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Malindisa, Sibusiso Tebogo

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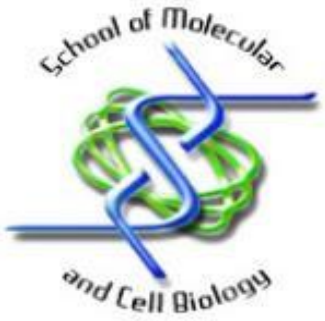
Genetic insights on the role of telomere dynamics in Chronic Kidney Disease (CKD) regardless of HIV status

Sibusiso Tebogo Malindisa

(480924)

Supervisor: Dr. B. T. Letsolo





A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree in Master of Science in the School of Molecular and Cell Biology.

Declaration

I, **Sibusiso Tebogo Malindisa** (**480924**), am a student registered for the degree of **MSc (Dissertation)** within the school of **Molecular and Cell Biology** in the academic year **2015**.

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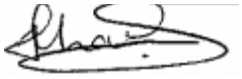
Signature_  _____ day of __4th March_____ **YEAR** __2016__

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Abstract

Telomeres play significant roles in maintaining genome stability, regulating cell proliferation and apoptosis. The role of telomere biology and telomerase reactivation has been studied extensively in cancers. Telomerase has been previously associated with driving chronic kidney disease (CKD) advancement and most frequently due to HIV infection. However, the mechanism by which telomerase activation contributes towards disease progression beyond its canonical function of telomere maintenance is poorly understood. Telomerase is a ribonucleoprotein whose main function is telomere maintenance. Telomerase activity is dependent on expression of the rate-limiting human telomerase reverse transcriptase (hTERT) component. In addition to telomere maintenance, *hTERT* is implicated in other non-telomere related functions that promote cellular proliferation. Expression of *hTERT* is predominantly regulated at the transcription level where variation in promoter and minisatellite (MNS16A) sequences alter its expression. This variation has been implicated to confer susceptibility to diseases such as cancer and ageing disorders in non-African populations. Data on variation and pathogenicity of telomere-associated genes in African populations is limited and warrants further research. Thus bioinformatics analysis was performed to elucidate variation within the human *TERT* gene and promoter in different populations. The promoter, MNS16A and relative telomere length (RTL) were also evaluated in 159 African study participants with and without CKD. TERT common variants are equally distributed across populations with limited data on connection to the effects of the variants in African populations. Further bioinformatics analyses revealed significant difference ($p<0.0001$) in distribution of promoter variant rs2853669 between African and non-African populations. No common promoter mutations were identified in our study population. Interestingly, the long MNS16A variant suggested to increase *TERT* expression was significantly overrepresented in individuals with CKD regardless of HIV status. For the first time, a strong association of the long MNS16A variant with CKD regardless of HIV status is reported, implicating MNS16A as a potential risk factor in CKD.

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List of Abbreviations

°C	Degrees Celsius
µL	microlitres
36B4	Acidic ribosomal protein
AA	Aplastic anaemia
AIDS	Acquired immune-deficiency Syndrome
AML	Acute myeloid leukemia
APOL1	Apolipoprotein 1
Brg1	Brahma-related gene 1
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CKD	Chronic kidney disease
DC	Dyskeratosis Congenita
DKC1	Dyskerin
DMEM	Dulbecco's modified medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNTPs	Deoxyribonucleotides
dsDNA	double stranded DNA
EDTA	Ethylene diamine tetra acetic acid
eGFR	estimated Glomerular Filtration Rate
ESRD	End stage renal disease
FBS	Foetal bovine serum
FFPE	Formalin Fixed Paraffin Embedded
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome wide association studies

HAART	Highly active antiretroviral treatment
HEK293	Human embryonic kidney cell line
HIV	Human Immunodeficiency Virus
HIVAN	HIV-associated nephropathy
hTERC	human telomerase RNA component
hTERT	human telomerase reverse transcriptase
IPF	Idiopathic pulmonary fibrosis
Kb	Kilobase
L	Litre
MDA-MB-231	Metastatic human breast cancer cell line
mL	millilitres
MNS16A	hTERT minisatellite
MRC5	Human lung fibroblast cell line
mRNA	Messenger RNA
MW	Molecular Weight
MYH9	Myosin heavy chain 9
NCBI	National Centre for Biotechnology Information
NF-KB	Nuclear Factor Kappa B
ng	Nanograms
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCA	Principle Component Analysis
POT1	Protection of Telomeres 1
RLUC	Universal negative control esiRNA
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
SD	Standard deviation
SDS	Sodium dodecyl sulphate
siRNA	Short interfering RNA

SNP	Single Nucleotide Polymorphism
ssDNA	single stranded DNA
STELA	Single telomere length analysis
TAE	Tris-Acetate EDTA
TB	Tuberculosis
TBE	Tris-Borate EDTA
TE	Tris-EDTA
UN	United Nations
UNAIDS	Joint United Nations Programme on HIV/AIDS
US	United States
V	Volts
VNTR	Variable number tandem repeats
WHO	World Health Organisation
β-Globin	Beta globin

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Chapter 1: Literature Review/Introduction

1.1. Background to the study

Human immunodeficiency virus (HIV) is still one of the leading causes of death in Africa (Sharp and Hahn, 2011). Ever since the emergence of HIV many diseases have become common and have been shown to be familiarly associated with HIV infection. Chronic kidney diseases (CKD) continue to be important determinants of morbidity and mortality in both HIV infected and uninfected individuals (Gupta et al., 2005). Telomeres and telomerase have been implicated in driving kidney disease progression especially in the presence of HIV infection (Shkreli et al., 2012). The introduction below highlights the relevance of telomere biology in kidney disease. However there is limited data regarding the role of telomeres and telomerase in disease progression and severity. Thus emphasizing the need of this study to assess telomere and telomerase genetics as potential biomarkers in kidney disease.

1.2. Human Immunodeficiency Virus

1.2.1. What is HIV?

HIV is a virus that causes Acquired Immunodeficiency Syndrome (AIDS). HIV has spread all over the world and has caused one of the deadliest pandemics in human history since its discovery in the 1980s (Barre-Sinoussi et al., 1983; Duesberg, 1988; Gallo et al., 1984). HIV is a retrovirus distinguished by its slow persistent replication (Gendelman et al., 1985). HIV has the ability to infect the immune system cells whose function is to protect the body against any foreign infectious molecules (Ho et al., 1988). This causes the immune system to fail and thus weakens the body and increases susceptibility to life-threatening infections. Destruction of the immune system, the main characteristic of the disease, causes patients to die from a range of opportunistic infections. As the opportunistic infections that occur reflect the prevalence of given pathogens in the afflicted population, tuberculosis has been one of the most common outcomes in Africa (Corbett et al., 2003; Girardi et al., 1997; Raviglione et al., 1997).

1.2.2. HIV: epidemiology, treatment and related diseases

Although HIV/AIDS has been recognised as a global epidemic, Africa still has the highest rates. The 2015 AIDS global epidemic report statistics released by the UNAIDS has revealed that Sub-Saharan Africa accounts for 70% of global HIV infections even though it has only 10% of the global population (http://www.unaids.org). This shows that the burden of HIV in this area is enormous. Moreover, in 2013 alone, South Africa had the second highest AIDS related deaths globally after Nigeria (**Fig. 1**).

The HI virus has different subtypes represented in two major groups namely; HIV type one (HIV-1) and type two (HIV-2). HIV-1 is the most common and pathogenic strain of the virus. Unlike HIV-1; HIV-2 is less pathogenic and is mostly found in West Africa (Gnann et al., 1987). The HIV-1 strain has several subtypes classified as HIV-1 subtypes A to K. Subtype C is the most common HIV strain in sub-Saharan Africa, India and Somalia. In Europe and America, subtype B and others are in high prevalence (Gnann et al., 1987).

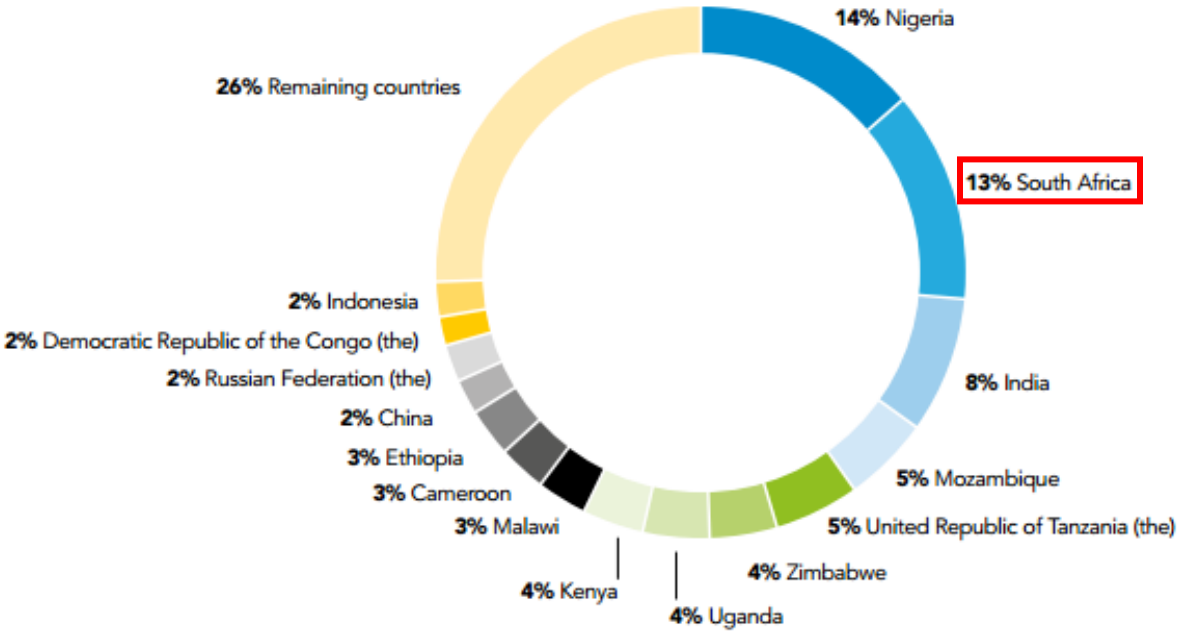


Figure 1: Chart showing the global AIDS related deaths with South Africa accounting for 13%, the second highest death rate in the world. Image obtained from the UNAIDS 2013 global report on the AIDS epidemic http://www.unaids.org (Accessed 03 March 2015).

HIV is not curable but can be managed by highly active antiretroviral treatment (HAART). These therapies combine inhibitors of the HIV protease and reverse transcriptase to slow down the viral progression (Autran et al., 1997). Although HIV-1 antiretroviral drugs were intended to treat the most common subtype in Europe and America, subtype B, they are currently used to treat subtype C. There has been limited evidence suggesting that these drugs are less effective on other HIV subtypes.

Despite the use of anti-retroviral drugs many diseases are still associated with HIV (Cohen, 2007). These diseases range from basic genital thrush to severe complex diseases such as cancer. Diseases that involve kidney malfunction have also been recognised as important susceptibility factors that lead to end stage renal disease (ESRD), a permanent state of kidney malfunction. ESRD is a severe major cause of death in HIV positive patients presenting with kidney disease (Rao et al., 1984). This has resulted in an increased burden of chronic kidney

disease in areas mostly affected by the HIV epidemic such as Sub-Saharan Africa (Fabian and Naicker, 2009; Naicker, 2010).

1.3. The Kidney

1.3.1. HIV and the Kidney

In 1984, a team of scientists described HIV-1–seropositive individuals who presented with renal disorder portrayed by gradual renal malfunction and proteinuria accumulation in their kidneys (Rao et al., 1984). However, many argued that the features were similar to those of renal failure caused by drug abuse such as heroin (Mazbar et al., 1990). Further studies were undertaken which revealed similar renal disease features in HIV patients with no drug abuse history (Bourgoignie et al., 1988).

Since then, it was shown that with HIV infection, patients develop an increased risk of acquiring acute, chronic and end-stage kidney disease. Furthermore, individuals of African ancestry have been shown to have four-fold increased risk of developing kidney disease compared to Caucasians (Naicker, 2010). The risk increases 18 to 50 fold in the presence of HIV infection (Fabian and Naicker, 2009; Fabian et al., 2013; Wyatt et al., 2007). These kidney diseases include thrombotic microangiopathy, immune complex-mediated glomerulonephritides, HIV-associated nephropathy (HIVAN) etc (Naicker, 2003, 2009). HIV infection is one of the common causes of chronic renal disease in HIV-1 infected individuals (Rao et al., 1984;(Winston et al., 1999). HIVAN, the severe form of CKD, is the third leading cause of end-stage renal disease (ESRD) in African Americans between the ages of 20 and 64 years (Winston et al., 1999).

It has also been indicated that the presence of opportunistic viral infections such as HIV could contribute to renal disease. The interaction of HIV proteins with renal cells such as that of the glomerulus and podocytes, direct infection of HIV-1 in renal cells as well as expression of HIV genes in kidneys (Fabian et al., 2013; Naicker, 2009, 2010; Wyatt et al., 2007). In addition, HIV infection has also been suggested to alter the expression of certain genes including the telomerase reverse transcriptase (*TERT*) gene which plays a role in kidney disease severity and progression (Chugh and Clement, 2012; Shkreli et al., 2012).

1.3.2. Chronic Kidney Disease (CKD)

Chronic kidney disease (CKD) is an umbrella term used to describe various disorders involving the disruption of the structure and function of the kidney (Levey and Coresh, 2012; Levey et al., 2003). The discrepancy in disease expression is related to cause and pathology, severity and rate of progression. CKD has become a worldwide public health problem. In the United States alone, the occurrences of kidney malfunction are rising. Similarly, developing countries in sub-Saharan Africa are also experiencing higher rates of CKD resulting in poor renal health outcomes and high costs of treatment (Naicker, 2003, 2009). Consequences of chronic kidney disease include not only kidney failure but also complications in heart health leading to cardiovascular disease. Existing evidence indicates that some of these adverse outcomes can be prevented or delayed by early detection and treatment. Unfortunately, CKD is under-diagnosed and undertreated in sub-Saharan Africa, in part as a result of lack of research identifying possible risk factors and treatment options (Kopp et al., 2010; Naicker, 2009, 2010).

1.3.2.1. Characteristics of CKD

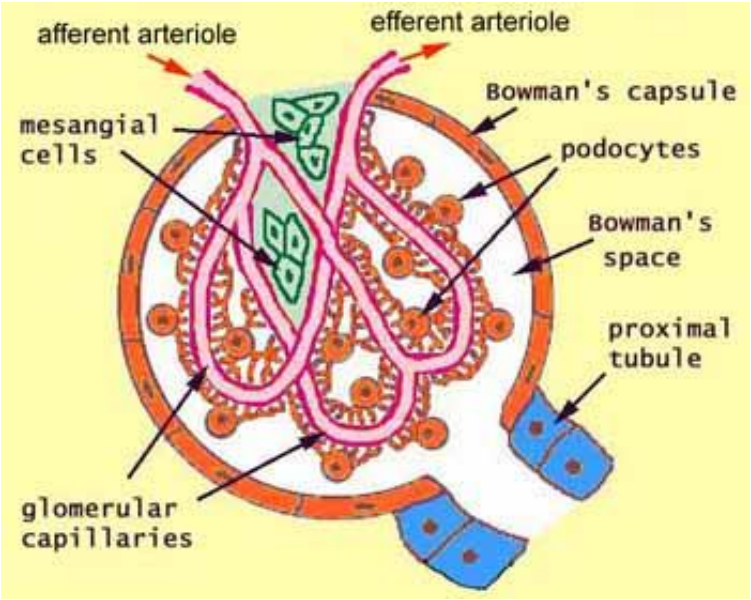


Figure 2: The renal corpuscle of the kidney showing the glomerular capillaries and the podocytes wrapped around them. The image shows an illustration of a normal kidney renal corpuscle. Image was adapted from Kidney and Urinary Tract Histology Study Guide accessed through http://www.siumed.edu/~dking2/crr/rnguide.htm (Accessed 30 April 2014). Image reproduced with permission from Professor David King (Southern Illinois University, Carbondale).

One mechanism in which CKD can arise is through podocyte malfunction. Podocytes are terminally differentiated epithelial cells that are located inside the glomerulus (Barisoni and Mundel, 2003; Mundel and Kriz, 1995; Zhu et al., 2013). They contribute to the formation of an intact glomerular barrier and their main functions include maintenance of glomerular capillaries, creation of glomerular basement membrane and regulation of glomerular filtrations (Barisoni et al., 2000; Barisoni et al., 1999). Podocytes normally leave gaps or thin filtrations allowing regulation of the filtration barrier (**Fig. 2**).

Podocyte proliferation is dependent on their state of differentiation. As they mature, they acquire a terminally differentiated phenotype (Nagata et al., 1998) i.e. they reach their finite ability to replicate. This maturation is accompanied by loss of proliferative activity and expression of Wilms tumor gene, WT1 a gene responsible for signalling cell maturation.

Podocytes are injured in many forms of glomerular malfunctions due to diseases that involve the immune system, toxin and metabolism associated injury and viral infection (Barisoni et al., 2000). Due to their restricted ability to proliferate, podocytes generally cease dividing and die out in most glomerular diseases resulting in glomerulosclerosis, a condition where filtration barrier is lost due to accumulation of fibrous scar tissue (Mundel and Kriz, 1995). However, studies have shown that in glomerular diseases that are idiopathic and those associated with HIV infection, podocytes lose their maturity and undergo dedifferentiation and re-enter the cell cycle causing interference within the filtration barrier (Barisoni et al., 2000). This interference results in glomerular collapse, a condition were renal epithelial cells over proliferate and cause renal structure abnormalities.

1.3.2.2. Pathogenesis of CKD

Patients with CKD usually present with proteinuria, a condition where there is an unusual amount of albumin in the urine, and reduced renal function (Klotman, 1999). Presence of renal tubular epithelial cells in the urinary residue and high levels of serum creatinine also indicate gradual loss of kidney function (Klotman, 1999). Hypertension is also common among most patients (Go et al., 2004).

1.3.2.3. Epidemiology of CKD

In the 2010 Global Burden of Disease, CKD was listed as the 18th among the diseases that cause death globally. In fact, according to the World Health Organisation (WHO), CKD escalated nine places up in the list from 27th in 1990. It is estimated that the prevalence of CKD ranges between 16 –18 % globally (Couser et al., 2011). The prevalence and incidence of CKD varies substantially in different regions or countries. For example, in the United States alone, 26 million individuals are estimated to suffer from CKD (Coresh et al., 2007; Couser et al., 2011). In sub-Saharan Africa, the overall estimated prevalence is 13.9% ranging from 2% in Ivory Coast to 30% in Zimbabwe and 14.3% in South Africa (Jha et al., 2013). There are different types of CKD which can vary according to pathological abnormalities of the kidney, proteinuria levels and estimated glomerular filtration rate (eGRF). These different types of CKD include thrombotic microangiopathy, immune complex-mediated glomerulonephritides (HIVICK) and HIV-associated nephropathy (HIVAN) (Naicker, 2003, 2009). The prevalence of these diseases differs. In fact a study performed on 99 biopsies from HIV seropositive patients presenting with kidney disease by the nephrology unit at Chris Hani Baragwanath Hospital, Johannesburg South Africa revealed that most of these patients (27%) presented with HIVAN, a severe type of CKD (**Fig. 3**). HIVAN shows a major increased tendency for HIV-infected individuals with African origin compared to other races (Abbott et al., 2001). However the current epidemiology of its prevalence in Africa is largely unknown due to under developed facilities.

1.3.2.4. Risk factors

The molecular mechanisms of CKD are poorly understood. Although considerable evidence indicates environmental factors as important role players in CKD progression, these do not completely explain the heterogeneity observed in CKD aetiology (Laouari et al., 2011). Disparity in CKD progression among individuals exposed to similar risk factors has been observed. Although non-communicable diseases such as Diabetes mellitus, obesity and hypertension are well-known triggers of CKD in developed countries (Coresh et al., 2007). In addition, in Asian and some sub-Saharan African countries, acute inflammation of the kidney and unknown triggers are recognised as major causes (Jha et al., 2013). Furthermore, rapid urbanisation and globalisation are believed to have accelerated the transition in disease burdens. Together with

continued high prevalence of infectious diseases and an increasing prevalence and severity of lifestyle disorders, such as diabetes and hypertension. In developing countries including South Africa incidences of CKD have increased over the years (Naicker at al., 2009).

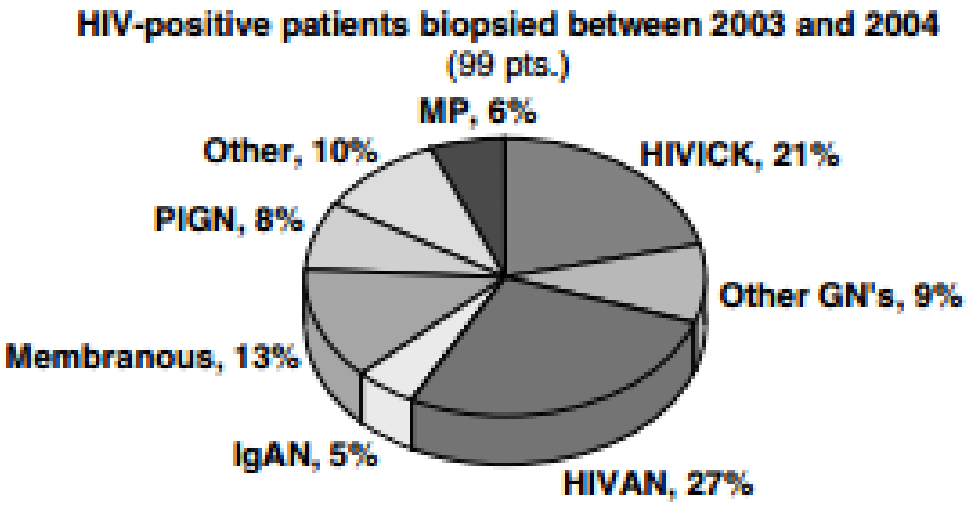


Figure 3: The prevalence of renal disease in HIV-positive patients biopsied between 2003 and 2004 at the Chris Hani Baragwanath Hospital (Gerntholtz et al., 2006). The most common CKD observed in HIV positive individuals was HIVAN accounting for 27% of CKD cases. Image reproduced with permission from the Nature Publishing Group.

Furthermore, ethnic differences have been detected in CKD progression suggesting that genetic factors may also contribute to susceptibility (Kopp et al., 2010; Laouari et al., 2011; Naicker, 2010). In the 1980s kidney diseases such as HIVAN was noticed mostly among African-Americans and Haitian immigrants in the United States (US). These findings suggested a strong racial discrepancy and genetic susceptibility that characterizes this distinctive kidney disease (Wyatt et al., 2012). Whilst several studies have investigated genetic components that confer susceptibility to CKD, the gene variants predisposing individuals to CKD are largely unknown. In fact, a strong association of the myosin gene *MYH9* and nephropathy was observed in African American individuals suffering from nephropathy (Kopp et al., 2008). Despite the compelling association of *MYH9* gene to HIVAN, several significant concerns remained ambiguous. One of which was the presence of the homozygous risk allele in one third of the African-American healthy individuals, suggesting that there were other genetic and environmental factors that

contribute to susceptibility to disease (Kopp et al., 2008). Further studies were performed to assess the susceptibility locus on chromosome 22q13.1. A stronger association was discovered with the apolipoprotein 1 gene (*APOL1*) (Genovese et al., 2010). Two *APOL1* significant alleles were only identified in African participants who were part of the 1000 Genomes Project and these were shown to be in strong linkage disequilibrium with the *MYH9* risk allele (Genovese et al., 2010; Wyatt, 2012). Furthermore, variation in MYH9 and APOL1 has been shown to be associated with non-diabetic chronic kidney disease in individuals of African origin with a significant association of *APOL1* risk alleles in black South Africans (Kasembeli et al., 2015).

More studies in mice have emphasised the role of genetic factors in CKD. In fact, a study by Shkreli and colleagues (2002) has shown that transgenic expression of the telomerase reverse transcriptase (TERT) in mice induced glomerular collapse resembling that in human CKD (**Fig. 4**). In contrast, silencing TERT in HIVAN mice models resulted in controlled podocyte proliferation and improved filtration barrier (Shkreli et al., 2012). This was an interesting finding as TERT has been previously implicated in disease involving de-differentiation and proliferation of cells (Gordon and Santos, 2010; Nakayama et al., 1998; Sekhri, 2014; Shay and Wright, 2001a). These findings further warranted an investigation of the role of telomerase in CKD.

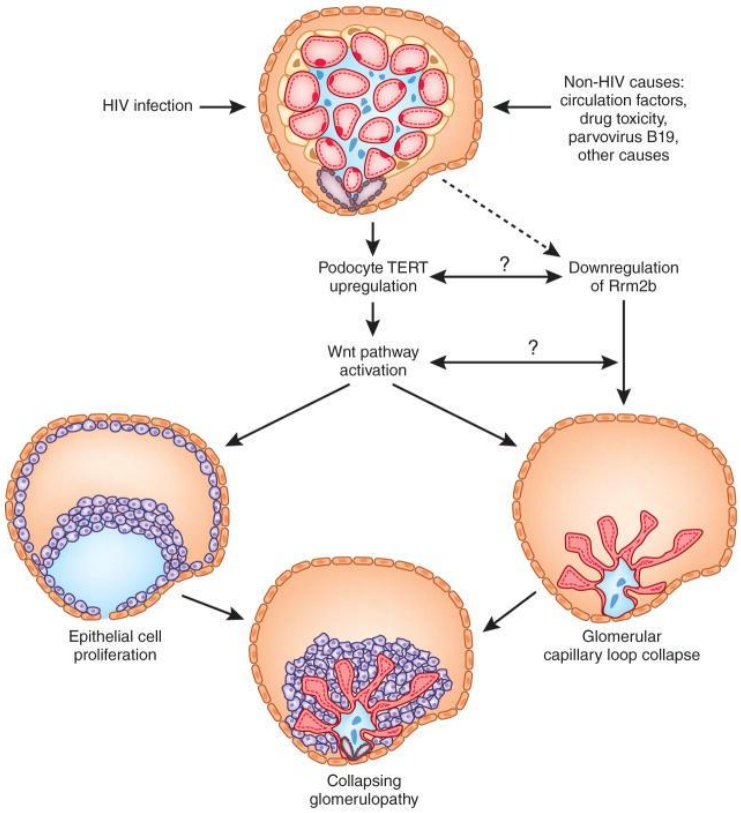


Figure 4: Mechanism of glomerular collapse caused by upregulation of TERT. The image illustrates the suggested mechanism by which HIV and non-HIV infections can induce up-regulation of TERT and the Wnt/β-catenin pathway. This inturn induces epithelial cell proliferation in the glomerulus resulting in glomerular collapse. Image obtained from (Chugh and Clement, 2012).

1.4. Telomere, Telomerase and Disease

One distinct feature observed in CKD is the proliferation and dedifferentiation of podoctyes leading to the glomerular collapse (D'Agati et al., 1989). In order to understand the ability of podocytes to dedifferentiate and re-enter cell cycle in CKD, pathways involved in cell proliferation need to be studied. One well defined pathway previously implicated in unlimited cellular proliferation is the telomerase reactivation (Shay and Bacchetti, 1997; Shkreli et al., 2012). Telomerase is an enzyme whose functions include regulation of cellular proliferation and viability. Exploring the role of telomere biology in CKD will help understand the pathogenesis of disease and ultimately allow identification of potential drug targets for the treatment or management of CKD. This will possibly improve renal health outcomes.

1.4.1. Telomeres and telomerase

1.4.1.1. Telomeres

Telomeres are DNA-protein complexes found at the end of eukaryotic chromosomes (Blackburn, 2000). Telomeres are composed of double-stranded (ds) and single-stranded (ss) G-rich DNA tandem repeats (TTAGGG in mammals) (**Fig. 5**). Telomeres are responsible for capping the ends of chromosomes, thus play a protective role as they prevent loss of essential genetic sequences (Chan and Blackburn, 2004). Telomeres achieve this through counteracting the effects of exonuclease activity and the "end-replication problem" i.e. without telomeric repeats; gene coding sequences would be lost (Wellinger et al., 1996).

Telomeres are bound by several telomeric proteins called the "Shelterin" complex (de Lange, 2005). This complex interacts together with several factors associated with DNA repair to alter the structure of the telomeric DNA (de Lange, 2005) (**Fig. 6**). This alteration allows protection of single-stranded telomeric DNA from events such as exonuclease activity, end-to-end fusion (Letsolo et al., 2010; Lin et al., 2010) with other telomeres and/or with broken DNA strands and unsuitable recombination (Plohl et al., 2002).

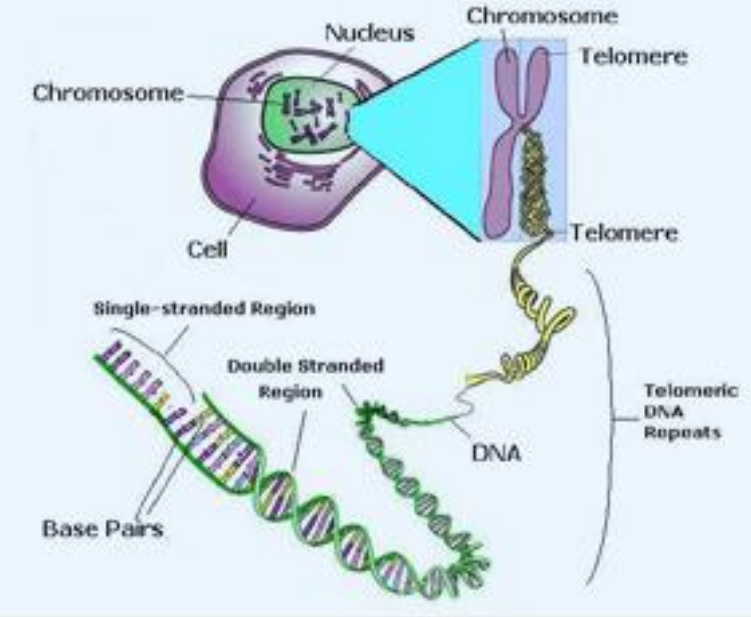


Figure 5: Diagram showing dsDNA and ssDNA repeats of terminal ends of eukaryotic chromosomes called telomeres. Image obtained from Pharminox accessed through http://www.pharminox.com/telomere_signalling.aspx.

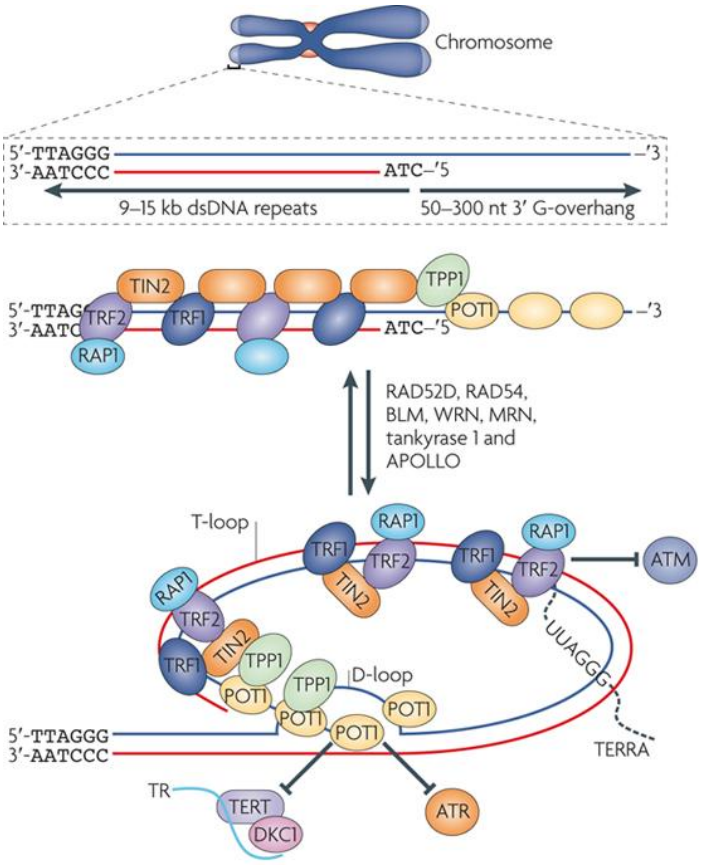


Figure 6: Diagram showing telomere DNA-protein complex and its formation. Proteins such as the protection of telomeres 1 (POT1) and Dyskerin (DKC1) are recruited to stabilise telomeres by forming a structure called the T-loop. Image obtained from O'Sullivan and Karlseder (2010).

Without the formation of the Shelterin complex, detrimental consequences that may lead to genomic instability may arise (O'Sullivan and Karlseder, 2010). These processes are potentially catastrophic; for example, fusions can lead to the formation of dicentric chromosomes, which are inherently unstable and result in imbalances in the genetic content of dividing cells progeny, or to a loss of genetic information (Artandi and DePinho, 2010; Letsolo et al., 2010; Lin et al., 2010).

To combat the loss of telomeric repeats, the shelterin complex not only works with DNA repair factors but also helps in recruiting the telomerase enzyme which is responsible for synthesis of telomeric DNA and counteracting the "end-replication problem" (section 1.4.1.2) (de Lange, 2005). In addition, telomeres also associate with several proteins to form a structure called the

telomeric loop or T-loop. This structural formation further allow chromosomes to prevent loss of genetic material and offer protection against DNA damage (Greider, 1999)

1.4.1.2. Telomeres and the "end-replication problem"

In addition to being ends of linear chromosomes, telomeres are not properly replicated during lagging-strand synthesis and thus shorten with every cycle of cell division, a phenomenon called "end replication problem" (Allsopp et al., 1992) (**Fig. 7**). During DNA replication, the DNA polymerase synthesises new DNA strand in the 5' to 3' direction. Due to its specificity, the DNA polymerase can successfully replicate the leading strand.

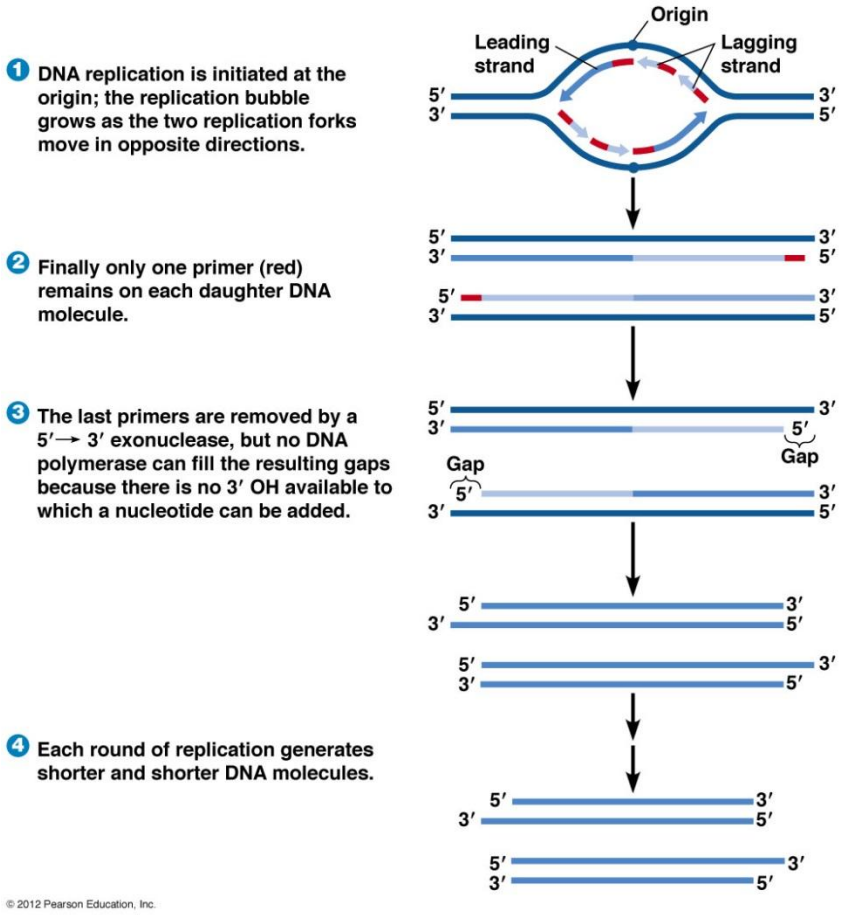


Figure 7: Diagram showing synthesis of DNA in both the leading and lagging strand. The lagging strand requires presence of Okazaki fragments and results in an incomplete 5' end. This mechanism is termed the "end-replication problem". http://www.mun.ca/biology/desmid/brian/BIOL2060/BIOL2060-19/CB19.html.

However, in the lagging strand, synthesis requires small DNA pieces called Okazaki fragments to initiate synthesis. These fragments work in conjunction with RNA primers. At the end of DNA synthesis, the last primer is removed leading to daughter molecules with a 5' terminal gap, this was termed the "end-replication problem" (Lingner et al., 1997; Watson, 1972).

Loss of these telomeric repeats would eventually prompt cells to exit the cell cycle, and enter replicative senescence; a state of permanent cell cycle arrest and a barrier to unlimited proliferation (Hayflick, 1965; Shay and Wright, 2005). Like senescent cells, differentiated cells have stopped dividing and thus reached their final non-replicative cell type. In some cases, cells bypass senescence and re-enter the cell cycle (Shankland and Wolf, 2000). This is often through the activity of the telomerase.

1.4.2. The telomerase enzyme

Telomerase is a holoenzyme and a multisubunit ribonucleoprotein (RNP) that consists of two essential components; the human telomere reverse transcriptase (hTERT) and the human telomerase RNA complement (hTERC) (**Fig. 8**) in addition to telomerase-associated proteins (Harrington et al., 1997). Telomerase maintains telomeres by synthesising telomeric repeats in a 5' to 3' fashion towards the distal end of the chromosome, thereby lengthening it (Blackburn, 2005; Blasco et al., 1999).

Telomerase is expressed in highly proliferating tissues such as germline cells, some stem cells and 85 - 90% of human tumour cells (Blasco et al., 1999; Kim et al., 1994). In contrast, telomerase activity is absent or expressed at very low levels in most adult somatic cells (Wright and Shay, 1992). The consequence of limited telomerase activity is progressive telomere shortening and restricted ability to allow cell division (Harley et al., 1990). Progressive telomere shortening eventually trigger cells to exit the cell cycle and enter replicative senescence or apoptosis (Blackburn, 2000; Blasco et al., 1999; Bryan et al., 1997). However, telomerase reactivation has been identified as one of the processes that permits cells to escape senescence. In fact, ectopic expression of TERT in somatic cells is sufficient to promote unlimited cell proliferation (Bodnar et al., 1998; Davis et al., 2003). Moreover, this reactivation

is associated with cellular immortality which is observed in 85% of cancers (Shay and Bacchetti, 1997). Furthermore, transfection of retinal pigment epithelial cells and foreskin fibroblasts with an expression vector containing the *TERT* gene resulted in substantial increase in telomere length and extended cell life span (Bodnar et al., 1998). This makes telomerase a perfect candidate for studies concerning cell cycle events that prevent cell cycle exit such as senescence or differentiation (Shankland and Wolf, 2000).

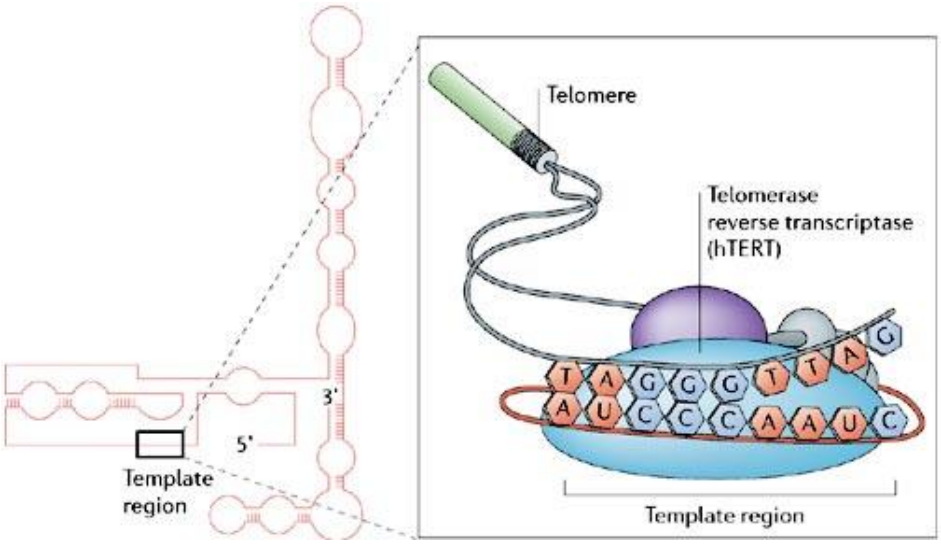


Figure 8: The process of telomere repeats addition by the telomerase enzyme (hTERT using hTERC as a template (Shay and Wright, 2006). Image reproduced with permission from the Nature Publishing Group.

1.4.2.1. The human telomerase RNA component (hTERC)

The human telomerase RNA component (hTERC) consists of an RNA sequence that is complementary to the telomeric DNA. This RNA sequence serves as a template for *de novo* addition of deoxynucleotides to the G-rich telomere strand by the major catalytic subunit of the telomerase enzyme; hTERT (Feng et al., 1995; Guilleret et al., 2002).

1.4.2.2. The human telomerase reverse transcriptase component (hTERT)

The hTERT utilises the hTERC as a template for telomere synthesis (Lingner et al., 1997). Unlike the *hTERC* which is present in all cell types, the *hTERT* mRNA is only detected in telomerase positive cells and therefore recognised as the rate limiting determinant of telomerase enzymatic activity (Counter et al., 1998; Kim et al., 1994). Indeed several studies have investigated the role of the hTERT component as the key regulator of telomerase activity. Nakayama and colleagues (1998) introduced the wild-type *hTERT* gene into normal differentiated fibroblast cells and observed a significant increase in telomerase activity (Nakayama et al., 1998).

The hTERT component is mainly known for its specialized role in telomere maintenance. The hTERT is primarily located in the nucleus where it elongates or stabilises telomeres. Reports have shown that it also localizes to mitochondria (Gordon and Santos, 2010) and the cell surface of HEK293 and MDA-MB321 cells (Naidoo et al., 2015). The function of hTERT in the cell surface and mitochondria is largely unknown. The available findings point to a non-telomere related role that encourages cell proliferation through interaction with various proteins including the 37kDa/67kDa laminin receptor (LRP/LR) (Naidoo et al., 2015). Several other studies have revealed other noncanonical functions of hTERT that influence cancer growth and progression. Two signalling pathways involved in development, oncogenesis and inflammation have been shown to interact with hTERT to induce cellular proliferation. These are the nuclear factor kappa B (NF-κB) and the Wnt/β-catenin pathways.

The transcription factor NF-κB is a crucial inflammation and development regulator (Biswas and Tergaonkar, 2007). Dysregulation of the NF-κB pathway has been associated with pathogenesis of various human diseases including inflammation and cancer (Akiyama et al., 2003). The NF-κB

transcription factor has been shown to interact with the TERT protein and regulate nuclear translocation of TERT in myeloma cells (Akiyama et al., 2003). Furthermore, studies have shown that upregulation of hTERT promotes activity of NF-κB and expression of NF-κB related genes (Ding et al., 2013; Ghosh et al., 2012). These findings suggest that hTERT may be a transcriptional regulator of the NF-κB pathway.

The hTERT has also been reported to regulate the Wnt/β-catenin pathway. This particular pathway is mainly involved in embryogenesis and regulation of cell polarity and potency (Clevers and Nusse, 2012). Alteration of the Wnt/β-catenin signalling has been implicated in susceptibility to cancer and developmental disorders (Clevers and Nusse, 2012; Martinez and Blasco, 2011). This was first observed in hair follicles of transgenic mice engineered to conditionally express inactive TERT. The induced *TERT* expression resulted in activation of hair follicle stem cells and ultimately triggering hair growth independently of the *TERT* catalytic function (Choi et al., 2008). Further analysis revealed that *TERT* upregulation induced expression of development-related genes closely controlled in the Wnt/β-catenin pathway (Choi et al., 2008).

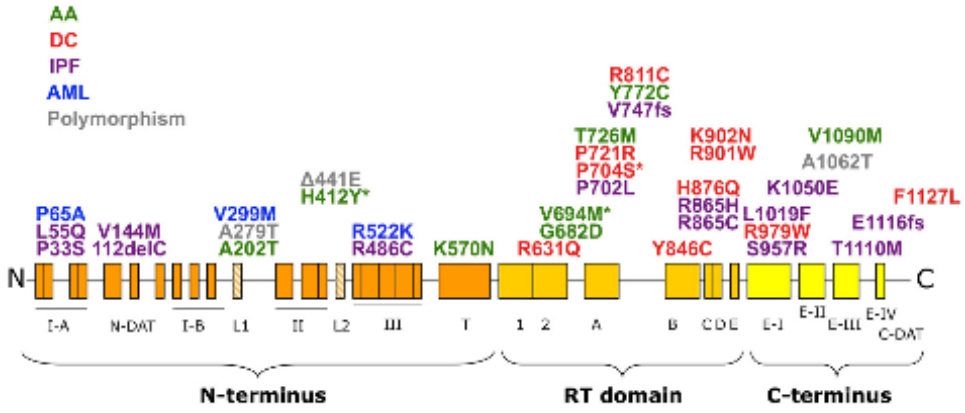
Another study by Park and colleagues (2009) revealed that introduction of TERT in *Xenopus* embryos and mice embryonic stem cells promoted over-proliferation of cells (Park et al., 2009). In addition, TERT was also shown to interact with transcription factor *Brahma-related gene 1* (Brg1); a role player in the Wnt/β-catenin pathway shown to maintain tumour initiation cell phenotype in cancers (Park et al., 2009). The Wnt/β-catenin pathway dysregulation via TERT expression has also been suggested to induce proliferation of renal epithelial cells in transgenic mice (*Section 1.3.2.4*.) (Shkreli et al., 2012). In contrast, defects associated with dysregulation of the Wnt/β-catenin pathways were absent in TERT deficient study organisms and cell lines (Choi et al., 2008; Clevers and Nusse, 2012; Martinez and Blasco, 2011; Park et al., 2009; Shkreli et al., 2012). These findings revealed that stimulation of NF-κB and Wnt/β-catenin pathways by TERT and vice versa may be the underlying mechanism that drives expression of genes involved in tumour survival and proliferation (Li and Tergaonkar, 2014).

1.5. Genome-wide Association Studies (GWAS) on telomere-associated genes

GWA studies were introduced in the late 1990s. At that time, most of the genetic studies used linkage analysis to detect susceptibility loci in complex diseases. Findings from linkage studies indicated that predisposition factors to majority of complex diseases are either relatively weak, highly heterogeneous or both. Due to the low success rates of linkage analysis, more statistical power was required to reliably determine these susceptibility factors. Researchers demonstrated that rather than studying affected individuals only, association studies provided a more reliable statistical power to detect weak effects by comparing affected and non-affected individuals. This would identify loci or alleles that were more prevalent in either group (Risch and Merikangas, 1996).

There is increasing evidence on the role of telomere dynamics in disease susceptibility as well as driving disease progression. To understand the variation of telomere biology genes and the implication in disease, several GWA studies have been performed. These studies set out to identify disease susceptibility factors in genes and pathways associated with telomeres. Several studies have been performed to date to assess the common variants in telomere related genes and their association with disease. It is noteworthy that most of these studies were only performed in non-African populations. Numerous variants have been identified within the *hTERT* gene and have been associated with cancers and ageing disorders. These include gliomas, lung and breast cancers as well as idiopathic pulmonary fibrosis (Mirabello et al., 2010; Pooley et al., 2010; Qu et al., 2014). For example, the variant or single nucleotide polymorphism (SNP) with reference SNP number rs2736100 located on chromosome 5: 1 284 601 (5: g. 1284601(A>C)). This SNP is found in intron 2 of the *hTERT* gene and has been suggested to alter *hTERT* expression and influence telomere length (Codd et al., 2013). This particular SNP results in an Adenine to Cytosine (A>C) nucleotide change. The rs2736100 has been reported to be associated with increased risk of developing several diseases including gliomas (Liu et al., 2010; Shete et al., 2009; Wrensch et al., 2009), lung and breast cancers (McKay et al., 2008; Shen et al., 2010; Truong et al., 2010; Wang et al., 2010) and interestingly, bladder cancers (Gago-Dominguez et al., 2011).

Similarly variation within the telomere associated genes has been implicated in several diseases. For example some germline and somatic mutations in the *dyskerin*, *hTERT* and *hTERC* genes are associated with telomere shortening, susceptibility to cancer and premature ageing (Carroll and Ly, 2009). Figure 9 highlights some of the mutations within the *hTERT* gene that have been associated with diseases such as acute myeloid leukemia (AML).



KEY

AA: Aplastic Anaemia

DC: Dyskeratosis Congenita

IPF: idiopathic pulmonary fibrosis

AML: acute myeloid leukemia

Figure 9: Different single nucleotide polymorphisms (SNPs) within the *hTERT* gene with clinical implications that confer susceptibility to certain cancers (Carroll and Ly, 2009). The SNPs outlined in the image are non-synonymous i.e. result in amino acid change. These SNPs confer susceptibility to several of the cancers and ageing disorders outlined.

Interestingly, GWA studies revealed somatic mutations that could also drive disease progression or increase disease risk. Importantly, more studies were channelled towards identifying these susceptibility factors within the critical component of telomerase; the *hTERT* gene and comparing average telomere length in affected and unaffected individuals.

1.5.1. Variation within the *hTERT* gene

The gene that codes for the catalytic reverse transcriptase subunit of the telomerase enzyme is located on chromosome 5p15.33 (Greider and Blackburn, 1985). The *hTERT* gene consists of 16 exons and 15 introns (Cong et al., 1999). Mutations within the gene itself were shown to be associated with high telomerase activity in different cancers (**Fig. 9**).

Several research groups have described associations between the rs2736100 variants and cancer risk, however most results were inconclusive of the allele conferring risk (Gago-Dominguez et al., 2011; Jin et al., 2009; Shen et al., 2010; Wang et al., 2010; Wrensch et al., 2009). In addition the most of the associations were reported non-African populations in Europe and Asia. Although the variant has been associated with adenocarcinoma risk in African-Americans (Spitz et al., 2013), a lack of association with breast cancer risk in women of African ancestry was reported (Zheng et al., 2012). This is the only study to date that has assessed the role of this particular gene in disease pathogenesis in African ancestry.

Currently available allele and genotype frequencies accessed through 1000 Genomes and HapMap Data, revealed no significant difference in the distribution of the alleles across different populations. The African populations genotyped for the SNP were assessed to annotate the variant and determine the distribution in different populations. No association studies to date have been performed to investigate the role of this particular variant in disease susceptibility. Our bioinformatics analyses have shown that there is still missing data on the pathogenicity and distribution of some of the *hTERT* variants which are clinically important risk alleles in non-African populations.

Another specific variant within hTERT is rs2736098, and has been previously shown to confer risk to lung cancer in Asian, African American and European populations, as well as bladder cancer in European populations. Even though the variant has been shown to have clinical importance in disease susceptibility, there is still limited data on the distribution of this rs2736098 in different populations. Most of the association studies were performed in Asian populations.

Similarly, studies have shown that rs401681 is not associated with telomere shortening, breast cancer colorectal cancer, or melanoma in Caucasians (Pooley et al., 2010). Furthermore, another study revealed association with decreased risk of oesophageal cancer in Chinese individuals (Yin et al., 2014). In contrast, a meta-analysis revealed that the T allele is associated with increased risk for pancreatic cancer in Chinese individuals (Liu et al., 2014).

However, components that influence the expression of *hTERT* such as the promoter and minisatellite (MNS16A) have become interesting candidates to be tested for association with several diseases including cancer and ageing disorders (Blasco, 2005). Numerous SNPs have been identified and found to be associated with increased telomere stability and telomerase activity in different cancers (Petersen et al., 2010; Prescott et al., 2011; Terry et al., 2012; Varghese and Easton, 2010).

1.5.1.1.1. The *hTERT* promoter

The promoter region of the *hTERT* gene consists of 300 bases upstream of the translation start site. These regions possess specific sequences that allow binding of transcription factors to initiate transcriptional activity. These regions control expression of genes and mutations in these regions may lead to a decreased or increased expression of specific genes. Recently, several studies have identified *hTERT* promoter variants and their role in disease progression. the analysis of whole genome sequence data of malignant tumours revealed two common *hTERT* promoter variants in 89% of the tumours analysed. These variants are located on chromosome 5 at positions 1 295 228 C>T and 1 295 250 C>T; hereafter termed C228T and C250T, respectively (Huang et al., 2013). These variants within the *hTERT* promoter influence cancer susceptibility (**Table 1**). Furthermore, these variants in the *hTERT* promoter have been hypothesised to play a role in altering *hTERT* expression (Shkreli et al., 2012). Another study verified the findings of Huang et al., 2013 and reported similar variants in different thyroid cancer types (Vinagre et al., 2013). Furthermore, promoter mutations were identified in bladder tumours and cell lines (Theodorescu and Cech, 2014).

In addition, studies have shown that *hTERT* variants are useful markers for the severity of diseases such as cancer and ageing disorders (Calado et al., 2009; Kinnersley et al., 2012). Indeed a significant association of the variants with high levels of *hTERT* mRNA expression has been reported (Vinagre et al., 2013). Most of these studies were performed in non-African population (Bellido et al., 2013; Yan et al., 2014). Thus identifying variants within the genetically diverse African population that influence *hTERT* expression may lead to better understanding of disease. Evidence from mice models suggests that TERT upregulation may play a role in CKD

susceptibility. Therefore determining variants that alter *hTERT* expression could be used as valuable biomarkers for testing susceptibility to CKD. This may identify individuals at risk and lead to alternative treatment strategies. In addition, understanding the role of telomerase and telomeres in CKD may give information regarding mechanisms that lead to the cell-cycle re-entry of differentiated podocytes.

Table 1: Presence of the C228T and C250T *hTERT* promoter mutations in different human cancers. Table adapted from (Vinagre et al., 2013).

Organ/tissue		Number	*TERT* mutation N(%)
Melanocytes			
	Nevi	9	0
	Skin Melanoma	56	16 (29%)
	Ocular melanoma	25	0
CNS			
	Pilocytic Astrocytoma	13	1 (8%)
	Diffuse Astrocytoma	20	3 (15%)
	Oligodendroglioma	22	10 (45%)
	Anaplastic Oligodendroglioma	24	13 (54%)
	Glioblastoma	39	24 (62%)
Thyroid			
	Benign	81	0
	PTC	169	13 (8%)
	FTC	64	9 (14%)
	PDTC	14	3 (21%)
	ATC	16	2 (13%)
	MTC	28	0
Bladder			
	Low grade	21	14 (67%)
	High grade	61	34 (56%)
Kidney			
	CCRCC	12	0
	CromRCC	4	0
	PRCC	10	0
Adrenal			
	Pheochromocytoma	17	0
GI			
	GIST	36	0
TOTAL		741	142 (19%)

PTC – papillary thyroid carcinoma; FTC - follicular thyroid carcinoma; PDTC – poorly differentiated thyroid carcinoma; ATC – anaplastic thyroid carcinoma; MTC – medullary thyroid carcinoma; CCRCC - clear cell renal cell carcinoma; CromRCC - chromophobe renal cell carcinoma; PRCC - papillary renal cell carcinoma; GIST – gastrointestinal stromal tumor.

1.5.1.1.2. The *hTERT* minisatellite (MNS16A)

In addition to gene promoters influencing expression of genes, minisatellites located upstream and/or downstream of genes play a crucial role as regulators of gene expression (Jeffreys et al., 1985). Minisatellites are variable number tandem repeats (VNTRs) that have differing number of repeats among different individuals. This difference in the number of repeats influences the level of gene expression in certain genes (Jeffreys et al., 1985; Vergnaud and Denoeud, 2000). Within the *hTERT* gene, several minisatellites and microsatellites have been identified. One specific *hTERT* minisatellite located downstream of the *hTERT* gene is the MNS16A. This particular VNTR has been suggested to affect the activity of the *hTERT* promoter, thus altering *hTERT* expression in lung cancer (Wang et al., 2003). Figure 10 shows the position of the MNS16A in the *hTERT* gene. The MNS16A has been suggested to influence the expression of *hTERT* through regulation of the hTERT mRNA expression (Hofer et al., 2011; Wang et al., 2003; Wang et al., 2008). In fact Wang et al., 2003 reported that the antisense *hTERT* mRNA has a biological function, which may be exerted through interference with *hTERT* levels.

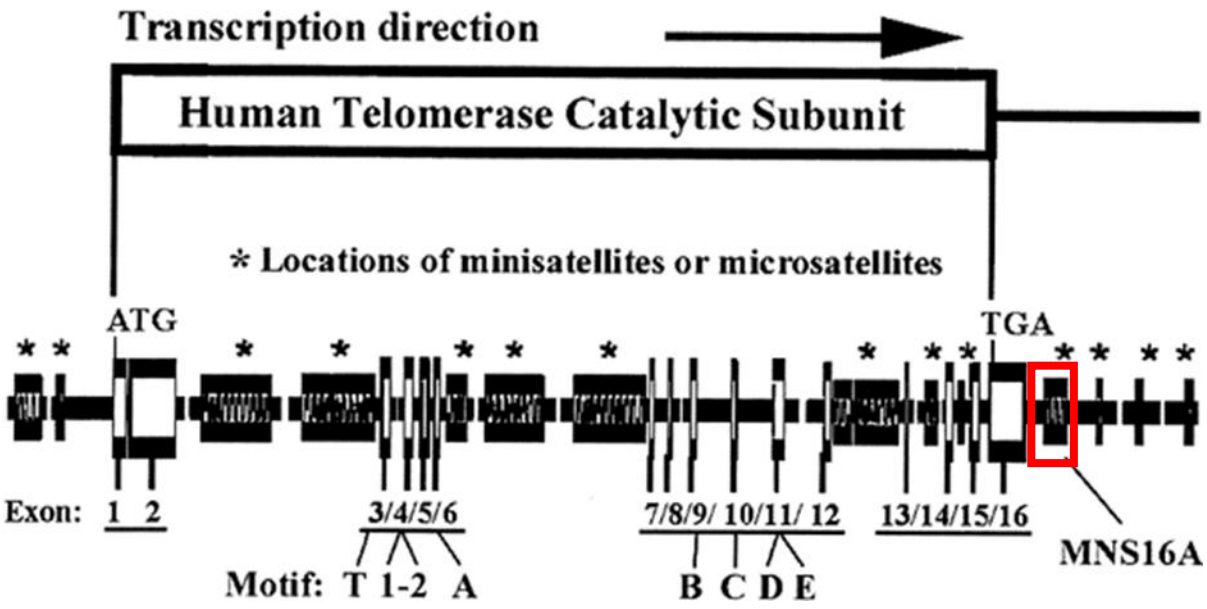


Figure 10: The genomic structure of the *hTERT* gene showing the exon and satellite DNA positions in the gene. Minisatellite MNS16A indicated by the red box. Obtained from Wang et al., 2003.

The regulation of the hTERT promoter by minisatellite is dependent on the size of the minisatellite. Several sizes (243 bp, 274 bp, 302 bp, 333 bp and 364 bp) of the MNS16A classified as either long (L) or short (S) have been previously identified (Hofer et al., 2011; Wang et al., 2003; Wang et al., 2008). These sizes have been used as a gold standard for characterizing the MNS16A. Some scientists argue that the long allele (L) is associated with high levels of *hTERT* expression whereas others argue that the short allele (S) is the high risk allele (Hofer et al., 2011; Hofer et al., 2013; Jin et al., 2011; Wang et al., 2003). However most of the studies revealed the long allele (L) as the associated risk factor (Xia et al., 2013).

These findings may be unique to different populations and/or tissues as most of these studies were replicated in different populations using different cancer types. No study as far as I know has assessed the role of the MNS16A in kidney diseases such as HIVAN which display over-proliferation of the podocytes which may link *hTERT* involvement in progression and severity of disease. Long MNS16A tandem repeats are associated with low promoter activity, thus the antisense *hTERT* mRNA does not interfere with normal *hTERT* expression leading to high *hTERT* activity which is then associated with cancer and "cancer-like" diseases.

Justification

Many studies have been successful at identifying variants found within telomere genes especially *hTERT* (Mirabello et al., 2009; Mirabello et al., 2010; Pooley et al., 2010; Prescott et al., 2011; Xia et al., 2013). However, the majority of these studies involved Asian and Caucasian populations with limited data available for African populations. With advancing technology, the cost of genotyping or sequencing has decreased dramatically over the past decade. Following this change, an unexpected increase in the sequence data available was observed. This included an increase in the African populations genotyped in the 1000 genomes project (http://www.1000genomes.org/). Despite the dramatic change, data associating detected variants to disease susceptibility in African populations is still limited. Identification of variation in telomere biology genes in Africans is crucial in understanding disease susceptibility and possible future medical intervention. The findings of this project will hopefully improve our understanding of the molecular pathogenesis of CKD and the role of telomere biology in disease

susceptibility. This will ultimately contribute to the body of knowledge in CKD improving renal health outcomes and offering new insight in the study of drug design for CKD treatment. The following aims and objectives were investigated.

1.6. Aims and Objectives

1.6.1. Aims

- To determine variation in the *hTERT* gene and promoter in different non-African populations and compare this to African populations.
- To determine whether telomere genetics play a role in chronic kidney disease progression and severity regardless of HIV status.

1.6.2. Objectives

- Identify common genetic variants within the *hTERT* gene and *hTERT* promoter in African and non-African populations.
- To optimise methodology using nucleic acids and protein extracted from HEK293 cell line.
- Determine chronic kidney disease progression and severity using patient clinical information.
- Identify South-African specific variants within the hTERT promoter.
- Determine variation within MNS16A in study population.
- Determine whether the *hTERT* promoter and MNS16A variants have an effect on *hTERT* expression and ultimately telomere length.

Chapter 2: Methods and Materials

2.1. Materials

2.1.1. Participant DNA

DNA was obtained from kidney biopsies of 98 CKD participants with HIVAN, HIV positive with kidney disease (nonHIVAN) and HIV negative with kidney disease (HIVnegK). DNA from 61 controls who are HIV positive without kidney disease (HIVPosNK) and HIV negative without kidney disease controls (CTRL) was provided. All individuals in the study identified themselves as black. The signed consent was obtained from control participants and patients that underwent kidney disease treatment at the Charlotte Maxeke Hospital, Johannesburg through a genetic study by Mr Alex Kasembeli. All patients were over the age of 18. Participants were males and females. They are all deceased. Ethical approval for this study was obtained from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand with ethics clearance number M140346 (Appendix A). These DNA samples were assigned new identification names (**Appendix C**).

2.1.2. Cell lines

Mammalian cells were cultured and used to extract nucleic acids and cell lysate for downstream applications. Table 2 shows the cell lines and culture media/conditions used for each cell line.

Table 2: Cell culture media used for the human embryonic kidney cell line (HEK293) in the study.

Cell line	**Culture Media**
HEK293 Human embryonic kidney cells	89% Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone) with 4500mg/L glucose, sodium pyruvate and 4mM L-glutamine (Sigma), supplemented with 10% Foetal Bovine Serum (FBS; Hyclone) and 1% penicillin/streptomycin (Sigma).

All plastics used to culture cells were obtained through Sigma. The T25 and T75 cell culture flasks (Sigma) were used to plate and grow cells.

2.2. Methods

2.2.1. Cell Culture

All cell culture work was performed in the ESCO Airstream Class II laminar flow hood (Life Technologies). Cell lines used in this research are listed in the left column in **Table 2.** These cell lines were incubated in a humid incubator with 5% CO_2 at 37 °C (NÜVE CO_2 incubator EC 160) and sub-cultured when confluent. Cells were regularly fed and re-seeded until confluent. The culture media components for the HEK293 cell line (**Table 2**)

The cells were either harvested for down-stream applications, sub-cultured, transfected with siRNA or frozen for long term storage at -80 °C. The cells were washed with 1-2 ml 1X PBS (Sigma Aldrich) and treated with 1-2 ml of pre-warmed Trypsin (Sigma Aldrich) at 37 °C to detach the cells from the culture flasks prior to splitting and sub-culturing. The trypsin reaction was terminated by the addition of respective pre-warmed culture media (4 ml in T25 culture flasks and 8 ml in T75 culture flasks). The cells were then counted using a haemocytometer. Based on the number of cells counted, a certain amount of cells was resuspended in warm culture media and plated in either a T25 of T75 cell culture flasks.

Successfully cultured cell lines (HEK293 and MDA-MB-231) were transfected with a universal negative control esiRNA RLUC (Sigma Aldrich). The purpose of this experiment was to learn the technique for future use. Briefly this involved preparing a transfection media by mixing 5 µL of 1000 ng/µL esiRNA with transfection media. One mL of the transfection media was added to 9 mL of freshly prepared warm cell culture media (**Table 2**). This mixture was added to the flask with cells and incubated for 24 hours at 37 °C and 5% CO_2 levels in a humid Nűve incubator. Following the 24 hours incubation, the media was discarded and fresh 10 mL media was added to the plate and incubated for a further 72 hours with constant media change.

To enable long-term storage, some of the cells were frozen in liquid nitrogen. Briefly this involved transferring 1 - 2 mL of cells resuspended in media into 2 mL cryovials. The cryovials were then inserted in a control rate freezer containing DMSO. This control rate freezer with vials containing cells was then stored at -20 °C overnight. After this, the control rate freezer (AEC-Amersham, United States) was then transferred to -80 °C fridge overnight. The vials were removed from the control rate freezer and transferred into a liquid nitrogen tank and left for long term storage. To thaw the cells, cell culture media was pre-warmed to 37 °C and cryovial removed from the liquid nitrogen. The cryovial containing cells were slowly defrosted at room temperature and 1 mL of cells added into the flask containing pre-warmed cell culture media.

2.2.2. DNA Extraction and Quantification

The DNA was extracted from the HEK293 cell line using the DNA Extraction Kit (Thermo Scientific) as the manufacturer's instructions. Briefly, the procedure involved removing the cell culture media from the confluent cells and treating them with trypsin (Sigma) for 1 – 2 minutes at 37 °C until they detached. The reaction was stopped by addition of 9 ml of culture media (media prepared as described in table 2). The cell suspension was transferred to a 15 ml falcon tube (Becton Dickson) and centrifuged at 1 500 rpm for 10 minutes. The supernatant was removed and pellet resuspended in 5–10 ml of 1X PBS depending on the pellet size. The suspension was transferred into six 1.5 ml Eppendorf tubes and centrifuged at 250 × g for 5 minutes. The supernatant was removed and DNA was extracted from cell pellets using the GeneJET Genomic DNA purification Kit (Thermo Scientific) as per manufacturer's instruction.

Briefly, the cell pellet was resuspended in lysis buffer to lyse the cells. Proteinase K solution was then added to digest proteins. The mixture was then incubated at 56 °C for 10 minutes. 20 µL of RNAse A solution was added to the mixture and incubated at room temperature for 10 minutes to allow RNA degradation. 400 µL of 50% ethanol was added and mixed by vortexing. The prepared lysate was then transferred into a spin column inserted in a collection tube. This was then centrifuged and subjected to two wash steps. In the final step, elution buffer containing Tris-EDTA was used to elute the extracted DNA. The DNA was then quantified using the

NanoDrop ND1000 system (Thermo Scientific) and stored at -20 °C for downstream applications.

2.2.3. RNA Extraction and Quantification

To extract RNA, the direct-zol RNA miniprep kit (Zymo Research) was used as per manufacturer's instructions. This procedure briefly involved resuspending cell pellet in 1 mL TRI-reagent (Sigma) and 1 mL 100% ethanol. The mixture was then transferred into a spin column containing a collection tube and centrifuged for 30 seconds at 13 000 xg centrifugation speed. The column was subjected to two washing steps using buffers diluted in 100% ethanol. The RNA was then eluted using 50 µL of DNAse/RNAse free water. The sample was ran on a 1% agarose gel to confirm successful extraction. The remainder volume of RNA was stored at -80 °C for further use.

2.3. Real-time PCR (qPCR)

2.3.1. *hTERT* gene expression

To optimise determination of *hTERT* gene expression, RNA extracted from HEK293 cell line was converted into cDNA using the Maxima cDNA synthesis kit (Thermo scientific) containing RNAse inhibitor and DNAse as the manufacturer's instructions. This briefly involved mixing the template RNA with a 5X reaction mixture containing reaction buffer, dNTPs, oligo dT and random hexamer primers, as well as the maxima enzyme mix containing the reverse transcriptase and RNAse inhibitor. Nuclease free water was added to make a 20 µL reaction. This mixture was briefly centrifuged and subjected to the following cycling conditions in a 9600 Thermocycler (BIORAD); incubation for 10 min at 25 °C followed by 15 min at 50 °C. To terminate the reaction, reaction mixture was incubated at 85 °C for 5 min. The samples were then stored at -20 °C for downstream applications.

The converted cDNA was subjected to a probe based application for detection by real-time quantitative PCR (qPCR). This was used to determine *hTERT* expression in the HEK293 cell line relative to the internal reference gene; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

(Schmittgen and Zakrajsek, 2000). GAPDH is used as a reference gene in many gene expression quantification studies (Suzuki et al., 2000). Reference genes are usually expressed in all cell types at a relatively constant expression level (Janssens et al., 2004).

Quantitative PCR is performed using a thermal cycler which allows elucidation of each nucleic acid sample with a beam of light at a specified wavelength permitting the detection of fluorescence emitted by the excited fluorophore (Freeman et al., 1999). The first step of the technique employs the conventional PCR procedure. This requires that each nucleic acid sample is in equivalent amounts and that each sample under analysis amplifies with the same efficiency to allow quantitative analysis (Freeman et al., 1999). The amplified products are then illuminated and normalised to the amplification of a reference gene prior to analysis.

2.3.2. Characterisation of the hTERT promoter and MNS16A

2.3.2.1. DNA Amplification

The University of California Santa Cruz (UCSC) genome browser accessed through the web address http://genome.ucsc.edu/ was used to determine the fragment sizes in base pairs (bp) of the expected sequence with nucleotides in the database that lie between and include the primer pair. The browser uses an application called *In-silico* PCR which searches a sequence database with a pair of PCR primers. When successful, the search returns a sequence output file containing all sequence in the database that lie between and include the primer pair as outlined in **table 4**. Primers used for this study were previously designed by authors cited in **table 3**.

Table 3: List of previously designed *hTERT* promoter and MNS16A primers used in this study.

Primer Name	Sequences (5'-3')	Region	Reference
MNS16A_F	AGGATTCTGATCTCTGAAGGGTG	MNS16A satellite	(Wang et al., 2003)
MNS16A_R	TCTGCCTGAGGAAGGACGTATG	MNS16A satellite	(Wang et al., 2003)
Promoter_F	CCAGGCCGGGCTCCCAGTGGAT	hTERT promoter	(Zhang et al., 2000)
Promoter_R	TCCTGCTGCGCACGTGGGAAGCC	hTERT promoter	(Zhang et al., 2000)

Table 4: The *hTERT* promoter fragment size and sequence obtained from the UCSC In-Silico PCR. The sequences of the pair of primers are underlined and bolded.

Sequence	size
Expected Promoter Sequence **CCAGGCCGGGCTCCCAGTGGAT**TCGCGGGCACAGACGCCCAGGACCGCGCTTCCCACGTGGCGG AGGGACTGGGGACCCGGGCACCCGTCCTGCCCCTTCACCTTCCAGCTCCGCCTCCTCCGCGCGGAC CCCGCCCCGTCCCGACCCCTCCCGGGTCCCCGGCCCAGCCCCCTCCGGGCCCTCCCAGCCCCTCCCC TTCCTTTCCGCGGCCCCGCCCTCTCCTCGCGGCGCGAGTTTCAGGCAGCGCTGCG**TCCTGCTGCGC** **ACGTGGGAAGCC**	275 bp
Expected Minisatellite Sequence **AGGATTCTGATCTCTGAAGGGTG**GGTAGGGTGGGGCAGTGGAGGGTGTGGACACAGG AGGCTTCAGGGTGGGGCTGGTGATGCTCTCTCATCCTCTTATCATCTCCCAGTCTCATCTC TCATCCTCTTATCATCTCCCAGTCTCATCTGTCTTCCTCTTATCTCCCAGTCTCATCTGTCATC CTCTTACCATCTCCCAGTCTCATCTCTTATCCTCTTATCTCCTAGTCTCATCCAGACTTACCT CCCAGGGCGGGTGCCAGGCTCGCAGTGGAGCTGGA**CATACGTCCTTCCTCAGGCAGA**	302 bp

2.3.2.2. The hTERT minisatellite

A gradient PCR was performed using 20 ng/µL DNA extracted from HEK293 cells and oligonucleotide primers previously (Wang et al., 2003). In the literature, different authors had used various annealing temperatures for the PCR amplification of the *hTERT* minisatellite (MNS16A). The recommended annealing temperatures were 60 °C, 61 °C and 65 °C. Thus to determine optimum annealing temperature the gradient was performed at 60-65 °C using Mini thermal cycler (BIORAD). To prepare the reaction, the Phusion Flash HiFi Master mix (Thermo Fisher Scientific) was prepared as the manufacturer's instruction. The master mix contained all the necessary reaction components for PCR except for template DNA and primers. The MNS16A forward (F) and reverse (R) primers used for MNS16A PCR amplification are listed on **Table 3**. The cycling conditions briefly involved initial denaturation at 98 °C for 1s, 35 cycles of 98 °C for 10s; 60 – 65 °C for 15s; 72 °C for 30s; and final extension at 72 °C for 1 minute. After completion of PCR run, the PCR products were resolved in a 2.5% agarose gel prepared as outlined in **Appendix B**.

The DNA obtained from participants was amplified by PCR to characterise the *hTERT* minisatellite (MNS16A). Successive cycles of denaturation of target DNA, annealing of primers, and extension of primers from their 3' ends by DNA polymerase, amplifies the region of interest. 10 µl PCR reactions were prepared and a no template control (NTC) was included to detect contamination (**Appendix B**). The reaction was carried out using the 9600 thermal cycler (BIORAD) and PCR products stored at – 20 °C until further use.

The amplified fragments were then resolved by 1 % agarose (Conda, Low EEO) gel electrophoresis using 1X Tris-Borate EDTA (TBE) buffer to validate the PCR amplified the correct fragment size.

2.3.2.3. Agarose Gel electrophoresis

Gel electrophoresis is a type of technique used to separate DNA fragments by size in an electric field. The technique utilises crosslinks that are formed when the agarose powder is mixed with Tris-EDTA buffer. These crosslinks determine whether a nucleic acid fragment can pass through. The larger the fragment size the less mobile the fragment will be.

The *hTERT* promoter and MNS16A amplified fragments were resolved in 1% and 2.2% agarose gel respectively in a 1X TBE buffer. The gel was left to run for 40 – 60 minutes at 80 volts and viewed using the gel doc imaging system (BIORAD). A 1Kb plus DNA ladder (GeneDirex) was used as a molecular marker to identify the size of fragments separated by gel electrophoresis.

2.3.3. Sequencing and Sequence Analysis

Prior to sequencing, samples (PCR products and gel slabs) were purified using PCR and Gel purification kits (Thermo Scientific) as the manufacturer's instructions. After the correct size fragment has been validated, the fragment of interest was extracted from the gel and purified using the GeneJet Gel extraction kit (Thermo K0691). This briefly involved cutting out the band of interest from the agarose gel and weighing it on a weighing scale. After determination of the weight of the gel, a 1:1 (binding buffer: gel weight) ratio of binding buffer is added to the gel (i.e. 100 µl of binding buffer for every 100 mg of agarose gel). This was then incubated at 60 °C for ten minutes to allow the gel to dissolve. Twice the volume of 100% isopropanol was added

to the mixture and transferred to a column with a collection tube. After several centrifugation and wash steps, the DNA was eluted in 25 µL using the elution buffer (10 mM Tris-HCl, pH 8.5).

Purified products were then validated by running 1 µl on a gel against a 1Kb ladder (Inqaba Biotech). The validated products were sequenced by Inqaba Biotec This procedure involves determining the order of the nucleotide bases in a DNA sequence. The type of sequencing used to carry out the reaction is the Sanger sequencing/Chain termination method which utilises dideoxynucleotides (ddNTPs) to terminate elongation of the sequence of interest (Sanger et al., 1977).

Sequence analysis was carried out using sequence alignment softwares such as Bioedit and/or Seqscanner. The softwares align and compare the sequence of interest with known human genome sequences in the *National Centre for Biotechnology Information* (NCBI) database GRCh37.p10 reference assembly (GCF_000001405.22). This allowed the detection of any variation within the *hTERT* promoter and minisatellite sequences in the study of black South African population.

2.3.4. Determination of telomere length

2.3.4.1. Telomere length determination by Single Telomere Length Analysis (STELA)

Telomere length was determined using the modified Single Telomere Length Analysis (STELA) (Baird et al., 2003). The first step involved PCR amplification of the telomere repeats and regions of DNA adjacent to telomeres (**Fig. 11**). The DNA was first diluted to 10ng/µL using 10 mM Tris_EDTA (TE) (Sigma). The diluted DNA was then diluted to 250 pg/µL containing 0.25 µM telorette 2 primer. This was prepared by mixing 1 µL of 10 ng/µL genomic DNA, 1 µL of 10 mM telorrette primer and 38 µL of 10 mM TE buffer (Sigma) and stored at - 20°C. To prepare the PCR reactions, 1X Phusion Flash High-Fidelity PCR master mix (Thermo Scientific) containing all the necessary reaction components for PCR (High fidelity Taq polymerase, dNTPs and buffers) was mixed with 0.5 µM of teltail primer with either 17pseq1rev or XpYpE primers outlined in **Table 5**. The PCR cycling conditions were as follows; 98 °C for 10 seconds, 23 cycles of 98 °C for

2 seconds; 65 °C (XpYpE)/ 59 °C (17 pseq1rev) for 30 seconds, 72 °C for 5 minutes and final elongation step at 72 °C for 10 minutes.

The amplified products were then resolved by submarine 0.5-0.8% agarose gel electrophoresis in Tris-Acetate EDTA (TAE) buffer for 18 hours. The separated fragments were transferred to a nylon membrane by alkaline Southern blotting (Koetsier et al., 1993). The DNA fragments were expected to maintain the separation pattern they had on the gel. It should be noted that the bands were not visible on the gel as expected.

Table 5: List of primer sequences for STELA PCR reactions.

Primer name	Primer sequence	Reference
Telorrette	5'-TGCTCCGTG CATCTGGCATCTAACCCT-3'	(Baird et al., 2003)
Teltail	5'-TGCTCCGTGCATCTGGCATC-3'	(Baird et al., 2003)
17pseq1rev	5'-GAATCCACGGATTGCTTTGTGTAC-3'	(Britt-Compton et al., 2006)
XpYpE	5'-TTGTCTCAGGGTCCTAGTG-3'	(Britt-Compton et al., 2006)

The transferred products were detected using the TeloTAGGG kit (Roche) as follows; the blotted DNA fragments were hybridised to a digoxigenin (DIG)-labelled probe specific for telomeric repeats. The membrane was then incubated with a DIG-specific antibody covalently connected to alkaline phosphate. Finally, the restrained telomere probe DIG was visualized by virtue of alkaline phosphatase metabolizing CDP-Star, a highly sensitive chemiluminescence substrate in a ChemiDoc (BioRad). The procedure was unsuccessful after several trials. Consequently the relative telomere length was assessed using real-time quantitative PCR as described in section 2.3.4.2.

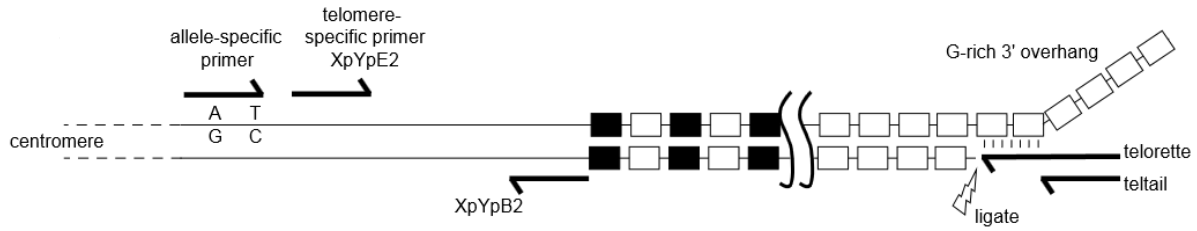


Figure 11: The principle of telomere extension using the STELA technique. Showing the primers and linkers involved in telomere extension. Reproduced with permission from the Nature Publishing Group (Baird et al., 2003).

2.3.4.2. Relative telomere length determination by quantitative real-time Polymerase Chain Reaction (qPCR)

Relative telomere length of participants was analysed using a modified method of quantitative PCR (qPCR) (Cawthon, 2002). All PCRs were performed on the light cycler 480 real time PCR system (Roche Diagnostics). Subsequently analysis was performed using the LC480 software version 1.5 (Roche Life Sciences). The experiment was prepared in triplicates for each DNA sample. Conditions are described in Appendix B. Genomic DNA extracted from HEK293 cell line and participants tissue was mixed with LC480 SYBR Green I Master (Roche diagnostics) containing all the necessary reaction components for PCR, the acidic ribosomal phosphoprotein (36B4) reference gene was amplified with primers **36B4u**, CAGCAAGTGGGAAGGTGTAATCC and **36B4d**, CCCATTCTATCATCAACGGGTACAA and nuclease free water. In a separate PCR reaction, respective primers pairs (Telomere length primers **tel 1**, GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT; **tel 2**, TCCCGACTATCCCTATCCCTATCCCTATCCCTATCC-CTA were used to determine telomere length.

The standard curve was generated using HEK293 extracted DNA. The relative telomere length was determined using the ratio between telomere length and 36B4 expression (Cawthon et al., 2003).

2.3.5. Bioinformatics

2.3.5.1. Determination of global variation in the hTERT gene and promoter

With the aid of the Sydney Brenner Institute of Molecular Biosciences (SBIMB) staff, SNPs within the *hTERT* gene and promoter were identified in different populations across the globe.

The data collection and analysis was done using the 1000 genomes and HapMap data accessed through www.1000genomes.org/ and hapmap.ncbi.nlm.nih.gov/ respectively. The distribution of the allele and genotype frequencies were assessed in European, Asian, African and mixed populations. These were then compared to the African populations genotyped in 1000 genomes to evaluate the differences observed in each population. In addition to African populations, sequence data from the black Sowetans in South Africa (BSO) (May et al., 2013) was also analysed.

2.3.5.2. DNA Amplification

The University of California Santa Cruz (UCSC) genome browser accessed through the web address http://genome.ucsc.edu/ was used to determine the fragment sizes in base pairs (bp) of the expected sequence with nucleotides in the database that lie between and include the primer pair. The browser uses an application called *In-silico* PCR which searches a sequence database with a pair of PCR primers. When successful, the search returns a sequence output file containing all sequence in the database that lie between and include the primer pair.

2.3.6. Statistical analyses

The MNS16A genotype and allele frequencies were determined by direct counting and deviation from Hardy-Weinberg Equilibrium (HWE) was tested. HWE was tested to establish whether genotype and allele frequencies in different populations remained constant in every generation with limited evolutionary influence. In order to determine whether a statistically significant association exists between CKD and MNS16A variants, analysis of variance (ANOVA and T-tests) and odd ratio (OR) was used. This briefly tests for the probability of presence or absence of disease in individuals with either long or short alleles of the MNS16A. The same tests were used to determine association between hTERT gene and promoter SNPs identified through bioinformatics analyses as described in section 2.3.5.1. All tests were performed at 95% confidence intervals (CI). To correlate MNS16A variants and clinical data to relative telomere length (RTL), Pearson Spearman's correlation was measured. Statistical analyses with a p value less than or equal to 0.05 were considered as statistically significant. All statistical analyses were performed using the GraphPad Prism statistics software version 5.1.

Chapter 3: Results

Section 1: Genetic variation within *hTERT* gene in African populations and non-African populations.

3.1. Chapter introduction

The primary aim of this study was to determine whether telomere genetics play a role in kidney disease progression and severity in the presence or absence of HIV infection. The *hTERT* gene has been identified as a genetic risk factor in several diseases. It has been shown that upregulation of *hTERT* at any point of the cell cycle, proliferation is induced. More evidence is suggestive of a non-telomere related function of *hTERT* that promotes cellular viability, proliferation and inflammation. Even more so, regulatory regions such as the *hTERT* promoter and MNS16A have become important role players as they have been shown to influence *hTERT* gene expression and ultimately telomere length (Huang et al., 2013; Wang et al., 2014; Wang et al., 2003).

It was predicted that participants who are HIV positive with kidney disease will present with variation in telomere length and telomere-related genes such as the *hTERT*. To test these predictions, the *hTERT* promoter was screened for mutations and MNS16A size was determined. The average telomere length was also measured. One of the aims of the study was to determine genetic variation within the *hTERT* gene in African population and to identify any unique single nucleotide polymorphisms in these populations.

3.2. *hTERT* gene and promoter (Bioinformatics)

To identify common genetic variants within the *hTERT* gene and promoter, data from genome-wide association studies was obtained with the assistance of the Wits bioinformatics/Sydney Brenner Institute for Molecular Biology (SBIMB). The obtained data was compared to other populations genotyped and reported in the literature. These were subsequently analysed using GraphPad prism to test for statistical significance and other bioinformatics tools including HapMap, 1000 genomes and Ensembl.

3.2.1. The *hTERT* gene

The *hTERT* gene located in chromosome 5p15.33 was assessed to identify variants in different populations. There was no significant differences in the distribution of the *hTERT* gene variants between African and non-African populations. **Table 6** below shows all the different populations analysed in this study. This included all the populations genotyped through HapMap and 1000 Genomes. Included in the analysis as well is a black South African population (n = 94) from Soweto (BSO) genotyped in the birth to twenty (B2T) longitudinal cohort (Richter et al., 2007). The BSO population consisted of 94 unrelated Southeastern Bantu-speakers residing in the Soweto-Johannesburg area in South Africa. These individuals are all of self-identify as of black ethnicity. The individuals were existing participants from a longitudinal study called the Birth to Twenty (B2T) cohort (Richter et al., 2007). This population is highlighted in bold in **Table 6**.

Table 6: Populations analysed for variation in common *hTERT* gene and promoter variants.

Population Code	Population Description	Super Population
CHB	Han Chinese in Bejing, China	East Asian (EAS)
JPT	Japanese in Tokyo, Japan	EAS
CHS	Southern Han Chinese	EAS
CDX	Chinese Dai in Xishuangbanna, China	EAS
KHV	Kinh in Ho Chi Minh City, Vietnam	EAS
CEU	Utah Residents (CEPH) with Northern and Western European Ancestry	European (EUR)
TSI	Toscani in Italia	EUR
FIN	Finnish in Finland	EUR
GBR	British in England and Scotland	EUR
IBS	Iberian Population in Spain	EUR
YRI	Yoruba in Ibadan, Nigeria	African (AFR)
LWK	Luhya in Webuye, Kenya	AFR
GWD	Gambian in Western Divisions in the Gambia	AFR
MSL	Mende in Sierra Leone	AFR
ESN	Esan in Nigeria	AFR
ASW	Americans of African Ancestry in SW USA	AFR
ACB	African Caribbeans in Barbados	AFR
BSO	**Black Sowetans in South Africa**	**AFR**
MXL	Mexican Ancestry from Los Angeles USA	Admix American (AMR)
PUR	Puerto Ricans from Puerto Rico	AMR
CLM	Colombians from Medellin, Colombia	AMR
PEL	Peruvians from Lima, Peru	AMR
GIH	Gujarati Indian from Houston, Texas	South Asian (SAS)
PJL	Punjabi from Lahore, Pakistan	SAS
BEB	Bengali from Bangladesh	SAS
STU	Sri Lankan Tamil from the UK	SAS
ITU	Indian Telugu from the UK	SAS

There was no significant difference in the distribution of most *hTERT* common variants among the different populations (all variants outlined in **appendix D**). Even more so, there is still missing data on distribution of some of the common variants especially among African populations. The majority of the variants reported previously in 1000 genomes and HapMap are not associated with phenotype as main aim of these two projects was to only detect variants present in different populations. Most research was carried out predominantly in non-African populations where some of these variants are shown to be significantly associated with disease susceptibility. As a result of African countries mainly focusing their research on infectious diseases, data is very limited on the distribution and pathogenicity of many of the *hTERT* variants. For the purpose of this result, three variants within the *hTERT* gene shown to play significant roles in disease susceptibility were analysed.

3.2.1.1. The rs2736100

The variant rs2736100 (A>C) is located in intron 2 of the *hTERT* gene and is suggested to influence *hTERT* expression and telomere length (Codd et al., 2013). This particular variant has been associated with several diseases including lung and bladder cancers (Gago-Dominguez et al., 2011; McKay et al., 2008). No significant difference in allele distribution among the different populations was observed. It should be noted that individuals in the South Asian populations had slightly higher frequencies of the minor allele C compared to all the other populations represented in this analysis (**Fig. 12**) represented in blue.

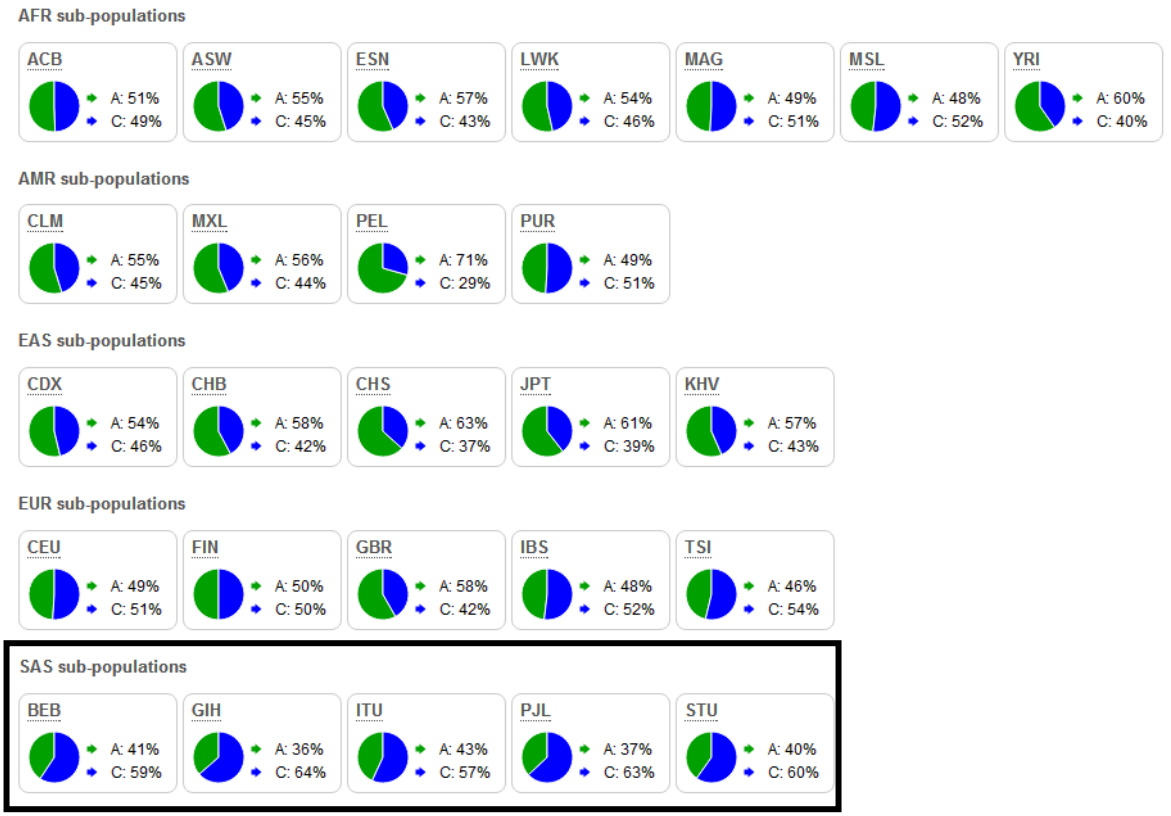


Figure 12: Allele frequencies of the rs2736100 SNP in different populations. The South Asian populations presented with higher frequencies of minor allele C indicated in blue and boxed in black rectangle.

3.2.1.2. The rs2736098

Another variant of importance located in the second exon of the *hTERT* gene is rs2736098. This variant results in a synonymous substitution (A305A) of cytosine to thymine (C>T) at position 1 293 971 on chromosome 5. Although synonymous variants do not alter the amino acid produced, the change in codon may influence the translational and protein folding kinetics. This change may result in a notable functional impact of the hTERT. All the African populations presented with very low frequencies of the minor allele T (10% or less) (**Fig. 13**). Interestingly, South Asian populations displayed significantly higher frequencies of the minor allele. These are highlighted in rectangular blocks (**Fig. 13**).

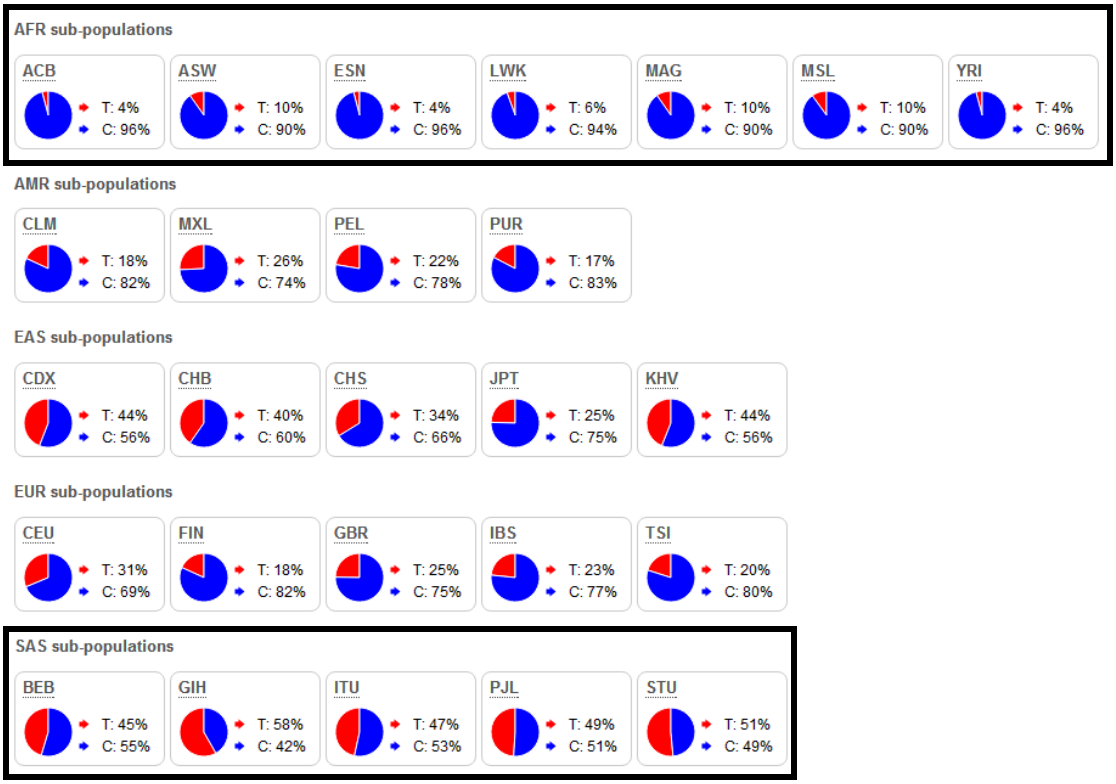


Figure 13: Allele frequencies of the rs2736098 SNP in different populations. The minor allele T is overrepresented in the South Asian populations.

3.2.1.3. The rs401681

Another variant of importance located in the *hTERT* gene is rs401681. This variant results in a cytosine to thymine (C>T). This particular variant has not been associated with risk to several diseases. In fact, the rs401681 is not associated with telomere shortening, breast and colorectal cancers, as well as melanoma in Caucasians (Pooley et al., 2010). The minor allele T is overrepresented in African populations (**Fig. 14**). Whereas in South Asian populations; the T-allele is in low frequencies. Highlighted in rectangular blocks (**Fig. 14**).

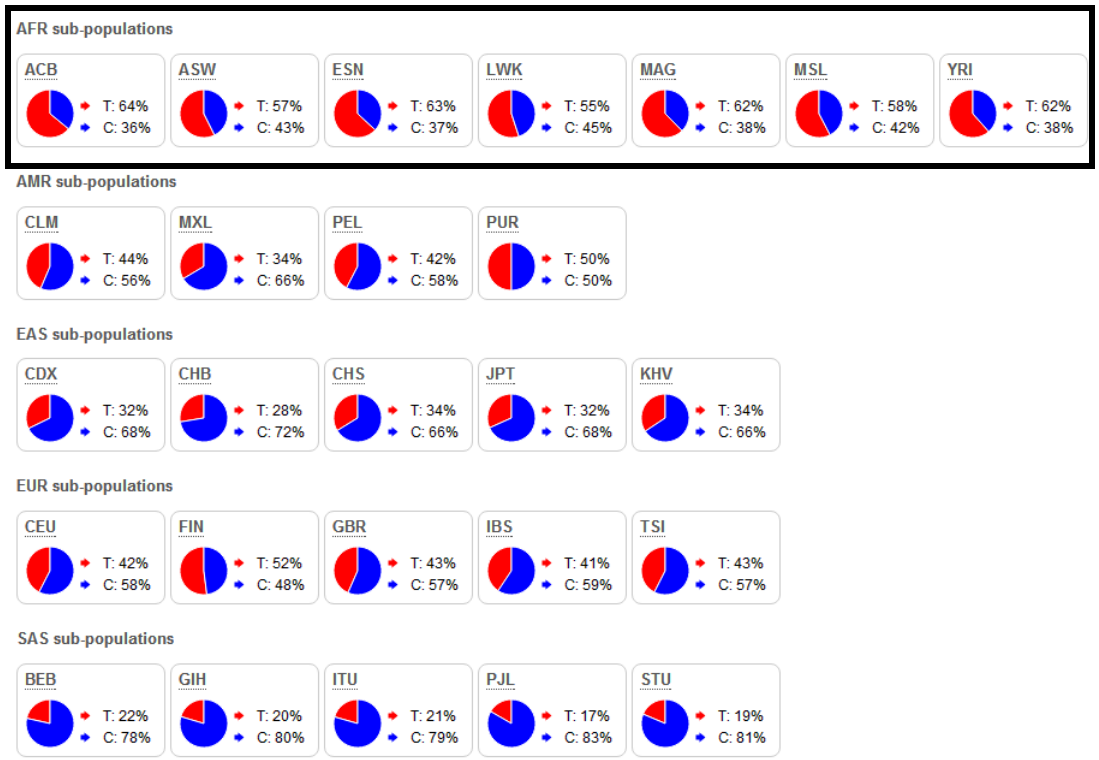


Figure 14: Allele frequencies of the rs401681 in different populations.

3.2.1.4. The Southeastern Bantu speakers

The three selected variants were also analysed in individuals genotyped in the Birth to Twenty (B2T) cohort (May et al., 2013). These included 94 unrelated Bantu speakers residing in the Soweto region of Johannesburg, South Africa. Allele frequencies were calculated to identify populations closely related to the Southeastern Bantu speakers (**Table 7**).

Table 7: Distribution of rs2736100, rs2736098 and rs401681 alleles in the Birth to Twenty cohort. The populations in bold had allele frequencies very similar to the black South African population (BSO).

Black Sowetans in South Africa (BSO) (n = 94)

	Ancestral allele frequency	Minor allele frequency	Populations closely related to:
rs2736100	**A**	**C**	**ACB** and **LWK**
	52%	48%	
rs2736098	**C**	**T**	**ACB**, ESN, **LWK** and **YRI**
	94%	6%	
rs401681	**C**	**T**	**ACB**, ESN, MAG and **YRI**
	38%	62%	

Analysis of the three SNPs in the South African population revealed similar allele distribution to three populations i.e. ACB, LWK and YRI. Comparison of the black South African population at a global scale has always clustered with the LWK and YRI populations. Interestingly analysis of these three SNPs within the *hTERT* gene suggests that BSO is similar to the ACB population.

3.2.2. The *hTERT* promoter (Bioinfomatics)

Bioinformatics data analyses of the common variants revealed no significant difference in the distribution of hTERT promoter SNPs in different populations (**Table 8**). Furthermore, the data obtained on these variants was strictly based on elucidating distribution of the variants among populations in different parts of the world (HapMap and 1000 Genomes). As a result, a phenotypic consequence of these variants is limited particularly in African populations.

Interestingly, a significant difference (**Fig. 15**) in distribution of one particular variant rs2853669 between African and non-African populations genotyped in the HapMap Project was observed. This particular variant results in a cytosine to thymine (C>T) change in 5: g.1295349(C>T) (GRCh37) and 5: g.1295234(C>T) (GRCh38). The rs2853669 has been associated with breast and oesophageal cancers in Caucasian and Asian populations but has not been associated with any disease susceptibility in Africans (Labussiere et al., 2014).

None of the common promoter variants were detected in the BSO population. The observed differences in distribution of rs2853669 variant in different populations warrants further research. This will increase the limited data on genetic variation in telomere-related genes and their potential role in disease susceptibility in Africans.

Table 8: Data showing different variants identified within the hTERT promoter in different populations and their genotype distributions. Variant **rs2853669** (Chr5: 1295349) highlighted in red had the major differences between African and non-African populations. Only the two variant positions have been allocated rs numbers i.e. Chr5:1295349 (rs2853669) and Chr5:1295373 (rs35226131).

SNP	Chr5:1295264 (A>G)			Chr5:1295277 (A>G)			Chr5:1295283 (A>G)			Chr5:1295297 (G>A)			Chr5:1295316 (T>G)		
Genotypes	A/A	A/G	G/G	A/A	A/G	G/G	A/A	A/G	G/G	G/G	G/A	A/A	T/T	T/G	G/G
CEU (n=99)	0	0	99	0	0	99	0	0	99	0	0	99	0	0	99
CHB (n=103)	0	0	103	0	0	103	0	0	103	0	0	103	0	0	103
ESN (n=99)	0	0	99	0	0	99	0	0	99	0	0	99	0	0	99
GWD (n=113)	0	0	113	0	0	113	0	1	112	0	5	108	0	0	113
LWK (n=101)	0	0	101	0	0	101	0	0	101	0	1	100	0	0	101
MSL (n=85)	0	0	85	0	0	85	0	0	85	0	2	83	0	0	85

SNP	Chr5:1295322 (C>G)			Chr5:1295349 (C>T)			Chr5:1295373 (T>C)			Chr5:1295399 (C>G)		
Genotypes	C/C	C/G	G/G	C/C	C/T	T/T	T/T	T/C	C/C	C/C	C/G	G/G
CEU (n=99)	0	0	99	10	49	40	0	5	94	0	0	99
CHB (n=103)	0	0	103	13	57	33	0	0	103	0	0	103
ESN (n=99)	0	0	99	2	5	92	0	0	99	0	0	99
GWD (n=113)	0	5	108	2	23	88	0	7	106	0	0	113
LWK (n=101)	1	8	92	0	11	90	0	0	101	0	0	101
MSL (n=85)	0	4	81	0	17	68	0	0	85	0	0	85

Populations

CEU: Northern Europeans from Utah

CHB: Han Chinese in Beijing, China

ESN: Esan in Nigeria

GWD: Gambian in Western Divisions in Gambia

LWK: Luhya in Webuye, Kenya

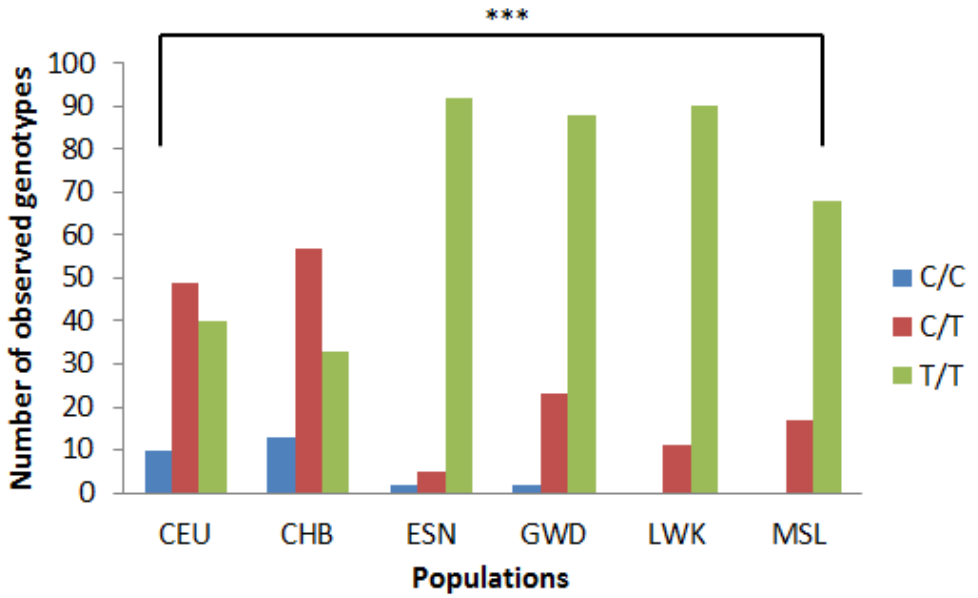


Figure 15: The hTERT promoter SNP **rs2853669** genotype frequencies in different populations. Significant difference in distribution of genotypes between the different populations (*** = p<0.0001). **CEU**: Utah Residents with Northern and Western European Ancestry; **CHB**: Han Chinese in Bejing, China; **ESN**: Esan in Nigeria; **GWD**: Gambian in Western Divisions in the Gambia; **LWK**: Luhya in Webuye, Kenya and **MSL**: Mende in Sierra Leone.

Section 2: The hTERT promoter and minisatellite (MNS16A) characterisation in study population.

3.3. Study population

DNA extracted from kidney biopsies of CKD participants and blood leucocytes of participants with no sign of CKD. Characteristics of the study participants are summarized in **table 9**. A total of 159 participants recruited from Charlotte Maxeke Hospital, Johannesburg: 98 patients with CKD (36 HIVAN; 32 HIV positive with CKD that is not HIVAN and 30 HIV negative with CKD) and 61 controls (31 HIV positive and 30 HIV negative with no clinical evidence of CKD). 62% (98/159) of the participants had kidney disease and of the 98, 69% (68/98) were HIV positive. The study also consisted of 58.5% (93/159) females and 41.5% (66/159) males. The female participants were over represented in the HIVAN and nonHIVAN histopathologic groups. There was no significant difference (p>0.05) in the mean age within all histopathologic groups.

Table 9: Clinical characteristics of study participants.

Characteristic	CKD		Controls			P value
	Histopathologic Groups					
	HIVAN (n=36)	nonHIVAN (n=32)	HIVnegK (n=30)	HIVposNK (n=31)	Controls (n=30)	
Men (%)	14(39%)	14 (44%)	18 (60%)	8 (26%)	12 (40%)	ns
Women (%)	22 (61%)	18 (56%)	12 (40%)	23 (74%)	18 (60%)	ns
Mean age (SD)	33.78 (8.563)	34.91 (9.848)	37.07 (11.31)	37.61 (8.049)	35.53 (11.68)	ns
Mean Serum creatinine (µmol/L)	577	285.1	337	108.5	-	***
Mean eGFR (ml/min)	15.31	38.59	98.5	181.2	-	*
Mean Urea (mmol/L)	16.92	15.18	19.9	-	-	ns

***SD** = standard deviation.*
***eGFR** = estimated glomerular flitration rate.*
***ns** = not significant at 95% confidence interval (p > 0.05).*
*Statistically significant when *** (p ≤ 0.001) ** (0.001 < p ≤ 0.01) * (0.05 ≤ p < 0.01)*

The mean age difference between all histopathological groups did not have a significant standard deviation (SD) ranging from 8.049 – 11.68). It is one of the recommendations that case-control studies involving telomere length measurement should consider age-matched participants as it is well know that progressive telomere shortening is a function of biological ageing (Blackburn, 2005b; Blackburn and Gall, 1978; Blasco et al., 1999; Greider and Blackburn, 1985; Harley et al., 1990; Shkreli et al., 2012). As result telomere length versus age analysis was performed.

Serum creatinine and urea levels are utilized in pathology as measures of kidney malfunction. Creatine and urea are usually excreted from the body through the kidneys. High levels of these two molecules in the blood indicate progressive kidney failure (Naicker, 2002; Wyatt, 2012; Wyatt et al., 2007). Using creatinine levels, age, gender and ethnicity; estimated glomerular filtration rate (eGFR) was calculated. The lower the levels of eGFR, the higher the risk of CKD developing into end-stage renal disease (ESRD), a major cause of death. Individuals presenting with eGFR between < 15 to 40 ml/min are usually at stages 3 – 5 of CKD where they suffer from moderate to severe loss of kidney function. This was mostly observed in the participants with CKD who are also infected with HIV.

Data regarding serum creatinine and urea levels in the control group was unavailable. This was because when control group participants were recruited, they were HIV negative and had no sign or symptom of CKD. Furthermore, urea levels were too low or undetectable in HIV seropositive group without CKD (HIVposNK). All clinical data is outlined in **Appendix C**.

3.4. The *hTERT* promoter

After completion of the gradient PCR procedure, the optimal annealing temperature for hTERT promoter amplification was determined. Using primers outline in the methods section 2.3.2.2.

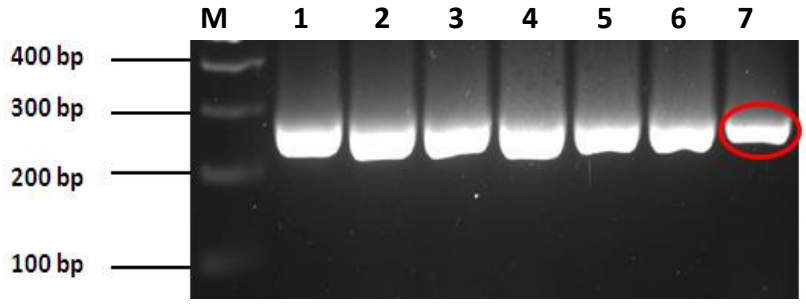


Figure 16: Gel image showing *hTERT* promoter amplification using gradient PCR. Lanes 1 – 7 represent HEK293 extracted DNA of 20ng/µl concentration. Lane 7 shows the desired amplicon size (approximately 275 bp) with limited smearing and non-specific amplification.

The annealing temperature for lane 7 was 67.1 °C and this was selected for amplification of *hTERT* promoter in participant.DNA.

3.5. The hTERT minisatellite (MNS16A)

The *hTERT* minisatellite (MNS16A) was amplified using DNA extracted from HEK293 cell line. The primers MNS16A_F and MNS16A_R (**Table 3**) and conditions are outlined in the methods section 2.3.2.2. The gradient PCR resulted in an amplification of a 243 bp MNS16A.

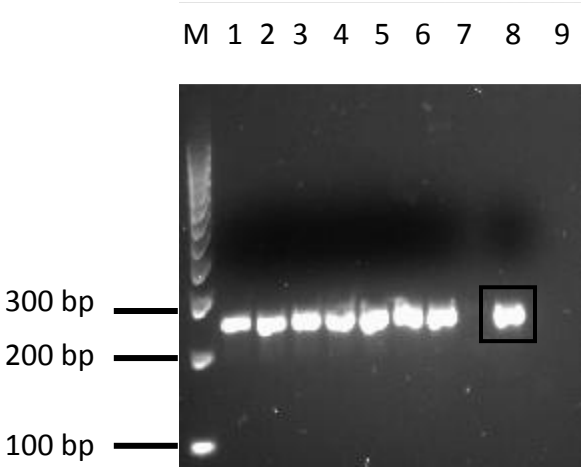


Figure 17: Gel image of a 2.5% agarose gel showing successful *hTERT* MNS16A amplification at eight gradient temperatures between 60°C and 65°C. Estimated size from UCSC Genome browser was 302 bp. Lane 8 was skipped due to suspicion of lane 8 product overflowing to lane 9. The temperature of 60 °C (lane 9) was selected for downstream PCR amplification of the MNS16A.

After all of these adjustments, another gradient PCR of temperatures 60-65 °C was performed and was successful at all temperatures. The PCR products were run on a 2.5 % agarose gel (**Fig. 17**). The 60–65 °C annealing temperatures showed successful amplification. However, a temperature of 60 °C was selected as the best annealing temperature for downstream analysis.

3.6. Promoter (Participant DNA)

To identify South African-specific variants in the *hTERT* promoter, the promoter region of approximately ~275 bp was amplified. The successfully amplified products were resolved on a 1% agarose gel in 1x TAE buffer (**Fig. 18**).

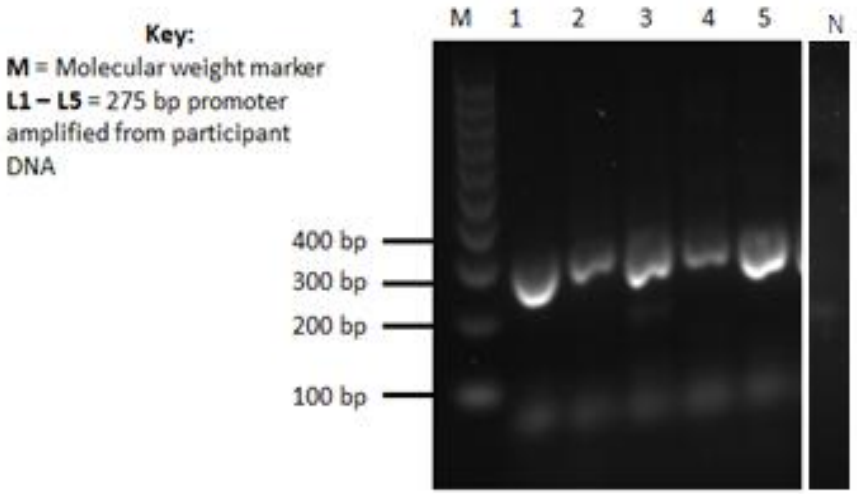


Figure 18: Gel image showing successful promoter amplification of participant DNA resolved in a 1% agarose gel. Lanes 1 – 5 shows successful amplification of the hTERT promoter ~275 bp. Lane N shows the negative control.

These were then purified and sent for direct sequencing at Inqaba Biotech. Figure 3 below shows alignment of sequencing results with the reference genome **hg19, GRCh37**. None of the reported promoter variants were detected in the study participant groups (**Fig. 19**). Neither did any new SNPs or SA-specific SNPS were identified.

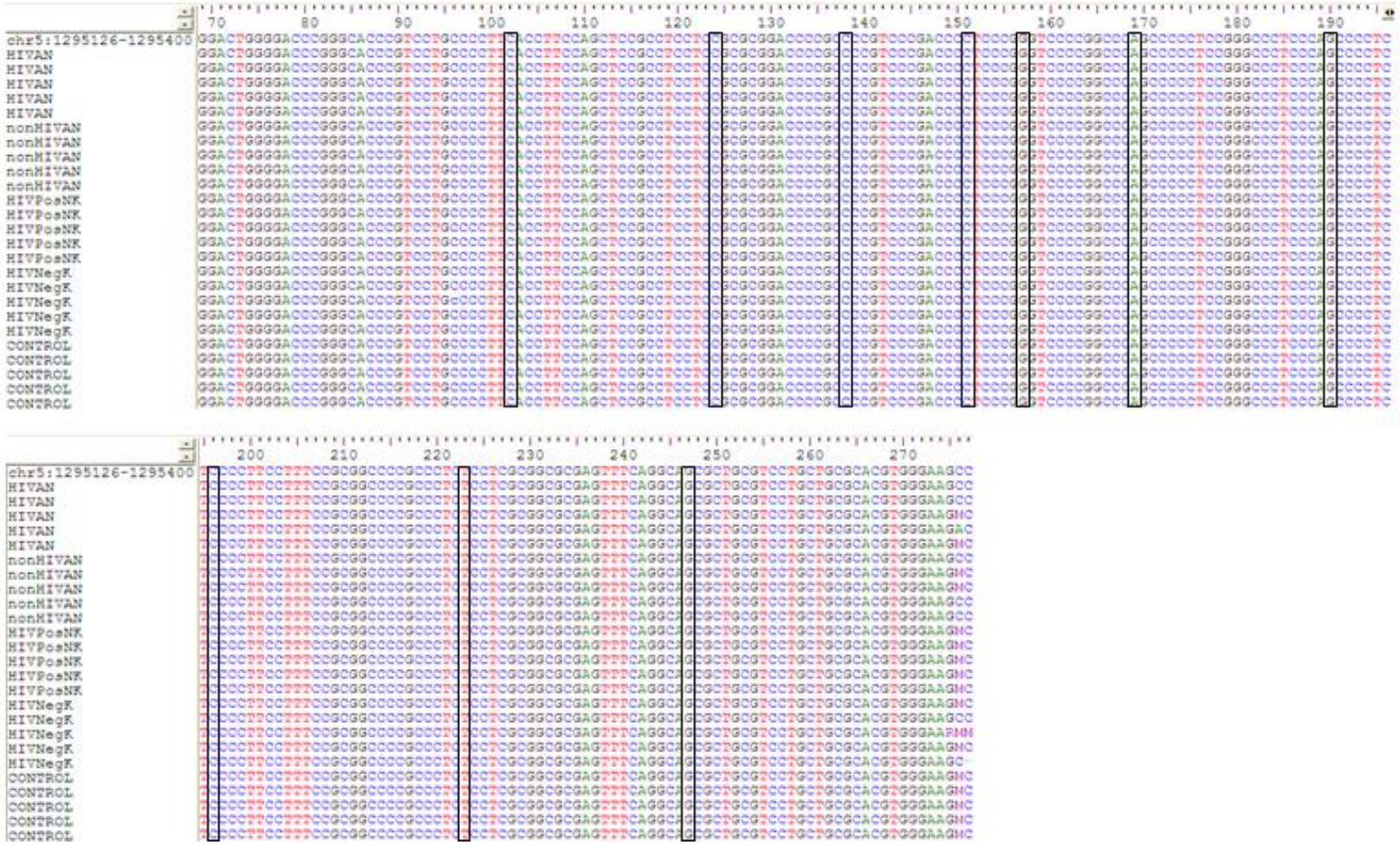


Figure 19: Sequence alignment of the promoter region. Highlighted with boxes are the common promoter variant positions. None of the common promoter variants were detected in the study population.

3.7. MNS16A genotyping

Genotyping for MNS16A allele sizes was successful in 150 study participants. In 9 participants amplification of the MNS16A was unsuccessful . However for these samples the promoter was successfully amplified. Since DNA amplification was successful at other loci, we ruled out DNA quality as the potential contributing factor. This observation suggests a possible deletion within the primer binding sites of the MNS16A region. The amplified products were successfully separated using agarose gel electrophoresis (**Fig 20-21**). In total, three different MNS16A genotypes were observed and classified as either long (302 bp) or short (243 bp and 274 bp). Amplified products were verified by direct sequencing.

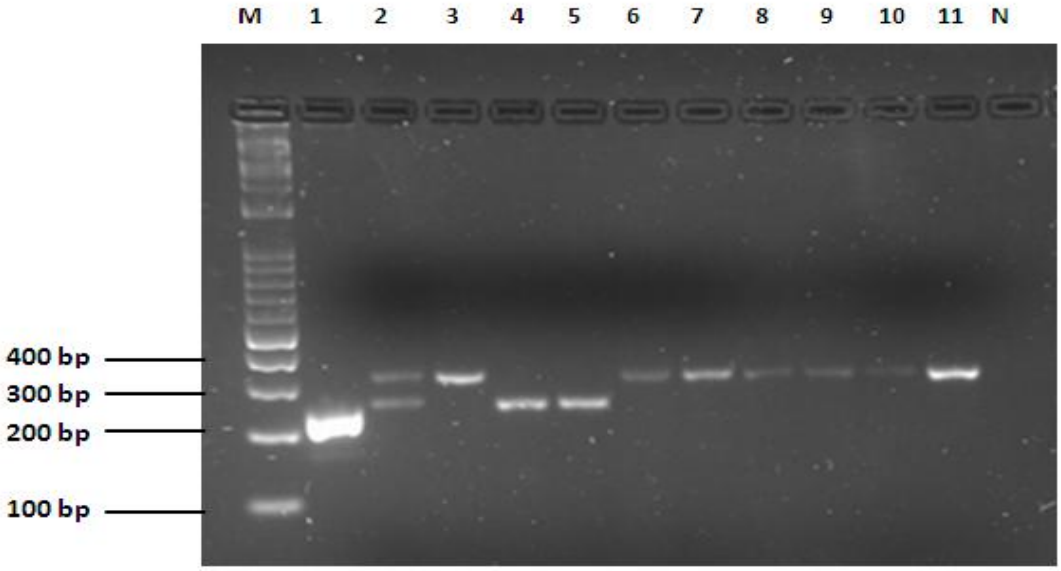


Figure 20: Agarose gel showing genotype patterns of the MNS16A in different individuals after PCR amplification. This shows the 302 bp and 243 bp MNS16A variants. Lane 1 = S/S (243/243 bp); Lane 2 = L/S (302/274 bp); Lane 3 = L/L (302/302 bp); Lanes 4-5 = S/S (274/274 bp); Lanes 6-11 = L/L (302/302 bp) and N = No template control.

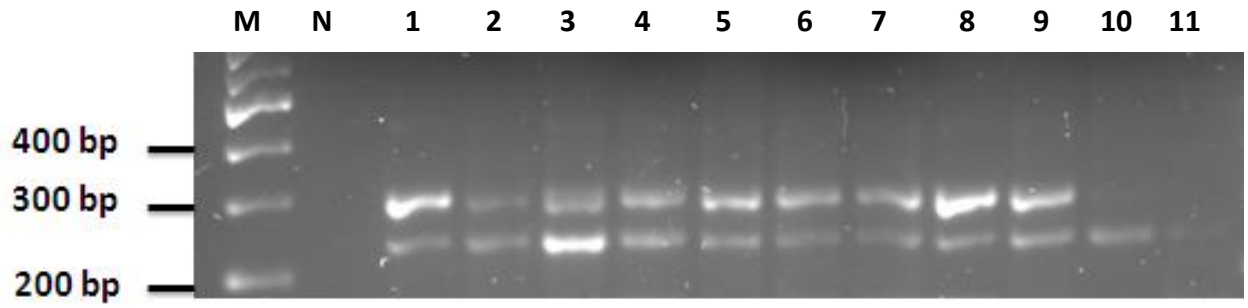


Figure 21: Agarose gel showing genotype patterns of the MNS16A in different individuals after PCR amplification. This shows the 302 bp and 243 bp MNS16A variants. Lanes 1 to 9 showing individuals with the heterozygous genotype **L/S** (302 bp/243 bp). Lanes 10 -11 showing homozygous genotype **S/S** (243bp/243 bp). The **M** and **N** lanes represent the molecular weight marker and negative control (**N**) respectively.

3.7.1. MNS16A genotype and allele frequencies

The MNS16A genotype and allele frequencies were determined in the CKD and control groups (**Table 10**). It was noted that the participants with CKD had an over representation of the long MNS16A variant. In contrast, the long MNS16A variant was underrepresented in the no CKD groups regardless of HIV status (**Fig. 22**). It is noteworthy that the participants with suspected deletion within the MNS16A were all HIV positive.

Table 10: The MNS16A genotype and allele distributions in study participants.

		Genotypes				**Alleles**	
Groups	Total (n)	L/L	L/S*	S*/S*	Deletion	L	S*
HIVAN	36	22 (61%)	4 (11%)	4 (11%)	6 (17%)	48 (80%)	12 (20%)
nonHIVAN	32	21 (66%)	4 (12%)	3 (9%)	4 (12%)	46 (76%)	16 (24%)
HIVnegK	30	13 (43%)	12 (40%)	5 (17%)	0	38 (63%)	22 (37%)
HIVposNK	31	1 (3%)	8 (26%)	21 (68%)	1 (3%)	10 (17%)	50 (83%)
Controls	30	1 (3%)	3 (10%)	26 (87%)	0	5 (8%)	55 (92%)

* represents either 243/272 bp short MNS16A sizes.

Deletion: Samples that did not amplify the minisatellite but were successful for promoter amplification and thus suggestive of a possible deletion within the MNS16A primer binding sites.

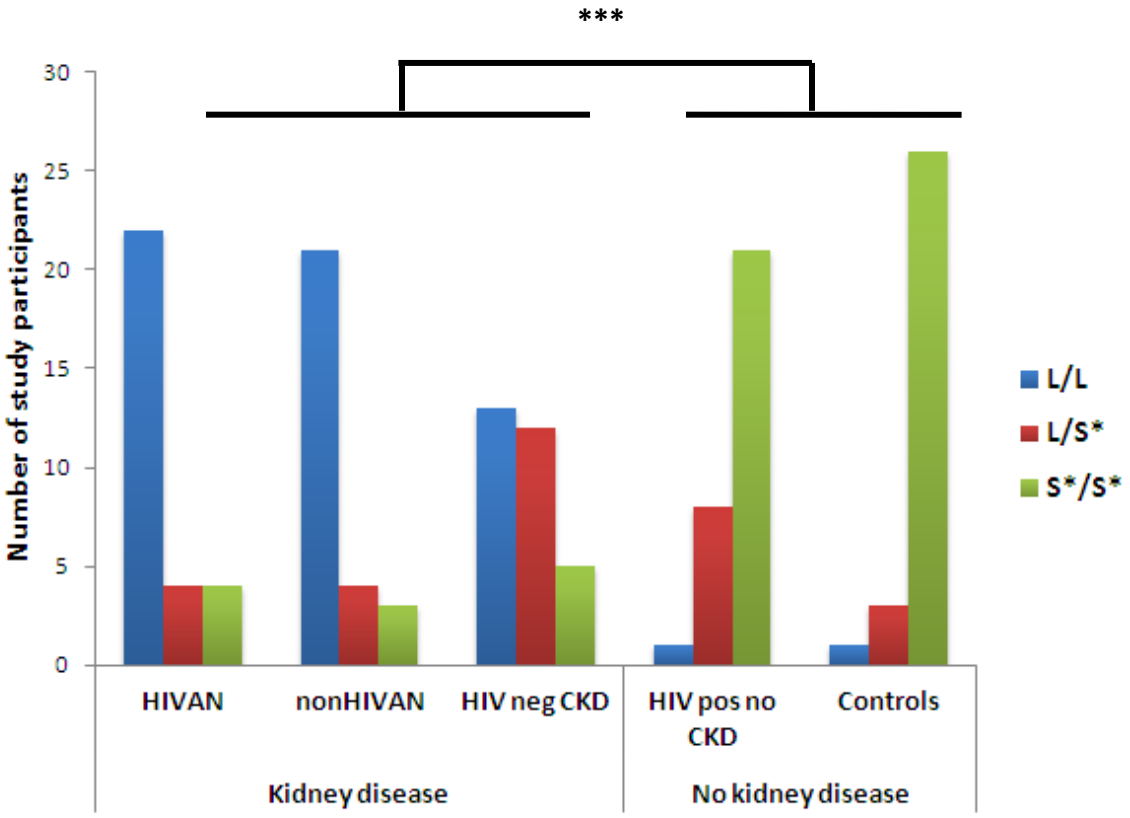


Figure 22: Distribution of MNS16A genotypes alleles between kidney disease and no kidney disease groups. There was a significant difference in the distribution of alleles between the two groups (*** $p<0.0001$). Analysis performed using the GraphPad Prism version 5.

Table 11: The difference in distribution of MNS16A genotypes and alleles with calculated odds ratio (OR) at 95% confidence interval (CI). Comparison 1 and 2 highlight the differences in distribution of MNS16A genotypes and alleles in different groups respectively.

	Comparison 1	**Comparison 2**
Groups	**Genotypes (L/L vs L/S* vs S*/S*)**	**Alleles (L vs S*)**
HIVAN vs nonHIVAN	OR = 0.7857 (0.1567 - 3.9386) p = 0.7693	OR = 1.3913 (0.5942 - 3.256) p = 0.4468
HIVAN vs HIVnegK	OR = 2.1154 (0.4802 - 9.3193) p = 0.322	OR = 2.3158 (1.10178 - 5.2692) p = 0.00453[c]
HIVAN vs HIVposNK	OR = 115.5 (11.915 - 1119.6) p < 0.0001[a]	OR = 20 (7.907 - 50.5882) p < 0.0001[a]
HIVAN vs Controls	OR = 143 (14.8669 - 1375.472) p < 0.0001[a]	OR = 44 (14.46 - 133.8867) p < 0.0001[a]
nonHIVAN vs HIVnegK	OR = 2.693 (0.5492 - 13.1991) p <0.0001[a]	OR = 1.6645 (0.7675 - 3.6099) p =0.1971
nonHIVAN vs HIVposNK	OR = 147 (14.1227 - 1530.0882) p < 0.0001[a]	OR = 14.375 (5.928 - 34.8576) p < 0.0001[a]
nonHIVAN vs Controls	OR = 182 (17.6173 - 188.2014) p <0.0001[a]	OR = 31.625 (10.7621 - 91.926) p < 0.0001[a]
HIVnegK vs HIVposNK	OR = 54.6 (5.72 - 521.01) p = 0.0005[b]	OR = 8.634 (3.6607 - 20.3748) p < 0.0001[a]
HIVnegK vs Controls	OR = 67.6 (7.1398 - 640.0436) p = 0.0002[b]	OR = 19 (6.613 - 54.5896) p < 0.0001[a]
HIVposNK vs Controls	OR = 1.2381 (0.073 - 20.9986) p = 0.8224	OR = 2.2 (0.7097 - 6.8776) p =0.1752

* represents either 243/272 bp short MNS16A sizes.

[a]significant at p< 0.0001, [b]significant at 0.0001<p<0.001 and [c]significant at p < 0.05.

Both genotype and allele distributions did not differ significantly in three CKD groups. Apart from allele distributions in HIVAN and HIVnegK groups (p = 0.00453) (**table 11**). This data also shows significant association MNS16A L/L genotype with CKD. The L/L genotype was significantly underrepresented in the participants without CKD. Furthermore, comparison of allele distributions revealed no significant difference in the groups without CKD. In addition, the MNS16A L/L was significantly over represented in the CKD groups (**Fig. 23**). This further supports our suggestion that the MNS16A long allele is significantly associated with CKD regardless of HIV status.

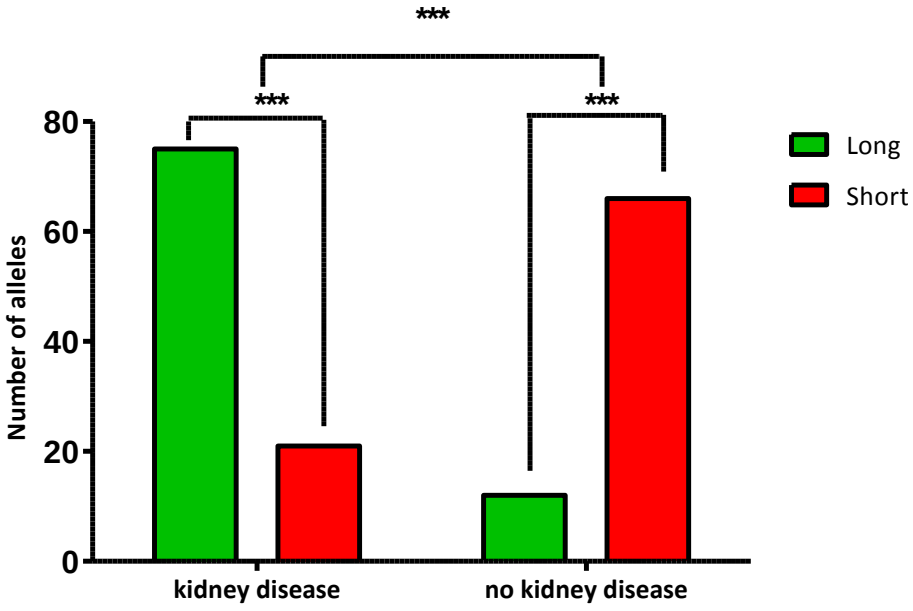


Figure 23: Distribution of long and short MNS16A alleles between kidney disease and no kidney disease groups. There was a significant difference in the distribution of alleles between the two groups (*** = p<0.0001). Analysis performed using the GraphPad Prism version 5.

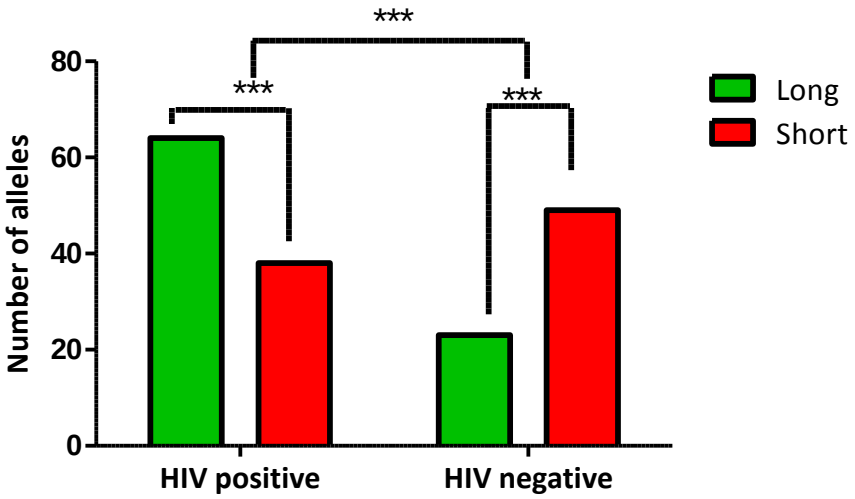


Figure 24: Distribution of long and short MNS16A alleles in HIV positive and HIV negative participants. There was a significant difference in the distribution of alleles between the two groups (*** = p<0.0001). Analysis performed using the GraphPad Prism version 5.

These findings indicate that the long MNS16A allele is significantly associated with kidney disease and HIV positive status. Although that seems to be the case, the long allele seems to occur mostly in CKD participants regardless of HIV status (**Fig. 24**).

3.8. MNS16A Sequencing

Sequencing of the MNS16A PCR products was performed to identify the variant sequences. More than 50 individuals were sequenced and sequences were compared to the human genome sequence Dec 2013 (GRCh38/hg38) through UCSC genome browser (accessed 12 August 2014). A BLAST search of the MNS16A sequence using previously designed (Wang et al., 2003) primers resulted in a 302 bp fragment with 100% homology the *hTERT* sequence (NG_009265.1 www.ncbi.nlm.nih.gov). Figure 25 shows the different sizes and position of the MNS16A variant.

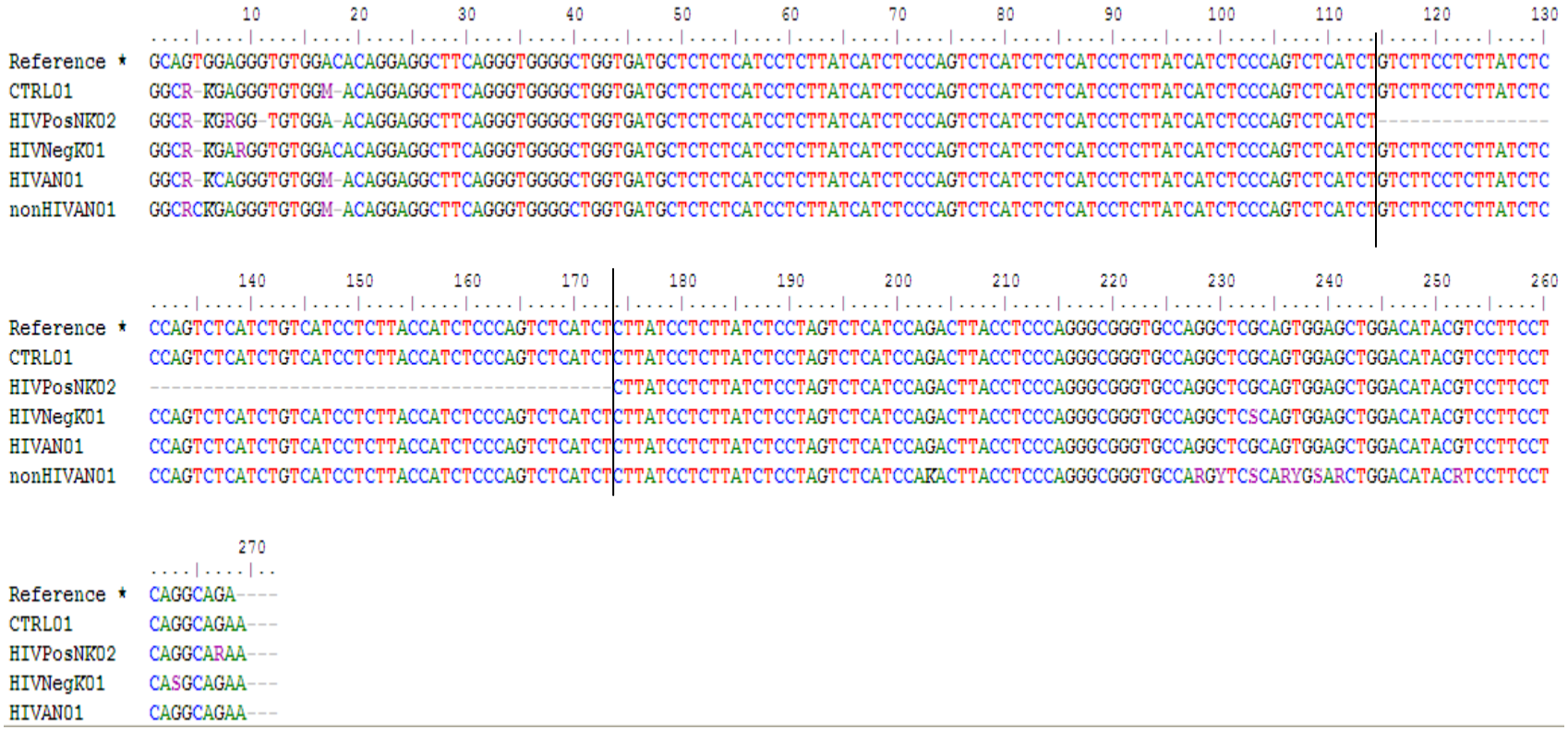


Figure 25: Alignment of MNS16A successful sequences showing the 274 and 243 bp sizes in different patient samples. The vertical lines show the missing repeats within the 243 bp size variant of the MNS16A. (*Reference sequence chr5: 1252360-1252651 GRCh37/hg19).

Section 3: Telomere length.

3.9. Relative telomere length (RTL) analysis

Relative telomere length (RTL) was analysed by qPCR as described earlier in the methods section 2.3.4.1. We examined the relationship between relative telomere length and MNS16A size with participant age, gender as well as HIV status. Only 20 DNA samples were used per histopathology group due to limited and poor quality DNA obtained from Formalin-fixed, paraffin-embedded (FFPE) kidney biopsies. Figure 26 shows the average RTL in each histopathology group. Although there was a statistically significant difference ($p<0.05$) in the average RTL between all the different histopathology groups, the HIVAN and HIV positive without kidney disease participants presented with noticeably shorter RTL.

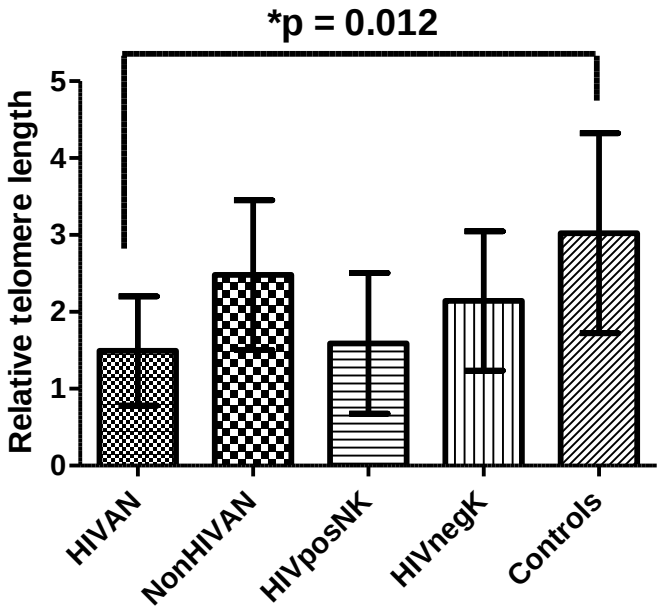


Figure 26: Graph showing the average RTL per histopathology group. The HIVAN and HIV positive without kidney disease groups presented with the lowest RTL. The healthy individuals (controls) presented with longer RTL.

3.9.1. Correlation of RTL with MNS16A variants

There was no significant association between minisatellite size and relative telomere length (RTL) ($p>0.05$). However, participants with L/S* genotype had relatively shorter telomere length compared to participants with the L/L and S*/S* MNS16A genotype (**Fig. 27**). Kruskal-Wallis test comparing the median RTL in different CKD groups revealed no significant association (**Fig. 27**) with MNS16A genotype.

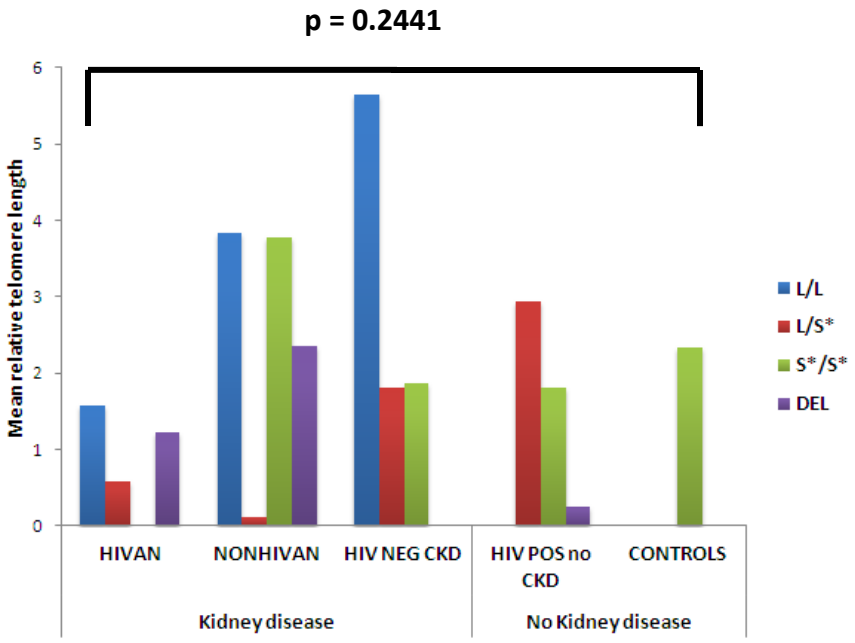


Figure 27: Graph representing the mean relative telomere length in participants with different MNS16A genotypes. DEL represents DNA samples suggested to have a deletion within the MNS16A chromosomal position in the primers lie.

Table 12: Dunns multiple comparisons test for RTL versus MNS16A genotype in different individuals. No statistically significant difference in the RTL per group.

Dunn's Multiple Comparison Test	Difference in rank sum	Significant when p < 0.05
HIVAN vs NONHIVAN	-6.625	p>0.05
HIVAN vs HIV NEG CKD	-4.250	p>0.05
HIVAN vs HIV POS no CKD	-1.750	p>0.05
HIVAN vs CONTROLS	2.000	p>0.05
NONHIVAN vs HIV NEG CKD	2.375	p>0.05
NONHIVAN vs HIV POS no CKD	4.875	p>0.05
NONHIVAN vs CONTROLS	8.625	p>0.05
HIV NEG CKD vs HIV POS no CKD	2.500	p>0.05
HIV NEG CKD vs CONTROLS	6.250	p>0.05
HIV POS no CKD vs CONTROLS	3.750	p>0.05

There was no statistically significant association between MNS16A genotype and RTL. Furthermore, there was no statistically significant difference in RTL between the five histopathology groups (**Table 12**). It should be noted that CKD participants with L/L MNS16A genotype presented with slightly longer telomere length compared to CKD individuals with L/S* and S*/S* genotypes. The participants in this group with the L/L genotype revealed relatively longer telomeres compared to other genotypes. This trend is consistent with the findings of Concetti and colleagues (2013). Even so, this needs to be validated with larger sample sizes.

3.9.2. Correlation of RTL with age

Several studies have analysed telomeres in relation to ageing. In this study, participants RTLs were grouped according to their different age ranges. Furthermore, RTL was correlated with age in the presence and absence of CKD. This revealed that telomere length is generally associated with ageing (**Fig. 28**). Again, this finding is coherent with known progressive telomere shortening and its association with ageing. The findings are not significant. This may

be due to the small sample size (n = 100) used for RTL analysis which further requires validation with larger sample sizes.

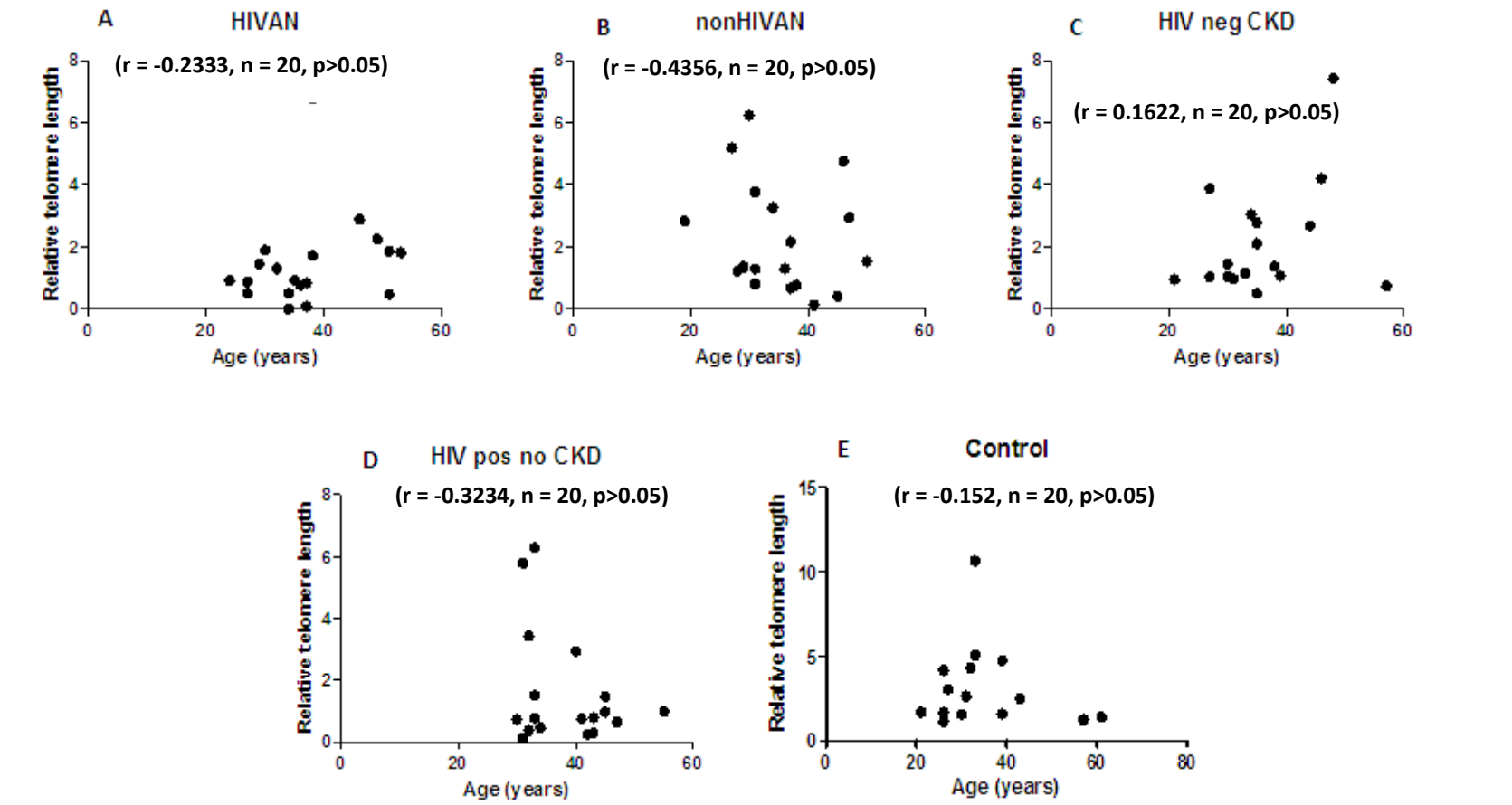


Figure 28: Correlation of relative telomere length and age in individuals with CKD regardless of HIV status. **A**) HIVAN, **B**) nonHIVAN, **C**) HIV negative CKD , **D**) HIV positive no CKD and **E**) HIV negative no CKD (Controls). Most of the HIVAN participants presented with shorter RTL (below 2) compared to other groups.

There was no significant correlation between age and relative telomere length (ns = $p > 0.05$ at 95% CI) within all the histopathology groups. In figure 28, individuals with the severe form of CKD i.e. HIVAN displayed on average shorter relative telomere length compared to other histopathology groups. Indeed a similar trend is observed in histopathology groups where younger individuals present with relatively longer telomere length with exceptions in the HIV negative with CKD group.

Spearman's correlation test was used to determine the relationship between RTL and age. Since telomere length is inversely proportional to age, a negative association was expected. All histopathology groups presented with a non-significant ($p>0.05$) weak negative correlation except for the HIV negative with CKD group, of which had a weak positive correlation (i.e. suggests telomere length increases with age. There are many factors that influence telomere length in different individuals. These factors include diet, exercise, economic status and viral infection (i.e. HIV). The above mentioned factors could partly explain the disparities in relative telomere length observed in the study group. Furthermore, no data regarding diet and lifestyle of study participants was recorded and hence data analysis was only restricted to available information.

3.9.3. Correlation of RTL with HIV status

A statistically significant association of HIV status with RTL was observed. However, shown also in figure 29 is HIV positive individuals presenting with generally shorter telomere length compared to HIV negative individuals in absence/presence of CKD. Further analysis using Dunns multiple comparison test revealed no significant difference in RTL in participants with CKD (**Fig. 29**).

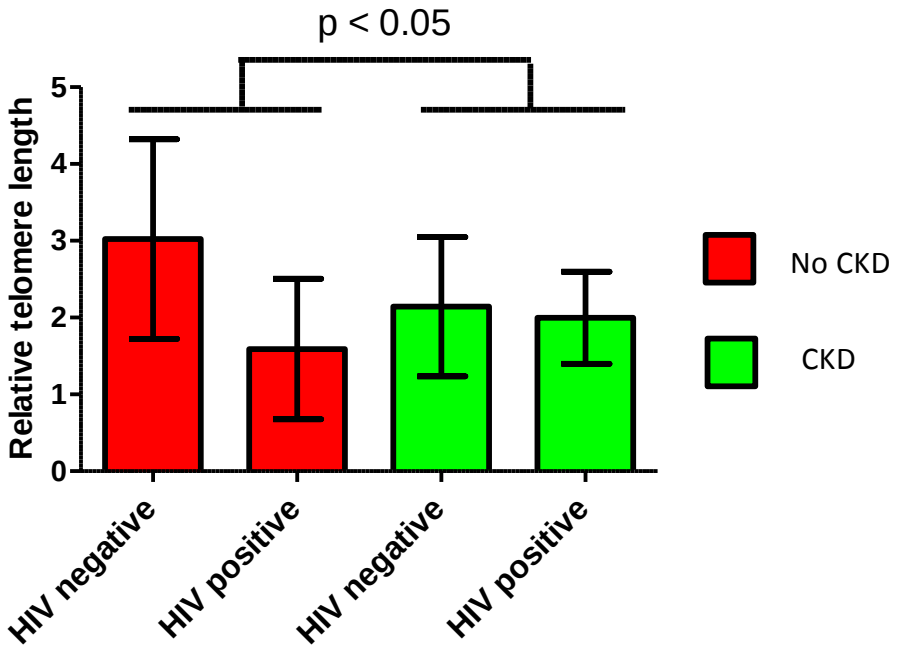


Figure 29: Correlation of RTL with HIV status. The green represents participants with CKD and the red represents participants without CKD. The HIV positive individuals presented with shorter RTL compared to HIV negative individuals, regardless of CKD status.

Chapter 4: Discussion

The current study was designed to elucidate the genetic variation in telomere biology related genes and their regulatory elements in a black South African cohort. In addition to identifying the variation, we also investigated the potential role of telomere dynamics in CKD. The objectives included using bioinformatics tools and literature to uncover the variation in telomere maintenance genes in different populations. To understand the molecular basis of diseases, studies are using genome wide association data to identify single nucleotide polymorphisms (SNPs) that either increase disease risk and/or affect drug metabolism (Agrawal et al., 2012; Shay, 2003; Shay and Wright, 2002). Telomere biology has been shown to play major roles in genome stability. It is well documented that during genome instability, some telomere-related genes are dysregulated. In this light, telomere biology is widely accepted as a biomarker of disease risk and possibly progression. Variation within telomere biology genes has been shown to confer risk to several diseases including ageing syndromes and cancer. In addition, telomere length and the consequence of telomere shortening varies among different individuals. Moreover, the genetic determinants of telomere length apart from paternal age are poorly understood. Therefore others have sort out to identify the possible variation within telomere associated genes.

Of the several telomere-related genes, *hTERT* is one of the most highly conserved with minimal variation across species and across populations. It is the *hTERT*, *hTERC* and the Shelterin associated genes that have been shown to display variation that increase disease risk and affect telomere length. This study sort out to investigate the variation within the *hTERT* gene, a gene that encodes for the protein that catalyses telomere elongation is involved in cell proliferation, cell signalling and viability. *hTERT* was the target gene in these analyses as it is one of the well-studied genes whose dysregulation has been implicated in several diseases. *hTERT* has been further suggested to be a critical role player in CKD. It has been suggested that *hTERT* provides terminally differentiated podocytes with unlimited proliferative potential resulting in glomerular collapse (Shkreli et al., 2012). The genetic elements that govern the expression of this particular gene have become an interesting area of research. Some of these regulatory elements such as the hTERT promoter and MNS16A have come up as notable.

The discussion that follows explores the implications of each finding. Firstly evidence of differences and similarities in distribution of common variant alleles in the *hTERT* gene and promoter. The lack of association studies in African populations in particular those in the telomere biology field. In addition, we report an association of MNS16A with CKD in the black South African cohort in this study. It should be noted that several studies have assessed the role of hTERT polymorphisms including promoter and MNS16A in many diseases. However no study has to date assessed the role of hTERT promoter and MNS16A in CKD. This is the first report to our knowledge that demonstrates an association of MNS16A with CKD in a black South African cohort.

4.1. Bioinformatics Data

Most large population studies done in African populations have been to explore variation but data regarding association of variation with disease susceptibility is limited.

4.1.1. The *hTERT* gene

Several studies have been performed to date to assess the common variants within the *hTERT* gene and their association with disease. It is noteworthy that most of these studies were only performed in non-African populations. Numerous variants have been identified within the gene and have been associated with cancers and ageing disorders. These include gliomas, lung and breast cancers as well as idiopathic pulmonary fibrosis (Mirabello et al., 2010; Pooley et al., 2010; Qu et al., 2014). The variants within the *hTERT* gene have been further shown to increase risk of cardiovascular disease and ultimately influence telomere length, a major determinant of cell life cycle (Blackburn, 2000)

Several research groups have described associations between the rs2736100 variants and cancer risk, however most results were inconclusive of the allele conferring risk (Gago-Dominguez et al., 2011; Jin et al., 2009; Shen et al., 2010; Wang et al., 2010; Wrensch et al., 2009). In addition the most of the associations were reported in non-African populations in Europe and Asia. Although the variant has been associated with adenocarcinoma risk in African-Americans (Spitz et al., 2013), a lack of association with breast cancer risk in women of African

ancestry was reported (Zheng et al., 2012). This is the only study to date that has assessed the role of this particular variant in disease pathogenesis in African ancestry.

Currently available allele and genotype frequencies accessed through 1000 Genomes and HapMap Data, revealed no significant difference in the distribution of the rs2736100 alleles across different populations. The African populations genotyped for the SNP were assessed to annotate the variant and determine the distribution in different populations. No association studies to date have been performed to investigate the role of this particular variant in disease susceptibility. Our bioinformatics analyses have shown that there is still missing data on the pathogenicity and distribution of some of the *hTERT* variants some of which are clinically important risk alleles in non-African populations.

Another specific variant within *hTERT* is rs2736098. This variant has been previously shown to confer risk to lung cancer in Asian, African American and European populations, as well as bladder cancer in European populations (Mirabello et al., 2009). Even though the variant has been shown to have clinical importance in disease susceptibility, there is still limited data on the distribution of this rs2736098 in different populations. Most of the association studies were performed in Asian populations.

Similarly, studies have shown that rs401681 is not associated with telomere shortening, breast and colorectal cancers or melanoma in Caucasians (Pooley et al., 2010). Furthermore, a study revealed association with decreased risk of oesophageal cancer in Chinese individuals (Yin et al., 2014). In contrast, a meta-analysis revealed that the T allele is associated with increased risk for pancreatic cancer in Chinese individuals (Liu et al., 2014).

4.1.2. The hTERT promoter

Increased telomerase activity has been identified as one of the hallmarks of disease progression and severity in 80-95% of human cancers (Hanahan and Weinberg, 2011; Shay et al., 2001). Moreover, the catalytic activity of the telomerase has been shown to be highly dependent on the expression of the human telomerase reverse transcriptase (hTERT), a critical component of the telomerase enzyme. Gene regulatory regions such as the *hTERT* promoter and minisatellite

(MNS16A) have become important targets for identifying variants that alter *hTERT* expression. The *hTERT* promoter plays a significant role in regulating *hTERT* expression as it has been shown to influence binding of several transcription factors that regulate *hTERT* expression (Kyo et al., 2008). In recent studies, *hTERT* promoter mutations have been shown to occur most often in melanoma, bladder cancer and hepatocellular carcinoma, and clear cell carcinomas (Hosen et al., 2015; Huang et al., 2015; Huang et al., 2013; Nault et al., 2013; Simon et al., 2015).

These variants include somatic promoter mutations **C228T** (Chr5:1295228 C>T) and **C250T** (Chr5: 1295250 C>T) which were both identified in human melanomas (Huang et al., 2013). Another common variant **rs2853669** (Chr5:1295349 T>C) which confers increased risk to several types of cancers including gliobastomas, breast cancers and esophageal squamous cell carcinoma (ESCC) (Park et al., 2014; Zhao et al., 2014). Furthermore, **rs35226131** (Chr5:1295373 C>T) a promoter variant also associated with ESCC (Zhao et al., 2014). In addition, more recent evidence also displays an association of TERT promoter mutations with sinonasal malignant melanoma (Jangard et al., 2015) bladder cancer (Li et al., 2015) as well as primary liver tumors (Nault and Zucman-Rossi, 2015). It would be interesting to assess whether these variants are associated with any of the above mentioned cancers in South African populations as they have not been reported before.

Some of the common reported variants (C228T and C250T) were not detected in all of the populations genotyped in HapMap Project and 1000 genomes. However, the variants **rs2853669** and **rs35226131** were observed in the HapMap populations. There was no significant difference in the distribution of **rs35226131** alleles within the different HapMap populations. Nevertheless, there was a difference observed in distribution of **rs2853669** alleles between African and non-African populations. In particular the African populations had higher frequencies of the minor allele.

The minor allele C of rs2853669 has been previously associated with several diseases was underrepresented in African populations. Data regarding the pathogenicity of this particular variant is limited in African populations. However it should be noted that the rs2853669 C/C genotype has been associated with lower *hTERT* expression in French individuals (Labussiere et

al., 2014). Among the complex regulation of telomerase expression, rs2853669 has been shown to modulate both *hTERT* expression and impact on prognosis in bladder cancer (Rachakonda et al., 2013)and poor outcomes in gliomas (Labussiere et al., 2014).

None of the common variants or SNPs within the *hTERT* promoter were detected in the BSO population. Low coverage SNP detection application was used in the BSO population. This may partly explain failure to detect common promoter variants in BSO population. Out of all the known *hTERT* gene variants, only a small percentage was detected in the BSO population. Furthermore, none of the promoter variants were detected.

The Luhya in Webuye, Kenya (LWK) and Yoruba in Ibadan, Nigeria (YRI) have always been used as the gold standard for genotype and allele distribution comparison in the South African black population. This is due to the fact that global scale analysis of the BSO reveals homogeneity with the two African populations LWK and YRI. According to May and colleagues (2013), these two populations appeared to cluster together when analysed using the multiple component analysis (MCA). Although LWK and YRI cluster together with BSO on a global scale, May et al (2013) discovered significant heterogeneity between the three populations. In addition to this observed heterogeneity, our data revealed that the BSO population may also be very similar to the ACB population. Furthermore, research into these variants has revealed that different populations display diverse distribution of variants. Some of these variants either increase of decrease susceptibility to different diseases in different populations.

4.2. Promoter

Telomere regulation has become a well-known biomarker of several diseases including ageing and cancer (Blackburn, 2005a; Blasco et al., 1999; Letsolo et al., 2010; Shay and Wright, 2001a, b; Shay et al., 2001). It has been postulated that regions that regulate expression of the critical component of telomerase, the *hTERT* are very important players in disease susceptibility involving dysregulation of *hTERT* (Counter et al., 1998; Park et al., 2014; Shkreli et al., 2012). None of the promoter variants were detected in our study population. This finding suggests that the *hTERT* promoter variants may be tissue/cell specific and thus also not associated with CKD, as they were not detected in our study population. However it should be noted that none

of the variants were detected in the BSO population either. This implies that these variants are regional and population specific.

4.3. MNS16A

Regulation of hTERT gene expression through MNS16A has been demonstrated as an important role player in transcription (Hofer et al., 2011; Hofer et al., 2013; Wang et al., 2003; Wang et al., 2008). Here, we are the first group to report MNS16A variation in a self-identified black South African population. We provided the evidence that shows significant association of long MNS16A allele (302 bp) with chronic kidney disease regardless of HIV status. This particular MNS16A variant seems to occur exclusively in participants with CKD. Interestingly, participants with CKD and are HIV negative had a higher frequency of the L/S* (* 243 bp or 272 bp) compared to all groups. According to the clinical data, HIV infected participants with CKD had progressed to the late stages of CKD (stages 3 – 5). Whereas uninfected participants with CKD were mostly in the early stages of CKD (stages 1 and 2). This finding suggests that the L/L MNS16A genotype may be associated with CKD progression and severity.

However it should be noted that there has been disparity on the role of the MNS16A in disease susceptibility. It has been shown that the MNS16A S* allele has been associated with increased risk of developing gliomas, breast and colorectal cancers (Hofer et al., 2011; Wang et al., 2008). On the contrary, the MNS16A L allele and L/L genotype were associated with increased risk in renal cell carcinomas (RCC), prostate and lung cancers (Hofer et al., 2013; Wang et al., 2003). There are no studies to date which have reported MNS16A variation in black South African individuals.

4.4. Relative Telomere Length (RTL)

Although DNA from blood samples for the no kidney disease group was used, it should be noted that telomere length determination has become routine practise (Sanders and Newman, 2013). The telomere length in leucocytes is often used as a biomarker of ageing in humans. In addition, control participants could not voluntarily donate their biopsies.

None of the correlations between RTL and MNS16A genotype, age and CKD status were statistically significant, it has been reported that progressive telomere shortening is associated with ageing, HIV status (Pathai et al., 2013), MNS16A L/L genotype (Concetti et al., 2013) and CKD (D'Agati and Appel, 1997; Pathai et al., 2013). However, mechanisms by that regulate telomere length in the presence or absence of disease is still poorly understood. It should be noted that although telomere length is largely influenced by age, other environmental factors including diet, exercise, viral infections and disease status increase the rate of telomere attrition (Lin et al., 2012; Mirabello et al., 2009).

The role of MNS16A variants in regulation of telomere length is poorly understood. It has been suggested that the L/L genotype is associated with progressive telomere shortening in individuals in Italy (Concetti et al., 2013). In contrast, no association between MNS16A variant sizes and telomere length was discovered in the Han Chinese population (Liu et al., 2014). Our study showed relatively shorter telomere length in CKD individuals with L/S* MNS16A genotype compared to individuals with L/L and S*/S* MNS16A genotypes in the same group. Additionally, CKD individuals displayed relatively longer/stabilised telomere length compared to all the other genotyped individuals. Despite that, the data was insignificant due to small sample size used for RTL analysis. Furthermore, the L and S* alleles were underrepresented in the no CKD and CKD groups respectively.

Telomere length is recognised as conventional biomarker of ageing (Blackburn, 2000). It is well documented that telomere length shortens with age (Chang and Harley, 1995). In a review of *in vitro* and *in vivo* studies, Martin-Ruiz and colleagues (2005) found that telomere length satisfied several criteria as a biomarker of ageing. Telomere length changes with age, has great inter-individual variability, is linked to basic biology of the cell, and correlates with ageing and ageing-related disease (Martin-Ruiz et al., 2005). Our findings revealed that although the data analysis was not significant, individuals with the severe form of CKD i.e. HIVAN displayed relatively shorter telomere length compared to all other individuals in the study. In addition, reduced telomere length has been shown to be associated with kidney (Wills and Schnellmann, 2011) and cardiovascular diseases (CVD). Another study investigated the association of telomere

length with progression of kidney disease in 132 patients with type 1 diabetes. They found that telomere length independently predicted progression to diabetic nephropathy (Wills and Schnellmann, 2011).

In the presence of HIV, telomere attrition has been suggested to progress faster compared to HIV uninfected individuals. One study revealed that high level of HIV viral load is associated with accelerated telomere attrition (Cote et al., 2012). In our study, a significant association was observed between HIV status and RTL. In addition, on close inspection HIV negative individuals presented with considerably longer RTL compared to HIV positive individuals regardless of age and disease presence. This is consistent with other studies. For example HIV infected individuals usually present with shorter telomere length in their leucocytes (Cote et al., 2012; Wolthers et al., 1996). Additionally a study in South Africa also reported a strong relationship of accelerated telomere length shortening in HIV infected individuals (Pathai et al., 2013).

It is noteworthy that the study had limitations and thus results should be taken with caution especially those pertaining to telomere length. This may be due to the sample size used and limited DNA availability and quality. Other factors to be considered are the environmental risk factors which play a significant role in CKD and telomere shortening. These were not taken into consideration as no information regarding the social or economic status, participant diet and whether participants suffered from any other disease besides CKD was obtained. All of the fore-mentioned has been suggested to influence both telomere length and disease progression. However, this data strongly demonstrates the association of the minisatellite with disease risk in CKD regardless of HIV status. It remains to be seen whether the MNS16A variants play a role in telomere length maintenance and *hTERT* gene expression.

Chapter 5: Conclusion

In conclusion, the findings of this study provide some evidence on the differences in the genetic variation of telomere biology genes between different populations. Notable differences in the distribution of some *hTERT* gene and promoter variants in African populations requires further investigations on the role of the variation in disease susceptibility.

This is the first study to report MNS16A stability in black South Africans with CKD in the presence and absence of HIV infection. Interestingly, a significant association of MNS16A L allele with CKD was detected. Despite the lack of conclusive evidence on the role of the MNS16A variation with RTL, data obtained is suggestive of a functional role that MNS16A plays in CKD severity and/or progression. Therefore this study has identified a potential role player in CKD. Although further investigation is required, this study supports the role of telomere dynamics in chronic kidney disease.

Study limitations and Recommendations

Due to low (DNA and RNA) concentrations and poor quality, telomere length could not be determined using a more accurate telomere length detection procedure i.e. STELA. Furthermore, RTL could only be determined in 100/159 individuals. Functional studies to investigate the potential role of the MNS16A variation in *hTERT* expression were unsuccessful at several trials, as RNA was of poor quality.

As much as the findings reveal a significant association of MNS16A long allele with CKD, validation with larger sample sizes and functional studies to address the role of telomere biology in CKD are recommended. This limited data availability warrants further research and encourages large scale association studies exploring the role of telomere biology variation and disease susceptibility in African populations. Since Africans have high genetic diversity, more

regional and ethnicity based research is advised. Furthermore, identification of these variants will assist in advancement of diagnostic and therapeutic measures to combat or reduce diseases that involve dysregulation of telomere biology genes.

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8. Appendices

8.1. Appendix A: Ethics Clearance Certificate

R14/49 Mr Sibusiso Malindisa et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140346

NAME: (Principal Investigator)	Mr Sibusiso Malindisa et al
DEPARTMENT:	School of Molecular and Cell Biology University of the Witwatersrand
PROJECT TITLE:	Telomere Dynamics and Genetic Variation within the Human Telomerase Reverse Transciptase Gene (hTERT) in Human Immunodeficiency Virus-Associated Nephropathy (HIVAN)
DATE CONSIDERED:	28/03/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Boitelo Letsolo
APPROVED BY:	Professor P Cleaton-Jones, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	08/09/2014

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

DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

8.2. Appendix B: Reagent and Solution preparation

Primer and probe Dilutions

Tris – EDTA (TE) buffer pH 7.0: 180 µL

100 µM primer/probe: 20 µL

Total of 200 µL of 10 µM primer

Primers stored at -20 °C.

Buffer Preparations

1X Tris-Borate EDTA (TBE) buffer

10X TBE (Sigma): 100 mL

ddH_2O: 900 mL

1L of 1X TBE buffer

1X Tris-Acetate EDTA (TAE) buffer

10X TAE (Sigma): 100 mL

ddH_2O: 900 mL

1L of 1X TAE buffer

Agarose gels

0.8% Agarose gel (600 mL)	1% Agarose gel (50 mL)	2.5% Agarose gel (50 mL)
1X TAE/TBE: 600 mL	1X TAE/TBE: 50 mL	1X TAE/TBE: 50 mL
4.8 g of Agarose (Seakem)	0.5 g of Agarose (Conda)	1.25 g of Agarose (Conda)

Mix the agarose powder with the 1X TAE/TBE buffer. Heat the mixture in the microwave until the agarose has completely dissolved. Wait for mixture to cool down and add 2.0 µL of GR-Green DNA stain per 50 mL of liquid agarose gel. Pour liquid gel on gel casting tray with comb already inserted. Leave gel to solidify for 30 – 45 minutes.

PCR preparations and conditions:

- **MNS16A and promoter**

Component	1X	Final concentration
Phusion Flash Master Mix (2X)	5 µL	1X
Forward Primer (10 µL)	0.5 µL	1 µM
Reverse primer (10 µL)	0.5 µL	1 µM
Nuclease free water	3 µL	-
Template DNA (50 ng/µL)	1 µL	5 µM
Total	10 µL	

8.3. Appendix C: Participant Clinical Data and MNS16A genotypes.

HIVAN Study IDs.		Age	Sex	MNS16A Genotypes	Creatinine (µmol/L)	Urea (mmol/L)	eGFR (ml/min)
1	HIVAN-01	46	M	L/L	540	22.5	10.6
2	HIVAN-02	34	F	L/L	266	12.3	18.9
3	HIVAN-03	36	F	L/L	534	20.4	8.4
4	HIVAN-04	53	F	Del	111	7.6	47.4
5	HIVAN-05	32	F	L/L	799	14.5	5.4
6	HIVAN-06	27	F	L/L	262	8.4	20.2
7	HIVAN-07	56	F	L/L	250	9.2	18.4
8	HIVAN-08	38	M	L/L	186	9.6	37.7
9	HIVAN-09	30	M	L/L	n/a	n/a	n/a
10	HIVAN-10	29	F	L/L	570	25.6	8.1
11	HIVAN-11	35	F	L/L	194	8.5	27.1
12	HIVAN-12	19	F	Del	152	6.9	40.6
13	HIVAN-13	29	M	L/S	869	20.5	6.7
14	HIVAN-14	36	F	L/L	469	26.9	9.7
15	HIVAN-15	27	M	L/L	733	20.4	8.3
16	HIVAN-16	30	F	L/L	419	19	11.5
17	HIVAN-17	26	F	L/L	1511	32	2.7
18	HIVAN-18	40	M	L/L	486	21	12.3
19	HIVAN-19	31	F	L/L	555	16	8.3
20	HIVAN-21	34	M	L/L	312	10.6	21.2
21	HIVAN-22	40	M	L/L	n/a	n/a	n/a
22	HIVAN-23	32	M	L/S	952	14.9	5.9
23	HIVAN-24	51	F	Del	n/a	n/a	n/a
24	HIVAN-25	32	F	Del	n/a	n/a	n/a
25	HIVAN-26	30	F	Del	811	27.8	5.4

n/a = Not available = Clinical data not provided for those particular participants.

HIVAN Study IDs.		Age	Sex	MNS16A Genotypes	Creatinine (µmol/L)	Urea (mmol/L)	eGFR (ml/min)
26	HIVAN-27	27	M	L/S	523	12.6	12.2
27	HIVAN-28	37	M	L/L	285	13	23.1
28	HIVAN-29	49	M	S/S	n/a	n/a	n/a
29	HIVAN-30	27	F	S/S	624	20.5	7.4
30	HIVAN-31	26	F	L/S	975	30	4.5
31	HIVAN-32	27	F	L/L	1963	30.4	2
32	HIVAN-33	24	M	L/L	648	15.4	9.8
33	HIVAN-34	38	M	L/L	915	18.5	6
34	HIVAN-35	32	F	Del	n/a	n/a	n/a
35	HIVAN-37	23	F	S/S	154	5.7	38.5
36	HIVAN-38	33	F	S/S	243	7	21.1

n/a = Not available = Clinical data not provided for those particular participants.

nonHIVAN Study IDs.		Diagnosis	Age (yrs)	Sex	MNS16A genotypes	Creatinine (µmol/L)	Urea (mmol/L)	eGFR (ml/min)
1	nonHIVAN-01	FSGS	30	F	L/L	n/a	n/a	n/a
2	nonHIVAN-02	IgA Nephropathy	29	M	L/S	485	29.2	13.2
3	nonHIVAN-03	FSGS	37	F	L/L	106	4	53.8
4	nonHIVAN-04	FSGS	34	F	L/L	388	16.7	12.2
5	nonHIVAN-05	FSGS	29	M	L/L	279	22.2	17.9
6	nonHIVAN-06	HIVICK	52	F	L/L	232	14	30.8
7	nonHIVAN-07	Minimal Change disease	38	M	DEL	39	2.9	228.5
8	nonHIVAN-08	Diabetic Nephropathy	38	F	L/L	659	27.4	6.5
9	nonHIVAN-10	HIVICK	27	M	L/L	46	3.4	150.2
10	nonHIVAN-11	Mebranoproliferative glomerulonephritis I	30	F	L/L	n/a	n/a	n/a
11	nonHIVAN-12	HIVICK	29	M	L/L	n/a	n/a	n/a
12	nonHIVAN-13	Benign nephrolosclerosis	41	M	L/S	n/a	n/a	n/a

13	nonHIVAN-14	Membranous glomerulonephritis	30	F	L/L	68	6	93.7
14	nonHIVAN-15	Global glomerulosclerosis with ischemic changes c/w ANS	31	F	L/L	692	18.7	6.4
15	nonHIVAN-16	FSGS	31	M	L/L	135	n/a	56.8
16	nonHIVAN-17	FSGS	48	F	L/S	n/a	n/a	n/a
17	nonHIVAN-18	HIVICK	41	F	L/S	234	13.6	21.1
18	nonHIVAN-19	HIVICK	27	F	L/L	n/a	n/a	n/a
19	nonHIVAN-20	Membranous glomerulonephritis	37	M	L/L	n/a	n/a	n/a
20	nonHIVAN-21	HIVICK	30	F	L/S	n/a	n/a	n/a
21	nonHIVAN-28	FSGS	31	M	S/S	227	13	31.2
22	nonHIVAN-29	HIVICK	26	F	L/L	618	22	7.6
23	nonHIVAN-33	Membranous glomerulonephritis	50	M	L/L	n/a	n/a	n/a
24	nonHIVAN-34	Membranous glomerulonephritis	47	F	DEL	n/a	n/a	n/a
25	nonHIVAN-35	FSGS	34	F	L/L	380	25	12.5

n/a = Not available = Clinical data not provided for those particular participants.

nonHIVAN Study IDs.		Diagnosis	Age (yrs)	Sex	MNS16A genotypes	Creatinine (µmol/L)	Urea (mmol/L)	eGFR (ml/min)
26	nonHIVAN-36	FSGS	36	M	DEL	n/a	n/a	n/a
27	nonHIVAN-37	Membranous glomerulonephritis	19	F	DEL	n/a	n/a	n/a
28	nonHIVAN-38	FSGS	25	M	S/S	n/a	n/a	n/a
29	nonHIVAN-39	Thrombotic microangiopathy	54	M	L/L	n/a	n/a	n/a
30	nonHIVAN-40	Membranous glomerulonephritis	39	F	L/L	n/a	n/a	n/a
31	nonHIVAN-41	Global glomerulosclerosis-low grade IgAN	66	F	L/L	105	16.2	48.3
32	nonHIVAN-42	HIVICK	32	F	S/S	153	8.5	36.3

n/a = Not available = Clinical data not provided for those particular participants.

HIV Negative with Kidney Disease Study IDs.		Diagnosis	Age (yrs)	Sex	MNS16A genotypes	Creatinine (µmol/L)	Urea (mmol/L)	eGFR (ml/min)
1	HIV_neg-01	IgA Nephropathy	39	M	L/L	n/a	n/a	n/a
2	HIV_neg-02	Membranous glomerulonephritis	57	M	L/L	n/a	n/a	n/a
3	HIV_neg-03	FSGS	30	M	L/L	6.4	21.9	1929.9
4	HIV_neg-04	Nodular Glomerulosclerosis	30	F	L/S	860	36	5
5	HIV_neg-05	Lupus nephritis	34	M	L/S	n/a	n/a	n/a
6	HIV_neg-06	Global glomerulosclerosis	33	M	L/S	n/a	n/a	n/a
7	HIV_neg-07	Lupus nephritis	26	F	L/S	92	2	68
8	HIV_neg-08	FSGS	43	F	S/S	n/a	n/a	n/a
9	HIV_neg-09	Membranous glomerulonephritis	39	M	L/S	n/a	n/a	n/a
10	HIV_neg-10	Membranoproliferative Glomerulonephritis I	31	M	L/S	n/a	n/a	n/a
11	HIV_neg-11	Membranous glomerulonephritis	18	F	L/S	18	5.2	481.6
12	HIV_neg-12	Membranous glomerulonephritis	35	M	S/S	115	5.9	66.7
13	HIV_neg-13	Lupus nephritis	44	F	L/L	n/a	n/a	n/a
14	HIV_neg-14	Benign Nephrolesclerosis	27	F	S/S	2047	39.1	1.9
15	HIV_neg-15	Minimal change disease	38	F	L/L	50	5.3	127.3
16	HIV_neg-17	Diabetic Nephropathy	57	M	L/S	89	8.3	81.2
17	HIV_neg-18	FSGS	27	M	L/L	104	5.9	79
18	HIV_neg-19	Membranous glomerulonephritis	61	F	L/S	316	100	13.8
19	HIV_neg-21	Global glomerulosclerosis	32	F	L/S	977	22	4.3
20	HIV_neg-22	Membranous glomerulonephritis	35	F	L/S	36	1.5	189.1
21	HIV_neg-25	Acute tubular necrosis (Not a CKD)	61	F	S/S	52	5.8	110.5
22	HIV_neg-26	Thrombotic microangiopathy	24	M	L/L	168	18.7	168
23	HIV_neg-28	Minimal change disease	21	M	S/S	152	13.9	53.6
24	HIV_neg-31	Extensive GS, hilar SS could be Oxalate (Oxalosis)	48	M	L/L	n/a	n/a	n/a
25	HIV_neg-36	Membranous glomerulonephritis	45	M	L/L	78	5	99.2

n/a = Not available = Clinical data not provided for those particular participants.

HIV-Pos Without Kidney Disease Study IDs.		Age (yrs)	Sex	MNS16A genotypes	Creatinine (µmol/L)	Urea (mmol/L)	eGFR (ml/min)
1	HIV_Pos-01	39	F	S/S	74	n/a	80.5
2	HIV_Pos-05	41	F	L/L	1274	n/a	3
3	HIV_Pos-06	31	F	S/S	51	n/a	129.7
4	HIV_Pos-15	45	F	L/S	76	n/a	75.9
5	HIV_Pos-16	33	F	L/S	66	n/a	95.1
6	HIV_Pos-17	60	F	S/S	62	n/a	90.5
7	HIV_Pos-18	47	M	S/S	88	n/a	85.6
8	HIV_Pos-19	45	F	L/S	84	n/a	67.6
9	HIV_Pos-27	27	F	S/S	41	n/a	171.6
10	HIV_Pos-28	38	F	S/S	72	n/a	83.6
11	HIV_Pos-29	26	F	L/S	53	n/a	128.5
12	HIV_Pos-30	46	M	S/S	77	n/a	100.3
13	HIV_Pos-31	44	M	S/S	78	n/a	99.7
14	HIV_Pos-32	39	M	S/S	142	n/a	51.2
15	HIV_Pos-33	32	M	S/S	65	n/a	131.2
16	HIV_Pos-34	42	F	S/S	52	n/a	119.2
17	HIV_Pos-40	35	M	S/S	92	n/a	86.3
18	HIV_Pos-41	32	F	S/S	53	n/a	123.2
19	HIV_Pos-42	31	F	S/S	46	n/a	146.1
20	HIV_Pos-43	34	F	S/S	73	n/a	84.1
21	HIV_Pos-44	33	F	S/S	55	n/a	117.4
22	HIV_Pos-45	43	F	S/S	49	n/a	127.1
23	HIV_Pos-46	42	F	del	73	n/a	80.6
24	HIV_Pos-47	31	F	S/S	62	n/a	103.5
25	HIV_Pos-48	40	F	L/S	78	n/a	75.4

n/a = Not available = Clinical data not provided for those particular participants.

HIV-Pos Without Kidney Disease Study IDs.		Age (yrs)	Sex	MNS16A genotypes	Creatinine (µmol/L)	Urea (mmol/L)	eGFR (ml/min)
26	HIV_Pos-49	24	F	S/S	66	n/a	101.4
27	HIV_Pos-50	38	F	L/S	70	n/a	86.3
28	HIV_Pos-51	21	M	L/S	60	n/a	156.8
29	HIV_Pos-52	40	F	S/S	72	n/a	82.7
30	HIV_Pos-53	45	M	S/S	88	n/a	86.3
31	HIV_Pos-54	42	F	L/S	71	n/a	83.2

n/a = Not available = Clinical data not provided for those particular participants.

Human Population Controls Study IDs.		Age (yrs)	Gender	MNS16A Genotypes
1	HPC-01	39	M	S/S
2	HPC-02	57	M	S/S
3	HPC-03	30	F	S/S
4	HPC-04	30	F	S/S
5	HPC-05	34	M	S/S
6	HPC-06	33	M	S/S
7	HPC-07	26	F	S/S
8	HPC-08	43	F	S/S
9	HPC-09	39	F	S/S
10	HPC-11	18	F	S/S
11	HPC-12	35	M	L/L
12	HPC-13	44	F	L/S
13	HPC-14	27	F	S/S
14	HPC-15	38	M	S/S
15	HPC-16	26	F	S/S
16	HPC-17	57	M	S/S
17	HPC-18	27	F	S/S
18	HPC-19	61	F	S/S
19	HPC-20	21	F	S/S
20	HPC-21	32	F	S/S
21	HPC-22	35	M	S/S
22	HPC-23	28	M	S/S
23	HPC-24	37	M	S/S
24	HPC-25	61	F	S/S
25	HPC-26	24	M	S/S
26	HPC-35	37	F	L/S
27	HPC-36	36	M	S/S
28	HPC-37	31	F	S/S
29	HPC-38	41	F	L/S
30	HPC-39	31	M	S/S

8.4. Appendix D: The genotype frequencies of the hTERT gene and promoter variants in different populations.

	hTERT variants detected in the BSO population (n = 94)							
Number	**Chr.**	**SNP**	**Allele 1**	**Allele 2**	**Homozygous Allele 1**	**Heterozygous**	**Homozygous Allele 2**	**MISSING**
1	5	rs5031049	A	G	0	5	89	0
2	5	rs2736122	T	C	6	36	52	0
3	5	rs2736118	A	G	12	42	40	0
4	5	rs11133715	A	G	4	30	60	0
5	5	rs35412024	C	T	2	4	88	0
6	5	rs4246742	T	A	9	35	50	0
7	5	rs33988305	T	C	0	8	86	0
8	5	rs34002187	A	G	0	8	86	0
9	5	rs11133719	T	C	0	8	86	0
10	5	rs4975605	A	C	13	44	37	0
11	5	rs56345976	A	G	8	44	41	1
12	5	rs114500100	C	A	0	8	86	0
13	5	rs10069690	C	T	11	44	39	0
14	5	rs7734992	T	C	7	43	44	0
15	5	rs34471035	A	G	1	11	82	0
16	5	rs13167280	A	G	0	12	82	0
17	5	rs6866456	C	T	9	42	43	0
18	5	rs7725218	A	G	17	48	29	0
19	5	rs35809415	A	G	0	7	87	0
20	**5**	**rs2736100**	**G**	**T**	**22**	**46**	**26**	**0**
21	5	rs2853677	G	A	11	39	44	0
22	5	rs2736099	A	G	4	30	60	0
23	5	rs2853676	T	C	12	42	40	0
24	5	rs2853672	A	C	19	51	24	0

	hTERT variants detected in the CEU population (n = 99)							
Number	Chr.	SNP	Allele 1	Allele 2	Homozygous Allele 1	Heterozygous	Homozygous Allele 2	MISSING
1	5	5:1253744	A	G	2	29	68	0
2	5	5:1255520	A	G	1	26	72	0
3	5	5:1256585	A	G	7	38	54	0
4	5	5:1257288	T	C	0	19	80	0
5	5	5:1257621	A	G	6	41	52	0
6	5	5:1259489	T	C	0	13	86	0
7	5	5:1260195	C	T	6	42	51	0
8	5	5:1261051	T	C	0	7	92	0
9	5	5:1261052	A	G	12	43	44	0
10	5	5:1264068	T	C	6	41	52	0
11	5	5:1264152	G	A	7	41	51	0
12	5	5:1265204	T	C	6	42	51	0
13	5	5:1265373	A	G	6	41	52	0
14	5	5:1265935	A	G	6	41	52	0
15	5	5:1266226	C	T	6	42	51	0
16	5	5:1266310	A	G	12	51	36	0
17	5	5:1267202	G	C	1	19	79	0
18	5	5:1267356	A	T	2	27	70	0
19	5	5:1270983	G	C	2	33	64	0
20	5	5:1271524	T	C	5	31	63	0
21	5	5:1271661	C	T	6	35	58	0
22	5	5:1273610	T	G	8	29	62	0
23	5	5:1273905	A	G	16	54	29	0
24	5	5:1275528	A	C	21	51	27	0
25	5	5:1275857	C	T	2	25	72	0
26	5	5:1276772	C	A	6	47	46	0
27	5	5:1276785	T	C	23	51	25	0
28	5	5:1276794	A	C	23	51	25	0
29	5	5:1276873	G	A	18	49	32	0
30	5	5:1277577	G	A	22	54	23	0
31	5	5:1278584	T	C	2	19	78	0
32	5	5:1279224	G	T	0	12	87	0
33	5	5:1279790	T	C	5	46	48	0
34	5	5:1279964	C	G	15	51	33	0
35	5	5:1280028	A	G	3	33	63	0
36	5	5:1280128	C	T	19	50	30	0
37	5	5:1280477	A	G	2	21	76	0

38	5	5:1280830	C	G	11	51	37	0
39	5	5:1280938	G	A	15	51	33	0
40	5	5:1282319	A	C	11	42	46	0

hTERT variants detected in the CHB population (n = 103)								
Number	**Chr.**	**SNP**	**Allele 1**	**Allele 2**	**Homozygous Allele 1**	**Heterozygous**	**Homozygous Allele 2**	**MISSING**
1	5	5:1255520	A	G	4	29	70	0
2	5	5:1256585	A	G	1	15	87	0
3	5	5:1257288	T	C	6	35	62	0
4	5	5:1257621	A	G	1	15	87	0
5	5	5:1260195	C	T	1	17	85	0
6	5	5:1260957	T	C	0	4	99	0
7	5	5:1266226	C	T	1	18	84	0
8	5	5:1266310	G	A	3	32	68	0
9	5	5:1267202	G	C	18	41	44	0
10	5	5:1267356	A	T	15	44	44	0
11	5	5:1270983	G	C	0	7	96	0
12	5	5:1271524	T	C	2	20	81	0
13	5	5:1271661	T	C	4	25	74	0
14	5	5:1273610	G	T	19	45	39	0
15	5	5:1273896	A	C	0	0	103	0
16	5	5:1273905	A	G	1	19	83	0
17	5	5:1283312	A	G	12	63	28	0
18	5	5:1283755	T	C	1	36	66	0
19	5	5:1284135	T	C	11	62	30	0
20	5	5:1284717	T	C	5	25	73	0
21	5	5:1285162	C	T	12	66	25	0
22	5	5:1285974	A	C	12	61	30	0
23	5	5:1287194	G	A	12	56	35	0
24	5	5:1287340	A	G	15	57	31	0
25	5	5:1287505	T	C	0	15	88	0
26	5	5:1288547	T	C	3	22	78	0
27	5	5:1289975	A	C	0	15	88	0
28	5	5:1291293	G	A	0	11	92	0
29	5	5:1291717	C	T	7	71	25	0
30	5	5:1291718	A	G	7	71	25	0
31	5	5:1291720	T	C	7	71	25	0

32	5	5:1291735	G	A	0	40	63	0
33	5	5:1291740	G	T	0	40	63	0
34	5	5:1292983	C	A	19	61	23	0
35	5	5:1294086	T	C	13	57	33	0

hTERT variants detected in the LWK population (n = 101)								
Number	**Chr.**	**SNP**	**Allele 1**	**Allele 2**	**Homozygous Allele 1**	**Heterozygous**	**Homozygous Allele 2**	**MIS**
1	5	5:1253744	A	G	14	50	37	
2	5	5:1254247	G	C	11	35	55	
3	5	5:1254251	T	C	10	36	55	
4	5	5:1254451	C	T	10	36	55	
5	5	5:1254943	T	C	10	36	55	
6	5	5:1255622	C	T	11	31	59	
7	5	5:1256228	C	T	9	33	59	
8	5	5:1256238	C	A	0	0	101	
9	5	5:1256256	C	T	1	6	94	
10	5	5:1256585	A	G	12	46	43	
11	5	5:1257024	T	C	4	29	68	
12	5	5:1257621	A	G	9	45	47	
13	5	5:1257905	T	C	3	29	69	
14	5	5:1259963	A	G	3	28	70	
15	5	5:1260033	C	T	16	34	51	
16	5	5:1260195	T	C	14	40	47	
17	5	5:1261052	A	G	5	27	69	
18	5	5:1264068	T	C	9	45	47	
19	5	5:1264143	T	C	8	33	60	
20	5	5:1264152	A	G	17	41	43	
21	5	5:1264156	A	G	8	34	59	
22	5	5:1264163	C	T	8	34	59	
23	5	5:1265204	C	T	17	45	39	
24	5	5:1265373	A	G	9	45	47	
25	5	5:1265664	T	C	13	33	55	
26	5	5:1265853	T	C	0	12	89	
27	5	5:1265935	A	G	9	44	48	
28	5	5:1266226	T	C	16	49	36	
29	5	5:1266310	G	A	19	49	33	
30	5	5:1266929	G	C	1	23	77	
31	5	5:1267202	G	C	1	21	79	

32	5	5:1267356	A	T	12	45	44	
33	5	5:1267497	A	G	1	17	83	
34	5	5:1267676	A	G	1	20	80	
35	5	5:1267973	C	T	1	10	90	
36	5	5:1268464	G	A	2	38	61	
37	5	5:1268847	C	A	2	38	61	
38	5	5:1268944	A	T	2	31	68	
39	5	5:1269365	A	G	3	38	60	
40	5	5:1270983	G	C	19	40	42	

hTERT variants detected in the LWK population (n = 101)								
Number	**Chr.**	**SNP**	**Allele 1**	**Allele 2**	**Homozygous Allele 1**	**Heterozygous**	**Homozygous Allele 2**	**MISSING**
41	5	5:1271661	C	T	19	49	33	0
42	5	5:1271714	C	T	2	39	60	0
43	5	5:1271861	A	G	0	20	81	0
44	5	5:1271879	A	G	1	19	81	0
45	5	5:1273610	T	G	7	35	59	0
46	5	5:1273905	A	G	6	40	55	0
47	5	5:1274983	G	T	2	18	81	0
48	5	5:1275528	A	C	23	53	25	0
49	5	5:1275857	C	T	5	36	60	0
50	5	5:1276772	C	A	3	22	76	0
51	5	5:1276785	T	C	2	25	74	0
52	5	5:1276788	G	A	0	0	101	0
53	5	5:1276794	A	C	2	25	74	0
54	5	5:1276873	A	G	3	34	64	0
55	5	5:1277577	A	G	4	37	60	0
56	5	5:1279790	C	T	10	50	41	0
57	5	5:1279964	G	C	15	45	41	0
58	5	5:1280022	C	T	4	26	71	0
59	5	5:1280028	A	G	3	28	70	0
60	5	5:1280128	T	C	8	45	48	0
61	5	5:1280252	A	G	0	11	90	0
62	5	5:1280830	C	G	4	38	59	0
63	5	5:1280938	A	G	10	44	47	0
64	5	5:1281079	A	G	2	21	78	0
65	5	5:1281693	C	T	18	50	33	0
66	5	5:1281743	T	C	2	18	81	0

67	5	5:1281890	C	T	18	48	35	0
68	5	5:1281975	C	G	18	50	33	0
69	5	5:1282159	C	T	6	30	65	0
70	5	5:1282165	C	T	6	31	64	0

hTERT variants detected in the LWK population (n = 101)								
Number	**Chr.**	**SNP**	**Allele 1**	**Allele 2**	**Homozygous Allele 1**	**Heterozygous**	**Homozygous Allele 2**	**MISSING**
71	5	5:1282194	T	C	1	17	83	0
72	5	5:1282319	A	C	4	40	57	0
73	5	5:1282414	A	G	28	42	31	0
74	5	5:1283095	T	C	1	12	88	0
75	5	5:1283312	A	G	28	44	29	0
76	5	5:1283755	T	C	1	40	60	0
77	5	5:1283841	A	C	8	33	60	0
78	5	5:1284046	T	C	8	33	60	0
79	5	5:1284135	T	C	3	44	54	0
80	5	5:1284976	T	C	1	25	75	0
81	5	5:1285162	T	C	6	46	49	0
82	5	5:1285238	A	G	6	18	77	0
83	5	5:1285974	A	C	3	40	58	0
84	5	5:1286516	C	A	22	51	28	0
85	5	5:1287194	G	A	11	46	44	0
86	5	5:1287340	A	G	3	33	65	0
87	5	5:1288547	T	C	5	42	54	0
88	5	5:1291293	G	A	6	27	68	0
89	5	5:1291717	C	T	12	72	17	0
90	5	5:1291718	A	G	12	72	17	0
91	5	5:1291720	T	C	12	72	17	0
92	5	5:1291735	G	A	0	28	73	0
93	5	5:1291740	G	T	0	28	73	0

94	5	5:1292446	T	C	1	26	74	0
95	5	5:1292649	G	C	1	26	74	0
96	5	5:1292714	C	G	0	6	95	0
97	5	5:1292983	A	C	24	52	25	0