk showed an autosomal-recessive inheritance rather than an additive pattern that is typical of most common complex disease. Although there still remains no evidence that *APOL1* polymorphisms are strongly associated with CKD and data from the human protein atlas suggest that APOL1 is not even expressed in the glomeruli (Johnstone *et al*, 2011), the inclusion of G1 and G2 SNPs from the *APOL1* gene would have been broadened the study.

The ancestry contribution in the mixed ancestry population needs to be considered in this study. The South African mixed ancestry population forms a unique, highly admixed population, resulting from the encounter of different people from Africa, Europe, and Asia, and more specifically, five different parental populations; Khoisan, Bantus, Europeans, Indians, and Southeast Asians (Quintana-Murci *et al*, 2010). The importance is the different contributions from the parental populations. The mixed race population in this study was confined to one, long-standing community, decreasing the likelihood of unbalanced genetic admixture between the participants. A comparison study could be done between the mixed ancestry groups within the different regions of South Africa, who undoubtedly have dissimilar ancestry contributions. Furthermore, due to the heterogeneity of the mixed race population, the inclusion of a homogenous group such as black South Africans warrants investigation.

Finally, to broaden the knowledge of the effect of the *MYH9* gene polymorphisms in the mixed ancestry population, larger studies in the future should include biopsy-confirmed HIVAN and FSGS participants, both highly prevalent in South Africa and both important in ESRD progression. HIV infected individuals, a prevalent finding in South Africa, are at risk of developing both HIVAN and secondary FSGS (Schrier, 2010). The E-1 haplotype of the *MYH9* gene has been associated with both HIVAN And FSGS in the African American population (Kopp *et al*, 2008; Freedman *et al*, 2009), opening the doors for this to be investigated in the mixed ancestry population in South Africa.

CHAPTER 4

[References]

Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, et al. Asymmetric dimethylarginine causes hypertension and cardiac dusfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2003;23:1455-1459.

Acharya VN. Diabetic and Hypertensive Nephropathy in India. *Journal of the Association of Physicians of India*. 2001;59:143.

Agarwal R. Vitamin D, proteinuria, diabetic nephropathy, and progression of CKD. *Clinical Journal of the American Society of Nephrology.* 2009;4:1523–1528.

Ainah U, Tan BS, Hoffman B, Rosas SE. Patient perception of risk factors associated with chronic kidney disease morbidity and mortality. *Ethnicity and Disease.* 2010:20:107-110.

Aiwan A, MacLean DR, Riley L, d'Espaignet ET, Mathers CD, Stevens GA, Bettcher D. Monitoring and surveillance of chronic noncummunicable diseases: progress and capacity in high-burden countries. *The Lancet.* 2010;376(9755):1861-1868.

Applied Biosystems. Real-Time PCR Systems: Chemistry Guide [Online]. 2005 [Citied 25 August 2012]. Available from: http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_041440.pdf.

Arita A, Kihara S, Ouch N, Takahashi M, Maeda K, Miyagawa J, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochemical and Biophysical Research Community.* 1999;257(1):79-83.

Ascherio A, Hennekens C, Willett WC, Sacks F, Rosner B, Manson J, et al. Prospective study of nutritional factors, blood pressure, and hypertension among US women. *Hypertension.* 1998;27(5):1065-1072.

Austin WJ, Bhalla V, Hernandez-Arce I, Isakson SR, Beede J, Clopton P, et al. Correlation and Prognostic Utility of B-Type Natriuretic Peptide ad Its Amino-Terminal Fragment in Patients With Chronic Kidney Disease. *American Journal of Clinical Pathology.* 2006;126:506-512.

Baboolal K, Ravine D, Daniels J, Williams N, Holmans P, Coles GA, et al. Association of the angiotensin I converting enzyme gene deletion polymorphisms with early onset of ESRF in PKD1 adult polycystic kidney disease. *Kidney International.* 1997;52(3):607-613.

Balkestein EJ, Staessen JA, Wang JG, van der Heijden-Spek JJ, van Bortel L, Barlassina G. Carotid and femoral artery stiffness in relation to three candidate genes in a white population. *Hypertension.* 2001;38:1190-1197.

Barnett AH, Barnett T, Kumar S. *Obesity and Diabetes.* New Jersey: John Wiley & Sons; 2009.

Barsoum RS. Chronic kidney disease in the developing world. *New England Journal of Medicine.* 2009;354:997-999.

Becker B, Kronenberg F, Kielstein JT, Haller H, Morath C, Ritz E, et al. Renal insulin resistance syndrome, adiponectin and cardiovascular events in patients with kidney disease: The Mild and Moderate Kidney. *Journal of the American Society of Nephrology.* 2005;16:1091–1098.

Behar DM, Rossett S, Tzur S, Selig S, Yudkovsky G, Bercovici S, et al. African ancestry allelic variation at the MYH9 gene contributes to increased susceptibility to non-diabetic end-stage kidney disease in Hispanic Americans. *Human Molecular Genetics.* 2010;19:1816-1827.

Bernt TJ, Schiavi S, Kumar R. "Phosphatonins" and the regulation of phosphorus homeostasis. *American Journal of Physiology – Renal Physiology.* 2005;289(6):F1170-F1182.

Berry AD, Ho C. One-sided sequential stopping boundries for clinical trials: a decision-theoretical approach. *Biometrics.* 1988;44:219-227.

Bethesda MD. Annual Data Report: Atlas of End-Stage Renal Disease in the United States, National Institute of Health, National Institute of Diabetes and Digestive and Kidney Diseases. *US Renal Data System.* 2007;1:Part 12.

Bethesda MD. Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States, National Institute of Health, National Institute of Diabetes and Digestive and Kidney Diseases. *US Renal Data System.* 2011;1(1):37-44.

Bidani AK, Griffen KA. Pathophysioogy of Hypertensive Renal Damage: Implications for Therapy. *Journal of the American Heart Association.* 2004;44:595-601.

Birn H, Verroust PJ, Nexo E, Hager H, Jacobsen C, Christensen EI, et al. Characterization of an epithelial approximately 460-kDa protein that facilitates endocytosis of intrinsic factor-vitamin B12 and binds receptor-associated protein. *Journal of Biological Chemistry.* 1997;272(42):26497-26504.

Bishop ML, Fody EP, Schoeff LE. *Clinical Chemistry; Princeples, Procedures, Correlations.* 5th ed. Baltimore: Lippincott Williams & Wilkins; 2005.

Bland JM, Altman DG. The odds ratio. *British Medical Journal.* 2000;320:1468.

Block GA, Klassen PS, Lazarus JM, Ofsthun N, Lowrie EG, Chertow GM. Mineral metabolism, mortality, and morbidity in maintenance hemodialysis. *American Society of Nephrology.* 2004;15:2208-2218.

Boes E, Fliser D, Ritz E, Konig P, Lhotta K, Mann JFE, et al. Apolipoprotein A-IV Predicts Progression of Chronic Kidney Disease: The Mild to Moderate Kidney

Disease Study. *Journal of the American Society of Nephrology.* 2006;17(2):28-536.

Böger CA, Heid IM. Chronic Kidney Disease: Novel Insights from Genome-Wide Association Studies. *Kidney and Blood Pressure Research.* 2011;34:225-234.

Böger CA, Chen M, Tin A, Olden M, Kottgen A, de Boer IH. CUBN Is a Gene Locus for Albuminuria. *Clinical Research.* 2011;22:555-570.

Bolignano D, Coppolino G, Romeo A, De Paola L, Buemi A, Lacquaniti A, et al. Neutrophil gelatinaseassociated lipocalin (NGAL) reflects iron status in haemodialysis patients. *Nephrology Dialysis Transplan.* 2009;24(11):3398-3403.

Botev R, Mallie JP, Couchoud C, Schuck O, Fauvel JP, Wetzels JFM, et al. Estimating Glomerular Filtration Rate: Cockcroft-Gault and Modification of Diet in Renal Diseases Formulas Compared to Renal Insulin Clearance. *Clinical Journal of the American Society of Nephrology.* 2009;4(5):899-906.

Boute N, Gribouval O, Roselli S, Benessy F, Lee H, Fuchshuber A, et al. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nature Genetics.* 2000;24(4):349-354.

Bradshaw D, Schneider M, Norman R, Bourne D. Mortality patterns of chronic disease of lifestyle in South Africa*. Chronic Diseases of Lifestyle in South Africa: Medical Research Council (MRC) Technical Report Tygerberg* [Online]. 1995*;*5-31. Available from: http://www.mrc.ac.za/chronic/cdlchapter2.pdf.

Brenner BM, Garcia DL, Anderson S. Glomeruli and blood pressure. Less of one, more of the other? *American Journal of Hypertension.* 1988;1(4):335-347.

Briscoe D, Stephens JC, O'Brien SJ. Linkage disequilibrium in admixed populations: application in gene mapping. *Journal of Heredity.* 1994;85:59-63.

Brown EJ, Schlondorff JS, Becker DJ, Tsukaguchi H, Uscinski AL, Higgs HN, et al. Mutations in the formin gene INF2 case focal segmental glomerulosclerosis. *Nature Genetics.* 2010;42(1):72-76.

Casas, JP, Chua, W, Loukogeorgakis S, Vallance P, Smeeth L, Hingorani AD, MacAllister RJ. Effects of inhibitors of the renin-angiotensin system and other anti-hypertensive drugs on renal outcomes: systemic review and meta-analysis. *Lancet.* 2005;366:2026-2033.

Cauchi S, El Achhab Y, Choquet H, Dina C, Krempler F, Weitgasser R, et al. TCF7L2 is reproducibly associated with type2 diabetes in various ethnic groups: a global meta-analysis. *Journal of Molecular Medicine.* 2007;85(7): 777-782.

Chagnac A, Weinstein T, Herman M, Hirsh J, Gafterand U, Ori, Y. The Effects of Weight Loss on Renal Function in Patients with Severe Obesity. *Journal of American Society of Nephrology.* 2003;14(6):1480-1486.

Christensen E, Verroust PJ, Nielsen R. Receptor-mediated endocytosis in renal proximal tubule. *Pflugers Archiv : European Journal of Physiology.* 2009;458(6):1039-1048.

City of Cape Town Census [Online]. 2001. [Citied 14 November 2011]. Available from: http://www.capetown.gov.za"http://www.capetown.gov.za

Clase CM, Garg AX, Kiberd BA. Prevalence of Low Glomerular Filtration Rate in Nondiabetic Americans: Third National Health and Nutrition Examination Survey (NHANES III). *Journal of the American Society of Nephrology.* 2002;13:1338-1349.

Coldits GA, Willett WC, Rotnitzky A, Manson JE. Weight as a risk factor for clinical diabetes in women. *American Journal of Epidemiology.* 1990;13(2):501-513.

Cooke JP. Asymmetrical dimethylarginine: The Uber marker? *Circulation.* 2004;109:1813-1818.

Crow JF, Dove WF. Anecdotal, Historical and Critical Commentaries on Genetics. *Genetics.* 1998;119:473-476.

Daniel KM. *Clinical significance of chronic kidney disease in the elderly*. Texas: ProQuest; 2008.

Darvasi A, Shifman S. The beauty of admixture. *Nature Genetics.* 2005;37:118-119.

Davids MR. Chronic kidney disease – the silent epidemic. *Continuing Medical Education.* 2007;25(8):378-382.

De Boer I, Ioannou GN, Kestenbaum B, Brunzell JD, Weiss NS. 25(OH)D levels and albuminuria in the Third National Health and Nutrition Examination Survey (NHANES III). *American Journal of Kidney Disease. 2007;*50:69–77.

De Jong PE, van der Velde M, Gansevoort RT, Zoccali C. Screening for Chronic Kidney Disease: Where Does Europe Go? *Clinical Journal of the American Society of Nephrology*. 2008;3:616-623.

Dean M, Santis G. Heterogeneity in the severity of cystic fibrosis and the role of the CFTR gene mutations. *Human Genetics.* 1994;93:304-368.

DeFilippi CR, Fink JC, Nass CM, Christenson R. N-terminal pro-B-type natriuretic peptide for predicting coronary disease and left ventricular hypertrophy in asymptomatic CKD not requiring dialysis. *American Journal of Kidney Diseases*. 2005;46(1):3-44.

Delfino RJ, Staimer N, Tjoa T, Gillen DL, Polidori A, Arhami M, et al. Air Pollution Exposures and Circulating Biomarkers of Effect in a Susceptible Population: Clues to Potential Causal Component Mixtures and Mechanisms. *Environ Health*

88

Perspectives. 2009;117(8):1232-1238.

Dieplinger H, Lobentanz EM, Konig P, Graf H, Sandholzer C, Matthys E, et al. Plasma apolipoprotein A-IV metabolism in patients with chronic renal disease. *European Journal of Clinical Investigation.* 1992;22:166–174.

Dieplinger B, Mueller T, Kollerits B, Struck J, Ritz E, von Eckardstein A, et al. Pro-A-type natriuretic peptide and pro-adrenomedullin predict progression of chronic kidney disease: the MMKD Study. *Kidney International.* 2009;75(4):408-414.

Dinarello CA. The interleukin-1 family: 10 years of discovery. *Federation of American Societies for Experimental Biology.* 1994;8(15):1314-1325.

Divers J, Freedman BI. Susceptibility genes in common complex kidney disease. *Current Opinion in Nephrology and Hypertension.* 2010;19(1):79-84.

Dronavalli S, Duka I, Bakris GL. The pathogenesis of diabetic nephrology. *Nature Clinical Practice: Endocrinology & Metabolism.* 2008;4(8):444-452.

El Nahas M. The global challenge of chronic kidney disease. *Kidney International.* 2005;68:2918-2929.

El Nahas M, Levin A. *Chronic Kidney Disease: A practical guide to understanding and management.* Oxford: Oxford University Press; 2009.

Ellis JW, Chen MH, Foster MC, Liu CT, Larson MG, de Boer I, et al. Validated SNPs for eGFR and their associations with albuminuria. *Human Molecular Genetics* [Online]. 2010 [Citied 15 May 2012]. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22492995/

Erasmus RT. The prevalence of impaired glucose tolerance and diabetes amongst the middle aged population of Bellville South Community, Cape Town, South Africa [unpublished data]. Cape Town: Stellenbosch University; 2008.

Ewing JA. Alcoholism: The CAGE Questionnaire. *Journal of American Medical Association.* 1984 ;252: 1905-1907.

Fassett RG, Venuthurupali K, Gobe GC, Coombes JS, Cooper MA, Hoy WE. Biomarkers in chronic kidney disease: a review. *Kidney International.* 2011;80:806-821.

Fervenza FC. *Nephrosclerosis* [Online]. 2012 [Citied 5 May 2012]. Available from: http://www.emedicine.medscape.com/article/244342-overview

Field AE, Coakley EH, Must A, Spadano JL, Laird N, Dietz WH, et al. Impact of Overweight on the Risk of Developing Common Chronic Diseases During a 10-Year Period. *Archives of Internal Medicine.* 2001;161(13):1581-1586.

89

Fishbane S, Chittineni H, Packman M, Dutka P, Ali N, Durie N. Oral paricalcitol in the treatment of patients with CKD and proteinuria: A randomized trial. *American Journal of Kidney Disease.* 2009;54:647 665.

Fliser D, Kronenberg F, Kielsen JT, Marash C, Bode-Boger SM, Haller H, et al. Asymmetric dimethylargine and progression of chronic kidney disease: The mild to moderate kidney disease study. *Journal of the American Society of Nephrology.* 2005;16(8):2456-2461.

Fliser D, Kollerits B, Never U, Ankerst DP, Lhotta K, Lingenhel A, et al. Fibroblast Growth Factor 23 (FGF23) Predicts Progression of Chronic Kidney Disease: The Mild to Moderate Kidney Disease (MMKD) Study. *Journal of the American Society of Nephrology.* 2007;18(9):2600-2608.

Fodinger M, Horl WH, Sunder-Plassmann G. Molecular biology of 5,10-methylenetetrahydrofolate reductase. *Journal of Nephrology.* 2000;*13(1):20-33.*

Forman JP, Giovannucci E, Holmes MD, Bischoff-Ferrari HA, Tworoger SS, Willett WC, et al. Plasma 25(OH)D levels and risk of incident hypertension. *Hypertension.* 2007;49:1063–1069.

Franke D, Volker S, Haase S, Pavicic L, Querfeld U, Ehrich JH, Zivicnjak M. Prematurity, small for gestational age and perinatal parameters in children with congenital, hereditary and acquired chronic kidney disease. *Nephrology Dialysis Transplantation.* 2010;25:3918-3924.

Friedman DJ, Pollak MR. Genetics of kidney failure and the evolving story of APOL1. *Journal of Clinical Investigation.* 2011;121(9):3367-3374.

Freedman BI, Volkova NV, Satko SG, Krisher J, Jurkovitz C, Soucie JM, et al. Population-Based Screening for Family History of End-Stage Renal Disease among Incident Dialysis Patients. *America Journal of Nephrology.* 2005;25(6):529-535.

Freedman BI, Hicks PJ, Bostrom MA., Comeau ME, Divers J, Bleyer AJ, et al. Non-muscle myosin heavy chain 9 gene MYH9 associations in African Americans with clinically diagnosed type 2 diabetes mellitus-associated ESRD. *Nephrology Dialysis Transplantation.* 2009;24(11): 3366–3371.

Freedman BI, Kopp JB, Winkler CA, Nelson GW, Rao DC, Eckfeldt JH, et al. Polymorphisms in the nonmuscle myosin heavy chain 9 gene (MYH9) are associated with albuminuria in hypertensive African Americans. *American Journal of Nephrology.* 2009;29:626-632.

Freedman BI, Langefeld CD, Lu L, Divers J, Comeau ME, Kopp JB, Winkler CA, Nelson GW, Johnson RC, Palmer ND, Hicks PJ, Bostrom MA, Cooke JN, McDonough CW, Bowden DW. Differential Effects of *MYH9* and *APOL1* Risk Variants on *FRMD3* Association with Diabetic ESRD in African Americans. PloS Genetics [Online]. 2011 [Citied June 16 2012]. Available from: doi: 10.1371/journal.pgen.1002150.

Gee GC, Payne-Sturges DC. Environmental health disparities: A framework integrating psychosocial and environmental concept. *Environ Health Perspect*. 2004;112(17):1645-1653.

Garrett MR, Pezzolesi MG, Korstanje R. Integrating Human and Rodent Data to Identify the Genetic Factors Involved in Chornic Kidney Disease. *Journal of the American Society of Nephrology*. 2010;21:398-405.

Genovese G, Tona SJ, Knob AU, Appel GB, Katz A, Bemhardy AJ, et al. A risk allele for focal segmental glomerulosclerosis in African Americans is located within the region contained APOL1 and MHY9. *Kidney International*. 2010;78(7): 698-704.

Genovese G, Friedman DJ, Ross MD, Lecordier L, Uzureau P, Freedman BI, et al. Association of Trypanolytic ApoL1 Variants with Kidney Disease in African-Americans. *Science*. 2010;329(5993):841-845.

Gillespie JH. Population genetics: A concise guide. Baltimore: John Hopkins University; 2004.

Gillespie KM. Type 1 diabetes: pathogenesis and prevention. *Canadian Medical Association Journal.* 2006;175(2):165-170.

Glassock RJ, Winearls C. An epidemic of Chronic Kidney Disease: fact or fiction? *Nephrology Dialysis Transplantation*. 2008;23:1117-1121.

Goldsmith D, Tayawardene S, Ackland P. *ABC of Kidney Disease*. New Jersey: Wiley-Blackwell; 2007.

Gouin-Thibault I, Pautas E, Mahe I, Descarpentries C, Nivet-Antoine V, Golmard JL, Siguret V. Is Modification of Diet in Renal Disease formula similar to Cockcroft-Gault formula to assess renal function in elderly hospitalized patients treated with low-molecular-weight heparin. *Journal of Gerontology Series: Biological Sciences and Medical Sciences.* 2007;62(11):1300-1305.

Goyette P, Summer JS, Milos R. Human Methylenetetrahydrofolate reductase: Isolation of cDNA, mappingand mutation identification. *Nature Genetics.* 1994;7:195-200.

Griffin KA, Kramer H, Bidani AK. Adverse renal consequences of obesity. *American Journal of Physiology – Renal Physiology*. 2008;294(4):F685-F696.

Gumienny TL, Brugnera E, Tosello-Trampont AC, Kinchen JM, Haney LB, Nishiwaki K, et al. CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, I srequired for phagocytosis and cell migration. *Cell.* 2001;107:27-41.

Gumprecht J, Zychma MJ, Grzeszczak W, Zukowska-Szczechowska E. Angiotensin 1-converting enzyme gene insertion/deletion and angiotensinogen

91

M235T polymorphisms: Risk of chronic renal failure. *Kidney International.* 2000;58(2):513-519.

Haiman M, Salvenmoser W, Scheiber K, Lingenhel A, Rudolph C, Schmitz G, et al. Immunohistochemical localization of apolipoprotein A-IV in human kidney tissue. *Kidney International.* 2005;68:1130-1136.

Hallan ST, Orth SR. The KDOQI 2002 Classification of chronic kidney disease: for whom the bell tolls. *Nephrology Dialysis Transplantation.* 2010;25:2832-2836.

Hanai K, Babazono T, Nyumaru I, Toya K, Tanaka N, Tanaka M, et al. Asymetric dimethylarginine is closely associated with the development and progression of nephrology in patients with type 2 diabetes. *Nephrology Dialysis Transplant.* 2009;24:1884-1888.

Hart TC, Gorry MC, Hart PS, Woodard AS, Shihabi Z, Sandhu J, et al. Mutations of the UMOD gene are responsible for medullary cyctic kidney disease 2 and familial juvenile hyperuricaemic nephropathy. *Journal of Medical Genetics.* 2002;39:882-892.

He J, Whelton PK. Elevated systolic blood pressure and risk of cardiovascular and renal disease: Overview of evidence from observational epidemiologic studies and randomized controlled trials. *American Heart Journal.*1999;138:5211-5219.

Hildebrandt F. Genetic kidney diseases. *Lancet.* 2010;10(375):1287-1295.

Hirschhorn JN, Daly MJ. Genome-Wide Association Studies for Common Diseases and Complex Traits. *Nature Reviews – Genetics.* 2005;6:95-108.

Hogg RJ, Furth S, Lemley KV, Protman R, Schwartz GJ, Coresh J, et al. National Kidney Foundation's Kidney Disease Outcomes Quality Initiative Clincical Practice Guidelines for Chronic Kidney Disease in Children and Adolescents: Evaluation, Classification, and Statification. *Pediatrics.* 2003;111(6)1416-1421.

Hojs R, Bevc S, Ekart R, Gorenjak M, Puklavec L. Serum cystatin C as an endogenous marker of renal function in patients with mild to moderate impairment of kidney function. *Oxford Journals.* 2006;21(7):1855-1862.

Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC, et al. Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *American Diabetes Association.* 2001;50(5):1126-1133.

Huda MN, Alam KS, Rashis HU. Prevalence of chronic kidney disease and its association with risk factors in disadvantages population. *International Journal of Nephrology* [Online]. 2012 [Citied 15 August 2012]. Available from: doi: 10.1155/2012/267329.

92

Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, et al. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. *The Journal of Biological Chemistry.* 1998;273:4135–4142.

Ichnose K, Kawasaki E, Eguchi K. Recent Advancement of Understanding Pathogenesis of Type 1 Diabetes and Potential Relevance to Diabetic Nephropathy. *American Journal of Nephrology.* 2007;27(6):554-564.

Imagawa A, Funahashi T, Nakamura T, Moriwaki M, Tanaka S, Nishizawa H, et al. Elevated serum concentration of adipose-derived factor, adiponectin, in patients with type 1. *Diabetes Care.* 2002;25:1665–1666.

Imanishi Y, Inaba M, Nakatsuka K, Nagasue K, Okuno S, Yoshihara A, et al. FGF-23 in patients with end-stage renal disease on hemodialysis. *Kidney International.* 2004;65:1943-1946.

Inomato S, Nukamoto Y, Inone M, Itoh M, Ohsawa Y, Masamuni O. Relationship between urinary excretion rate and renal histology in non insulin dependent diabetes mellitus: with reference to clinical significance of microalbuminuria. *Journal of Diabetic Complication.* 1989;3:172-8.

International Diabetes Federations. Global diabetes plan 2011-2021 [online]. 2011 [Citied 11 April 2012]. Available from http://www.idf.org/sites/default/files/Global_Diabetes_Plan_Final.pdf.

Isobe T, Saitoh S, Takagi S, Takeuchi H, Chiba Y, Katoh N, et al. Influence of gender, age and renal function on plasma adiponectin level: the Tanno and Sobetsu study. *European Journal of Endocrinology. 2005;*153: 91–98.

Iwai N, Kajimoto K, Kokubo Y, Tomoike H. Extensive Genetic Analysis of 10 Candidate Genes for Hypertension in Japanese. *Hypertension.* 2006;48:901-907.

Jaaskelainen E, Keski-Nisula L, Toivinen S, Paattiniemi EL, Helisalmi S, Punnonen K, et al. Polymorphism of the interleukin 1 receptor antagonist (IL1Ra) gene and placental abruption. *Journal of Reproductive Immunology.* 2008;79:58-62.

Jafar TH, Stark PC, Schmid CH, Landa M, Maschio G, de Jong PE, de Zeeuw D, Shahnaz S, Toto R, Levey AS. Progression of Chronic Kidney Disease: The Pole of Blood Pressure Control, Proteinuria, and Angiotensin-Converting Enzyme Inhibition. *Annals of Internal Medicine. 2*003;139:244-252.

Jha V. Herbal medicines and chronic kidney disease. *Nephrology.* 2009;15(s2):10-17.

Johnson DW, Jones GRD, Becker GJ, Mathew TH. Automated reporting of eGFR: a useful tool for identifying and managing kidney disease. *The Medical Journal of Australia.* 2009;190(4):200-203.

Johnstone DB, Zhang J, George B, Leon C, Gachet C, Wong H, et al. Podocyte-Specific Delection of Myh9 Encoding Nonmucle Myosin Heavy Chain 2A Predisposes Mice to Glomerulopathy. *Molecular and Cellular Biology.* 2011;31(10);2162-2170.

Kagami S, Border WA, Miller DE, Noble NA, Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor – beta expression in rat glomerular mesangial cells. *Journal of Clinical Investigation.* 1994;93:24321-2437.

Kaplan JJM, Kim SH, North KN, Rennke H, Correia LA, Tong HA, et al. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nature Genetics.* 2000;24(3):251-256.

Kandula P, Dobre M, Schold JD, Schreiber MJ, Mehrotra R, Navaneethan SD, et al. Vitamin D Supplementation in Chronic Kidney Disease: A Systematic Review and Meta-Analysis of Observational Studies and Randomized Controlled Trials. *Clinical Journal of the American Society of Nephrology.* 2011;6:50-62.

Kao L, Klag MJm Meoni LA, Reich D, Berthier-Schaad Y, Li M, et al. A genome-wide admixture scan identifies MYH9 as a candidate locus associated with non-diabetic end stage renal disease in African Americans. *Nature Genetics.* 2008;40(10):1185-1192.

Karin SSA, Churchyard GJ, Karim QA, Lawn SD, et al. HIV infection and tuberculosis in South Africa: an urgent need to escalate the public health response. *The Lancet.* 2009;9693(374): 921-933.

Katz I. Kidney & Kidney related Chronic Diseases in South Africa and Chronic Disease intervention program experiences. *Advances in Chronic Kidney Diseases.* 2005;12(1):14-21.

Khurana. Essentials of Medical Physiology. India: Elsevier India; 2008.

Kiberd BA, Clase CM. Cumulative risk for developing end-stage renal disease in the US population. Journal of the American Society of Nephrology. 2002;13:1635–1644.

Kollerits B, Fliser D, Heids IM, Ritz E, Kronenberg F. Gender-specific association of adiponectin as a predictor of progression of chronic kidney disease. *Kidney International.* 2007;71:1279-1286.

Komura N, Kihara S, Sonoda M, Maeda N, Tochino Y, Funahashi T, et al. Increment and impairment of adiponectin in renal failure. *Cardiovascular Research.* 2010;86(3):471-477.

Kopp JB, Smith MW, Nelson GW, Johnson RC, Freedman BI, Bowden DW, et al. MYH9 is a major-effect risk gene for focal segmental glomerulosclerosis. *Nature Genetics.* 2008;40(10): 1175-1184.

94

Kopp JB, Nelson GW, Sampath K, Johnson RC, Genovese G, An P, et al. APOL1 Genetic Variants in Focal Segmenatl Glomerulosclerosis and HIV-Associated Nephropathy. *Journal of the American Society of Nephrology.* 2010;22:2129-2137.

Köttgen A, Hwang J, Rampersaud E, Coresh J, North KE, Pankow JA, et al. TCF7L2 Variants Associate with CKD Progression and Renal Function in Population-Based Cohorts. *Clinical Epidemiology.* 2008;19:1989-1999.

Köttgen A, Glazer NL, Dehghan A, Hwang SJ, Katz R, Li, M, et al. Multiple Novel Loci are Associated with Indices of Renal Function and Chronic Kidney Disease. *Nature Genetics.* 2009;41(6):712-717.

Köttgen A, Hwang SJ, Larson MG, Van Eyk JE, Fu Q, Benjamin EJ, et al. Uromodulin Levels Associate with a Common UMOD Variant and Risk for Incident CKD. *Journal of the American Society of Nephrology.* 2010;21:337-344.

Kramer H, Bidani LA, Cao G, Cooper R, McGee D. Obesity and prevalent and incident CKD: the Hypertension Detection and Follow-up Program. *American Journal of Kidney Diseases.* 2005;46:587-594.

Kronenberg F, Konig P, Neyer U, Auinger M, Pribasnig A, Lang U, et al. Multicenter study of lipoprotein(a) and apolipoprotein(a) phenotypes in patients with end-stage renal disease treated by hemodialysis or continuous ambulatory peritoneal dialysis. *Journal of the American Society of Nephrology.* 1995;6:110–120.

Kronenberg F, Kuen E, Ritz E, Junker R, Konig P, Kraatz G, et al. *A*polipoprotein A-IV serum concentrations are elevated in mild and moderate renal failure. *Journal of the American Society of Nephrology.* 2002;13:461–469.

Kronenberg F. Emerging risk factors and markers of chronic kidney disease progression. *Nature Reveiws – Nephrology.* 2009;5:677-689.

Ksiazek P, Bednareck-Skublewska A, Buraczynska M. The C677T methylenetetrahydrofolate reductase gene mutation and nephrology in type 2 diabetes mellitus. *Medical Science Monitor.* 2004;10:BR47-BR51.

Kumada M, Kihara S, Sumitsuji S, Kawamoto T, Matsumoto S, Ouchi N, et al. Association of hypoadiponectinemia with coronary artery disease in men. *Arteriosclerosis Thrombosis Vascular Biology.* 2003;23:85–89.

Kurella M, Lo JC, Chertow GM. Disease amoung Nondiabetic Adults. *Journal of the American Society of Nephrology.* 2005;16:2134-2140.

Larsson T, Nisbeth U, Ljunggren O, Juppner H, Jonsson KB. Circulating concentration of FGF-23 increases as renal function declines in patients with chronic kidney disease, but doen not change in response to variation in phosphate intake in healthy volunteers. *Kidney International.* 2003;64:2272-2279.

95

LeRoith D. *Insulin-like Growth Factors and Cancer: From Basic Biology to Therapeutics.* Heidelberg:Springer; 2011.

Levey AS, Coresh J, Balk E, Kausz AY, Levin A, Steffes MW, et al. National Kidney Foundation Practice Guidelines for Chronic Kidney Disease: Evaluation, Classification, and Statification. *Annals of Internal Medicine*. 2003;139(2):137-149.

Levey AS, Eckardt KU, Tsukamoto Y, Levin A, Corech J, Rossert J, et al. Definition and Classification of chronic kidney disease: A position statement from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney International*. 2005;67:2089-2100.

Levey AS, Stevens LA, Schmid CH, Zhang L, Castro AF, Feldman HI, et al. A New Equation to Estimate Glomerular Filtration rate. *Annals of Internal Medicine.* 2009;150:604-612.

Levitt NS, Steyn K, Lambert EV, Reagon G, Lombard CJ, Fourie JM, et al. Modifiable risk factors for type 2 diabetes mellitus in a peri-urban community in South Africa. *Diabetes Medical.* 1999;16:946-950.

Li N, Stephens M. Modeling Linkage Disequilibrium and Identifying Recombination Hotspots Using Single-Nucleotide Polymorphism Data. *Genetics.* 2003;165:2213-2233.

Lindeman RD, Tobin J, Shock NW. Longitudinal studies on the rate of decline in renal function with age. *Journal of the American Geriatric Society*. 1985;33:278-285.

Locatelli F, Pozzini P, Vecchio L. Renal manifestations in the metabolic syndrome. *Journal of the American Society of Nephrology*. 2006;17:S81-S85.

Lunetta KL. Statistical Primer for Cardiovascular Research. *Circulation.* 2008;118:96-101.

Luttropp K, Lindholm B, Carrero JJ, Glorieux G, Schepers E, Vanholder R, et al. Genetics/Genomics in Chronic Kidney Disease - Towards personalized medicine. *Seminars in Dialysis*. 2009;22(4):417-422.

Luychx VA, Steenkamp V, Stewart MJ. Acute Renal Failure Associated with the Use of Traditional Folk Remedies in South Africa. *Renal Failure*. 2005;1:35-43.

Mackay IM. Real-time PCR in Microbiology: From Diagnosis to Characterization. United Kingdom: Horizon Scientific Press; 2007.

Madero T, Sarnack MJ. Creatinine-based formulae for estimating glomerular filtration rate: is it time to change to chronic kidney disease epidemiology collaboration equation? *Current Opinion in Nephrology & Hypertension.* 2011;20(6): 622-620.

Maki DD, Ma JZ, Louis TA, Kasiske BL. Long-term Effects of Antihypertensive Agents on Proteinuria and Renal Function. *Archives of Internal Medicine.* 1995;155(10):1073-1080.

Mañalich R, Reyes L, Herrera M, Melendi C, Fundora I. Relationship between weight at birth and the number and size of renal glomeruli in humans: A histomorphometric study. *Kidney International.* 2000;58:770-773.

Mandal AK. *Textbook of Nephrology for the Asian Pacific Physicians.* New Delhi: Jaypee Brothers Publishers; 2004.

Marculescu R, Endler G, Schillinger M, Lordanova M, Exner M, Hayden E, et al. Interleukin-1 Receptor Antagonist Genotype IS Associated With Coronary Atherosclerosis in Patients With Type 2 Diabetes. *Diabetes.* 2002;51(12):3582-3585.

Menon V, Li L, Xuelei W, Greene T, Balakrishnan V, Madero M, et al. Adiponectin and Mortality in Patients with Chronic Kidney Disease. *Journal of the American Society of Nephrology.* 2006;17:2599-2606.

Marks V, Mesko D. *Differential Diagnosis by Laboratory Medicine*, Berlin: Springer; 2002.

McCarthy MI, Hirschhorn JN. Genome-wide association studies: potential next steps on a genetic journey. *Human Molecular Genetics.* 2008;17(R2):R156-R165.

McKeigue PM. Mapping genes that underlie ethnic differences in disease risk methods for detecting linkage in admixed populations, by conditioning on parental admixture. *American Journal of Human Genetics.* 1998;63:241-251.

Mehrotra R, Kermah D, Fried L, Adler S, Norris K. Racial Difference in Mortality Among Thos with CKD. *Journal of the American Society of Nephrology.* 2008;19(7):1403-1410.

Mehrotra R, Kermah DA, Salusky IB. Chronic kidney disease, hypovitaminosis D, and mortality in the United States. *Kidney International.* 2009;76:977–983.

Michels WM, Grootendorst DC, Verduijn M, Elliot EG, Dekker FW, Krediet RT. Performance of the Cockcroft-Gault, MDRD, and New CKD-EPI Formulas in Relation to GFR, AGE, and Body Size. *Clinical Journal of the American Society of Nephrology.* 2010;5(6): 1003-1009.

Molony DA, Craig JC. *Evidence-based Nephrology - Volume 23 of evidence based medicine series*. New Jersey: John Wiley & Sons; 2008.

Morton NE. Sequential Tests for the Detection of Linkage. *American Journal of Human Genetics.* 1955;7(3):227-318.

Musso CG, Oreopoulos DG. Aging and Physiological Changes of the Kidneys Including Changes in Glomerular Filtration Rate. *Nephron Physiology*. 2011;119(1):1-5.

National Institute for Health and Clinical Excellence. *Chronic kidney disease Early identification and management of chronic kidney disease in adults in primary and secondary care* [Online]. 2008 [Citied 16 September 2011]. Available from: http://www.nice.org.uk/nicemedia/live/12069/42119/42119.pdf.

National Kidney Foundation. *KDOQI Clinical Practice Guidelines for Chronic Kidney Disease: Evaluation, Classification, and Stratification: Part 7 – Stratification of risk for progression of kidney disease and development of cardiovascular disease: Guideline 13* [Online]. 2006 [citied 16 April 2012]. Available from: http://www.kidney.org/professionals/kdoqi/guidelines_ckd/p7_risk_g13.htm

National Kidney foundation. *Chronic Kidney Disease [Online]*. 2010 [cited 10 October 2011] Available from: http://www.kidney.org/kidneydisease/ckd/index.cfm

Nelson GW, Freedman BI, Bowden DW, Langefeld CD, An P, Hicks PJ, et al. Dense mapping of MYH9 localizes the strongest kidney associations to the regions of introns 13 to 15. *Human Molecular Genetics.* 2010;19(9):1805-1815.

Naicker, S. End-stage renal disease in Sub-Saharan Africa. *Ethnicity and Disease.* 2009;19(1);S113-S115.

Nicholas SB, Tareen N, Zadshir A, Martines D, Pan D, Norris KC. Management of early chronic kidney disease in indigenous populations and ethnic minorities*. Kidney International.* 2005;68:S78-S81.

Nugenta RA, Fathima SF, Feigl AB, Chyung D. The Burden of Chronic Kidney Disease on Developing Nations: A 21st Century Challenge in Global Health. *Nephrology Clinical Practical*. 2011;188:c269-c277.

Nvengaard JR, Bendtsen TF. Glomerular number and size in relation to age, kidney weight and body surface in normal man. *The Anatomical Record*. 1992;232(2):194-201.

Okpechi I, Swanepoel C, Duffield M, Mahala B, Wearne N, Alagbe S, et al. Patterns of renal disease in Cape Town South Africa: A 10 year review of a single-centre renal biopsy database. *Nephrology Dialysis Transplant*. 2010;26(6): 1853-1861.

Orth SR, Hallan SI. Smoking: A risk factor for progression of Chronic Kidney Disease and for Cardiovascular Morbidity and Mortality in Renal patients - Absence of evidence or evidence of absence. *Clinical Journal of the American Society of Nephrology*. 2008;3:226-236.

98

O'Seaghdhal CM, Parekh RS, Hwang SJ, Li M, Kottenegn A, Coresh, J, et al. The MYH9/APOL1 region and chronic kidney disease in European-Americans. *Human Molecular Genetics* [Online]. 2011 [cited 10 May 2012]. Available from http://hmg.oxfordjournals.org/content/early/2011/04/12/hmg.ddr118.full

Perry MC. *The chemotherapy source book*. Pennsylvania: Lippincott Williams & Wilkins; 2008.

Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB, Rimm EB. Plasma adiponectin levels and risk of myocardial infarction in men. *The Journal of the American Medical Association.* 2004;291:1730–1737.

Pittas AG, Dawson-Hughes B, Li T, Van Dam RM, Willett WC, Manson JE, et al. Vitamin D and calcium intake in relation to type 2 diabetes in women. *Diabetes Care.* 2006;29:650–656.

Praga M, Hernandez E, Morales E, Campos AP, Valero MA, Martinez MA, et al. Clinical features and long-term outcome of obesity-associated focal segmental glomerulosclerosis. *Nephrology Dialysis Transplant.* 2001;16:1790-1798.

Provan D. *Oxford Handbook of Clinical and Laboratory Investigation,* Oxford: Oxford University Press; 2010.

Puone T, Steyn K, Bradshaw D, Laubscher R, Fourie J, Lambert V, et al. Obesity in South Africa: The South African Demographic and Health Survey. *Obesity Research.* 2002;10(10):1038-1048.

Puri D. *Textbook of Medical Biochemistry*. India: Elsevier; 2005.

Quintana-Murci L, Harmant C, Quach H, Balanovsky O, Zaporozhchenko V, Bormans C, et al. Strong Maternal Khoisan Contribution to the South African Coloured Population: A Case of Gender-Biased Admixture. *The American Journal of Human Genetics.* 2010;86(4):661-620.

Rao P, Reddy GC, Kanagasabapathy AS. Malnutrition-inflammation-atherosclerosis syndrome in chronic kidney disease. *Indian Journal of Clinical Biochemistry.* 2008;23(3):209-217.

Ravani P, Tripepi G, Malberti F, Testa S, Mallamaci F, Zoccali. Asymmetrical Dimethylarginine Predicts Progression to Dialysis and Death in Patients with Chronic Kidney Disease: A Competing Risks Modeling Approach. *Journal of the American Society of Nephrology.* 2005;16(8):2449-2455

Ravani P, Malberti F, Tripepi G, Pacchini P, Cutrupi S, Pizzini P, et al. Vitamin D levels and patient outcome in chronic kidney disease. *Kidney International.* 2009;75:88-95.

Reyes L, Mañalich R. Long-term consequences of low birth weight. *Kidney International.* 2005;68:S107-S111.

99

Richards M, Nicholls G, Espiner EA, Lainchbury JG, Troughton RW, Elliot J, et al. Comparison of B-Type Natriuretic Peptides for Assessment of Cardiac Function and Prognosis in Stable Ischemic Heart Disease. *Journal of the American College of Cardiology.* 2006;47(1): 52-60.

Ronco C, Brendolan A, Levin N. *Cardiovascular disorders in haemodialysis - Volume 149 of Contributions to Nephrology*. Basil: Karger Publishers; 2005.

Rule AD, Gussak HM, Pond GR, Bergstralh EJ, Stegall MD, Cosio FG, et al. Measured and estimated GFR in healthy potential kidney donors. *American Journal of Kidney Diseases.* 2004;43(1):112-119.

Sabolić I, Asif AR, Budach WE, Wanke C, Bahn A, Burckhardt G. Gender differences in kidney function. *European Journal of Physiology.* 2007;445(3):397-429.

Saleem MA, Zavadil J, Bailly M, McGee K, Witherden IR, Pavenstadt H, Hsu H, Sanday J, Satchell CS, Lennon R, Ni L, Bottinger EP, Peter M, Mathieson PW. The molecular and functional phenotype of glomerular podocytes reveals key features o contractile smooth muscle cells. *American Journal of Physiological: Renal Physiology.* 2008;295(4):F959-F970.

Saraheimo M, Forsblom C, Fagerudd J, Teppo AM, Pettersson-Fernholm K, Frystyk
J, et al. Serum adiponectin is increased in type 1 diabetic patients with nephropathy. *Diabetes Care.* 2005;28:1410–1414.

Saraheimo M, Forsblom C, Thorn L, Wad'en J, Rosengård-Bärund M, Heikkil O, et al. Serum Adiponectin and Progression of Diabetic Nephropathy in Patients With Type 1 Diabetes. *Diabetes Care.* 2008;31:1165:1169.

Sayin BE, Bayram B, Turkoglu Z, Kelestemur U, Mutlu F. Angiotensin Converting Enzyme (ACE) Gene I/D Polymorphism Genotypes and ACE Levels of Serum and Synovial Fluid of Patients with Osteoarthritis. *Journal of Pharmaceutical Sciences.* 2009;34:74-81.

Schliwa M. *Molecular Motors.* New Jersey: John Wiley & Sons; 2006.

Schmitz PG, O'Donnell MP, Kasiske BL, Katz SA, Keane WF. Renal injury in obese Zucker rats: glomerular hemodynamic alterations and effects of enalapril. *American Journal of Physiology – Renal Physiology.* 1992;263(3):F496-F502.

Schrier, RW. Renal and Electrolyte Disorders. Baltimore: Lippincott Williams & Wilkins; 2010.

Schulze MB, Shai I, Rimm EB, Li T, Rifai N, Hu FB. Adiponectin and future coronary heart disease events among men with type 2 diabetes. *Diabetes.* 2005;54: 534–539.

Seishima M, Muto Y. An increased apo A-IV serum concentration of patients with chronic renal failure on hemodialysis. *Clinica Chimica Acta.* 1987;167:303–311.

Sharma K, Ramachandrarao S, Qiu G, Usui HK, Zhu Y, Dunn SR, et al. Adiponectin regulates albuminuria and podocyte function in mice. *Journal of Clinical Investigation.* 2008;118:1645–1656.

Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Research and Clinical Practice.* 2010;87(1):4-14.

Shen Y, Peake PW, Kelly JJ. Should we quantify insulin resistance in patients with renal disease? *Nephrology.* 2005;10:599–605.

Shils ME, Shike M, Ross C, Caballero B, Cousins RJ. Modern Nutrition in Health and Disease. Baltimore:Lippincott Williams and Wilkins; 2005.

Shimazaki A, Kawamura Y, Kanazawa A, Sekine A, Saito S, Tsunoda T, et al. Genetic Variations in the Gene Encoding ELMO1 Are Associated With Susceptibility to Diabetic Nephropathy. *Diabetes.* 2005;54(4):1171-1178.

Silbiger SR, Neugarten J. The impact of gender on the progression of chronic renal disease. *American Journal of Kidney Diseases.* 1995;25(4):515-533.

Silha JV, Krsek M, Skrha JV, Sucharda P, Nvomba BL, Murphy LJ. Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. *European Journal of Endocrinology.* 2003;149(4):331-335.

Silva FG. The ageing kidney: a review. *International Urology and Nephrology.* 2005;37:185-205.

Sinclair AM, Isles CG, Brown I, Cameron H, Murray GD, Robertson JW. Secondary hypertension in a blood pressure clinic. *Archives of Internal Medicine.* 1987;147:1289-1293.

Snyder S, Pendergraph B. Detection and Evaluation of Chronic Kidney Disease. *American Family Physician.* 2005;72(9):1723-1732.

Smith MW, O'Brien SJ. Mapping by admixture linkage disequilibrium; advantages, limitations and guidelines. *Nature Review Genetics.* 2005;6(8):625-632.

South African Dialysis and Transplantation Registry. *Combined report on maintainance dialysis and transplantation in the Republic of South Africa,* Cape Town: SADTR. 1994.

Southern African Hypertension Society. *Hypertension* [Online]. 2011[citied 5 September 2011]. Available from: http://www.hypertension.org.za/"http://www.hypertension.org.za/

Stengel B, Terver-Carr ME, Powo NR, Eberhardt MS, Brancati FL. Lifestyle factors, obesity and the risk of chronic kidney disease. *Epidemiology.* 2003;14:479-487.

Stevens LA, Coresh J, Schmid CH, Feldman HI, Froissart M, Kusek J, et al. Estimating GRF using Serum Cystatin C Alone and in Combination with Serum Creatinine: A Pooled Analysis of 3418 Individuals with CKD. *American Journal of Kidney Disease.* 2008;51(3):395-406.

Stevens LA, Levey AS. Current Status and Future Perspectives for CKD Testing. *American Journal of Kidney Diseases.* 2009;53(S3):S17-S26.

Straussman R, Acov AB, Woolfson DN, Ravid S. Kinking the Coiled Coil-Negatively Charged Residues at the Coiled-coil Interface. *Journal of Molecular Biology.* 2007;366(4):1232-1242.

Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. *The Lancet.* 2005;365(9467):1333-1346.

Sturtevant A. The linear arrangement of six sex-linked factors in Drosophila, as shown by their mode of association. *Journal of Experimental Zoology.* 1913;14:43-59.

Takami Y, Horio T, Iwahima Y, Takiuchi S, Kamide K, Yoshihara F, et al. Diagnostic and prognostic value of plasma brain natriuretic peptide in non-dialysis-dependent CRF. *American Journal of Kidney Disease.* 2004;44(3):420-428.

Tzur S, Rosset S, Shemer R, Yudkovsky G, Selig S, Taregn A, et al. Missense mutations in the APOL1 gene are highly associated with end stage kidney disease risk previously attributed to the MYH9 gene. *Human Genetics.* 2010;128(3):345-350.

Vallance P. Importance of asymmetrical dimethylarginine in cardiovascular risk. *Lancet.* 2001;358:2096-2097.

Van Deventer HE, Goerge JA, Paiker JE, Becker PJ, Katz IJ. Estimating Glomerular Filtration Rate in Black South Africans by Use of the Modification of Diet in Renal Disease and Cockcroft-Gault Equations. *Clinical Chemistry.* 2008;54(7):1197-1202.

Van Guilder HD, Vrana KE, Freeman WM. Twenty-five years of quantitative PCR for gene expression analysis. *BioTechniques.* 2008;44(5):619-626.

Van Pelt-Verkuil E, van Belkum A, Hays JP. Principles and Technical Aspects of PCR Amplification. Germany: Springer; 2008.

Van Timmeren MM, van den Heuvel MC, Bailly V, Bakker SJL, van Goor H, Stegeman CA. Tubular kidney injury molecule-1 (KIM-1) in human renal disease. *The Journal of Pathology*. 2007;212(2):209-217.

Variava Y, Du Toit E, Lawley CG, Milne FJ, Reinach SG. Hypertension as a cause of end stage renal failure in South Africa. *Journal of Hypertension*. 1990;4:379-383.

Waanders F, van Timmeren MM, Stegeman CA, Bakker SJL, van Goor H. Kidney injury molecule-1 in renal disease. *Journal of Pathology*. 2010;220:7–16.

Wadsworth ME, Cripps HA, Midwinter RE, Colley JR. Blood pressure in a natural birth cohort at the age of 36 related to the social and familial factors, smoking, and body mass. *British Medical Journal – Clinical Research Edition*. 1985;291(6508):1534-1538.

Weinberg RB, Spector MS. Human apolipoprotein A-IV: displacement from the surface of triglyceride-rich particles by H DL2-associated C-apoproteins. *Journal of Lipid Research*. 1985;26:26-37.

White S. How can we achieve global equity in provision of renal replacement therapy. *Bulletin of World Health Organisation*. 2008;86(3):161-240.

Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*. 2004;27:1047-1053.

Wimm MD, Conlon PJ, Lynn KL, Faskalakis N, Kwansy E, Berviller S, et al. A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science*. 2005;308(5729):1801-1804.

Wolf G. After all those fat years: renal consequences of obesity. *Nephrology Dialysis Transplant*. 2003;18(12):2417-2474.

Wolf M. Active Vitamin D and survival. *Journal of the American Society of Nephrology*. 2008;19(8):1442-1443.

Wong C, Kanetsky P, Raj D. Genetic polymorphisms of the RAS-cytokine pathway and chronic kidney disease. *Paediatric Nephrology*. 2008;23:1037-1051.

World Health Organization. *Obesity: preventing and managing the global epidemic. Report of a consultation on obesity* [Online]. 1998 [citied 24 June 2012]. Available from: http://libdoc.who.int/trs/WHO_TRS_894.pdf

World Health Organisation. International Society of Hypertension Guidelines for the Management of Hypertension. *Journal of Hypertension*. 1999;17:151-183.

World Health Organization. *GLOBAL HEALTH RISKS: Mortality and burden of disease attributable to selected major risks* [Online]. 2004 [Citied 16 September 2011]. Available from:

103

http://www.who.int/healthinfo/global_burden_disease/GlobalHealthRisks_report_full.pdf.

World Health Organization. *Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia* [Online]. 2006 [Citied 13 September 2011]. Available from:
http://www.who.int/diabetes/publications/Definition%20and%20diagnosis%20of%20diabetes_new.pdf.

World Health Organization. *Obesity and Overweight* [Online]*.* 2006 [citied 24 June 2012]. Available from: www.who.int/dietphysicalactivity/publications/facts/obesity/en/

Yoshida H, Mitarai T, Kawamura T, Kitajma T, Miyazaki Y, Nagasawa R, et al. Role of the deletion polymorphism of the angiotensin-converting enzyme gene in the progression and therapeutic responsiveness of IgA nephropathy. *Journal of Clinical Investigation.* 1995;96:2162-2169.

Zehnder D, Bland R, Walker EA, Bradwell AR, Howie AJ, Hewison M, et al. Expression of 25-Hydroxyvitamin D3-1a-Hydroxylase in the Human Kidney. *Journal of the American Society of Nephrology.* 1999;10(12):2465-247.

Zhang QL, Rothenbacher D. Prevalence of chronic kidney disease in population-based studies: systemic review. *Biomed Central: Public Health.* 2008;8(117).

Ziyadeh F. Mediators of Diabetic Renal Disease: The Case for TGF-β as the Major Mediator. *Journal of American Society of Nephrology.* 2004;115:S55-S57.

Zoccali C, Bode-Böger S, Mallamaci F, Benedetto F, Tripepi G, Malatino L, et al. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. *Lancet.* 2001;358(9299):2113-2117.

Zoccali C, Mallamaci F, Tripepi G, Benedetto F, Cutrupi S, Parlongo S, et al. Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease. *Journal of the American Society of Nephrology.* 2002;13:134–141.

Zoccali C, Mallamaci F, Panuccio V, Tripepi G, Cutrupi S, Parlongo S, et al. Adiponectin is markedly increased in patients with nephrotic syndrome and is related to metabolic risk factors. *Kidney International Supplementary.* 2003;63(84):S98–S102.

104

CHAPTER 5

[Addenda]

ADDENDUM A – ETHICS CLEARANCE

01 August 2012   **MAILED**

Prof RT Erasmus
Department of Pathology
Division Chemical Pathology
9th Floor
Tygerberg Hospital

Dear Prof Erasmus

Occurrence of CKD and its association with the MYH9 gene in a South African population

ETHIC REFERENCE NO: N10/05/142

RE : APPROVED

It is a pleasure to inform you that a review panel of the Health Research Ethical Committee has approved the above-mentioned project on 1 August 2012, including the ethical aspects involved, for a period of one year from this date.

This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in ALL future correspondence. You may start with the project. Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary.

Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No. 61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Please note that for research at primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Hélène Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

106

Approval date: 1 August 2012   Expiry date: 1 August 2013

Yours faithfully,

<u>MRS MERTRUDE DAVIDS</u>
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / Email: mertrude@sun.ac.za
Fax: 0219313352

107

ADDENDUM B – MAIN STUDY QUESTIONNAIRE

Cape Peninsula University of Technology

GLUCOSE INTOLERANCE AND DIABETES QUESTIONNIARE

Name of Interviewer: …………………………….

Date of Interview: ……./…..../….... **Ref No ……**

To the respondent:
Thank you very much for your willingness to participate in the completion of this questionnaire. The information obtained on this questionnaire will provide us with information on all the possible health, family, life style and dietary risk factors within your house hold that might influence the development of diabetes. This is because many health conditions develop slowly over time yet could be prevented if diagnosed early or if pre determined. This questionnaire therefore aims at getting information which may be used to determine the extent of diabetes and those likely to develop diabetes in the future. The questionnaire should not take long and we hope you find it interesting and enjoyable. All answers provided will be treated as confidential and anonymous.

Note
No special knowledge is needed to fill this questionnaire. Please feel free to ask for clarification if needed.

Postal Address: --

Residential address: --

Telephone OR
Cell phone Contacts: --

108

A. PERSONAL DATA

Instructions:
Please complete the following general information about yourself by ticking in the box next to the appropriate answers. Please take your time and read through questions carefully.

1. What is your date of birth? ☐☐☐☐☐☐

2. What is your gender?

     Male ☐   Female ☐

3. What is your marital status?

     Married ☐   Single ☐   Widowed ☐

     Divorced ☐   Other ☐

4. How would you describe yourself?

     Black ☐   White ☐

     Colored ☐   Asian ☐

5. What is the highest level of education you have completed?
     (a) Primary School or less ☐
     (b) High School (Not Completed) ☐
     (c) High School graduate ☐
     (d) College Or Technical College (Not Completed) ☐
     (e) College or Technical College Graduate ☐
     (f) University or technikon (Not Completed0 ☐
     (g) University or technikon graduate ☐

6. What is your Profession/Occupation?
Please state………………………………………………………………..
7 How long have you been living in Bellville South?

     Less than 6 Months ☐   Less than 1 Year ☐

     1-5 Years ☐   5 years and above ☐

109

B. FAMILY HEALTH HISTORY

Instructions:

The following questions will tell us about your family health history. Please complete all the questions by placing a tick next to the appropriate answer or writing in the answer.

8. Are you currently on any medication? Yes ☐   No ☐

9. If Yes, Please list………………………………………………………………
…………………………………………………………………………………………
…………………………………………………………………………………………
…………………………………………………………………………………………

10. Have you ever been told that you have diabetes?

Yes ☐   No ☐

11. Have any of the following in your family ever had or are being treated for   diabetes?

(a) Mother
      Yes ☐   No ☐

(b) Father
      Yes ☐   No ☐

(c) Sister(s)
      Yes ☐   No ☐

(d) Brother(s)
      Yes ☐   No ☐

(e) Husband/Wife
      Yes ☐   No ☐

(f) Children
      Yes ☐   No ☐

(g) Grandchildren
      Yes ☐   No ☐

110

12. Have any of the following extended family members ever suffered or are suffering from diabetes?

 (a) Paternal (Fathers Side)
   (i) Uncles

                    Yes ☐   No ☐

   (ii) Aunties

                    Yes ☐   No ☐

  (iii) Grandparents

                    Yes ☐   No ☐

 (b) Maternal (mothers Side)
     (i) Uncles

                    Yes ☐   No ☐

   (ii) Aunties

                    Yes ☐   No ☐

  (iii) Grand Parents

                    Yes ☐   No ☐

13. Have you or any of the following ever been treated for heart problems?

   (a) Yourself
                    Yes ☐   No ☐

   (b) Spouse
                    Yes ☐   No ☐

   (c) Mother
                    Yes ☐   No ☐

   (d) Father
                    Yes ☐   No ☐

111

(e) Children

    Yes ☐   No ☐

(f) Grandparents

    Yes ☐   No ☐

(g) Sisters

    Yes ☐   No ☐

(h) Brothers

    Yes ☐   No ☐

14. Have any of the following ever been treated for High Blood pressure?

(a) Yourself

    Yes ☐   No ☐

(b) Spouse

    Yes ☐   No ☐

(c) Mother

    Yes ☐   No ☐

(d) Father

    Yes ☐   No ☐

(e) Children

    Yes ☐   No ☐

(f) Grandparents

    Yes ☐   No ☐

(g) Sisters

    Yes ☐   No ☐

(h) Brothers

    Yes ☐   No ☐

112

15. Did either of your natural parents ever die of a heart attack?

  (a) Before the age of 60?

          Yes ☐   No ☐

  (b) After the age of 60?

          Yes ☐   No ☐

16. Have you or any of the following ever been treated for High Cholesterol?

   (a) Yourself

          Yes ☐   No ☐

   (b) Spouse

          Yes ☐   No ☐

   (c) Mother

          Yes ☐   No ☐

   (d) Father

          Yes ☐   No ☐

   (e) Children

          Yes ☐   No ☐

   (f) Grandchildren

          Yes ☐   No ☐

   (g) Brother

          Yes ☐   No ☐

   (h) Sisters

          Yes ☐   No ☐

113

E. ALCOHOL USE

22. Have you ever consumed any alcoholic drinks (Wine, Beer, and Spirits)?

Yes ☐   No ☐

23. Do you still consume alcoholic drinks?

Yes ☐   No ☐

24. If you consume or consumed alcohol, how old were you when you first   started drinking?
Please state.   …………years

25. If you stopped, how old were you when you stopped drinking?
Please state.   ………

26. Which type of alcohol do you or did you drink?

Wine ☐   Beer ☐   Spirits ☐

☐ Others, please indicate………………………

27. When you drink or drank alcoholic drinks, how many drinks or glasses
do you or did you consume daily? Indicate the number…..

28. How many days a week do you or did you consume alcohol?

1-2 ☐   3-4 ☐   5-6 ☐ every day   ☐

29. Have you or did you ever feel you should cut down your drinking?

Yes ☐   No ☐

30. Have people ever annoyed you by criticizing your drinking?

Yes ☐   No ☐

31. Have you ever felt bad about your drinking?

Yes ☐   No ☐

32. Have you ever had a drink first thing in the morning to steady your nerves or get rid of a hangover (Eye Opener)

Yes ☐   No ☐

114

F. BODY MEASUREMENTS.   **Interviewers Name……………………**

33. Weight and Height.

Body Weight (kg)			
Body height (cm)			
Body Mass Index			

34. **CALLIPERS MEASUREMENTS**

Biceps 1 (cm)		
Biceps 2 (cm)		
Biceps 3 (cm)		
Total		
Triceps 1 (cm)		
Triceps 2 (cm)		
Triceps 3 (cm)		
Total		
Sub-Scapular 1 (cm)		
Sub-Scapular 2 (cm)		
Sub-Scapular 3 (cm)		
Total		
Suprailiac 1 (cm)		
Supra-iliac 2 (cm)		
Supra- Iliac 3 (cm)		
Total		

35. CIRCUMFERENCE MEASUREMENTS
36.

Waist Circumference 1 (cm)			
Waist Circumference 2 (cm)			
Waist Circumference 3 (cm)			
Total			

Hip Circumference (cm)			
Hip Circumference (cm)			
Hip Circumference (cm)			
Total (cm)			

37. **BLOOD PRESSURE MEASUREMENTS. Interviewers Name………**

Systolic Pressure 1 (mmHg)			
Systolic Pressure 2 (mmHg)			
Systolic Pressure 3 (mmHg)			

Diastolic Pressure 1 (mmHg)			
Diastolic Pressure 2 (mmHg)			
Diastolic Pressure 3 (mmHg)			

115

Pulse 1 (Beat per Minute)			
Pulse 2 (Beat per Minute)			
Pulse 3 (Beat per Minute)			

G. BLOOD ANALYSIS.

Interviewers Name…………………..

38. (a) Fasting State Measurements

(i) Did subject eat this morning?........... If yes state time…………..

(ii) When did subject eat the last meal last evening?..........................

(iii) Please indicate the time when fasting blood taken………………

(iv) Please indicate the time when Glucose was given...…………...

Glucose mmol/l		
Cholesterol mmol/l (L=1, N=2, H=3)		
Triglycerides mmol/l (L=1, N=2, H=3)		

(b) Post Prandial Measurements

Please indicate time when post pradial blood was taken…….

Glucose mmol/l		
Cholesterol mmol/l		
Triglycerides mmol/l		

39. **URINALYSIS**

Glucose (N=Negative, P= Positive)		P		N	
Protein (N=Negative, P=Positive)		P		N	

116

H. Researchers Check list.

Please make a tick or cross against each of the following questions

1. Were all personal data questions answered?

2. Were all Family Health History questions answered?

3. Were all questions on Tobacco use answered?

4. Were all questions on Alcohol use answered?

5. Were all dietary questions answered?

6. Were all body measurements carried out?

7.
(a) Was fasting blood taken?

(b) Was Glucose Given?

8. Was Post Pradial Blood taken?

9. Was B.P Taken

ADDENDUM C – CONSENT FORM FROM MAIN STUDY

Cape Peninsula University of Technology

THE BELLVILLE SOUTH DIABETES SURVEY CONSENT FORM

Investigators:	Prof Matsha (CPUT)
	Mr. Shafick Hassan (CPUT)
	Prof Rajiv Erasmus (University of Stellenbosch)
	Mr Soita David Jonah (MTech student)
Address:	Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology (CPUT), Bellville Campus Symphony Way, 7535
	Chemical Pathology Department, Faculty of Health Sciences, University of Stellenbosch (Tygerberg Campus), Tygerberg, 7505.
Contact Numbers:	Mr SD Jonah – 072 555 8628
	Mr Hassan – 021 959 6274
	Prof Erasmus – 021 938 4107
	Prof Matsha – 021 959 6366

Dear Participant,

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied and that you clearly understand what this research entails and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do initially agree to take part.

This study has been approved by the Committee for Human Research at Cape University of Technology and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

Worldwide new causes of certain diseases or conditions are continuously being discovered by research on the cells and molecules of the body. For research to be carried out on

certain diseases it is necessary to first establish the incidence and prevalence of the disease. This project aims to determine the incidence and prevalence of diabetes and glucose intolerance. Additionally, this project aims to collect genetic material (blood) to analyze for certain variants and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

Why have you been invited to participate?

The prevalence of diabetes of diabetes in South Africa is not well documented and few studies have been conducted since 1994. Many subjects with diabetes are unknown to the health service, often because they are not yet diagnosed. In order to assess the magnitude of the problem, you have been approached to participate in this project to determine the incidence of diabetes amongst our adult population.

You have randomly been selected by means of a computer program to participate in the above-mentioned study. Adults of all races, gender, age (between ages 35 and 60 years) and weight will be approached as subjects.

What will your responsibilities be?

The participant will be requested to provide information about his/her medical history, family history and information on eating, drinking and smoking habits. Completion of the questionnaire will take no longer than 10 minutes. Measurement such as weight, height, waist and hip will be done. Fasting Venous Blood will be collected thereafter you will be asked to drink a glucose solution (glucose content 75g). After two hours another venous blood will be collected. The blood will be used to determine whether you have diabetes or glucose intolerance. The other tests that will be determined from your blood sample are: Cholesterol and Triglycerides levels. The remainder of the blood sample will be used for genetic and future research studies. The DNA may be stored for several years until the technology for meaningful analysis becomes available. No pharmaceutical agent (medication) will be tested in the study.

Will you benefit from taking part in this research?

You will be notified of your glucose tolerance state or whether you are diabetic by the medical nurse or doctor. Thereafter, you will be referred to your local health centre or general practitioner for further investigations and treatment.

In the unlikely event that the research may lead to the development of commercial applications, the participant or the participant's heirs will not receive any compensation, but profits will be reinvested into supporting the cause of further research which may bring benefits to my/*the participant's family and to the community, such as health screening, medical treatment, educational promotions, etc.

Are there any risks involved in my taking part in this research?

A slight bruising might occur after blood has been drawn from the arm but this will heal quickly. After the administration of the glucose solution, you may feel nauseous and dizzy in which case you must notify the medical personnel. A medical nurse will be present on all occasions. In addition, the research team will be in contact with medical doctors should you need emergency care by a medical doctor.

Who will have access to your medical records?

119

The participant's identity will be kept confidential throughout. Information will not be associated with the participant's name. The research staff will use only a coded number, access will be limited to authorized scientists and any scientific publications, lectures or reports resulting from the study will not identify me/*the participant.

Some insurance companies may mistakenly assume that taking part in research indicates a higher risk for disease. Thus no information about you or your family will be shared with such companies.

Will you or your child be paid to take part in this study and are there any costs involved?

You will not be paid to take part in the study, but your transport, if required will be covered for each study visit. It is envisaged that you may be hungry since you would have come fasting, therefore, biscuits or fruit will be provided. There will be no costs involved for you if you take part in the project.

Is there anything else that you should know or do?

You should inform your family practitioner or usual doctor that you are taking part in a research study.
You will receive a copy of this information and consent form for your own records if it is requested

DECLARATION BY PARTICIPANT:

I declare that:

I have read or had read to me this information and consent form and that it is written in a language with which I am fluent and comfortable.
I have had a chance to ask questions and all my questions have been adequately answered.
I understand that taking part in this study is voluntary and I have not been pressurized to take part.
I may choose to withdraw from the study at any time and will not be penalized or prejudiced in any way.
I may be asked to leave the study before it has finished if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan as agreed to.

I also consent that my blood may be:
- ♦ **Used and stored for future research studies** ☐

- ♦ **Used and discarded** ☐

Signed at (*place*) ....…......……………….. on (*date*) …………...……….. 2007

Signature of participant_____ **Signature of witness**_____

120

DECLARATION BY THE INVESTIGATOR

I *(name)* …………………………………………..……… declare that:

I explained the information in this document to (*Names of participant*)……………………………….………
I encouraged him/her to ask questions and took adequate time to answer them.
I am satisfied that he/she adequately understand all aspects of the research, as discussed above

Signed at (*place*) ....…......……………. on (*date*) …………....……….. 2007.

Signature of investigator_____   **Signature of witness**_____

ADDENDUM D – SOLUTIONS

Lysis Buffer- pH 7.4

31 ml from NH_4Cl (1M stock)

1 ml of $KHCO_3$ (1M stock)

100µl of EDTA (100mM stock)

Phosphate Buffered Saline (PBS)- pH 7.4

0.2 g   KCl

8.0 g   NaCl

0.2 g KH_2PO_4

1.15 g NA_2HPO_4

Add all components, one at a time to 900 ml of dH_2O, then dissolve by adding dH_2O to 1L,

Nuclear Lysis Buffer (500 ml)- pH 8.2

11.5 g NaCl

10 ml Tris (1M stock)

10 ml EDTA (10mM stock).

1% (w/v) Sodium Dodecyl Sulphate (pH 7. 2)

1 g of electrophoresis-grade SDS was dissolved in 100 ml dH_2O.

The solution was heated and stirred with a magnet stirrer to assist dissolution.

122

6 M Sodium Chloride (NaCl)

350.64 g of NaCl was dissolved in 800 ml of dH$_2$O and then the volume was adjusted to 1 L with dH$_2$O.

1X Tris EDTA (TE) Buffer

10 mM Tris   (10 ml 1 mM stock)

1 mM EDTA (2 ml of 0.5M stock)

Made up to 1 L with dH$_2$O

Ethidium Bromide (EtBr) Stain (10 mg/ml)

1 g EtBr in 100 ml dH$_2$O was added together and stored in a dark bottle

Loading Buffer (Bromophenol blue)

0.2 g (2%) BPB powder

1 ml (10 mM) of 1M Tris stock (pH 8.0)

50 ml (50%) Glycerol

49 ml dH$_2$O

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