INVESTIGATING THE ASSOCIATION BETWEEN LEUCOCYTE TELOMERE LENGTH AND GLUCOSE INTOLERANCE

By

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Declaration

I, Cecil Jack Weale, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

24/12/2017

Signed **Date**

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Dedication

To my father, R.C Weale. My rock.

Abstract

Background: Telomeres are DNA-proteins situated at the ends of linear chromosomes, responsible for genome stabilization. A link has been previously described between leucocyte telomere length (LTL) and age-related inflammatory disorders such as atherosclerosis, rheumatoid arthritis and cancer. Since diabetes mellitus has been described as a chronic inflammatory condition, it has been hypothesized that there is significant LTL shortening in individuals with dysglycaemia.

Aim: To investigate leucocyte telomere length in patients with pre-diabetes, newly diagnosed, known diabetics on treatment and to compare the results to normoglycaemic individuals.

Methods: A total of 205 eligible subjects (78% women) median age 56 years, from the Bellville-South community were followed-up between 2008 and 2011. Baseline and follow-up data collections included glucose tolerance status, anthropometric, blood pressure, lipids, insulin, γ-glutamyl transferase, cotinine, and HbA1c. In all participants, telomere length was measured using the absolute telomere q-PCR method performed on a Bio-Rad MiniOpticon Detector.

Results: Although there was a change in individuals' glycaemic status over the 3 years, no significant differences were observed in LTL across glycaemic status: (Baseline $p = 0.7618$, 3 Year Follow-up $p = 0.2204$). However, in a multiple regression model, adjusted for age and gender, LTL was negatively associated with age and GGT, and positively associated with high density lipoproteins (HDL) (all $p < 0.05$).

Discussion and conclusion: This research study was the first longitudinal study of LTL in Africans. We show that LTL shortening is not evident within three years, nor is it associated with glycaemia. Our findings also corroborate previous notions associating LTL with age. The lack of association between LTL and glycaemia has been previously reported, however further studies are required using larger sample and broader BMI spread.

Key words

Pre-diabetes, type 2 diabetes, inflammation, telomeres, leucocytes.

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List of Abbreviations

8-OHdG - 8-hydroxy-desoxyguanosine

AGEs - advanced glycation end-products

aTL - absolute telomere length

BMI – body mass index

CAD - coronary artery disease

CVDs - cardiovascular diseases

DBP – diastolic blood pressure

DM - diabetes mellitus

DNA – deoxyribonucleic acid

EDTA - ethylenediaminetetraacetic acid

FBG – fasting blood glucose

FFAs - free fatty acids

G6PDH - glucose-6-phosphate dehydrogenase

GPx - glutathione peroxidase

GR - glutathione reductase

GSH - glutathione

HbA1c – glycated haemoglobin

HDL – high-density lipoprotein

HF - heart failure

HP - hip

HPLC - high performance liquid chromatography

HSC - haematopoietic stem cells

hsCRP - high sensitive C-reactive protein

IDDM - insulin dependent diabetes mellitus

IFG - impaired fasting glucose

IFN - interferon

IGT - impaired glucose tolerance

IL - interleukin

LDL – low-density lipoprotein

LTL – leucocyte telomere length

MCP - monocyte chemo-attractant peptide

MDA - malondialdehyde

MetS - metabolic syndrome

MHC - major histo-compatibility

MI - myocardial infarction

MODY - Maturity onset diabetes of the young

MW - molecular weight

NGT - normal glucose tolerance

NIDDM - non-insulin dependent diabetes mellitus

NTC - negative template control

OGTT - oral glucose-tolerance test

Post BG – post blood glucose

QC – quality control

q-PCR – quantitative-polymerase chain reaction

RA - rheumatoid arthritis

ROS - reactive oxygen species

S Cotinine – serum cotinine

S Creatinine – serum creatinine

SADHSR - South African Demographic and Health Survey Report

SBP – systolic blood pressure

SCG – single copy gene

SOD - superoxide dismutase

SOPs - standard operating procedures

STD - standard

T1DM - type 1 diabetes mellitus

T2DM - type 2 diabetes mellitus

TC – total cholesterol

TERT - telomere reverse transcriptase

TG - triglycerides

TL – telomere length

TNF - tumor necrosis factor

TR - telomerase RNA component

WC – waist circumference

WHR – waist hip ratio

Definitions

- **Cellular senescence**: the irreversible state of G1 cell cycle arrest in which cells are refractory to growth factor stimulation.
- **Inflammation**: the response of living tissue to injury. It involves a well-organized cascade of fluid and cellular changes within living tissue.
- **Leucocyte**: any of the various large unpigmented cells in the blood of vertebrates, also called a white blood cell.
- **Pre-diabetes**: a condition whereby a person’s blood glucose levels are higher than normal but not high enough to be classified as diabetic.
- **Telomere attrition**: telomere shortening.

Chapter 1: Introduction and Literature Review

1.1 Introduction

Diabetes Mellitus (DM) is a group of metabolic disorders characterized by hyperglycaemia, resulting from defects in insulin secretion, insulin action or both (Ozougwu et al., 2013). Approximately 382 million people world-wide were diagnosed with diabetes in 2013 and this number is expected to rise to 592 million by the year 2035. The increase in population size, increased aging, as well as westernized lifestyle changes are all likely to lead to a 55% increase in diabetics by 2035 (Li et al., 2015).

The opinions on the source and etiology of DM differ greatly depending on the type of DM. These include several pathogenic processes involved in its progression, ranging from autoimmune destruction of the pancreatic β-cells resulting in insulin deficiency, to other abnormalities which lead to a resistance to the action of insulin (Baynest, 2015; American Diabetes Association, 2014). The foundation of the abnormalities involving carbohydrate, fat, and protein metabolism in diabetes occur as-a-result of insufficient action of insulin on target tissues. This insufficient insulin action is caused by inadequate insulin secretion and/or reduced tissue responses to insulin in the hormone-action pathways (American Diabetes Association, 2014).

1.2 Classification of Diabetes

The first accepted classification of DM was published by the World Health Organization (WHO) in 1980 and two major classes were proposed, namely: Insulin Dependent Diabetes Mellitus (IDDM) or Type 1 diabetes mellitus, and Non-insulin dependent Diabetes Mellitus (NIDDM) or Type 2 diabetes mellitus. This was however revised to include the various stages of hyperglycaemia as well as other categories (World Health Organization, 1999). The current classification of DM includes: Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), gestational diabetes and "other specific types" such as monogenic diabetes) which are represented in Table 1.1 (Maraschin et al., 2010).

Table 1.1: Etiologic classification of diabetes mellitus (Maraschin et al., 2010)**.**

Classification
Type 1 diabetes mellitus • Immunologically mediated • Idiopathic
Type 2 diabetes mellitus
Gestational diabetes
Other specific types: • Genetic disorder of β-cell function (MODY, mitochondrial DNA) • Genetic disorders in insulin action (lipoatrophic diabetes) • Exocrine pancreas diseases (pancreatitis, haemochromatosis) • Endocrinopathies (acromegaly, Cushing's syndrome) • Drug-induced (glucocorticoids, tiazidics) • Infections (cytomegalovirus, congenital rubeola) • Uncommon immunological forms (insulin receptor antibodies) • Other genetic syndromes (Down, Turner, Prader-Willi syndrome)

1.2.1 Type 1 diabetes mellitus (T1DM)

Type 1 diabetes mellitus (T1DM) is also known as insulin dependent DM and occurs at any age but is more commonly diagnosed in children, adolescents and young adults. It accounts for approximately 10-15% of all diabetes cases worldwide (American Diabetes Association, 2015; Kgasi, 2017).

T1DM is characterized by the complete absence of insulin secretion from the β-cells of the pancreas as a consequence of autoimmune processes (Tamura et al., 2016). These autoimmune processes are attributed to the presence of anti–glutamic acid decarboxylase, islet-cell or insulin antibodies, which lead to β-cell destruction, hence inhibiting the production and secretion of insulin. As a result, all Type 1 patients rely entirely on insulin therapy to maintain a normoglycaemic state (Baynest, 2015; Tamura et al., 2016).

1.2.2 Type 2 diabetes mellitus (T2DM)

Type 2 diabetes mellitus (T2DM) is also known as non-insulin dependent DM, and accounts for about 90–95% of all diabetes cases (Tamura et al., 2016; American Diabetes Association, 2015). T2DM is the most common form of DM and is recognized as a major epidemic in most developed countries, however the epidemic is now beginning to manifold in developing countries as well (Hu, 2011).

It develops as-a-result of decreased sensitivity of target tissues to insulin which is also known as insulin resistance (Ozougwu et al., 2013). It is a chronic metabolic disease dependent on several factors or causes due to a combination of genetic factors associated with impaired insulin secretion, insulin resistance, and other environmental factors (Abdul & Hassan, 2013; KAKU, 2010). Most individuals with T2DM are of an older age group, display visceral obesity (which is linked to insulin resistance and lack of exercise) and have a family history of DM. They also present with hypertension and dyslipidemia (high triglyceride and low HDL-cholesterol levels; postprandial hyperlipidemia), obesity, lack of exercise, stress as well as aging (Baynest, 2015).

Apart from lifestyle (namely stress obesity and lack of exercise) genetic factors also play a role in the onset of T2DM. Previous studies have shown that there are several genes accountable for, or linked with occurrence the of T2DM, however these genes have not been confirmed as the chief critical factor in the incidence of this type of diabetes (Abdul & Hassan, 2013).

1.3 Epidemiology and Etiology of Type 2 diabetes mellitus (T2DM)

The prevalence of DM is increasing at an alarming rate, however, most governments and public health professionals remain unaware of the current impact of DM and its associated complications (Tracey et al., 2016; Kearns et al., 2014). Although previously only considered an issue in developed countries, trends of DM are extending to developing countries, and concerns are being raised in Africa regarding the issue (International Diabetes Federation, 2015). More than half of all DM-affected persons in Africa come from only 4 countries: Nigeria, Ethiopia, the United Republic of Tanzania and South Africa, largely due to their large population sizes (International Diabetes Federation, 2013; Mbanya et al., 2014). In South Africa, the increase in the prevalence of T2DM may be attributed to rapid urbanization and economic change, particularly among the non-white population groups. Amongst many others, this westernization could also be one of the many factors contributing towards the increase in metabolic syndrome in the country (Erasmus et al., 2012).

Findings in South Africa have shown that there is a rising incidence of T2DM with age, more specifically after 35 years of age in both males and females, with age >35 years being recognized as an independent risk factor for DM (Gezawa et al., 2015). T2DM-related mortality is reported to be 1.7 times higher in women than in men, with 50.5% of women in Southern Africa having T2DM, as opposed to an estimated 49.5% of men (International Diabetes Federation, 2015). This can be attributed to women having a greater risk of obesity. This occurs as a consequence of cultural beliefs that obese women are more attractive and are unlikely to have HIV, but more notable, sociocultural factors such as inactive lifestyles (Goedecke et al., 2017). According to the WHO 2011, approximately 90% of T2DM cases are as a result of excess body weight due to a sedentary lifestyle.

Lack of exercise, smoking and alcohol consumption are examples of contributing lifestyle factors, and epidemiological studies have revealed that obesity is the most important of these in the development of insulin resistance and the consequent progression to T2DM (Bi et al., 2012; Wu et al., 2014).

T2DM is known to demonstrate a strong familial trait. It has been stated that individuals who have one parent affected by T2DM have a 40% risk of developing the disease while those with both parents affected have a 70% lifetime risk of developing it. Also, first degree relatives of individuals with T2DM are about 3 times more likely to develop the disease as opposed to subjects without a positive family history (Gezawa et al., 2015; Ali, 2013).

Genetic factors are known to play an important part in the development of T2DM, as demonstrated by the rare monogenic subtype group (MODY) and studies revealing genetic variation amongst different ethnic groups provide further evidence for the genetic foundation of this disease (Singh, 2015). It has long been established that obesity too is affected by genetic factors, and there has been amassing evidence implicating genetics playing a role in the risk of becoming obese. Furthermore, coupled with the genetic aspect, chronic low-grade systemic inflammation has also been associated with obesity and is considered a crucial element in the pathogenesis of insulin resistance and T2DM (Xia & Grant, 2013; Makki et al., 2013)

1.4 Obesity and Type 2 diabetes mellitus (T2DM)

Obesity is a disease characterized by an abnormal accumulation of body fat, and is well known to contribute to the onset of metabolic disorders. Longitudinal studies have shown obese people are likely to develop T2DM (World Health Organization, 2006; Cao, 2014). Body Mass Index (BMI), is the most frequently used measurement of relative weight (Noor et al., 2005; Ellulu et al., 2014). Adults with a BMI of >25 kg/m^2 are considered overweight, those with a body mass index (BMI) of >30 kg/m^2 are deemed obese, and those with a BMI of >40 kg/m^2 are categorized as severely obese (Feakins, 2016; Chan & Woo, 2010). Reports have estimated that by the year 2030, approximately one-third of the western world will be obese and this increase in the global prevalence of obesity has led to soaring rates of DM (Knaapen et al., 2013; Monteiro & Azevedo, 2010).

Obesity with metabolic syndrome has been linked to endothelial dysfunction, insulin resistance and low-grade inflammation. The effects of obesity on the immune processes are related to alterations in lymphoid tissue structure and integrity, as well as changes in leucocyte populations and inflammatory phenotypes (Andersen et al., 2016). Adipose tissue, which has previously been understood to mainly play a role in fat storage, is becoming increasingly recognized as a complex

endocrine organ (Balasubramanyam, 2013). In obese individuals, adipose tissue is the site for an inflammatory cascade, subsequently leading to insulin resistance. Although many components of the immune system have been found to play a role in either endorsing or reducing adipose tissue inflammation, macrophages are key players (Balasubramanyam, 2013).

Obese people have a greater visceral adiposity (adipose deposits in or around internal organs) in comparison to subcutaneous adiposity (adipose deposits beneath the skin), and within these deposits there tends to be an increased concentration of macrophages. Increased macrophage accumulation has been confirmed in the adipose tissue of obese people with T2DM (Balasubramanyam, 2013).

In states of obesity and glycaemia, glycation occurs (Nowotny et al., 2015). This is a non-enzymatic reaction between ketones or aldehydes with various amino acids and contributes to protein aging. Protein glycation may also take place under normal conditions; however, it is accelerated in hyperglycaemia due to increased levels of circulating glucose, resulting in the formation of irreversible advanced glycation end-products (AGEs) (Nowotny et al., 2015). These resultant AGEs possess toxic properties associated with inflammation and oxidative stress. They result in the stimulation of accumulated macrophages, in turn promoting cell death, impaired cell adhesion, cell differentiation, as well as further migration of immune cells to the site of inflammation (Nowotny et al., 2015).

Another possible cause for the increased accumulation of these adipose macrophages may be changes in the adipose metabolic function, resulting in increased concentrations of free fatty acids (FFAs) (Kosteli et al., 2010). Also suggested, is that obesity causes elevated basal lipolysis, and the resultant increase in FFAs initiates the release of chemotactic stimuli which attract the macrophages (de Heredia et al., 2012). The macrophages tend to accumulate around the areas of necrotic adipocytes forming 'crown-like structures', and stimulated by AGEs and reactive intermediate molecules, such as glycolaldehyde, large amounts of pro-inflammatory cytokines are secreted e.g. tumor necrosis factor- α (TNF-α), interleukin-1 β (IL-1β) and IL6. These as well as chemo-attractants, such as monocyte chemo-attractant peptide 1 (MCP-1), contribute to local and systemic inflammation, as well as insulin resistance (Yamamoto & Yamamoto, 2012; Nowotny et al., 2015; Exley et al., 2014).

Apart from macrophages, T lymphocytes play a pivotal role in promoting inflammation in adipose tissue. These lymphocytes, together with macrophages are key to the pathogenesis of T2DM and associated inflammation (Ross, 1994; Xia et al., 2017). Recent reports have pointed towards a potential involvement of CD4+ T lymphocytes in obesity and T2DM, demonstrating increased levels of activated CD4+ T lymphocytes in the visceral adipose tissue of obese mice (Shirakawa et al.,

2016). Also reported was the expression of major histo-compatibility MHC class II on adipocytes, which in turn results in the activation of the CD4+ T lymphocytes into further differentiated effector cells which initiate inflammation in the adipose tissue. These effector cells, more notably T-helper-1 (Th-1) and T-helper-17 (Th17) cells are pro-inflammatory cells. The Th1 cells produce and release cytokines such as interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumour necrosis factor- β (TNF-β), which initiate cell-mediated immunity and phagocyte-dependent inflammation. The Th17 cells, which have been reported to be important in the pathogenesis of T2DM and obesity, secrete interleukin-17 (IL-17). This in turn stimulates the production of tumour necrosis factor- α (TNF-α), which chemotactically attracts other inflammatory cells, elevating the inflammatory state (Xia et al., 2017). The intensified inflammatory response promotes increased oxidative stress, increased AGE production and macrophage stimulation, and the cycle continues. The circulating pro-inflammatory cytokines, adipokines and chemokines have a direct impact on the induction of glucose intolerance and insulin resistance by antagonizing insulin's metabolic actions at peripheral tissue-sites such as the liver, as well as skeletal muscle (Wang et al., 2007).

1.5 Structure and function of Telomeres in normal cells

T2DM is understood to be a polygenic disease developing due to a combination of multiple genomic interactions as well as other factors, more notably obesity and aging. Aging plays a key role in the development of many other disorders such as cancers and cardiovascular diseases, however the biological processes associating aging and gene interaction to produce T2DM is still poorly understood. It has been reported though, that a possible indicator could be found in repeat sequences of DNA known as telomeres (Ali, 2013; Elks & Scott, 2014). Aging is a biological process affecting most cells, organisms and species. It has been suggested that telomeres act as a "biological clock," shortening with the aging of cells. Telomeres are DNA-protein complexes situated at the ends of linear chromosomes in eukaryotic cells that consist of non-coding, double-stranded, G-rich tandem repeats ranging in length from a few hundred base pairs in yeast to several kilo-base pairs in vertebrates (Oeseburg et al., 2010; Serrano & Andrés, 2004).

Telomeres are responsible for genome stabilization, protecting the genome from nucleolytic degradation, recombination, repair, and inter-chromosomal fusion, thus leading to the conservation of the vital coding genes (Shammas, 2011). Telomere shortening occurs as cells replicate and it is thought that the telomere DNA is sacrificed to preserve the coding regions of the genome. This process continues until eventually a critical threshold is reached called the "Hayflick limit". When the "Hayflick limit" is reached, it results in cell senescence and/or apoptosis (Muzumdar & Atzmon, 2012).

Figure 1.1: Illustration showing the position of telomeres at the end of our chromosomes. (Genome Research Limited. http://www.yourgenome.org/facts/what-is-a-telomere)

Telomere homeostasis is maintained by the enzyme telomerase, which is responsible for the addition of the telomere repeats TTAGGG at the end of chromosomes, and other telomere associated proteins which assist in minimizing telomere shortening. The other telomere associated proteins include: telomere reverse transcriptase (TERT), and a telomerase RNA component (TR) which all help regulate mechanisms involving cell survival, gene regulation, cell signaling, proliferation and differentiation, as well as DNA repair (Tamura et al., 2016; Bellot & Wang, 2013).

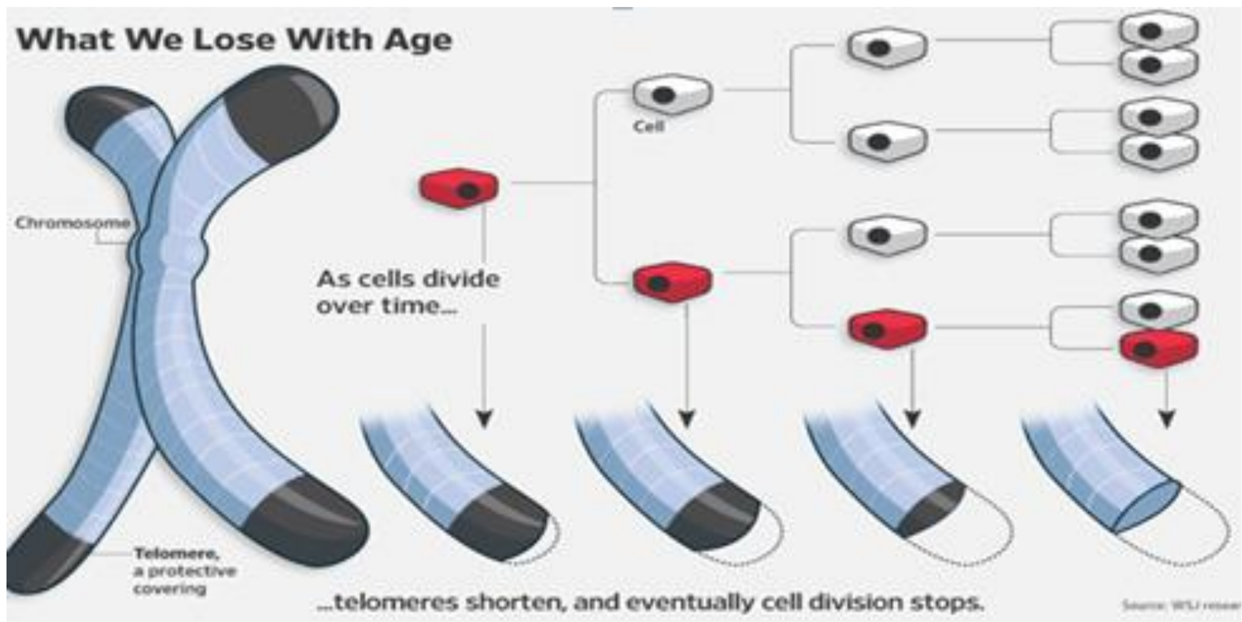

Figure 1.2: Telomere shortening with age. (Telomere Restoration. http://telomeres-aging.com/)

Certain agents associated with specific lifestyles may accelerate telomere shortening by inducing DNA damage in telomeres, hence affecting the health and lifespan of an individual. Telomere shortening has not only been linked to aging but also to inflammation and several age-related diseases including tumorigenesis, coronary artery disease, heart failure as well as DM (Shammas, 2011).

1.6 Telomere length in inflammation and age-related disease

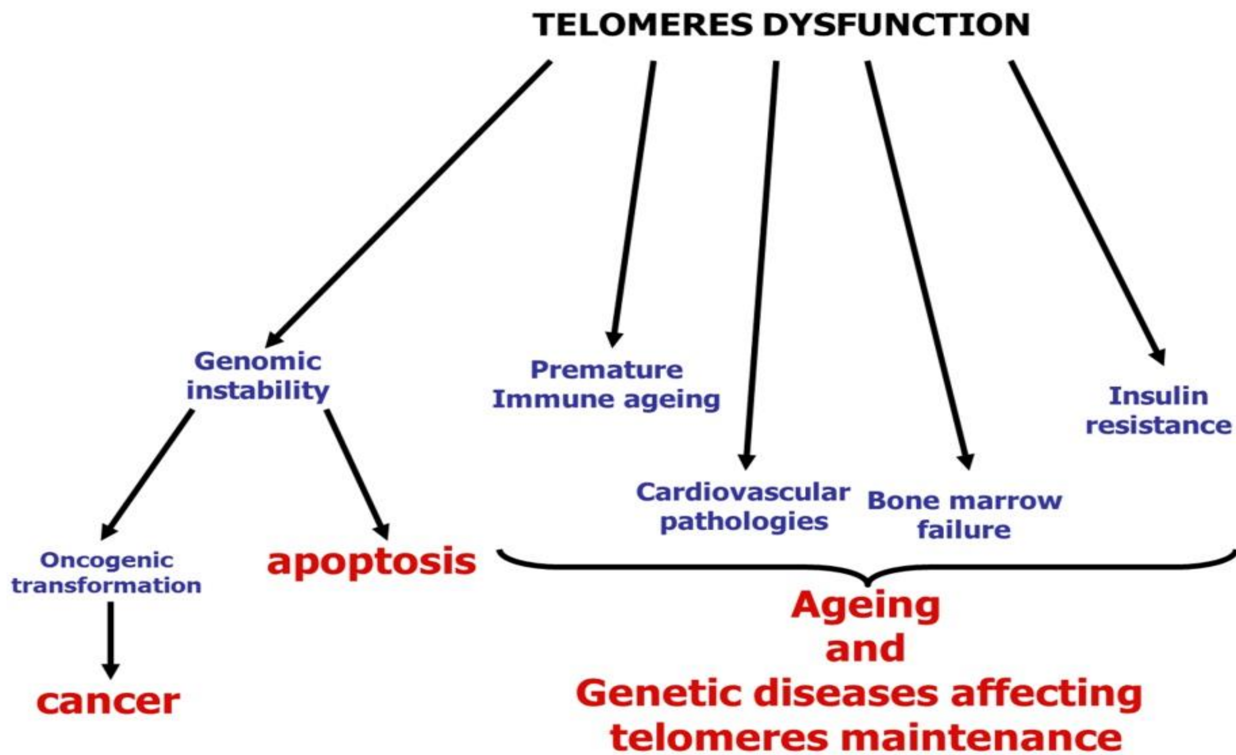

Figure 1.3: Relationship between telomeres dysfunction and pathologies. (Bellot & Wang, 2013).

1.6.1 Telomere length in inflammation

Inflammation is a normal response to harmful stimuli such as injury and infection. The process may either be acute, as a result of temporary stimuli, or chronic, as a result of recurrent exposure and failure of the acute response to resolve the infection or injury (Ueha et al., 2012). Chronic inflammation forms the basis of numerous diseases such as cancer, myocardial infarction, diabetes and lung failure. Persistent immune activation and inflammation leads to tissue injury, cell death, scarring, and fibrosis. The consequence of this is eventual permanent loss of function of the affected organs (Wong et al., 2014).

The immune system is very intricate, involving many cells and lymphoid organs. It consists of two components that act together to combat inflammation: the innate immune system and the adaptive immune system, and the participating cells of both responses (B lymphocytes, T lymphocytes, monocytes and dendritic cells) which all originate from stem cells in the bone marrow (Effros, 2012). The adaptive immune response is highly dependent on both T and B lymphocyte function. The T cells, consist of CD4+ (helper) and CD8+ (cytotoxic) T cells, with the role of CD4+ T cells being

stimulation of the CD8+ T lymphocytes to attack and kill target cells (e.g. cells infected by pathogens such as viruses and cancer cells), as well as stimulation of B cells to produce pathogen-recognizing antibodies (Effros, 2012; Kaszubowska, 2015).

During a typical immune response, antigen presentation occurs. CD8+ T cells identify peptide fragments bound to class I MHC molecules while CD4+ T cells identify them in association with class II MHC molecules (Udono et al., 2007). Thereafter, there is differentiation from naive T lymphocytes to numerous activated effector cells. There is also maturation of naive B lymphocytes into antibody-producing plasma cells in the lymphoid nodules (Udono et al., 2007). In T lymphocytes, these numerous cell divisions, which occur to bring about a target-specific differentiation, result in subsequent telomere erosion. In B lymphocytes however, the telomere shortening occurs at a slower rate. Studies of B cells have revealed that during clonal expansion, there are certain mechanisms in place for telomere maintenance during proliferation (Kaszubowska, 2015). The main mechanism accountable for the maintenance of telomere length is the stimulation of telomerase activity, and it has been shown that telomerase activity is highly regulated during B cell differentiation (Hohensinner et al., 2011; Kaszubowska, 2015).

Inflammation results in the release of cytokines, and among them are interleukins (IL). Interleukins are a group of cytokines which initiate various immune responses in different cell types. IL-1, which is a pro-inflammatory cytokine, is produced by macrophages and stimulates an inflammatory immune response and haematopoiesis, while IL-8, which is also secreted by macrophages acts as a chemotactic agent for neutrophils during acute inflammation (Wong et al., 2014). IL-8 has also been shown to encourage rapid mobilization of haematopoietic stem cells (HSC) as well as differentiation into leucocytes. On estimate, 15-20 cell divisions take place from a naive state to millions of activated clones of effector cells in the leucocyte lineage (Kaszubowska, 2015). The resultant cell divisions result in a degree of telomere shortening and initiate a course of replicative senescence. This characteristic of IL-8 may have important implications in telomere erosion since increased mitotic division is necessary to replenish used up haematopoietic stem cells (HSC) but could also lead to telomere shortening (Wong et al., 2014).

1.6.2 Telomere length and telomerase in age-related disease

Numerous reports have observed short leucocyte telomere length (LTL) in patients with inflammatory diseases such as hepatitis C, liver cirrhosis, chronic kidney disease and chronic obstructive pulmonary disease in comparison to normal healthy individuals (Carrero et al., 2008; Kinouchi et al., 1998; Aikata et al., 2000; Steer et al., 2007). These findings support the notion that inflammation promotes leucocyte telomere shortening (Liu et al., 2014). In addition, others have demonstrated a

link between the shortening of leucocyte telomere length and age-related inflammatory diseases. These include diseases such as autoimmune disorders, cancer, atherosclerosis and other cardiovascular diseases, and diabetes (O'Donovan et al., 2011; Wong et al., 2014; Hoffmann & Spyridopoulos, 2011). An example is rheumatoid arthritis (RA) which is a chronic autoimmune disease of the synovial joints and despite being relatively poorly understood, occurs as a result of both genetic and environmental factors (Wasserman, 2011).

It has been determined that telomere shortening, which triggers premature cell-senescence, could play a role in the pathogenesis of autoimmune diseases (Blinova et al., 2016). Premature cell-senescence is a key feature of RA, and studies have demonstrated telomere shortening in T and B lymphocytes in patients with the disorder (Costenbader et al., 2011; Blinova et al., 2016). As a result, there is an alteration in the adaptive immune response, causing a decrease in naive T lymphocytes, an increase in memory T lymphocytes and impaired T lymphocyte signaling. This affects the immune system's ability to distinguish between "self" and "foreign" antigens, promoting the autoimmune reactivity (Lee & Bae, 2016).

Cancer is also an age –related disease. Exposure to endogenous and environmental factors alters telomere length and integrity leading to disease (Zhang et al., 2015; Gu, 2015). There has been increasing evidence linking telomere dysfunction to cancer initiation and development (Gu, 2015). Telomerase activity appears to be suppressed in human normal cells, but can be reactivated in cancer cells and is a vital pre-requisite for the immortalization of these abnormal cells (Gu, 2015). This reactivation has been reported to be associated with TERT, encoding the reverse transcriptase subunit of telomerase (Akincilar et al., 2016). TERT expression is reactivated in approximately 85% of cancers, and although the molecular events leading to this are still yet to be fully deciphered, postulations are that this may be chiefly due to the occurrence of point mutations in TERT promoter regions. These mutations have been reported to be highly recurrent in various carcinomas such as melanoma, urothelial and bladder cancer, as well as glioblastoma (Akincilar et al., 2016; Li & Tergaonkar, 2016).

Aging is a key risk factor for the development of cardiovascular diseases (CVDs) such as myocardial infarction (MI), stroke, hypertension, chronic heart failure (HF), with the incidence of CVDs significantly increasing with age (Saliques et al., 2010). More specifically, it has been found that there is a link between telomere length in vascular cells and the development of coronary artery disease (CAD) (Saliques et al., 2010). Telomere length and structure are regulated by complex mechanisms involving the interactions between telomerase and other associated proteins. It has however been observed that telomerase activity decreases with age but increases in states of inflammation and injury. The mechanisms promoting this increased activity include increased levels of reactive oxygen

species (ROS), decreased nitric oxide and decreased TERT activity all of which have been reported to be associated with the process of aging and atherogenesis (Wang & Yeh, 2016). Exposure to ROS results in premature endothelial and smooth muscle cellular senescence by promoting continued cell proliferation. In heightened states of inflammation and oxidative stress, it has been seen that the above mechanisms contribute to progressively shortened LTL. This process has been reported in atherosclerosis, as well as chronic or ischaemic heart failure (Wang & Yeh, 2016).

1.7 Telomere length in Obesity and Type 2 diabetes (T2DM)

1.7.1 Mechanisms of telomere shortening

Apart from increased inflammatory activity and elevated cell turnover and division, hastened telomere erosion could also be caused by the release of ROS which may cause damage to telomeric DNA by oxidative stress (Wong et al., 2014). Both obesity and T2DM have been recognized as states of increased oxidative stress, and it is understood that this could play a key role in telomere attrition in these individuals (Kim et al., 2009; Choi et al., 2009). In these patients, there is an increase in the production of ROS, which in obesity-associated T2DM can be attributed to increased proliferation of adipocytes. The adipocytes produce adipokines, leading to localized inflammation and consequently, increased apoptosis and necrosis. Potent ROS such as superoxides, hydroxyl radicals, and hydrogen peroxide are released from the mitochondria of cells into the surrounding tissues. These circulating oxidizing agents may cause double-stranded DNA breakages in the genome and chemical changes in the telomere DNA sequences (Mundstock et al., 2015; Choi et al., 2009). The release of ROS from the mitochondrial electron transport chain, increased glucose auto-oxidation as well as polyol and protein kinase C pathway activation have been described in individuals with hyperglycaemia. It is well known that hyperglycaemia provokes an increase in ROS production, due to increased activation of reducing equivalents into the mitochondrial electron transport chain which in turn leads to an acceleration in telomere shortening (Salpea et al., 2010).

It has been reported that there is a negative correlation between LTL and increased 8-hydroxy deoxyguanosine concentrations (an oxidized nucleoside of DNA) (Wong et al., 2014). 8-hydroxy deoxyguanosine is a key marker of DNA damage resulting from oxidation, and is caused by chemical changes in the purines and pyrimidines of telomeric oligonucleotides in the form of 8-oxoguanine adducts. The presence of 8-oxoguanine adducts obstruct telomerase activity and reduce the binding of enzymes (TRF1 and TRF2) to the telomere sequence which is required for the control of telomere homeostasis. They also cause single or double strand breaks and GC-TA mutations, resulting in

genomic instability. This instability contributes to disruption of telomere length, maintenance and function (Mi et al., 2008; Coluzzi et al., 2014).

The oxidative stress observed in the white cells of patients with DM, is also present in the pancreatic β-cells. These factors all result in the shortening of β-cell telomeres leading to premature β-cell senescence and dysfunction of insulin secretion (Wong et al., 2014; Tamura et al., 2016).

Patients with T2DM develop numerous complications which include increased infections, cardiovascular disorders and thrombosis. The increased inflammation associated with the disease could result in increased cell division and therefore the steady shortening of telomeric length. This phenomenon could explain the evolution of the disease and the development of complications such as immune exhaustion.

1.7.2 Research linking telomere shortening with glucose intolerance and diabetes.

Leucocyte telomere shortening has been observed in individuals with impaired glucose tolerance, however, no solid link has been demonstrated between telomere shortening and the risk of developing DM (Adaikalakoteswari et al., 2007; J. Zhao et al., 2014). A recent study was carried out to determine the relationship between telomere length, fasting plasma glucose and two-hour post blood glucose, otherwise called the oral glucose-tolerance test (OGTT) (Khalangot et al., 2017). This study was based on an earlier project by (Menke et al., 2015) performed to determine the association between LTL and DM using fasting plasma glucose, in which no relationship was determined. Therefore, the OGTT was incorporated to reveal and compare LTL between diabetes categories: normal glucose tolerance (NGT), impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and diabetes. The conclusions made were that the OGTT was inversely associated with LTL in the NGT, IFG/IGT and DM groups, while fasting plasma glucose was negatively correlated with LTL only in the DM people (Khalangot et al., 2017).

It has been commonly postulated that telomere attrition may play a role in the onset and development of metabolic syndrome and the association of telomere length and T2DM development has been explored in several well-designed studies (Zhou et al., 2016; Kirchner et al., 2017). In a prospective population-based approach, LTL and the incidence of T2DM was studied (Willeit et al., 2014). Within a 15-year follow-up, 44 of 606 participants without T2DM at baseline developed DM and it was estimated that individuals who had a shorter LTL at baseline had an average risk of 31% of progressing to DM. In a similar study, Zhao *et al.*, 2014 assessed the association of LTL with the future risk of diabetes over a five-year timeframe. Individuals within the lowest LTL quartile had a twofold risk of developing T2DM, portraying a nonlinear relationship between telomere length and

DM risk, independent from other risk factors (Zhao et al., 2014). In contrast though, an earlier study portrayed a weak association between LTL and diabetes risk. You *et al.*, 2012 examined whether any modest associations occurred between telomere length and diabetes using genetic variants that were associated with changes in telomere length. The study assessed both allele counts and multiple variables as separate covariates in the same regression model as instruments in the Mendelian randomization analysis. No significant association was detected between genetically determined telomere lengths and diabetes risk in the large multiethnic cohort followed for 6 years (You et al., 2012).

Although studies have identified a link between chronic inflammation and shortened telomeres, evidence in T2DM remains scarce and controversial, and it is still yet to be established whether there is a relationship between LTL and the development of T2DM (Tamura et al., 2016; Willeit et al., 2014). It is evident that there is a need for further research to provide additional knowledge on how telomere length and accelerated shortening could contribute to the progression of the T2DM and the development of complications. In this study therefore, we hypothesize that LTL is associated with T2DM and that a relationship exists between telomere length and varying degrees of glucose tolerance.

1.8 Aim of this study

The aim of this study was to longitudinally assess the leucocyte telomere length in participants across all glucose tolerance groups at baseline and after three years, and investigate if telomere length could be correlated with markers of glucose metabolism and disease progression.

1.9 Objectives of this study

The objectives of this study were to:

1. To compare LTL in patients with hyperglycaemia with normoglycaemic individuals.
2. To investigate the association of LTL with the progression of the disease by comparing the LTL at baseline and at three-year follow up.
3. To correlate LTL with patient demographics, markers of inflammation and markers of glucose metabolism.

Chapter 2: Methodology

2.1 Ethical considerations

This study formed part of the Bellville South study that has been previously described (Matsha et al., 2013). Both the baseline and the three-year follow-up studies were approved by the Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences Ethics Committee (Reference Number: CPUT/HW-REC 2008/002 and CPUT/HW-REC 2010 respectively). The study was carried out according to the Code of Ethics of the World Medical Association (Declaration of Helsinki 2013). This sub-study also required ethical clearance which was sought from and granted by the Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics Committee (CPUT/HW-REC 2014/H08).

2.2 Study design

This was a longitudinal study involving participants from the Bellville South study (Matsha et al., 2013).

2.3 Study setting

The setting for the study was the Township of Bellville South, Western Cape, Cape Town, South Africa. Bellville South is a township formed in the late 1950s and, according to the 2011 population census, it has a population of approximately 29 301 people, with an average household size of 4.84 individuals. The population is comprised of predominantly coloured or mixed ancestry individuals (76%), followed by black Africans (18.5%) and Caucasian and Asians making only 1.5% (Lehohla, 2011). Most residents in this community are of lower to middle-class socio-economic status, having resided there for over five years and some their entire lives, with 37% of households having a monthly income of R3 200 or less. Recent statistics involving individuals from Bellville South show an increased number of inhabitants with diabetes (Statistics South Africa, 2012). The target population for this project included participants 18 years or older, which comprised 16 168 individuals, 14 352 of which were of mixed ancestry origin.

2.4 Study population

Individuals were mixed ancestry members of a cohort study conducted in Bellville South, in the metropolitan city of Cape Town in South Africa, and who had undergone both baseline and three-

year follow-up evaluations. The mixed ancestry population which is commonly referred to as "Coloured", is a South African population group comprising of 32 – 43% Khoisan, 20 – 36% Bantu-speaking African, 21 – 28% European and 9 – 11% Asian ancestry (De Wit et al., 2010). Between January 2008 and March 2009, suitable participants from a multistage random sample of 1000 households were invited to take part in the baseline evaluation.

2.4.1 Inclusion criteria

For the baseline evaluation, only participants residing in the Bellville South, Cape Town were included in this study. The participants were between 18 and 70 years, not acutely ill and in the case of female participants, only women who were not pregnant were included. Also, only those who consented to genetic analysis were included.

2.4.2 Exclusion criteria

For the baseline evaluation, individuals who did not reside in Bellville South were excluded. Individuals younger than 18 years of age, who were acutely ill, as well as pregnant females, were excluded. Also, participants who did not consent to genetic analysis were excluded.

946 participants were recruited and the three-year follow-up survey commenced in February 2011 on the same participants that took part in the Baseline survey.

2.5 Study procedure – Baseline and Three-Year Follow-up

2.5.1 Demographics

An extensive protocol describing data-collection procedures (questionnaires as well as anthropometric measurements) was established. The questionnaire requested information about the participant's demographics such as gender, age, previous medical history of diabetes, dietary, as well as lifestyle aspects. The four components of the questionnaire were: 1) personal demographics, 2) family health history, 3) diet and 4) cigarette smoking. The questionnaire was adopted from other standard sources such as the CAGE Questionnaire (Ewing J, 1984), the South African Demographic and Health Survey Report by the South African Medical Research Council (SADHSR) (Bradshaw & Steyn, 2005; Steyn et al., 2006). The SADHSR questionnaire focuses on chronic and lifestyle diseases by addressing the diabetes status, nutritional status, hypertension and physical activity of the participants. The CAGE questionnaire is mainly used in alcohol related studies.

The questionnaire used for this study was also adopted from a previous study to measure the prevalence of DM amongst learners attending schools in the Belhar, Delft and Mfuleni suburbs in Cape Town (Somers et al., 2006). The family health history section of the questionnaire was used to identify the presence or previous treatment of family members, up to third degree relatives, for associated inheritable diseases. The section on cigarette smoking focused on: a) the specific habit, b) duration of smoking, c) quantity of cigarettes smoked, and d) when the habit was stopped. A detailed medication history was obtained by asking participants, as well as examining their clinic cards, and recording the drugs which they brought to the study site.

2.5.2 Clinical measurements

The clinical measurements that were performed included: height, weight, waist circumference, hip circumference and blood pressure measurements. All anthropometric measurements were performed in triplicate and the average was calculated and used for the final analysis.

2.5.2.1 Height

Measurement of height was in centimeters (cm) and performed using a portable stadiometer. A participant was required to stand bare-foot on the base of the stadiometer, at right angles to the sliding lever. His/ her head would then be placed in the Frankfort plane with hands freely at the sides. The scapular and buttocks were placed as close to the vertical sliding metallic bar as possible to ensure accurate readings and the sliding metallic bar was then adjusted to gently rest on the subject's head. If the participant happened to be taller than the investigator, the investigator stood on a platform to enable an accurate reading. The height of wheelchair-bound participants was however excluded.

2.5.2.2 Weight

Weight was measured in kilograms (kg) using a calibrated Sunbeam EB710 digital bathroom scale. It was necessary for individuals to be wearing light clothing and be barefoot. The weight of all participants was recorded except those who happened to be wheelchair-bound or who were posturally impaired. The scale would be zeroed and the participant asked to stand on the middle of the flat surface of the scale. Hands were placed on the sides and after ensuring that the subject's weight was evenly distributed, the reading was taken. Readings less than 0.5 kg were rounded off to the nearest lower kilogram while those above 0.5 kg were rounded off to the nearest higher kilogram. The body mass index (BMI) was then calculated by dividing the weight and height squared, BMI = $\frac{Body\ mass\ (Kg)}{height\ (m)^2}$ (Hall & Cole, 2006).

2.5.2.3 Waist circumference (WC)

A non-elastic tape was used to measure the waist circumference (WC). Subjects were asked to stand in an erect position with hands placed on their sides and with their feet and abdominal muscles relaxed. Measurements were taken with the investigator in front of the participant, and by placing the measuring tape around the natural waist (narrowest part of the torso as seen from the anterior view). For obese participants, the narrowest circumference between the ribs and the iliac crest was measured (Tolonen et al., 2002).

2.5.2.4 Hip circumference (HP)

Hip circumference (HP) was measured at the maximal circumference over the buttocks using a non-elastic tape. The investigator would ensure eye-level with the region to be measured, by either sitting or squatting, then place the tape placed around the buttocks on the widest region and the measurement taken. Care was taken not to press tightly against the skin, for accuracy. If the hip circumference exceeded the tape size, the measurement was not recorded, but noted (Tolonen et al., 2002).

2.5.2.5 Blood pressure

Systolic blood pressure and diastolic blood pressure were used to assess the blood pressure. Systolic blood pressure (SBP) is the highest arterial pressure created by force of the blood upon artery walls as a result of the pumping action of the left ventricle of the heart. Diastolic blood pressure (DBP), on the other hand, refers to the lowest arterial blood pressure when the heart muscles contract after a systolic event. The ratio of SBP over DBP, is used to express blood pressure and it is represented as a fraction, and the units are millimeters of mercury (mm Hg) (Pickering, 2005).

Blood pressure measurements were performed according to WHO guidelines (World Health Organization, 1999). Measurements were taken using a calibrated baumanometer (Rossamax; Berneck, Switzerland) with the participant in a relaxed, seated position. Participants were seated, with back support, while their arms were extended and rested on the table at the same level as their heart. It was necessary that the correct adult cuff size was placed 2 cm above the elbow joint to ensure accurate readings. Triplicate readings, at one-minute intervals, were recorded, with the lowest reading chosen as the participant's blood pressure. Participants were also asked to remain silent while taking measurements as this has been found to affect readings (Adams et al., 2002).

2.5.2.6 Blood collection

For each participant six blood tubes were used for collection: three fasting and three postprandial. Self-reported T2DM participants (who were confirmed by either medical records or medication) had

only the fasting blood samples taken and no 2-hour bloods were drawn. Collection of both the fasting and postprandial blood samples was done in one grey-capped (sodium fluoride) tube, one plain tube (no clotting factors) and one purple-capped, ethylenediaminetetraacetic acid (EDTA) tube. The grey-capped tubes were used for blood glucose measurements, while the plain tubes were used to obtain serum for serological tests: serum cotinine, insulin and lipid profile. The purple-capped (EDTA) tubes were used for glycosylated haemoglobin (HbA1c) level measurements, as well as for DNA extraction. Blood samples collected were transported daily in an ice-box for processing at the Metropolis Private Pathology Laboratory (Century City, Cape Town). The purple-capped tubes for DNA extraction were stored in a -20 ^{0}C freezer.

2.5.3 The oral glucose tolerance test (OGTT)

It was necessary that all participants, excluding the self-reported diabetic subjects, undertook the oral glucose tolerance test (OGTT). Subjects were required to fast overnight, after which the OGTT was performed according to WHO guidelines (World Health Organization, 1999): 1) investigators asked participants whether they had fasted, 2) collected fasting blood samples, 3) gave participants 75 grams of anhydrous glucose dissolved in 250 - 300ml of water, which was drunk within 3 - 5 minutes, and the time recorded, 4) collected a second blood sample after 2 hours (2h - PG). Phlebotomy was performed by trained nurses.

2.5.4 Biochemical data

Biochemical measurements (summarized below in Table 2.1) such as fasting blood glucose (FBG) and 2h - PG plasma concentrations (Post BG), glycosylated haemoglobin (HbA1c), serum insulin (S insulin), triglycerides (TG), cholesterol, gamma glutamyl-transferase (GGT) enzyme and serum cotinine (S cotinine) were conducted as previously described (Matsha et al., 2012).

Table 2.1: Biochemical parameters measured

Analyte	Method	Equipment used
Plasma glucose (mmol/L)	Enzymatic Hexokinase	Cobas 6000, Roche Diagnostics; Mannheim, Germany
HbA1c (%)	High Performance Liquid Chromatography	Cobas 6000, Roche Diagnostics; Mannheim, Germany
Insulin (mmol/L)	Paramagnetic Particle Chemiluminescence Assay	Beckman DXI (Beckman Coulter, Miami, USA)
Lipids Profile (mmol/L)	Friedwald’s Formula	Cobas 6000 Roche Diagnostics (Indiana, USA)
Ultra-sensitive (U-CRP)	Latex Particle Immunoturbidimetric	Beckman AU (Beckman Coulter, Miami, USA)
GGT (IUL)	IFCC - Rate	Beckman AU (Beckman Coulter, Miami, USA)
S Cotinine (ng/mL)	Competitive Chemiluminescent	Immulite 1000, Siemens
S. Creatinine (umol/L)	Modified Jaffe - Kinetic	Beckman AU (Beckman Coulter, Miami, USA)

2.5.5 Diabetes status classification

Classification of participants was done in accordance with the revised WHO criteria of 1999 (World Health Organization, 1999). Participants were categorized as either: Normoglycaemic, Pre-diabetic (IGT and/or IFG), Diabetic (screen detected diabetics) and Known diabetics on treatment, diagnosed in accordance with the World Health Organization criteria (WHO, 2006). A diagnosis of DM was dependent on a history of diabetes, as well as fasting and postprandial glucose concentrations, and was done using the following specifications: fasting plasma glucose ≥ 7mmol/L or 2-hour plasma glucose ≥11.1mmol/L).

2.5.6 Quality control (QC)

The study was conducted observing strict standard operating procedures (SOPs). The field workers and nurses involved were briefed on how to take blood pressure, blood collection, anthropometric measurements and questionnaire management. Researchers were required to sign off on questionnaires, blood pressure and collection, as well as anthropometric measurements that they conducted so as to have a trace history, as well as to validate measurements and data entries. Spot field checks were also conducted by project supervisors in order to verify the accuracy of measurements and enhance quality control (Adams et al., 2002).

Equipment was routinely calibrated as per manufacturer’s specifications. Scales were routinely assessed for accuracy using standard weights. The work-load of all staff involved in the study was

kept within reasonable limits to avoid false measurements due to fatigue. Each team had a supervisor responsible for overseeing performance and who was also responsible for equipment calibration. Weekly meetings were held to evaluate progress, problem-solving, and retraining if necessary.

2.6 DNA Isolation

Of the original 946 participant blood samples available, samples from only 205 participants were used for DNA extraction to determine telomeric length. The DNA was extracted from 1 - 2ml of whole blood collected in EDTA blood tubes and stored at -20°C using the salt extraction method (www.genomics.liv.ac.uk, 2001). Blood cells were lysed by adding a five times volume (~ 5 – 10ml) of lysis buffer (155mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA; pH 7.4) to the blood. The tubes were then vigorously vortexed for about 10 - 15 seconds and placed on ice for 5 minutes. This step was repeated three times. Thereafter, the tubes were centrifuged at 1500rpm for 10 minutes using a Beckman General Purpose centrifuge (Beckman Coulter Inc., CA, USA). The resultant pellets were washed with 10ml of phosphate buffered saline (PBS; 2.68mM KCl, 136mM NaCl, 1.47mM KH_2PO_4, and 8.1mM Na_2HPO_4, pH 7.4) and centrifuged twice for 10 minutes at 1500rpm. 3ml of nucleic lysis buffer (10mM Tris, 400mM NaCl, 2mM EDTA; pH 8.2) and 300µl of 10 % sodium dodecylsulfate (SDS) was then added and the tubes vortexed to dissolve the pellets. 30µl of 10mg/ml proteinase K was added, mixed well, and the tubes were incubated at 55°C overnight to digest proteins.

The next day, 1ml of 6M NaCl was added to the tubes. They were then vortexed and centrifuged at 2500rpm for 10 minutes. Care was taken to transfer the supernatant of each tube into a clean tube, leaving behind the white protein precipitate. The supernatants were re-centrifuged and two volumes (~ 9ml) of 100 % ethanol were added to the tubes to precipitate the DNA. Due to the fact that DNA is insoluble in ethanol, it forms a thread-like precipitate. The tubes were then centrifuged at 8000rpm for 30 minutes and the resultant supernatants were discarded.

The DNA pellets were washed with 1ml of 70% ethanol and the DNA-ethanol mixture transferred to a 1.5ml eppendorf tube. Transfer of the DNA pellet to the eppendorf tube was confirmed by visual inspection. Eppendorf tubes were then centrifuged at 15000rpm for 30 minutes in a microcentrifuge (Beckman Coulter Inc., CA, USA) and the supernatants discarded. The DNA pellets were dried by inverting the tubes on a paper towel and air drying them for 15 minutes. 100 – 200µl of nuclease free water was added to the tubes to dissolve the pellets, this volume being dependent on the size of the pellet. Tubes were then placed on a mixer (Elmi Intelli-Mixer RM-2) (Elmi Ltd, Riga, Latvia) overnight to aid suspension of the pellet. The newly extracted DNA was then stored at -80ºC

2.7 Freeze-thawing of DNA Samples

DNA was freeze-thawed. Samples were taken out of storage at -80°C and kept at 4°C. After the DNA required was removed, the remainder was placed back in the fridge at -80°C. Before analysis took place the concentration and quality of the freeze-thawed DNA was assessed using a nanodrop (Nanodrop Technologies, Wilmington, USA) and samples included for analysis all had an OD (optical density) ratio $^{A260}/_{A280}$ >1.8.

2.8 Telomere length measurement

This particular method of telomere length measurement was adopted from a protocol devised by (O'Callaghan & Fenech, 2011)

2.8.1 Standards and primers

The standards and primers used for the measurement of leukocyte telomere length are recorded in table 2.2.

Table 2.2: Standards and primers used for q-PCR (Sigma-Aldrich/ Merck)

	Oligomer Name	Species	Oligomer sequence (5' – 3')	Length
Standards	Telomere standard	Human/ rodent	(TTAGGG)14	84 bp
	36B4 standard	Human	CAGCAAGTGGGAAGGTGTAATCCGTCTCCACAGACAAGGCCAGGACTCG TTTGTACCCGTTGATGATAGAATGGG	75 bp
PCR Primers	TeloF	Human/ rodent	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGG TTTGGGTT	>76 bp
	TeloR	Human/ rodent	GGCTTGCCTTACCCTTACCCTTACCC TTACCCTTACCCT	
	36B4F	Human	CAGCAAGTGGGAAGGTGTAATCC	75 bp
	36B4R	Human	CCCATTCTATCATCAACGGGTACAA	

2.8.2 Oligomer standard working solutions

It was necessary that all oligomers were purified using HPLC (high performance liquid chromatography). Long oligomers (>50 mers) have a high failure rate during synthesis and therefore these were removed to maintain the accuracy of the oligomer standards. It was necessary that the oligomers were diluted in the appropriate volume of Tris-EDTA to a concentration of 100µM. The standards were then vortexed, centrifuged at 15000rpm for 1 minute and thereafter stored at -20ºC until required.

2.8.3 Primer working solutions

Working solutions of primers also had to be made up by dilutions of the original primer concentrations with Tris-EDTA buffer solution. For both the SCG primers and the Telomere primers, the appropriate volume of buffer was added to give a final working concentration of 100µM. The primers were then vortexed, centrifuged at 15000rpm for 1 minute and thereafter stored at -20ºC until required.

2.8.4 The standard curve for human SCG, 36B4

Dilutions of known amounts were made using PCR-grade water, from the working solution of the synthesized 84 mer oligonucleotide containing TTAGGG repeats and these were used to create a standard curve. The standard curve was used to calculate the number of repeats in each standard. A single copy gene (SCG) was used as a control for amplification with all samples. This control was used to determine the number of genome copies per sample. The choice of SCG was essential for the consistency of results as any change in copy number could have significantly affected TL measurements. The 36B4 SCG was used, as this is the most commonly used SCG and it encodes the acidic ribosomal phosphoprotein (P0).

The Genome copy number per reaction was calculated as follows:

The synthesised 36B4 oligomer standard was 75bp in length with a molecular weight (MW) of 23268.1. The weight of one molecule was MW/ Avogadro's number and therefore the weight of the synthesised 36B4 oligomer standard was: $2.32681 \times 10^{4} / 6.02 \times 10^{23} = 0.38 \times 10^{-19}$g. The highest concentration standard, 36B4 single copy gene standard (36B4 SCG STD), had 200pg of 36B4 oligomer (200×10^{-12}g) per reaction and hence contained: $200 \times 10^{-12} / 0.38 \times 10^{-19} = 5.26 \times 10^{9}$ copies of 36B4 amplicon. The 36B4 SCG STD was equivalent to 2.63×10^{9} diploid genome copies, as there are two copies of 36B4 per diploid genome. A standard curve was produced by performing serial dilutions of the 36B4 SCG STD (10^{-1} to 10^{-6} dilution) (see Fig 2.1). Plasmid DNA (pBR322) was added to each standard to maintain a constant 20ng of total DNA per reaction tube. The standard curve was used to measure the diploid genome copies per sample (see Figure 2.2B).

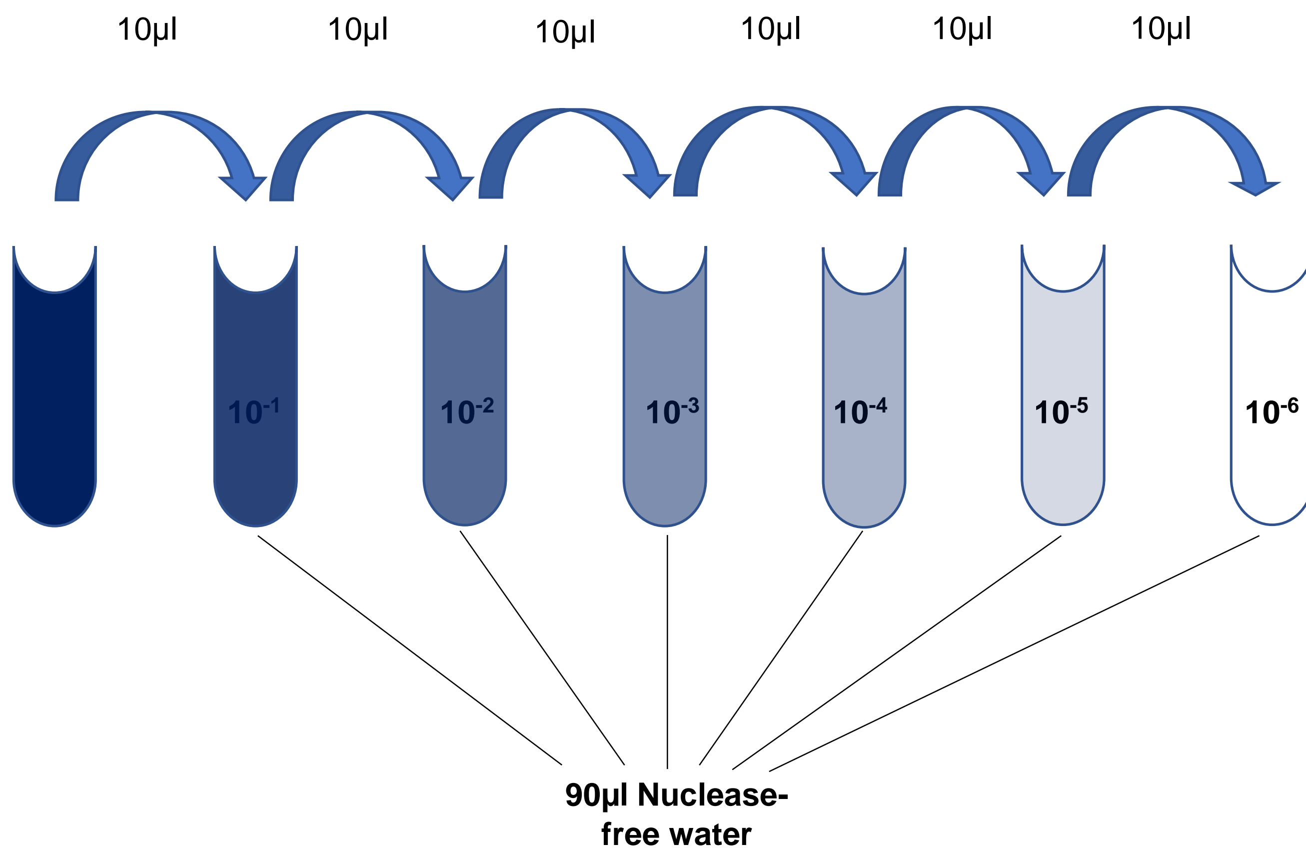

Figure 2.1: Serial dilutions for the SCG standard and the Telomere standard from the original prepared working solutions.

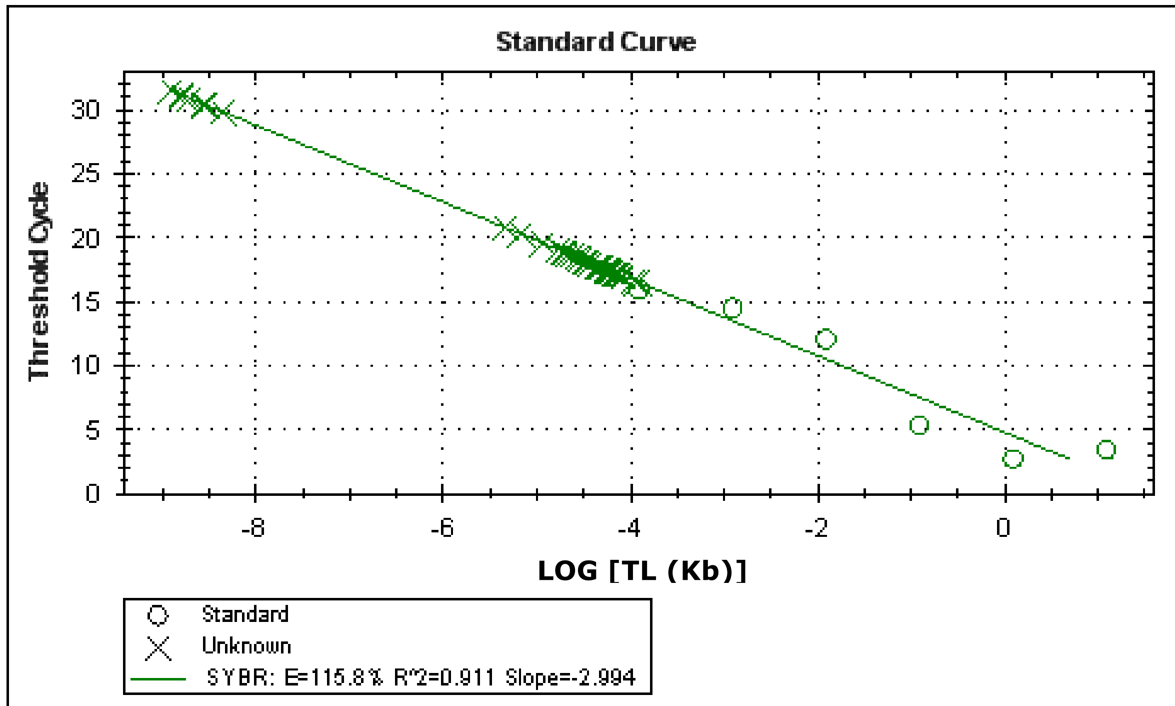

Figure 2.2A: Standard curve used to calculate the absolute telomere length. The C_t (cycle threshold) is the number of PCR cycles for which enough SYBR green fluorescence was detected above the background. The chart shows the standard curve for calculating the length of telomere sequence per reaction tube. The X-axis represents the amount of telomere sequence in Kb per reaction. The graph shown represents the linear range of the PCR and the value generated from the experimental samples was equal to Kb of telomere sequence per sample.

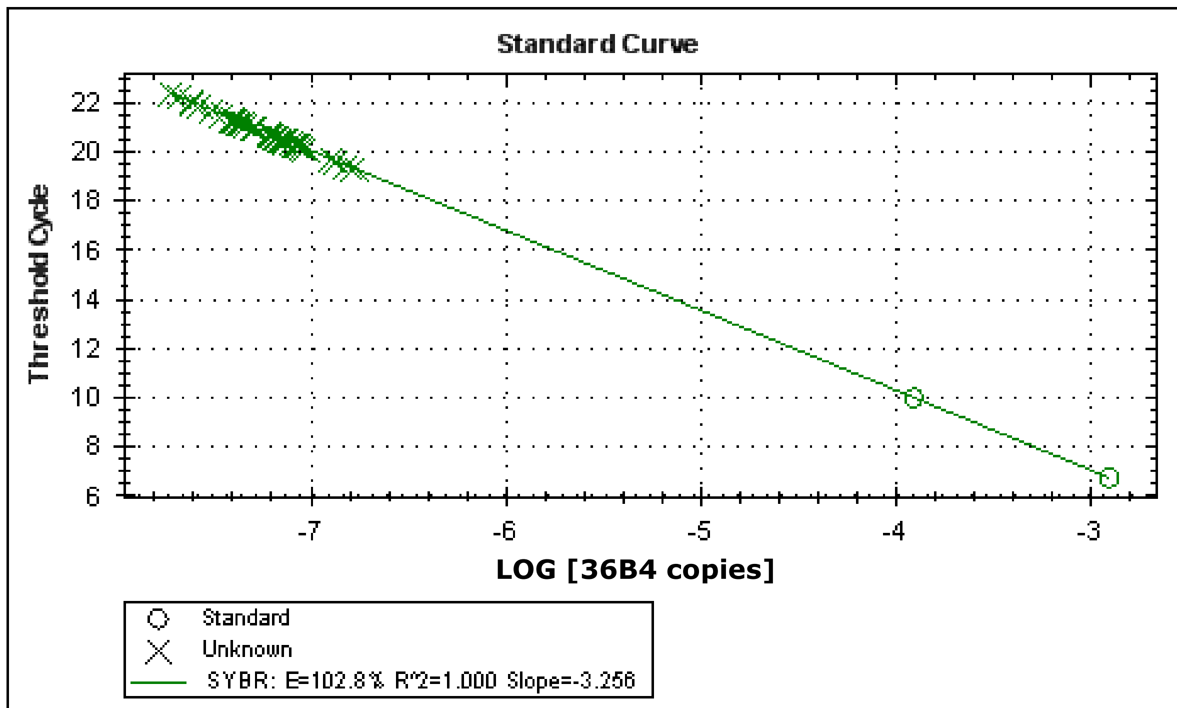

Figure 2.2B: The graph shows the standard curve for calculating the genome copies using the 36B4 STD copy number. Standard curves were generated using the Bio-Rad MiniOpticon Detector Detection System with the CFX Manager ™ Software Ver. 1.6 (Bio-Rad Laboratories, Inc, Hercules City, CA).

2.8.5 q-PCR for Absolute Telomere Length (aTL) using Telomere Standard

The SYBR Green KiCqStart ReadyMix (Sigma-Aldrich/ Merck) was prepared as per the manufacturer's specifications and after preparation, the master mix was mixed. Thereafter 16µl of master mix was added into each of the 48 wells. In the wells reserved for the standards, 5.2µl of diluted standard (10^{-1} through to 10^{-6}) were added together with 2.8µl of plasmid DNA (pBR322). 4µl of the COLO 699 N cell-line positive control and 4µl of nuclease-free water was aliquoted into a separate well as the negative template control (NTC). Lastly, 4µl of the unknown DNA samples were aliquoted into the remaining reaction wells and the plate was sealed with cap-strips. To ensure all PCR components mixed at the bottom of each well, the plate was gently tapped a few times on the work-bench-top (See Table 2.3 below for the 48-well plate layout).

Table 2.3: 48-well plate layout for aTL-qPCR using the Telomere STD

	1	**2**	**3**	**4**	**5**	**6**
A	STD 1	STD 1	STD 2	STD 2	STD 3	STD 3
B	STD 4	STD 4	STD 5	STD 5	STD 6	STD 6
C	Positive control	Positive control	A1	A1	A2	A2
D	A3	A3	A4	A4	A5	A5
E	A6	A6	A7	A7	A8	A8
F	B1	B1	B2	B2	B3	B3
G	B4	B4	B5	B5	B6	B6
H	B7	B7	B8	B8	NTC	NTC

Positive control = COLO 699 N cell-line, STD 1 to STD 6 = Telomere STD dilutions (10^{-1} – 10^{-6}), NTC = No template control, A1 to A20 = Baseline samples; B1 to B20 = corresponding three-year follow-up samples.

2.8.5.1 PCR program (Timing: 2 hours)

The cycling conditions for both the telomere and the 36B4 amplicons were: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, followed by a dissociation curve.

2.8.5.2 Quality Control of results (Timing 0.5 hours)

After processing, the positive and no signal controls in the NTC were checked for amplification together with the standards and samples. The standard curve was constructed using results from the Telomere STD dilutions. Using these concentrations, the linear range of the reaction was observed and all target samples fell within the linear range; those that amplified outside this linear range were removed from further analysis. After amplification was completed the CFX Manager ™ Software produced a LOG starting value for each reaction that was equivalent to Kb/reaction based on the telomere standard curve values.

2.8.6 q-PCR for 36B4 SCG

The above procedure was repeated, using the 36B4 SCG STD, and a master mix with the SCG primers in the reaction, with the value generated from the SCG being the number of genome copies per reaction. Processing and analyzing of the data was done by exporting the values (Kb/reaction for telomere and genome copies/reaction for SCG) to csv format. The Kb/reaction value obtained was then used to determine the total telomere length in Kb per human diploid genome. This was

done by dividing the telomere Kb/ per reaction value by the diploid genome copy number to give a total telomeric length in Kb per human diploid genome.

2.9 Statistical analysis

Data was captured in Microsoft Excel (Microsoft Office Professional 2010) and Statistica (Dell™ Statistica™ 13.2, 2013) software was used for all statistical analysis. Medians and/ or means were used as measures of central location for ordinal and continuous variables. Relationships between two continuous variables were analysed with regression analysis and the strength of the relationship measured with the Pearson correlation. In instances where the continuous variables were not normally distributed, Spearman correlation was used. When one continuous response variable was related to several other continuous input variables, multiple regression analysis was used and the strength of the relationship was measured with multiple correlations. For randomized non-parametric designs, the Kruskal-Wallis test was used and data was presented as median and interquartile ranges. A p-value < 0.05 represented statistical significance in hypothesis testing.

Chapter 3: Results

3.1 Data available

A total of 946 participants took part in the baseline evaluation, of which 580 did not consent for follow-up evaluations. Of those that remained, 15 moved from the study sites or died before follow-up evaluation. Therefore 351 participants underwent both baseline and follow-up evaluation. Of these, 146 either did not consent or had insufficient DNA material for this analysis. The final analytic sample therefore comprised 205 participants. Table 3.1 illustrates the baseline profile of participants in the final analytic sample and those who were potentially eligible participants, but were excluded. Generally, the excluded participants were similar in the male to female ratio, but the excluded group was significantly older. The glucose tolerance status profile, anthropometric measurements as well as the lipid profile were similar in both groups (Table 3.1).

Table 3.1: Baseline profile of the participants excluded and those in the final analytic sample

Characteristics	Participants included	Participants excluded	
	Median (25Q;75Q)		p-value
Number	205	146	
Men, %	22.0%	22.0%	0.37
Age	57.0 (48.0; 62.0)	60.0 (48.0; 69.0)	**0.04**
Current smoking, %	39.7%	32.9%	0.20
Glucose tolerance status, %			0.60
Normal	37.5%	47.0%	
Impaired fasting glycaemia (IFG)	2.5%	1.5%	
Impaired glucose tolerance (IGT)	15.0%	10.6%	
IFG/IGT	4.0%	3.0%	
Body Mass Index (BMI) kg/m^2	31.8 (27.3; 36.5)	31.1 (26.9; 34.5)	0.33
Waist circumference (WC) cm	101.5 (93.0; 111.0)	101.0 (89.3; 109.9)	0.26
Hip (HP) cm	112.0 (104.0; 122.7)	110.5 (102.0; 122.0)	0.43
Waist/ hip ration (WHR)	0.89 (0.85; 0.95)	0.89 (0.83; 0.95)	0.33
Systolic blood pressure (SBP) mmHg	123.0 (112.0; 135.0)	120.0 (113.0; 135.0)	0.62
Diastolic blood pressure (DBP) mmHg	76.0 (69.0; 83.0)	73.0 (67.0; 80.0)	**0.03**
Fasting blood glucose (FBG) mmol/l	6.00 (5.00; 7.70)	5.90 (5.00; 7.05)	0.62
Post blood glucose (Post BG) mmol/l	7.40 (6.00; 10.15)	6.90 (6.00; 8.30)	0.08
Insulin, IU/ml	9.30 (3.80; 14.6)	7.50 (2.35; 11.7)	**0.04**
Glucose/insulin	0.70 (0.43; 1.62)	0.85 (0.50; 2.50)	**0.02**
HbA1c, %	6.10 (5.70; 6.80)	6.00 (5.60; 6.40)	0.07
Total cholesterol (TC) mmol/l	5.56 (4.88; 6.33)	5.73 (5.03; 6.49)	0.21
Triglycerides (TG) mmol/l	1.37 (1.06; 1.92)	1.43 (1.06; 1.96)	0.56
High-density lipoprotein (HDL) mmol/l	1.14 (0.99; 1.39)	1.18 (0.99; 1.39)	0.81
Low-density lipoprotein (LDL) mmol/l	3.65 (2.93; 4.35)	3.84 (3.13; 4.56)	0.17

3.2 Baseline and three-year follow-up general characteristics of participants by gender

The general characteristics of participants according to gender at both baseline and follow up are summarized in Table 3.2. The median (25th, 75th percentile) age of the 205 participants (78% women) at baseline and three-year follow up was 57.0 (48.0; 62.0) and 59.0 (51.0; 66.0), respectively. The anthropometric measurements, body mass index (BMI), hip circumference (HP) and waist to hip circumference ratio (WHR) were significantly higher in females compared to their male counterparts at baseline and three-year follow-up, all $p \leq 0.0001$. Similarly, fasting insulin at baseline was significantly higher in females, $p=0.0290$, with a corresponding high prevalence of diabetes in females (Figure 3.1). Although high-density lipoprotein (HDL) was higher in females, this did not reach significance at three-year follow up, $p=0.0640$ as it did at baseline, $p=0.0293$. On the other hand, in males, gamma glutamyl-transferase (GGT) and serum creatinine (S creatinine) were significantly raised, respectively, $p \leq 0.0158$ and $p \leq 0.0001$ at both baseline and three-year follow-up, whilst triglycerides (TG) were only significantly raised at baseline, $p=0.0377$.

Table 3.2: General characteristics of participants by gender

	Total (N205)	Male (N45)	Female (N160)	p-value	Total (N205)	Male (N45)	Female (N160)	p-value
		Baseline				Three-Year Follow-up		
Age (Years)	57.0 (48.0; 62.0)	58.0 (53.0; 64.0)	56.0 (46.5; 62.0)	0.1606	59.0 (51.0; 66.0)	61.0 (53.0; 67.0)	59.0 (50.0; 65.5)	0.2891
BMI	31.7 (27.4; 36.5)	27.5 (23.6; 32.3)	32.8 (28.5; 37.8)	**<0.0001**	31.2 (26.9; 36.5)	27.3 (23.0; 29.9)	32.9 (28.5; 38.2)	**<0.0001**
WC (cm)	101.5 (93.0; 111.0)	99.0 (88.0; 112.0)	102.0 (93.5; 110.5)	0.3092	97.4 (88.3; 107.0)	93.5 (88.3; 107.0)	98.0 (88.1; 107.1)	0.4668
HP (cm)	112.0 (104.0; 122.7)	103.0 (98.0; 110.2)	114.3 (106.0; 126.0)	**<0.0001**	107.4 (98.3; 118.2)	100.3 (94.0; 107.3)	109.6 (101.1; 120.5)	**<0.0001**
WHR	0.89 (0.85; 0.95)	0.96 (0.92; 1.03)	0.88 (0.84; 0.93)	**<0.0001**	0.89 (0.83; 0.94)	0.94 (0.90; 1.00)	0.88 (0.82; 0.92)	**<0.0001**
SBP (mmHg)	123 (112; 135)	126 (120; 136)	121 (111; 135)	0.0878	136 (123; 155)	142 (124; 159)	135 (123; 152)	0.4756
DBP (mmHg)	76.0 (68.0; 83.0)	77.0 (71.0; 86.0)	75.0 (67.0; 83.0)	0.2221	82.0 (74.0; 90.0)	80.0 (77.0; 91.5)	82.0 (73.0: 90.0)	0.3354
FBG (mmol/L)	6.00 (5.00; 7.75)	5.85 (5.00; 7.15)	6.00 (5.10; 7.85)	0.4007	5.60 (5.00; 7.40)	5.60 (4.80; 7.40)	5.65 (5.00; 7.35)	0.5995
PostBG (mmo/L)	7.40 (6.00; 10.10)	7.10 (5.70; 10.00)	7.45 (6.05; 10.15)	0.5022	6.50 (5.30; 8.10)	5.75 (4.30; 8.00)	6.65 (5.50; 8.10)	0.1284
Fasting Insulin (mIU/L)	9.2 (3.8; 14.6)	6.9 (2.9; 11.9)	9.7 (5.1; 15.2)	**0.0290**	12.2 (6.9; 17.8)	10.4 (6.2; 15.5)	13.1 (7.2; 18.1)	0.1537
Gluc/Insulin ratio	0.71 (0.43 1.62)	0.84 (0.58; 2.64)	0.66 (0.40; 1.32)	**0.0423**	0.56 (0.33; 0.88)	0.64 (0.38; 0.99)	0.55 (0.32; 0.81)	0.2305
HbA1c (%)	6.10 (5.70; 6.80)	6.00 (5.65; 6.95)	6.10 (5.75: 6.75)	0.7171	6.20 (5.80; 7.00)	6.00 (5.70; 6.80)	6.20 (5.80; 7.00)	0.1722
CRP (mg/L)	5.50 (1.60; 10.40)	4.30 (0.90; 9.40)	5.65 (1.75; 10.65)	0.3093	5.30 (2.00; 9.20)	5.20 (1.30; 7.90)	5.40 (2.35; 9.35)	0.6007
GGT (IUL)	30.0 (22.0; 47.0)	37.0 (27.0; 47.5)	28.0 (21.0; 45.5)	**0.0158**	27.0 (20.0; 45.0)	36.5 (25.5; 48.0)	25.5 (19.0; 43.0)	**0.0034**
TC (mmol/L)	5.56 (4.88; 6.33)	5.50 (4.75; 6.27)	5.57 (4.89; 6.43)	0.3859	5.40 (4.71; 6.21)	5.34 (4.85; 6.19)	5.42 (4.65; 6.25)	0.9875
TG (mmol/L)	1.37 (1.06; 1.92)	1.64 (1.10; 2.21)	1.34 (1.06; 1.83)	**0.0377**	1.36 (0.99; 1.75)	1.52 (0.92; 1.99)	1.36 (1.00; 1.71)	0.3515
HDL (mmol/L)	1.13 (0.99; 1.39)	1.08 (0.94; 1.21)	1.15 (1.00; 1.40)	**0.0293**	1.33 (1.12; 1.61)	1.20 (1.05; 1.49)	1.34 (1.14; 1.62)	0.0640
LDL (mmo/L)	3.65 (2.93; 4.35)	3.37 (2.82; 4.22)	3.68 (3.01; 4.41)	0.1506	3.28 (2.68; 4.10)	3.20 (2.73; 4.10)	3.29 (2.66; 4.10)	0.8802
Chol/HDL ratio	4.78 (3.98; 5.69)	5.11 (4.10; 5.93)	4.73 (3.95; 5.48)	0.2129	3.96 (3.13; 4.98)	4.26 (3.02; 5.33)	3.88 (3.15; 4.90)	0.1753
S Cotinine (ng/mL)	9.0 (9.0; 261.0)	9.0 (9.0; 340.5)	9.0 (9.0; 242.0)	0.2464	9.0 (9.0; 289.0)	9.0 (9.0; 282.0)	9.0 (9.0; 294.0)	0.6336
S Creatinine (umol/L)	83.0 (73.0; 93.5)	96.0 (86.0; 105.5)	79.0 (71.0; 89.0)	**<0.0001**	72.0 (63.0; 85.0)	87.0 (80.0; 97.0)	68.0 (60.0; 78.5)	**0.0001**

3.3 Baseline and three-year follow-up general characteristics of participants by glycaemic status

At baseline, the crude prevalence of pre-diabetes and screen-detected diabetes was higher in females when compared to their male counterparts. For instance, the prevalence of pre-diabetes in males and females were, 20.0 and 21.3% respectively; whilst in screen-detected diabetes it was 17.8 and 22.5%, respectively. However, in subjects with doctor-diagnosed diabetes and on treatment, the prevalence of diabetes was higher in males compared to females, 22.2%, and 19.4% respectively (Figure 3.1). The baseline glucose tolerance status of participants was distributed as followed [n (%)]: Normal 77 (37.6%), pre-diabetes (IFG and/or IGT) 43 (21.0%), screen-detected diabetes 44 (21.4%) and known diabetes 41 (20.0%). Although borderline significant, the participants with screen-detected or known diabetes were older, $p<0.0869$ (Table 3.3). As expected, the indices of glucose homeostasis, fasting blood glucose (FBG), post 2-hour blood glucose (PostBG), and glycated haemoglobin (HbA1c), were significantly higher in subjects with diabetic or non-diabetic hyperglycaemia, $p \leq 0.0001$. Fasting insulin was however, reduced in subjects with diabetes and on treatment but higher in those with pre-diabetes and screen-detected diabetes at baseline, however this did not reach statistical significance, $p=0.5562$.

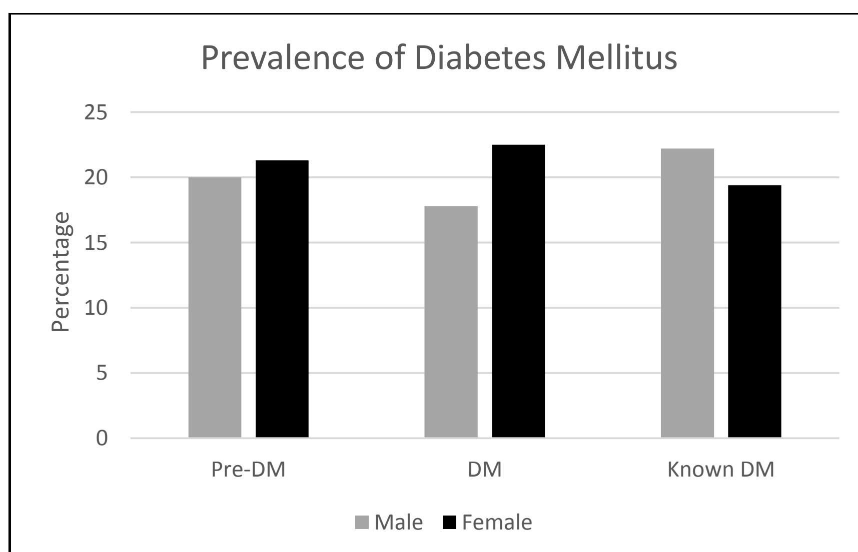

Figure 3.1. Baseline crude relevance of prediabetes and diabetes. Pre-DM, prediabetes (includes impaired fasting glucose, impaired glucose tolerance status or both); DM, screen-detected diabetes or undiagnosed diabetes; Known DM, doctor diagnosed diabetes and on glucose lowering treatment.

At three-year follow-up, fasting insulin was significantly higher in all subjects with pre-diabetes, screen-detected or known diabetes, $p=0.0029$. Both at baseline and three-year follow-up, systolic blood pressure (SBP), BMI, waist and hip circumferences, waist to hip ratio, and triglycerides were

significantly lower in normo-tolerant subjects, p≤0.0371, whilst high density lipoprotein cholesterol was significantly raised, p≤0.0382. At baseline only, high sensitive C-reactive protein (hsCRP) and GGT were significantly higher in subjects with prediabetes or screen-detected diabetes P≤0.0050. The above-mentioned results are summarized below in Table 3.3.

3.3.1 Glucose tolerance status and progression/regression during follow-up

The change in status during the three-year follow-up across these subgroups is described in Table 3.3, which shows that 19 participants (cumulative incidence rate: 9.3%) acquired a status of 'progression' during follow-up, including 10 (cumulative incidence rate: 4.9%) who acquired a diabetes status. On the other hand, 37 participants (18.0%) acquired a status of 'regression' during the same period, including 10 (4.9%) subjects who were previously diagnosed with diabetes at baseline and regressed to pre-diabetes. Therefore, at three-year follow up, prevalence of diabetic or non-diabetic hyperglycaemia was present in 114 (55.6%) compared to 128 (62.4%) at baseline.

3.3.2 Baseline metabolic syndrome and progression/regression during follow-up

At baseline, metabolic syndrome (MetS) was more prevalent in males compared to female participants, respectively, 65.9 and 62,5% (Figure 3.2). The higher prevalence of Mets in males is driven by the significantly higher levels of triglycerides and lower high-density lipoproteins in the male participants (Table 3.3).

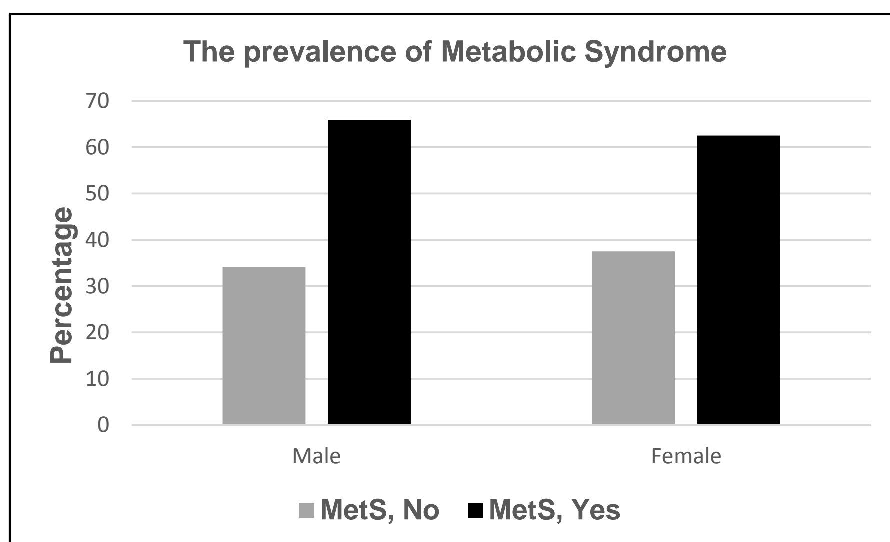

Figure 3.2. The prevalence of metabolic syndrome by gender at baseline**.** MetS, metabolic syndrome.

Table 3.3: Differences in the biochemical and anthropometric measurements according to the glycaemic profile

	Total N205	Normoglycaemic N77	Pre-Diabetic N43	New Diabetic N44	Known Diabetic N41		Total N205	Normoglycaemic N91	Pre-Diabetic N37	New Diabetic N13	Known Diabetic N64	
			Baseline						**Three-Year Follow-up**			
			Median (25Q;75Q)			**p-value**			**Median (25Q;75Q**			**p-value**
Age (Years)	57.0 (48.0; 62.0)	55.0 (46.0; 61.0)	55.0 (45.0; 62.0)	56.0 (47.5; 62.5)	59.0 (54.0; 64.0)	0.0869	59.0 (51.0;66.0)	58.0 (47.0;65.0)	57.0 (48.0;64.0)	60.0 (53.0;65.0)	61.5 (55.0;67.5)	0.0579
BMI	31.7 (27.4;36.5)	29.4 (25.0;33.4)	32.5 (28.7;36.3)	35.0 (30.5;38.1)	31.1 (26.5;37.3)	0.0002	31.3 (27.0;36.6)	29.4 (24.6;35.3)	34.5 (29.2;37.0)	32.9 (30.3;40.3)	31.9 (28.5;36.4)	0.0023
WC (cm)	101.5 (93.0;111.0)	96.0 (87.5;106.0)	105.0 (97.0;112.0)	107.7 (101.0;117.5)	101.0 (93.0;114.5)	0.0001	97.3 (88.3;107.0)	92.5 (81.1;105.0)	101.5 (92.3;108.0)	100.0 (99.3;117.3)	98.3 (91.5;109.0)	0.0047
HP (cm)	112.0 (104.0;122.7)	109.0 (103.0;116.0)	115.0 (108.0;122.7)	116.5 (108.7;127.0)	111.0 (99.8;125.5)	0.0036	108.8 (98.3;119.3)	105.1 (96.5;113.4)	114.7 (106.1;121.6)	110.7 (104.1;116.0)	110.0 (100.0;120.6)	0.0190
WHR	0.89 (0.85;0.95)	0.88 (0.84;0.92)	0.89 (0.83;0.95)	0.90 (0.86;0.98)	0.92 (0.86;0.96)	0.0371	0.88 (0.83;0.94)	0.88 (0.82;0.93)	0.87 (0.82;0.92)	0.94 (0.88;1.01)	0.91 (0.84;0.96)	0.0098
SBP (mmHg)	123.0 (112.0;135.0)	119.0 (111.0;129.0)	121.0 (112.0;132.0)	130.5 (116.0;140.5)	127.0 (114.0;142.0)	0.0259	136.0 (123.0;155.0)	131.0 (120.0;150.0)	142.0 (130.0;157.0)	130.0 (124.0;167.0)	139.0 (126.0;159.0)	0.0264
DBP (mmHg)	76.0 (68.0;83.0)	76.0 (67.0;85.0)	76.0 (68.0;82.0)	77.5 (69.0;86.0)	74.0 (68.0;83.0)	0.7670	82.0 (74.0;90.0)	80.0 (72.0;85.0)	87.0 (77.0;93.0)	84.0 (79.0;97.0)	81.0 (75.0;90.0)	0.0286
FBG (mmol/L)	6.00 (5.00;7.75)	5.10 (4.80;5.60)	6.00 (5.00;6.10)	7.90 (7.10;9.00)	11.05 (6.40;13.05)	**<0.0001**	5.60 (5.00;7.4)	5.00 (4.60;5.4)	5.80 (5.40;6.4)	8.90 (7.50;11.0)	8.55 (6.45;10.9)	**<0.0001**
PostBG (mmo/L)	7.40 (6.00;10.10)	6.00 (5.40;6.50)	8.60 (8.00;9.20)	13.00 (10.60;17.50)	NA	**<0.0001**	6.50 (5.30;8.10)	5.70 (4.90;6.60)	8.40 (7.90;9.00)	15.10 (11.80;19.00)	NA	**<0.0001**
Fasting Insulin (mIU/L)	9.20 (3.80;14.60)	8.30 (3.80;13.70)	10.20 (6.00;14.10)	10.70 (3.60;16.10)	7.80 (2.40; 14.15)	0.5562	12.20 (6.90;17.80)	10.40 (5.60;15.10)	13.20 (6.60;18.80)	17.90 (14.20;27.70)	13.20 (8.75;18.85)	0.0029
Gluc/Insulin ratio	0.71 (0.43;1.62)	0.60 (0.37;1.24)	0.56 (0.40;0.95)	0.74 (0.54;2.22)	1.16 (0.74;3.16)	0.0029	0.56 (0.33;0.88)	0.48 (0.34;0.89)	0.46 (0.30;0.78)	0.50 (0.23;0.75)	0.64 (0.34;0.89)	0.5199
HbA1c (%)	6.10 (5.70;6.80)	5.70 (5.40;6.10)	5.90 (5.80;6.20)	6.75 (6.25;7.70)	7.35 (6.50;8.95)	**<0.0001**	6.20 (5.80;7.00)	5.90 (5.70;6.20)	6.20 (5.95;6.55)	7.40 (6.60;8.80)	7.20 (6.50;8.75)	**<0.0001**
CRP (mg/L)	5.50 (1.60;10.40)	4.00 (0.90;7.70)	7.60 (1.80;14.60)	7.10 (2.35;16.90)	4.10 (1.80;8.45)	0.0050	5.30 (2.00;9.20)	5.30 (1.80;11.80)	6.50 (3.90;10.10)	8.20 (4.40;10.40)	3.90 (1.40;7.00)	0.0874
GGT (IUL)	30.0 (22.0;47.0)	27.0 (18.0;39.0)	31.0 (24.0;47.0)	39.0 (25.0;58.5)	29.5 (20.0;41.5)	0.0056	27.0 (20.0;45.0)	26.0 (19.0;45.0)	27.0 (20.0;46.0)	42.0 (36.0;47.0)	27.0 (20.0;41.5)	0.1010
TC (mmol/L)	5.56 (4.88;6.33)	5.52 (4.92;6.16)	5.53 (4.59;6.30)	5.82 (5.14;6.74)	5.43 (4.74;6.30)	0.3082	5.40 (4.71;6.21)	5.48 (4.76;6.29)	5.46 (5.00;6.08)	5.97 (4.85;6.13)	4.92 (4.53;6.36)	0.4994
TG (mmol/L)	1.37 (1.06;1.92)	1.23 (0.94;1.46)	1.44 (1.10;1.90)	1.58 (1.18;1.96)	1.70 (1.27;2.15)	0.0003	1.36 (0.99;1.75)	1.23 (0.88;1.69)	1.28 (1.01;1.68)	1.53 (1.36;1.94)	1.43 (1.13;1.95)	0.0099
HDL (mmol/L)	1.13 (0.99;1.39)	1.16 (1.00;1.42)	1.15 (1.06;1.39)	1.15 (1.00;1.36)	1.01 (0.86;1.23)	0.0212	1.33 (1.12;1.61)	1.39 (1.20;1.68)	1.21 (1.04;1.46)	1.34 (1.17;1.49)	1.25 (1.09;1.54)	0.0382
LDL (mmo/L)	3.65 (2.92;4.35)	3.63 (3.02;4.16)	3.63 (2.82;4.26)	3.88 (3.16;4.64)	3.38 (2.81;4.47)	0.3836	3.28 (2.68;4.10)	3.28 (2.73;4.03)	3.41 (3.03;4.21)	3.64 (2.75;4.32)	3.00 (2.38;4.06)	0.1795
Chol/HDL ratio	4.78 (3.98;5.70)	4.52 (3.70;5.39)	4.51 (3.79;5.12)	4.89 (4.31;6.07)	5.05 (4.32;6.39)	0.0278	3.96 (3.13;4.98)	3.87 (2.97;4.73)	4.26 (3.27;5.52)	3.96 (3.40;5.19)	4.02 (3.19;5.01)	0.2066
S Cotinine (ng/mL)	9.00 (9.00;261.0)	10.00 (9.00;308.0)	9.00 (9.00;317.0)	9.00 (9.00;184.5)	9.00 (9.00;120.0)	0.0983	9.0 (9.0;289.0)	9.0 (9.0;339.0)	9.0 (9.0;163.0)	212.0 (9.0;334.0)	9.0 (9.0;40.6)	0.0665

3.4 Telomere length

3.4.1 Telomere length by gender, glucose tolerance status and metabolic syndrome: Baseline

We observed no significant difference between the telomere length by either gender, (p=0.3290), glucose tolerance status, (p=0.4576) and metabolic syndrome, (p=0.4901) (Figure 3.3 A-C). For example, the median (25Q; 75Q) telomere length (Kb) for males was 7.90 (7.60; 8.40), and for females 7.80 (7.50; 8.40), p=0.3290.

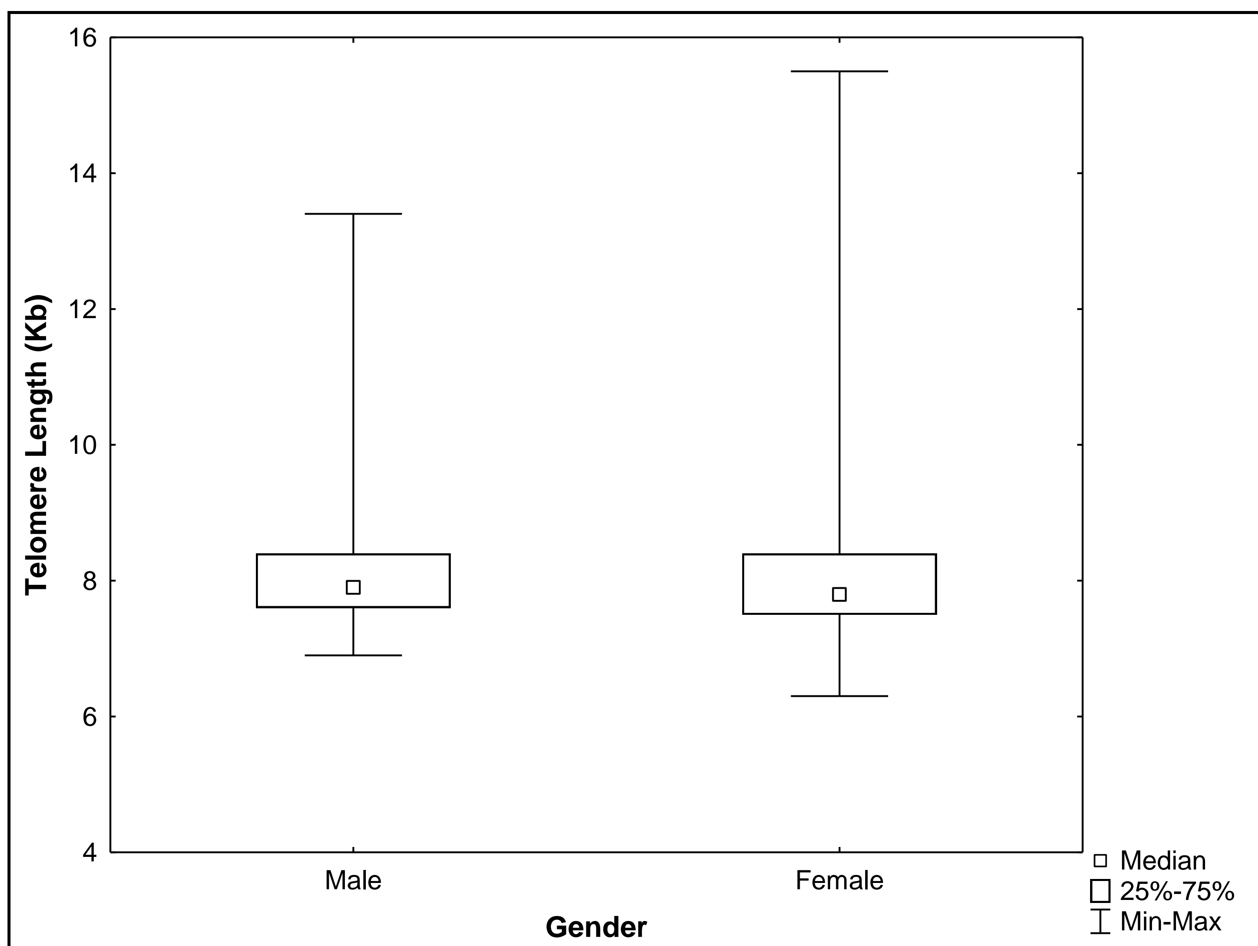

Figure 3.3A. Telomere length by gender. Median (25Q; 75Q) telomere length between male and female subjects, respectively, 7.90 (7.60, 8.40), in females 7.80 (7.50, 8.40); p=0.3290

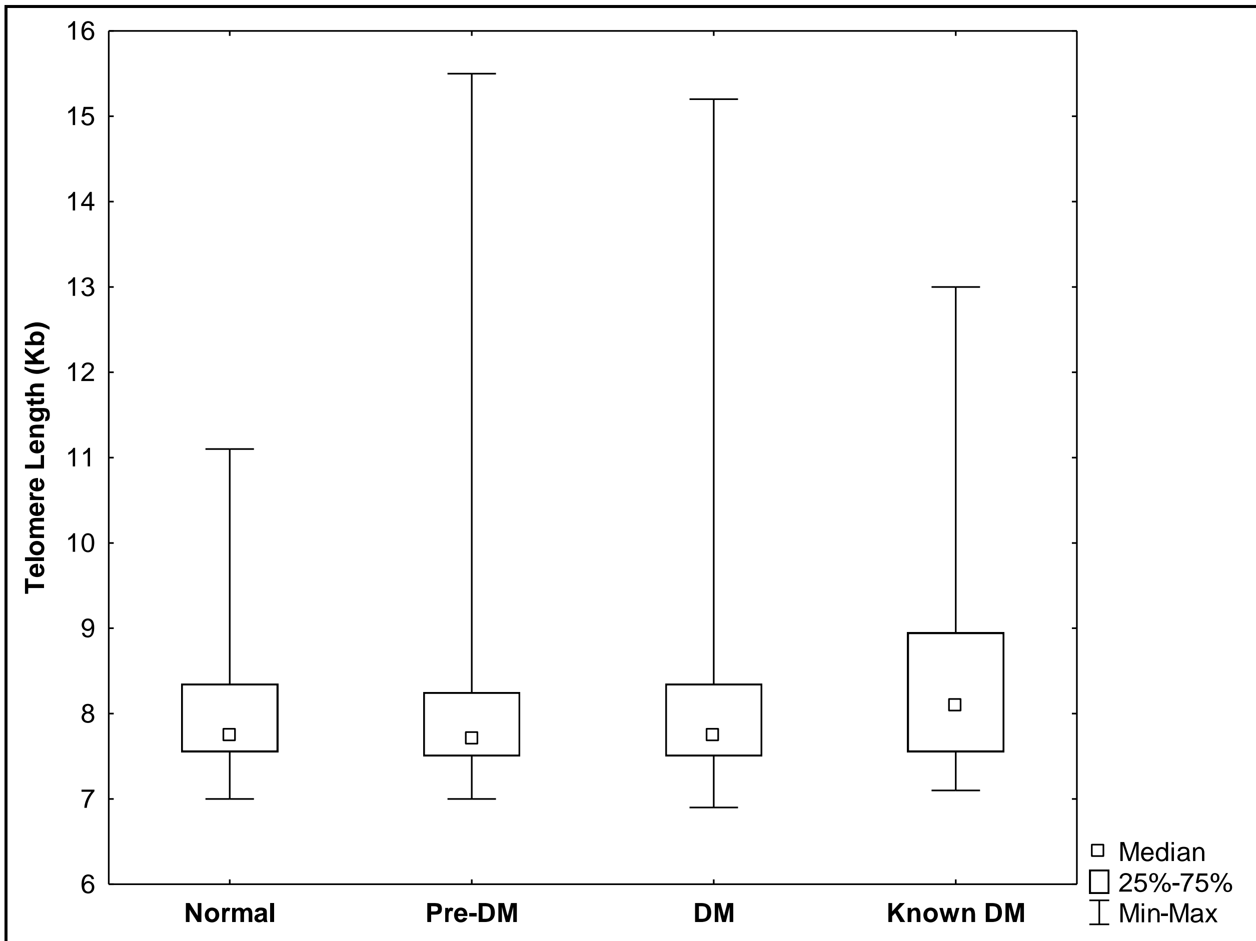

Figure 3.3B: Telomere length by glucose tolerance status. Median (25Q; 75Q) telomere length in normal, pre-DM, DM and Known DM, respectively, 7.75 (7.55, 8.35); 7.70 (7.50, 8.25); 7.75 (7.50, 8.35); 8.10 (7.55, 8.95); p=0.4576

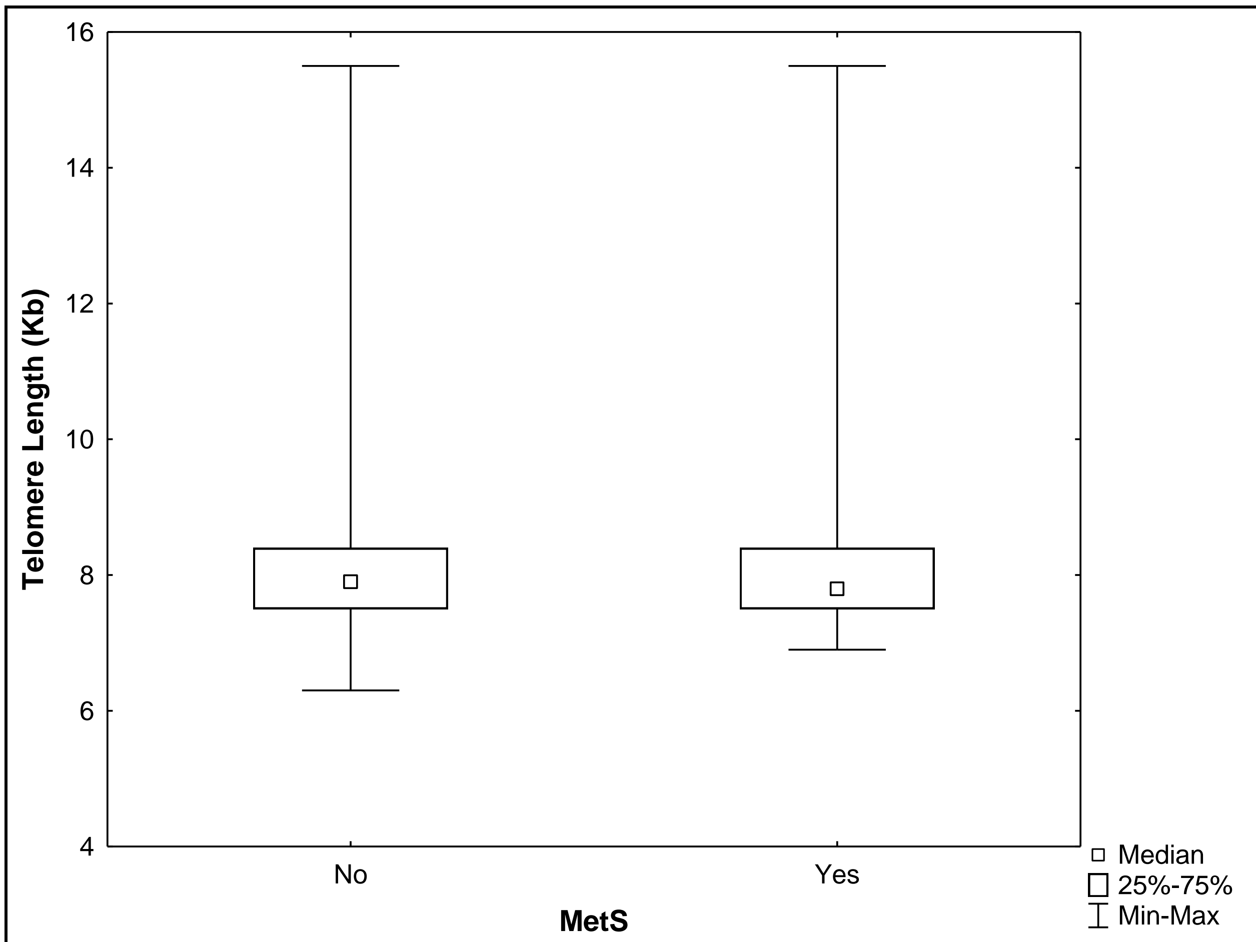

Figure 3.3C. Telomere length in participants with metabolic syndrome. Median (25Q; 75Q) telomere length between subjects with Mets and those without, respectively, 7.90 (7.50, 8.40), with MetS 7.80 (7.50, 8.40); p=0.4901.

3.4.2 The telomere length (kb) as measured against the progression/regression of glucose tolerance status

Although the telomere length was raised at three-year follow-up, it was not significant. Similarly, the telomere length was not significantly different between the two time-frames when tested for progression or regression of glucose tolerance status, $p>0.05$ (Figure 3.4). However, in subjects who had pre-diabetes and remained in the same status after three years, the telomere length was significantly increased at three-year follow-up, $p=0.0159$ (Table 3.4).

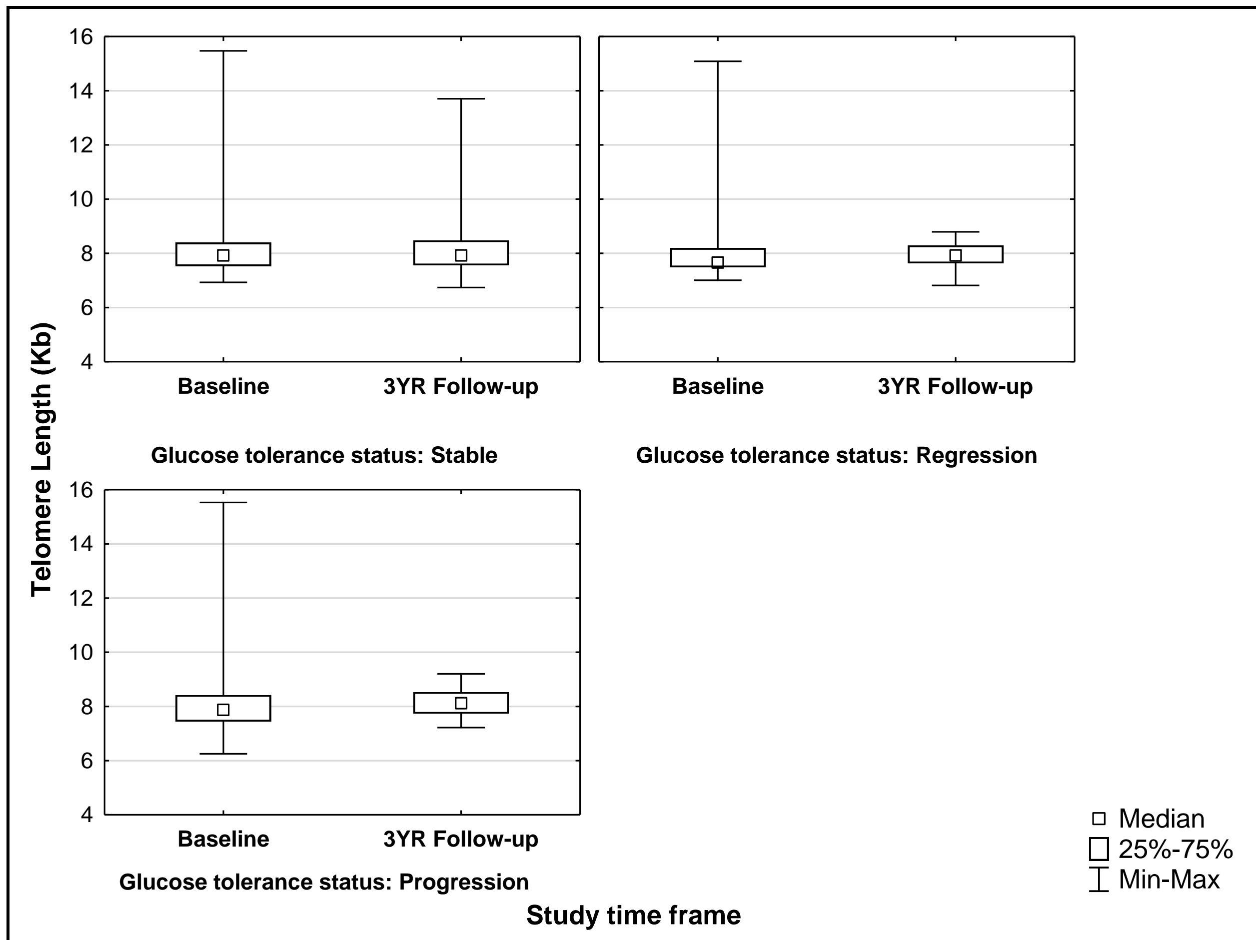

Figure 3.4: Telomere length as measured against the progression/regression of the glucose tolerance status. There was no significant difference in the telomere length in subjects who showed progression, regression or those who remained stable as tested against glucose tolerance status (Table 3.3). Subjects who have progressed: median (25Q; 75Q): 7.88 (7.45; 8.42) and 8.12 (7.74; 8.53), respectively for baseline and three-year follow-up, p=1.0000; Subjects who have regressed: median (25Q; 75Q): 7.66 (7.49; 8.19) and 7.93 (7.64; 8.29), respectively for baseline and three-year follow-up, P =1.000; Subjects who have remained stable: median (25Q; 75Q): 7.93 (7.53; 8.39) and 7.93 (7.57; 8.47), respectively for baseline and three-year follow-up, p=0.6695.

Table 3.4: Telomere length as measured against the progression/regression of the glucose tolerance status

Glucose tolerance status	Telomere length Baseline study	Telomere length Three-Year Follow-up study	
	Median (25,75Q)		p-value
Progression/regression			
Normoglycaemic to Normoglycaemic, N64	7.90 (7.55, 8.35)	7.90 (7.60, 8.50)	0.9568
Normoglycaemic to Pre-diabetic, N10	7.90 (7.50, 8.30)	7.90 (7.60, 8.20)	0.8385
Normoglycaemic to Diabetic, N3	8.40 (7.40, 11.10)	8.40 (8.00, 8.60)	0.5930
Pre-diabetic to Normoglycaemic, N21	7.80 (7.50, 8.40)	8.10 (7.80, 8.50)	0.5328
Pre-diabetic to Pre-diabetic, N16	7.60 (7.45, 7.95)	7.95 (7.50, 8.65)	**0.0159**
Pre-diabetic to Diabetic, N6	8.05 (7.60, 14.80)	8.15 (7.60, 9.00)	0.7532
Diabetic to Normoglycaemic, N6	7.75 (7.20, 8.30)	8.10 (7.60, 8.40)	1.000
Diabetic to Pre-diabetic, N10	7.50 (7.30, 7.70)	7.80 (7.40, 7.90)	0.2411
Diabetic to Diabetic, N69	8.10 (7.50, 8.50)	7.90 (7.60, 8.50)	0.5044
Total to Total, N205	7.80 (7.50, 8.40)	7.90 (7.60, 8.50)	0.3456

3.4.3 The telomere length (kb) as measured against the progression/regression metabolic syndrome status

Over the three-year timeframe, 28 participants progressed from a normal status to a MetS status, while 33 participants regressed from a MetS to a normal status. The rest either retained a normal status (without developing MetS), or retained their MetS status from baseline. No significant differences in the telomere length were observed when tested for progression or regression of metabolic syndrome status, $p>0.05$ (Table 3.5).

Table 3.5: Telomere length as measured against the progression/regression of Metabolic Syndrome status

Metabolic Syndrome status	Telomere length Baseline study	Telomere length Three-Year Follow-up study	
	Median (25,75Q)		p-value
Progression/regression			
Normal. remained Normal, N46	7.90 (7.50; 8.30)	7.95 (7.60; 8.50)	0.7425
Normal, changed to MetS, N28	7.95 (7.55; 8.70)	7.90 (7.45; 8.45)	0.4502
MetS: remained MetS, N95	7.80 (7.50; 8.40)	8.00 (7.60; 8.50)	0.3387
MetS, changed to Normal, N33	7.70 (7.50; 8.40)	7.90 (7.70; 8.80)	0.2772
Total to Total, N202	7.85 (7.50; 8.40)	7.90 (7.60; 8.50)	0.3402

3.4.4 Correlation between telomere lengths and anthropometric and biochemical parameters according to glycaemic status

For the correlations, the subjects were assessed in total, and categorized into those with hyperglycaemia (diabetes and prediabetes), as well as those with diabetes only (screen-detected or known diabetes). We observed a significant negative correlation with age and telomere length at both baseline and three-year follow up which was more pronounced in subjects with diabetes. At baseline this correlation was significantly evident whether participants were grouped as hyperglycaemia r=-0.18, p=0.04 or diabetes only, r=-0.28, p=0.01. In addition, telomere length correlated negatively with GGT in the overall sample at baseline, r=-0.16, p=0.02, positively with HBA1c in hyperglycaemia, r=0.18, p=0.04 and diabetes, r=0.22, p=0.04. At three-year follow-up, the only other significant correlation was between diastolic blood pressure in subjects with hyperglycaemia, r=0.19, p=0.04. However, after multiple linear regression analysis adjusted for age and gender, only the association between telomere length, age and GGT remained (Table 3.6).

Table 3.6 Correlation between telomere lengths and anthropometric and biochemical parameters according to glycaemic status

	Baseline						Three-Year follow-up					
Variable	**Overall**		**Hyperglycaemia**		**Diabetes**		**Overall**		**Hyperglycaemia**		**Diabetes**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Age (Years)	-0.11	0.13	**-0.18**	**0.04**	**-0.28**	**0.01**	**-0.14**	**0.04**	**-0.19**	**0.04**	**-0.24**	**0.03**
BMI	-0.09	0.18	-0.11	0.20	-0.16	0.14	-0.04	0.57	-0.01	0.89	-0.05	0.65
WC (cm)	-0.07	0.32	-0.11	0.24	-0.07	0.50	-0.02	0.74	0.09	0.33	0.04	0.72
HP (cm)	-0.05	0.47	-0.08	0.34	-0.12	0.26	-0.00	0.98	0.03	0.78	0.03	0.81
WHR	-0.03	0.70	-0.01	0.93	0.11	0.30	-0.06	0.41	0.06	0.56	-0.04	0.75
SBP (mmHg)	-0.04	0.59	-0.06	0.50	-0.01	0.93	0.02	0.81	0.13	0.17	0.09	0.42
DBP (mmHg)	-0.01	0.84	-0.02	0.83	0.09	0.44	0.10	0.18	**0.19**	**0.04**	0.10	0.40
FBG (mmol/L)	-0.02	0.80	-0.01	0.88	-0.01	0.94	-0.00	0.95	0.09	0.36	0.08	0.48
PostBG (mmo/L)	-0.01	0.87	0.09	0.44	0.06	0.69	0.07	0.41	0.22	0.14	0.47	0.10
S. Insulin (mIU/L)	0.07	0.34	0.11	0.23	0.12	0.27	-0.04	0.59	0.00	0.96	-0.08	0.50
Gluc/Insulin ratio	-0.06	0.41	-0.11	0.23	-0.14	0.19	0.06	0.41	0.03	0.74	0.13	0.27
HbA1c (%)	0.09	0.22	**0.18**	**0.04**	**0.22**	**0.04**	-0.03	0.70	0.10	0.27	0.05	0.69
CRP (mg/L)	-0.01	0.89	0.06	0.48	0.04	0.73	0.00	0.98	0.04	0.71	0.00	0.99
GGT (IUL)	**-0.16**	**0.02**	-0.11	0.20	-0.11	0.34	0.07	0.32	0.07	0.43	0.13	0.26
TC (mmol/L)	0.09	0.21	0.12	0.18	0.13	0.25	0.06	0.40	0.11	0.25	0.11	0.34
TG (mmol/L)	-0.04	0.54	-0.05	0.56	-0.04	0.72	0.01	0.89	0.01	0.88	-0.06	0.62
HDL (mmol/L)	0.09	0.22	0.06	0.47	-0.01	0.93	0.01	0.85	-0.02	0.82	0.01	0.95
LDL (mmo/L)	0.06	0.43	0.12	0.16	0.14	0.19	0.04	0.56	0.12	0.19	0.12	0.29
Chol/HDL ratio	0.02	0.81	0.07	0.45	0.14	0.20	0.01	0.83	0.11	0.24	0.10	0.40
S Cotinine (ng/mL)	-0.04	0.56	-0.07	0.45	-0.01	0.89	-0.04	0.59	-0.12	0.22	-0.11	0.35
S. Creatinine (umol/L)	0.03	0.71	0.01	0.91	0.09	0.42	-0.00	0.96	-0.11	0.23	-0.14	0.21

Chapter 4: Discussion

The objectives of this study were to assess LTL in patients with hyperglycaemia and to compare this to normoglycaemic individuals. A further aim was to investigate the association of LTL with the progression of the disease by comparing the LTL across all glucose tolerance groups at baseline and at three years follow up. The principal findings of this longitudinal study are (i) LTL showed no significant differences in gender, glucose tolerance status or metabolic syndrome; (ii) Although high rates of deterioration of metabolic syndrome and glucose status over time were found in this population, there was no significant difference in the telomere length after three years including subjects who showed progression or regression. However, in subjects with prediabetes at baseline who remained in the same glucose tolerance status, LTL was significantly increased after three years. (iii) A significant negative correlation was observed between LTL and GGT as well as with age at both baseline and three-year follow, and this was more pronounced in subjects with diabetes. This association between age and GGT remained after multiple regression analysis adjusted for age and gender.

Telomeres, the tandem repeats of TTAGGG DNA sequence situated at the ends of linear chromosomes in eukaryotic cells shortens after each cell division (Riethman, 2008). As a result, the telomere length is an indicator of biological aging (Aubert & Lansdorp, 2008) as well as age-related disorders such as type 2 diabetes (Z. Zhao et al., 2014; Willeit et al., 2014; Wang & Yeh, 2016; Sethi et al., 2016). In our study we included participants with normal glucose tolerance, prediabetes (IFG, IGT or both), undiagnosed diabetes and those with diabetes and on treatment and telomere length was not significantly different in all these groups of glucose tolerance status. Although a number of studies have shown an association between telomere length and diabetes (Adaikalakoteswari et al., 2005; Sampson et al., 2006; Olivieri et al., 2009; Salpea et al., 2010; Shen et al., 2012; You et al., 2012; Ma et al., 2013) in some, this association was either weak (You, 2012) or cell type dependent (Sampson, 2006). Sampson *et al.*, for example measured telomere length in peripheral venous monocyte and T-cells of 21 type 2 diabetes and 29 controls by fluorescent in situ hybridization and reported significantly lower mean monocyte telomere length in the diabetic group, but not in the lymphocytes (Sampson et al., 2006). On the other hand, similar to our findings, some studies have failed to show an association between telomere length and diabetes. In a study involving 501 patients with Type 2 diabetes, of whom 284 had at least one complication and 217 were without complications and 400 control subjects, leucocyte telomere length was not significantly different between type 2 diabetes without complication and control subjects (Testa et al., 2011). Similarly, a recent study that investigated leucocyte telomere length in patients with type 2 diabetes with a median time since

diagnosis of 1 year and without complications reported no difference in peripheral leucocyte relative TL between these patients and age-matched nondiabetic control subjects with normal glucose tolerance (Rosa et al., 2017).

It is well recognized that telomere length shortens with age and cross-sectional studies have verified that this shortening occurs at a constant rate (Muzumdar & Atzmon, 2012). We however, did not find a significant difference in the telomere length after three years in subjects who progressed to a worse glucose tolerance status. It could be that a three-year timeframe is not a sufficient period of time to assess change in telomere length. In a study conducted in 2014, Zhao *et al.*, 2014 examined whether LTL at baseline predicted incident diabetes independent of acknowledged diabetes risk factors in American Indians. Of the total 2328 participants that were free of diabetes at baseline, 292 individuals developed diabetes during an average five-and-a-half-year follow-up period. It was determined that shorter LTL significantly and independently predicted increased risk of development of diabetes (Zhao et al., 2014). In a similar study of 606 participants, 44 who were initially free of diabetes developed T2DM over a 15-year follow-up period (Willeit et al., 2014). The study also concluded the existence of a significant association between shorter leucocyte telomeres and risk of T2DM development.

In contrast to these positive findings, an earlier study evaluated 1675 incident diabetic case participants over a six-year follow-up timeframe and compared them to 2382 control participants matched by age. Findings from the study portrayed a weak association between LTL and diabetes risk and no statistically significant association was found (You et al., 2012). While there is clear evidence for the effect of the diabetic state and its metabolic effects on telomere integrity such as low-grade inflammation with associated increased oxidative stress, the conflicting conclusions of these studies are difficult to reconcile and it is therefore imperative that further large-scale prospective studies are necessary (Kirchner et al., 2017).

There is substantial evidence illustrating the adverse effects of hyperglycaemia on biochemical pathways. The resultant processes, such as glucose oxidation, the formation of advanced glycation end products (AGE), and activation of polyol pathways, are associated with the production of reactive oxygen species (ROS), eventually leading to increased oxidative stress in body tissues (Brownlee, 2001; Ceriello, 2003; Son, 2012). In a Canadian study conducted to examine the oxidative stress-related parameters in T2DM, blood serum samples were collected from diabetic patients and nondiabetic control individuals. The study showed elevated levels of oxidative stress markers such as glucose-6-phosphate dehydrogenase (G6PDH), malondialdehyde (MDA), glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx) and superoxide dismutase (SOD). The

conclusions confirmed previous postulations that there is an increased oxidative stress state in T2DM patients compared with healthy individuals (Aouacheri et al., 2015).

Links have also been described between oxidative stress and subsequent promotion of increased telomere attrition, with a negative correlation between levels of oxidative stress and LTL (Salpea & Humphries, 2010; Liu et al., 2014; Tian et al., 2017). Ma *et al*., carried out an investigation to determine whether telomere shortening occurs in Type 1 and Type 2 diabetes in 62 T2DM individuals and 34 T1DM subjects, against a non-diabetic control group of 40 persons. The antioxidant status of the participants was estimated by human 8-hydroxy-desoxyguanosine (8-OHdG) quantization, and results showed that the LTL in Type 1 and Type 2 diabetics were significantly shorter than in the control group. It was therefore observed from the study that there was an association between LTL shortening and increased oxidative stress in both T1DM and T2DM (Ma et al., 2013). These findings shed light on a limitation of our study in which oxidative stress markers were not included. Literature has described that in states of inflammation, accelerated telomere erosion may occur due to the release of ROS, which may cause damage to telomeric DNA by oxidative stress (Choi et al., 2009; Wong et al., 2014; Mundstock et al., 2015). For a more comprehensive understanding of the intra-cellular mechanisms behind the oxidative burden and associated DNA oxidation in chronic inflammatory diseases, it is therefore crucial to evaluate markers of inflammation as well as oxidative stress markers such as 8-OHdG.

It is known that telomeres are progressively lost with each cell division, however minimal extension does occur. This extension is attributed to the activity of telomerase, the reverse transcriptase enzyme that lengthens telomeres (Zhao et al., 2014). Telomerase activity is suppressed in normal human cells however can become reactivated in cancer cells and is a vital pre-requisite for the immortalization of these abnormal cells (Gu, 2015). This reactivation has been reported to be associated with the TERT gene which encodes the reverse transcriptase subunit of telomerase. TERT expression has been reported to be reactivated in approximately 85% of cancers, although the molecular events leading to this are still yet to be fully comprehended (Akincilar et al., 2016). In our study we observed that in subjects with pre-diabetes at baseline, who remained pre-diabetic at three-year follow-up, LTL was significantly increased after the three years. It has been described that a positive relationship exists between exercise and physical fitness and telomere length maintenance and subsequent lengthening (Denham et al., 2016). In 2012, a study was carried out to investigate the influence of the lifestyle intervention on LTL over an average four-and-a-half-year follow-up period. The study showed a significant increase in LTL in about two thirds of the individuals both in the intervention and in the control groups during follow-up. This confirmed that LTL can increase with time even in obese people with impaired glucose metabolism (Hovatta et al., 2012). It

is therefore evident that exercise training may prevent and manage symptoms of cardio-metabolic diseases whilst simultaneously maintaining telomere length. This may provide a possible explanation for the results we obtained, with individuals adopting these intervention measures, subsequently retaining their glucose tolerance status and increasing their LTL (Denham et al., 2016). Taken together, we believe that the increase in LTL in prediabetes could indirectly indicate that the disease awareness in our population had increased and thus it is likely that some participants may have engaged in preventive strategies within the three-year period. This is said, because one of the benefits for participating in the study was to obtain information on glucose status and referral to a health care center or doctor for management.

Gamma-glutamyl transferase (GGT) is a very sensitive but nonspecific index of liver dysfunction and a biological clue of excessive alcohol intake (Pavanello et al., 2012). In our study, we found a significant negative correlation between LTL and GGT as well as with age at both baseline and three-year follow, which was more pronounced in subjects with diabetes. This association remained after multiple regression analysis adjusted for age and gender. A link has been previously described between LTL and alcohol consumption. In 2012, a study confirmed that excessive alcohol abuse was associated with shortened telomeres, suggesting premature aging at a cellular level. The study, conducted by Pavanello *et al.*, found shorter leucocyte telomere lengths particularly amongst individuals who consumed heavy amounts of alcohol (that is, more than 4 drinks per day). GGT, has also been postulated to be strongly linked to the development of T2DM. In a two-year prospective, randomized, controlled primary prevention study of diabetes, among 537 IGT men aged 35-55 years, 123 incident diabetes cases occurred. The risk of DM was found to be significantly increased with increasing baseline GGT after adjusting for confounders such as BMI and alcohol drinking (Nanditha et al., 2014). In contrast to this, a separate study, conducted by Lee et al., portrayed weak genetic evidence that GGT levels may have a causal role in the development of type 2 diabetes (Lee et al., 2016). It can therefore be seen that the underlying causal relationship between GGT and T2DM onset remains unclear.

Metabolic Syndrome (MetS) is a group of interconnected factors, namely: abdominal obesity, dyslipidemia (low HDL-cholesterol levels and high triglyceride levels), hypertension, as well as hyperglycaemia. MetS is known to be one of the major risk factors for the development and onset of age-related diseases including cardiovascular diseases and DM (NCEP, 2002). Several studies have shown significant associations between shorter TL and dysregulated MetS components (Révész et al., 2014; Monickaraj et al., 2012). In one study which took place in the Netherlands, the authors hypothesized that shorter baseline LTL is associated with a worse metabolic profile over a six-year follow-up timeframe. 2848 participants aged between 18 and 65 years of age were assessed

and LTL was found to be associated with a higher metabolic risk profile. In our study, despite the high MetS progression in participants, we did not find an association between LTL and MetS. In accordance with our findings, other studies too have not been able to determine a solid link between the LTL and components of MetS (Fitzpatrick et al., 2007; Bekaert et al., 2007). These contrasting results bring to light the need for future studies to determine and help researchers understand the complex relationship between the telomere maintenance mechanisms and the components of MetS.

Chapter 5: Conclusion and limitations

Patients with T2DM develop numerous complications which include increased infections, cardiovascular disorders and thrombosis. The increased inflammation associated with the disease could result in increased cell division and therefore the steady shortening of telomeric length. This phenomenon could explain the evolution of the disease and the development of complications such as immune exhaustion. Shortened telomeres have been associated with chronic inflammation, increased cell turnover and immune cell senescence, and studies revolving around telomere length in relation to age-related inflammatory disorders are fast becoming a major area of growth in the research world. Findings linking telomere attrition and many health conditions have highlighted the need for methods to accurately and consistently measure telomere length.

5.1 Limitations of this study

Our results showed no association between LTL with the progression of the T2DM over the three-year timeframe with no significant correlations between LTL and the various markers of glucose metabolism. A major limitation encountered in this investigation was the short follow-up period. Other similar studies have shown positive associations between LTL and glucose intolerance with a much longer follow-up period and this factor could have influenced the overall results of our study. Another limiting factor to be considered is the small sample size. When carrying out investigations of the same design, small sample sizes may tend to dilute important inter-group relationships, and may not allow for an accurate representation of the changes in LTL among the study population. A further limitation of this study was that most of the participants were obese which could explain why no significant differences could be found between the different study populations. A full blood count, white cell differential, markers of oxidative stress as well as measurements of inflammatory cytokines were not performed on the study population. These measurements are important in the evaluation of inflammation and immune activation and therefore should be included in future studies. Also, the use of whole blood as the source of DNA rather than the layer of white blood cells (buffy coat), could be another explanation for the non-significant findings of this study.

5.2 Future perspectives

Although this longitudinal study could not definitively associate LTL shortening with glycaemic status over a three-year follow-up period, light has been shed on factors that may be easily overseen by investigators when trying to determine links between LTL and any disease. Possible adjustments for

future assessments of LTL in relation to progression of T2DM, would be to conduct larger, prospective longitudinal studies with a longer follow-up timeframe. When selecting the study population, consideration should be taken to ensure that there is a large enough sample size to analyze a broader age-range; and to allow for a more representative number across all glucose tolerance statuses.

Future work should include additional markers of inflammation and oxidative stress such 8-OHdG. In this current study the leucocyte telomere length of blood cells was analysed however previous research has shown that telomere length can differ between cell populations (Sampson et al., 2006). It may there be important to further analyse telomere length in various cell types including the pancreatic β-cells.

5.3 Conclusion

This study could not detect any significant difference in LTL over a three-year period, however, the early detection or identification of additional risk factors for DM would assist in the risk assessment as well as identification of potential novel treatment strategies. The importance of telomere length as a marker of aging has been well established, but its function as a predictor of disease progression is poorly understood. Further studies should be carried out to provide additional knowledge on how accelerated shortening of telomere length could contribute to the progression of T2DM and the development of complications. This may lead to the development of therapies which could prevent chronic inflammation and telomere shortening leading to a slowing or prevention of many of the complications seen in patients with T2DM.

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Appendix A

Participant Information and Consent Form

TITLE OF RESEARCH PROJECT: PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA

REFERENCE NUMBER:

PRINCIPAL INVESTIGATORS:	Professor Tandi Matsha (Cape Peninsula University of Technology) Professor Rajiv Erasmus (Stellenbosch University) Professor Andre Kengne (SA Medical Research Council)
Project manager:	Dr Gloudina Maria Hon (Cape Peninsula University of Technology)
ADDRESS:	Obesity and chronic diseases of lifestyle Department of Biomedical Sciences Faculty of Health & Wellness Sciences Cape Peninsula University of Technology, Bellville
CONTACT NUMBER:	Prof T Matsha 021 959 6366 or email: matshat@cput.ac.za
Ethics approval:	Cape Peninsula University of Technology Ethics Reference number: CPUT/SW-REC 2015/H01 University of Stellenbosch Ethics Reference number: N14/01/003

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

This research study has been approved by the ethics **Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology** and it will be conducted according to international and locally accepted ethical guidelines for research, namely the Declaration of Helsinki, and the SA Department of Health's 2004 Guidelines: *Ethics in Health Research: Principles, Structures and Processes.*

1. What is Genetic research?

Genetic material, also called DNA or RNA, is usually obtained from a small blood sample. Occasionally genetic material is obtained from other sources such as saliva or biopsy specimens. (A biopsy is a tiny piece of tissue that is cut out e.g. from the skin or from a lump, to help your doctor make a diagnosis.) Genes are found in every cell in the human body. Our genes determine what we look like and sometimes what kind of diseases we may be susceptible to. Worldwide, researchers in the field of genetics are continuously discovering new information that may be of great benefit to future generations and also that may benefit people today, who suffer from particular diseases or conditions.

2. What does this particular research study involve?

This research study seeks to address the increasing problem of diabetes and cardiovascular diseases such as heart attack and stroke amongst the mixed ancestry or coloured population of South Africa. In this study we shall identify people with diabetes and those at high risk of diabetes as well as investigate the environmental and genetic risk factors that predispose some individuals to the development of diabetes and cardiovascular diseases. Examples of environmental factors include body weight, diet, and physical activity. Additionally, this project aims to investigate whether oral health is a risk factor for diabetes and cardiovascular diseases. In this study we shall investigate whether some individuals have early cardiovascular diseases by using an ultrasound machine. This project also aims to collect genetic material (blood) to analyze for certain variants and to store excess material for future research. When a large group of patients with similar diseases has been collected, meaningful research into the disease processes may become possible.

3. Why have you been invited to participate?

Our research team has previously conducted a similar research study involving the coloured community and found out that more that 18 out of 100 individuals had diabetes but did not know. We also found that some of the risk factors associated with diabetes in other populations were not necessary the same as those affecting the coloured population of South Africa. You have therefore been invited to take part in this research study to assist in establishing the risk factors for diabetes and cardiovascular diseases affecting the coloured people of South Africa.

4. What procedures will be involved in this research?

A. You will be requested to provide information about your medical history, family history and information on eating, drinking and smoking habits. Completion of the questionnaire will take no longer than 30 minutes.

B. You shall be requested to provide a record of the medication you are currently taking, therefore if you are taking chronic medication, you shall be requested to provide this to the research team to record the medication.
C.Measurement such as weight, height, waist and hip will be done.
D. Fasting Venous Blood (20ml) will be collected thereafter you will be asked to drink a glucose solution (glucose content 75g). After two hours another venous blood (10ml) will be collected. The blood will be used to determine whether you have diabetes or you are at high risk for developing diabetes.
E. The other tests that will be determined from your blood sample are: Cholesterol, triglycerides, creatine levels to assess your kidney function, liver enzymes to assess your liver, and biochemical markers for inflammation.

A finger prick blood sample (a drop of blood), to be taken at the same time of the first venous blood sample, may also be required from you. The finger prick blood sample will be used to test for diabetes or the risk of developing diabetes on a point-of-care test instrument. Researchers will compare the finger prick point-of-care diabetes test with that of the send away venous blood laboratory test and would be able to establish whether the point-of-care test provides the same accurate results as that of the laboratory. Point-of-care testing may in the future be used to provide fast and accurate results without the need to send blood away to a laboratory for processing. This may be of benefit to people undergoing testing for diabetes as results would be available within a few minutes.

G. The remainder of the blood sample will be used for genetic and future research studies. The serum and DNA may be stored for several years until the technology for meaningful analysis becomes available. No pharmaceutical agent (medication) will be tested in the study.

H. For oral health, research study personnel will extract wooden toothpick, flocked brush, and mouthwash saliva samples from you to test for the presence of Porphyromonas gingivalis as an indicator for periodontal disease. Flocked brush and wood toothpick sampling will involve inserting devices in the subgingival crevice between the last upper premolar and the first upper molar. The device will sweep down the anterior surface of the first upper molar with the direction of motion away from the gum to minimize any potential discomfort. Mouthwash sampling will involve rinsing with 10 ml sterile saline solution for 20 seconds.

I. Early cardiovascular diseases will be performed by means of an ultrasound machine.

J. The research team will follow up on you on a yearly basis and some of these tests may be repeated. The investigators wish to follow you up for your entire life. In the unfortunate event that you are deceased during the study period. The study team will review stats SA data and/or medical records to ascertain whether the cause of death was due to diabetes or cardiovascular diseases.. ***If you do not wish to be followed up on a yearly basis and your Statistics SA and/or medical records not to be accessed in the unfortunate event that you are deceased whilst being a participant in the study, you will have an opportunity to request that it be not accessed when you sign the consent form.***

K. Radio imaging techniques will be done on consenting subjects. These include **(i)** ultra sound to assess whether you have signs of early cardiovascular diseases, **(ii)** computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases **(iii)** Dual-energy X-ray absorptiometry (DXA) devices will be used to study the morphology of the liver. These radio imaging techniques involve radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant.. ***If you do not wish to undergo any of these radio imaging techniques, you will have an opportunity to decline when you sign the consent form.***

L. An eye examination will be done to test your eye vision and any other abnormalities in the eye. For this examination, drops placed in your eyes widen (dilate) your pupils to allow the doctor to better view inside your eyes. The drops may cause your close vision to blur for a short while.

5. Are there any risks involved in genetic research?

A slight bruising might occur after blood has been drawn from the arm but this will heal quickly. After the administration of the glucose solution, you may feel nauseous and dizzy in which case you must notify the medical personnel. A medical nurse or doctor will be present on all occasions. You may also learn that you have diabetes, in which case you will be referred to your health care giver with the results for further treatment and management. If during the study it is discovered that you have changes in your genes that may lead to a serious disease, a genetic counsellor at the expense of the principal investigators will counsel you. Radio imaging techniques such as the CT-scan involves radiation which can be harmful if one is exposed excessively. For this study a low dose radiation will be used for acquisition of the images thereby minimizing radiation exposure to the participant.

6. Are there any benefits to your taking part in this study and will you get told your results?

Your personal results will be made known to you ***only if they indicate*** that you may:

- Have diabetes, thereafter, you will be referred to your local health centre or general practitioner for further investigations and treatment.
- Have a condition or predisposition to developing diabetes that is treatable or avoidable e.g. by a lifestyle modification.
- Need genetic counselling.

- **However, participants with normal results who wish to know their results are free to contact the research team and their results will be given upon written request.**

7. How long will your blood be stored and where will it be stored?

The blood samples may be stored ***indefinitely*** to accommodate new technologies that may develop. In the event that a technology is not available in South Africa to analyse your blood sample, your blood specimen may be sent to another country with the technology either now or at a later date. However, if your specimen is to be sent to another country, permission to do so will be sought from relevant bodies. Your blood specimen will be stored at the Cape Peninsula University of Technology.

8. If your blood is to be stored is there a chance that it will be used for other research?

Your blood will only be used for genetic research that is directly related to Diabetes and cardiovascular diseases. Also, if the researchers wish to use your stored blood for ***additional research in this field*** they will be required to apply for permission to do so from the ethics

Faculty of Health & Wellness Sciences of the Cape Peninsula University of Technology. If you do not wish your blood specimen to be stored after this research study is completed you will have an opportunity to request that it be discarded when you sign the consent form.

_9._ How will your confidentiality be protected?

Your identity will be recorded once and kept confidential throughout. This is to allow the principal investigators to convey information that may be beneficial to you. Access will be limited to the principal investigators by assigning a special study code to all your data and blood samples. This means that your sample will be identified with a special study code that will remain linked to your name and contact details. However, during the entire research study, your blood specimens will be anonymized and the research staff won't be able to associate it with your name and contact details. You shall also be supplied this code so that if at any time the investigators need to contact you, you may only identify yourself using your special code. Any scientific publications, lectures or reports resulting from the study will not identify you.

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

10 Will you or the researchers benefit financially from this research?

You will not be paid to take part in this study ***although your out-of-pocket expenses may be reimbursed.*** The expenses that will be covered by the research team are those that include transportation to a hospital radiography department should you consent to radio imaging.

Important information: In the unlikely event that this research leads to the development of a commercial application or patent, you or your family will not receive any profits or royalties, but profits will be reinvested into supporting the cause of further research which may bring benefits to you or your family and to the community, such as health screening, medical treatment, educational promotions, etc.

11 Is there anything else you should know or do?

You should inform your family practitioner or usual doctor that you are taking part in a research study. You can contact Prof T Matsha at 021 959 6366 or matshat@cput.ac.za,

If you have any further queries or encounter any problems, you can also contact the Cape Peninsula University of Technology Health and Wellness Sciences Research Ethics Committee, Chairperson: Prof Engel-hills at 0219596570 or EngelhillsP@cput.ac.za or

You will receive a copy of this information and consent form for your own records if it is requested.

12 Declaration by participant

By signing below, I …………………………………..…………. agree to take part in a research project that includes

genetic research study entitled (**PROGRESSIVE RESEARCH ON RISK FACTORS OF TYPE 2 DIABETES AND CARDIOVASCULAR DISEASES IN SOUTH AFRICA**).

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is voluntary and I have not been pressurized to take part.
- I have received a signed duplicate copy of this consent form for my records.

13. Tick the option you choose:

I agree that my blood or tissue sample can be stored ***indefinitely*** after the project is completed but that it is anonymized with all possible links to my identity removed, and that the researchers may then use it for additional research in this or a related field. Once my sample is anonymized, my rights to that sample are waivered. My sample may be shipped to another laboratory in SA or abroad to be used in other research projects in this or a related field

OR

I agree that my blood or tissue sample can be stored ***indefinitely,*** but I can choose to request at any time that my stored sample be destroyed. My sample will be identified

☐

with a special study code that will remain linked to my name and contact details. I have the right to receive confirmation that my request has been carried out.

OR

Please destroy my blood sample as soon as the current research project has been completed.

14. Tick the option you choose:

☐ I ***consent*** that the research team may follow me up for yearly check-up ***AND*** in the unfortunate event that I am deceased whilst still part of the study, I ***consent*** that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

☐ I ***do not consent*** to follow me up for yearly check-up ***BUT*** in the unfortunate event that I am deceased whilst still part of the study, I ***consent*** that the team may access Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

OR

☐ I **do not consent** to follow me up for yearly check-up ***AND*** in the unfortunate event that I am deceased whilst still part of the study, I ***do not consent*** that the team accessing Statistics SA and/or my medical records to ascertain whether the cause of my death was due to diabetes or cardiovascular diseases.

15. Tick the option you choose: Radio Imaging

☐ I ***consent*** to ultra sound techniques to assess if I have early cardiovascular diseases

☐ I ***do not consent*** to ultra sound techniques that assess if I have early cardiovascular diseases

AND

☐ I ***consent*** computed tomography scan (CT-scan) to accurately assess the fat content that is dangerous for cardiovascular diseases

☐ I ***do not consent*** to computed tomography scan (CT-scan) that accurately assess the fat content that is dangerous for cardiovascular diseases

AND

☐ I ***consent*** to Dual-energy X-ray absorptiometry (DXA) used to study body composition.

☐ I ***do not consent*** Dual-energy X-ray absorptiometry (DXA) used to study body composition

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

Finger print

....

Signature of participant **Signature of witness**

16 Declaration by investigator

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

- I explained the information in this document to …………………..……………...

- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research as discussed above.
- I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) .... on (*date*) .... 2016.

....

Signature of investigator **Signature of witness**

17. Declaration by Interpreter

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

- I assisted the investigator (*name*) .... to explain the information in this document to (*name of participant*) .... Using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) .... on (*date*) .... 2016.

....
......

Signature of interpreter

....

Signature of witness

Appendix B

Ethical Clearance Certificate

HEALTH AND WELLNESS SCIENCES RESEARCH ETHICS COMMITTEE (HW-REC) Registration Number NHREC: REC- 230408-014

P.O. Box 1906 • Bellville 7535 South Africa

Symphony Road Bellville 7535

Tel: +27 21 959 6917

Email: sethn@cput.ac.za

21 May 2018

REC Approval Reference No:

CPUT/HW-REC 2017/H2 - renewal

Faculty of Health and Wellness Sciences – Biomedical Sciences

Dear Mr Cecil Jack Weale

Re: APPLICATION TO THE HW-REC FOR ETHICS CLEARANCE

Approval was granted by the Health and Wellness Sciences-REC on 30 March 2017 to Mr Weale for ethical clearance. This approval is for research activities related to student research in the Department of Biomedical Science at this Institution.

TITLE: The association between telomere length and glucose intolerance

Supervisor: Prof Davison and Prof Matsha

Comment:

Data collection permission is required and has been obtained.

Approval will not extend beyond 22 May 2019. An extension should be applied for 6 weeks before this expiry date should data collection and use/analysis of data, information and/or samples for this study continue beyond this date.

The investigator(s) should understand the ethical conditions under which they are authorized to carry out this study and they should be compliant to these conditions. It is required that the investigator(s) complete an **annual progress report** that should be submitted to the HWS-REC in December of that particular year, for the HWS-REC to be kept informed of the progress and of any problems you may have encountered.

Kind Regards

Mr. Navindhra Naidoo

Chairperson – Research Ethics Committee

Faculty of Health and Wellness Sciences