Leukocyte telomere length and its relation to nitric oxide metabolites in a bi-ethnic sample: the SABPA study

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Preface

This dissertation is based on a study that formed part of the program for the degree *Magister Scientiae* in Physiology. The article format was used to compile the dissertation. This format is endorsed by the North-West University and contains a manuscript for publication and a detailed literature review. The style of this document is in accordance with the specifications of the *Aging Journal*.

Chapter 1 includes the background and the motivation for conducting the study. Chapter 2 contains a literature review of telomere length and nitric oxide as well as other biochemical and cardiovascular markers related to cardiovascular disease. This chapter also includes a short summary, aims, objectives and hypotheses of the study. Chapter 3 consists of a detailed description of the methods carried out to collect the data. Chapter 4 contains the manuscript that will be submitted to the *Aging Journal*. The general conclusions and recommendations are discussed in Chapter 5.

Summary

Motivation

Aging of the cardiovascular system is associated with cardiovascular morbidity and mortality. One of the measures for biological aging is telomere length. Telomere length is considered to be more closely related to cardiovascular disease than chronological age. If telomere length declines, so does the protective mechanisms of nitric oxide (NO) in the cardiovascular system. Additionally, different population groups also have different telomere lengths and NO bioavailability profiles which may indicate a variation in risk of cardiovascular disease in different populations.

NO plays a critical role in the maintenance of a healthy cardiovascular system by counteracting endothelial senescence. It is known that when NO declines, so does telomerase activity. When NO bioavailability decreases, telomere shortening may accelerate, because of the association between telomerase activity and NO. Shortened telomere length is age dependent, but might also indicate declining cardiovascular health, through a cycle of negative impact. This cycle could reduce NO, which decreases telomere length, thus increasing cell senescence that could again decrease NO production.

The burden of cardiovascular disease is an enormous challenge worldwide. This is also true for the South African population. The association between telomere length and cardiovascular disease is well known, but minimal studies have been done on the South African population. Globally, studies conducted in black populations on telomere length, NO and telomerase activity have shown contradictory results. To date, there are no publications that focus on the association between telomere length and NO metabolites (NOx) in a South African population. With this study, an attempt was therefore made to increase our understanding of the relationship between telomere length and the cardiovascular system, by investigating associations of telomere length with NOx in a bi-ethnic population.

Aim

Shorter leukocyte telomere length is associated with increased cardiovascular risk and decreased NO bioavailability. Decreased telomere length, increased cardiovascular risk and decreased NO are also linked with increased oxidative stress and inflammation. The aim of this study was to compare telomere length, NOx, blood pressure and inflammatory- and oxidative stress markers

in a bi-ethnic population and to investigate the associations of telomere length with NOx and markers of oxidative stress.

Methodology

Participants in this cross-sectional study were 152 black and 186 white school teachers aged between 23 and 68 years, with complete datasets for the main variables of the study. Ambulatory blood pressure was measured and standard methods used to analyze leukocyte telomere length. NOx as well as glutathione peroxidase (GPx), as a marker of oxidative stress, were measured. The inflammatory markers investigated were interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α).

Study population

This study form part of the follow-up phase of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study, a prospective cohort study. The initial phase of the study, which was conducted between February and May of 2008 and 2009, included a total of 409 participants, aged 20-65 years. Initial exclusion criteria for the study were pregnancy, lactation and blood donation or vaccination three months prior to the commencement of the study. The follow-up phase of the SABPA study was conducted between February and May of 2011 and 2012. A total of 359 school teachers from the North West Province in South Africa participated in the follow-up phase of the study. After applying additional exclusion criteria such as participants infected with the human immunodeficiency virus (HIV) and non-valid leukocyte telomere lengths, the data of 338 participants were used. Only data of the follow-up phase was analyzed cross-sectionally for this sub-study, as telomere lengths were not determined during the baseline phase.

A general health questionnaire was completed by participants with the assistance of trained fieldworkers. A registered nurse obtained a blood sample from fasting participants with a sterile winged infusion set from the ante-brachial vein branches. Serum and plasma were prepared according to standard procedures.

Characteristics of black men and women were compared with their white counterparts with T-tests, while proportions were compared with Chi-square tests. Partial correlations were performed to evaluate the associations between telomere length and NOx levels while adjusting for age. Multiple regression analyses were performed to evaluate the independent associations of telomere length with NOx and oxidative stress markers in the bi-ethnic groups considering sex, age, waist circumference, total cholesterol, glycated hemoglobin (HbA1c), gamma glutamyl transfers (GGT), tumor necrosis factor alfa (TNF-α), symmetric dimethylarginine (SDMA) and ambulatory systolic blood pressure (BP).

Results and conclusion

Black men and women had higher BP ($p < 0.001$), higher IL-6 ($p \leq 0.016$) and shorter telomeres ($p < 0.001$), but similar NOx levels than their white counterparts. GPx activity was higher in black groups compared to white groups ($p \leq 0.002$). Independent positive associations of telomere length with NOx (adj $R^2 = 0.21$; $\beta = 0.249$; $p = 0.03$) and GPx activity (adj $R^2 = 0.21$; $\beta = 0.23$; $p = 0.03$) were indicated in white men, while telomere length was negatively associated with TNF-α (adj $R^2 = 0.33$; $\beta = -0.249$; $p = 0.010$) in white women. These associations were absent in the black groups. Furthermore, the lack of an association between telomere length and NOx in the black men was independent of lifestyle factors such as smoking, alcohol abuse and physical activity. To conclude, telomere length of black men was not associated with NOx or markers of oxidative stress, as observed in white men. The less favorable, cardiovascular and inflammatory profiles of the black population were unrelated to shorter telomere lengths. Further investigations on early life exposures as potential contributor to shorter telomere length in black populations are proposed.

List of abbreviations

ADMA: Asymmetric dimethyl arginine

AIDS: Acquired immune deficiency syndrome

ANG II: Angiotensin II

BH4: Tetrahydrobiopterin

BMI: Body mass index

BP: Blood pressure

CRP: C-reactive protein

CAD: Coronary artery disease

CVD: Cardiovascular disease

DBP: Diastolic blood pressure

ER: Estrogen receptor

ET-1: Endothelin-1

ETC: Electron transport chain

FAD: Flavin adenine dinucleotide

FFA: Free fatty acids

FMN: Flavin mononucleotide

GGT: γ-glutamyl transferase

H_2O_2: Hydrogen peroxide

HAART: Highly active antiretroviral therapy

HbA1c: Glycosylated hemoglobin A1c

HBD: Heparin binding domain

HDL-C: High density lipoprotein cholesterol

HIV: Human immunodeficiency virus

HR: Heart rate

hTERT: Human telomerase reverse transcriptase

hTR: Human telomerase RNA

IL-6: Interleukin-6

LDL-C: Low density lipoprotein cholesterol

L-NMMA: NG-monomethyl-L-arginine

LTL: Leukocyte telomere length

Mg^{2+}: Magnesium

NADPH: Nicotinamide-adenine-dinucleotide phosphate

NOx: Nitric oxide metabolites

NOX NAD(P)H oxidase

MDA: Malondialdehyde

PP: Pulse pressure

PWV: Pulse wave velocity

ROS: Reactive oxygen species

SBP: Systolic blood pressure

SDMA: Symmetric dimethyl arginine

sGC: Soluble guanylate cyclize

SOD: Superoxide dismutase

suPAR: Soluble urokinase plasminogen activator receptor

TBARS: Thiobarbituric acid reactive substances

TNF: Tumor necrosis factor

VSMC: Vascular smooth muscle cells

WC: Waist circumference

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Affirmation of Authors

The following researchers contributed to this dissertation:

Mr J Combrink

Mr Combrink performed the literature research, compiled the proposal, applied for ethics approval, performed the statistical analyses and wrote this dissertation, including the manuscript for publication.

Prof HW Huisman

Prof Huisman was the study supervisor, who supervised the proposal writing, ethics application, statistical analyses and the compilation of this dissertation. He also reviewed all work leading to and included in this dissertation. Prof Huisman made professional recommendations to improve the manuscript.

Prof CMC Mels and Prof AE Schutte

Proff Mels and Schutte were the co-supervisors. They provided with statistical analyses, reviewed the manuscript, assisted with proposal writing and gave constructive criticism that aided in improving on the documentation of the results.

Mr J Combrink

Prof HW Huisman

Prof CMC Mels

Prof AE Schutte

Chapter 1

Introduction

Chapter 1 Introduction

Background and motivation

Telomere length is used to measure an increase in biological aging, which has a closer relation to cardiovascular disease than chronological aging [1-3]. A decline in telomere length is associated with a decrease in the protective properties of nitric oxide (NO) in the vascular system [4]. In addition, different ethnic groups also present different telomere lengths, which may indicate a variation in cardiovascular disease risk in different ethnic groups [5, 6].

Telomeres are composed of nucleoproteins and are found on the terminals of eukaryotic chromosomes. These telomeres have a repetitive TTAGGG structure over thousands of base pairs that shorten over time in somatic cells, due to cell division [6]. Cell division is however not infinite and has a limit, called the Hayflick limit [7]. The Hayflick limit is brought about by the suppressed telomerase activity in somatic cells [8]. The enzyme telomerase assists by adding the telomere repeats (TTAGGG) at the end of telomeres during cell division [9]. If telomerase is absent or inactive, telomeres will shorten [9]. Suppressed telomerase activity occurs in most physiological systems except for the reproductive germinal cells which have active telomerase and do not have shortened telomeres [10]. Some studies have found that the offspring of older fathers tend to have longer telomere lengths, suggesting that telomerase not only maintains telomere length in germinal cells, but can also lengthen telomeres in the sperm cells of older men [10, 11].

Aging of the cardiovascular system, measured by shorter telomeres, is regarded as the progressive decline in the cardiovascular system's physiological function [3, 12]. Genetic and lifestyle factors influence both the rate of shortening of telomere length, as well as vascular function [13, 14]. Lifestyle factors, which will be discussed later, may lead to increased inflammation, oxidative stress and DNA damage, which may further increase the rate of senescence of endothelial cells [15-17]. Senescent endothelial cells still have the ability to function, but they lose their ability to duplicate due to short telomere lengths [18]. These senescent cells accumulate over time and cause lower regeneration ability of tissue and hinder the homeostasis of endothelial cells [19].

Shorter telomeres are closely associated with cardiovascular disease. Cardiovascular pathologies associated with short telomere length include atherosclerosis, which is observable as carotid plaque and thickening of the carotid intima media, heart failure, coronary heart disease and myocardial infarctions [20-23]. In hypertensive patients, carotid plaques are also associated with shorter telomere length [24]. In another study, carotid intima media thickness (cIMT) had the tendency to increase with shorter telomeres [25]. Some studies have found that increased pulse pressure as well as high blood pressure are associated with shorter telomeres in hypertensive men [26, 27].

Telomere length is strongly influenced by non-modifiable genetic factors such as sex and ethnicity [28]. At birth, the telomere lengths of men and women are relatively similar, but over time, the telomere lengths of men shorten at a faster rate than those of women [29]. One study has found that pulse pressure and mean arterial pressure are significantly associated with telomere length in men but not in women [30]. It is proposed that estrogen plays a protective role in the maintenance of telomere length, acting as an antioxidant [31]. Other possible explanations are that men generally have more lifestyle risk factors, since men are more likely to smoke and abuse alcohol, than women [32, 33]. Not only is sex related to telomere length, but ethnic differences also tend to be gene pool specific. African Americans have longer telomeres than their white counterparts of the same age, while black South Africans have shown to have shorter telomere lengths in comparison to white South Africans [32-34]. This study focused on ethnic differences and the design of the proposed study was therefore on a bi-ethnic group of adults from South Africa.

South Africa is a country with some of the highest occurrences of HIV-infected people in the world [35]. In 2013, a South African study provided evidence that HIV accelerates biological aging and thus shortens telomeres [36]. In the present study, with a focus on telomere shortening and NOx, it is justified to exclude participants infected with HIV. This phenomenon of HIV accelerating telomere shortening is brought about by increased inflammation and oxidative stress as well as a decrease in NO [37].

NO plays a critical role in the maintenance of a healthy cardiovascular system by counteracting endothelial senescence [38]. When NO synthesis is inhibited, acceleration in telomere shortening and decreased telomerase activity are evident [39]. Some studies have found that a low activity of telomerase exists in endothelial cells, but the activity of telomerase decreases as NO decreases, leading to endothelial cell senescence [40-41]. In contradiction, a study in the United Kingdom found no association between telomerase activity and NO [42].

Cardiovascular disease is an immense problem in South Africa [43]. The black population of Southern Africa has a heavy burden of cerebral incidence and mortality due to cardiovascular disease [44]. It is known that telomere length is associated with cardiovascular diseases, but minimal studies have been done on the South African population. Studies regarding telomere length as well as studies regarding NO and telomerase activity in black populations have been contradictory [30-32, 37, 40]. No studies on South Africans, focussing on telomere length and its association with NO metabolites (NOx), have been conducted to date. With this study, an attempt was therefore made to increase our understanding of the relationship between telomere length and the cardiovascular system by investigating associations of telomere length with NOx in a bi-ethnic population.

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Chapter 2

Literature study

Chapter 2: Literature study

2.1 Telomeres

2.1.1 Introduction

Telomeres are quantifiable biomarkers of biological aging [1]. The measurement of telomere length can identify potential age-related cardiovascular diseases [2]. Telomeres are protective structures at the end of DNA strings that consist of nucleoproteins with none functional gene coding as seen in Fig 2.1 [3]. These telomeres have a constant repeat (TTAGGG) and are shown to protect against DNA damage [3].

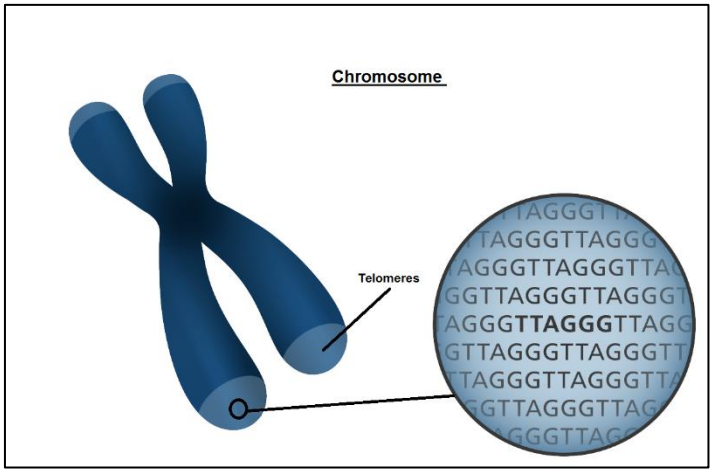

Fig 2.1 Telomere end caps on DNA [4]. Adapted from www.yourgenome.org.

With each cell division, somatic cells have a shortening of telomeres due to the minimalistic activity of telomerase in these cells [5]. When telomeres reach a critical length, the cell will go into senescence and be unable to undergo further cell division [5]. This inability of senescent cells to regenerate new cells could lead to pathology in those physiological systems, with the cardiovascular system being very sensitive to aging [6, 7].

The rate of telomere shortening has been shown to vary between different sexes and ethnicities [7-9]. In this study, part of those biochemical processes associated with telomere length will be investigated [10]. Telomeres are not only influenced by sex and ethnicity, but environmental- and lifestyle factors also play a crucial role [11]. One mechanism through which lifestyle- and environmental factors influence telomere length is oxidative stress [12].

2.1.2 Telomerase

Telomerase is an enzyme that consists of a catalytic subunit, namely human telomerase reverse transcriptase (hTERT) as well as an RNA component known as human Telomerase RNA (hTR) as shown in Fig 2.2 [20, 21]. The hTR is responsible to form the template for the TTAGGG sequence which telomeres consist of [20, 21].

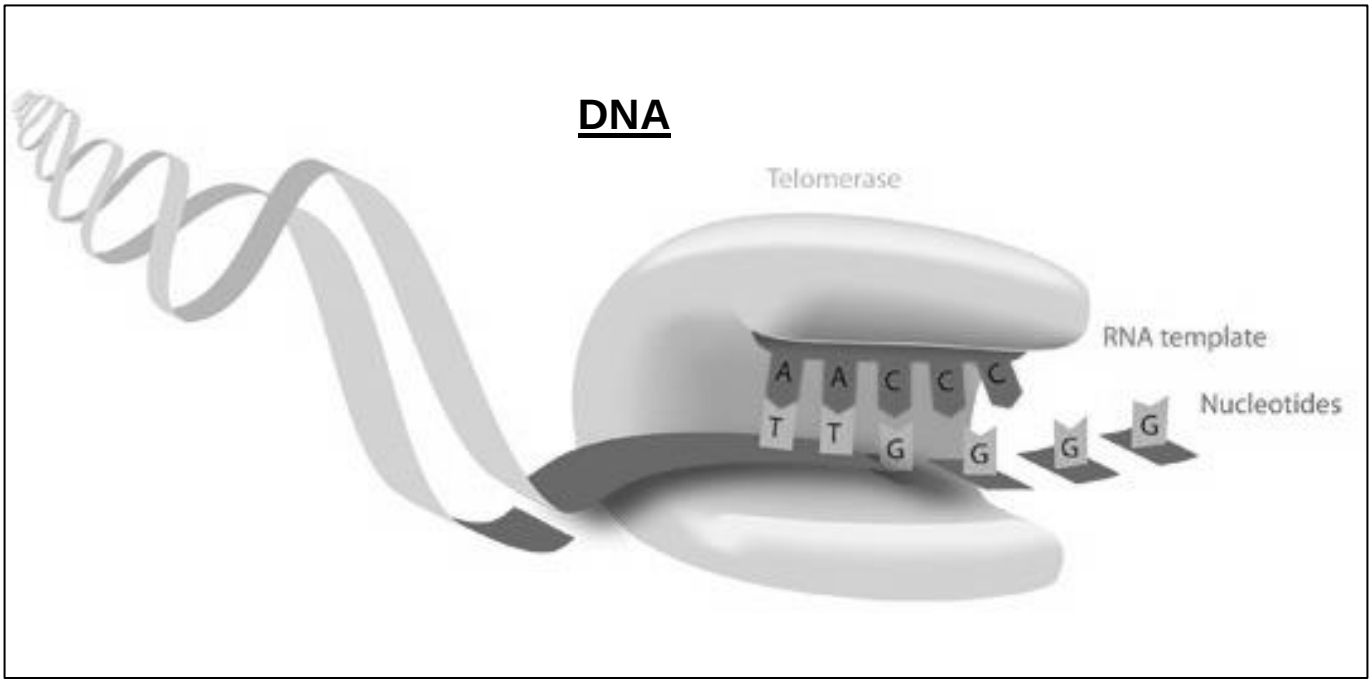

Fig 2.2 Telomerase [22]. Adapted from counter et al.

Telomerase protects the DNA by assisting telomeres to maintain their length [5]. Telomerase inactivation can thus potentially be associated with cardiovascular disease due to early myocyte senescence as well as atherosclerosis and other cardiovascular diseases and may also lead to a shorter lifespan [8, 23]. Telomerase activity starts to decrease in somatic cells in the fetal stage [24]. Although somatic cells have a decrease in telomerase activity with aging, the germline cells from the reproductive system continue to have high telomerase activity [25, 26].

Telomerase was tested on mice treated with a telomerase reverse transcriptase over expression virus [27]. The mice showed better overall health, less osteoporosis and insulin resistance and increased cognitive and metabolic function as well as an increase in physical activity [27]. The mice also had an increase in telomere length [27]. However, the effect of increased telomerase is not risk free, since early studies indicated an increased incidence of cancer in mice [28]. Except for the cancer pathologies, these mice were of overall better health [28].

Studies found that numerous life factors influence telomerase activity [11, 29]. These factors can be pinned to both psychological and physical stressors [30]. Studies found that less physiological stress, meditation, positive psychological changes and lower level of depression symptoms are all related to an increase in

telomerase activity [30, 32]. Higher telomerase activity was also evident in individuals who had lower cortisol and glucose levels, who followed low fat diets combined with exercise and those who consumed Mediterranean diets [11, 29, 33].

2.1.3 Telomeres and life expectancy

Individuals with shorter telomeres have shorter life expectancies [13]. This phenomenon is also true for twins, where the twin with shorter telomeres has a higher risk of mortality [14]. Although strong evidence exists which supports the association between shorter telomeres and a decreased life expectancy, some studies could not find such an association [15]. It must also be mentioned that a great deal of these telomere studies was cross-sectional and in other studies, the mortality rates were only investigated over short periods of time [16]. Most studies that investigated incidences of mortality were not only of short time spans, but were also done in older populations [15, 16]. In a study conducted by Martin-Ruiz et al., it was found that the usefulness of telomere length as a biomarker declines with age [15,17]. This might explain the reason why some of the studies did not find a causal link between telomere length and mortality. Although studies showed that individuals with shorter telomeres suffered from poorer health than their counterparts, mortalities associated with shorter telomere are more prominent in men and mostly cardiovascular related [18, 19].

2.1.4 Telomeres and cardiovascular disease

Relating to pathology of the cardiovascular system, there are studies that have found associations between telomere length and cerebral incidents [34-36]. Controversially, studies exist that could not establish such an association [37, 38]. However, the studies that could not find an association with cerebral incidents such as strokes had a few shortcomings. Firstly, they were statistically underpowered and secondly, they did not divide different ethnic groups and followed different study designs [37, 38]. Longer telomeres are associated with a decrease in deaths due to cardiovascular disease as well as major adverse cardiac event outcomes [39, 40]. Patients that have type 2 diabetes and who have had a myocardial infarction, have shorter telomeres than controls [41]. D'Mello et al. further state that there may rather be a link between telomere length and the rupture of plaques and thrombosis leading to myocardial infarctions and stroke than an association between telomere length and atherosclerotic growth [40].

2.1.5 Telomere length, ethnicity and sex

Limited and contradictory literature exists on ethnic differences in telomere length, but Hunt et al. found that African Americans had longer telomeres than their counterparts of European origin [42]. The study by Hunt et al. included two studies, namely the National Heart, Lung, and Blood Institute (NHLBI-FHS) Family Heart Study and the Bogalusa Heart Study (BHS) [42]. In the NHLBI-FHS (n = 1968) the average age of men was 56.1 ± 13.1 years and of women 57.1 ± 12.6 years [42]. The BHS (n = 485) was a younger group with an average age of 31.1 ± 4.4 and 30.0 ± 4.9 years for men and women respectively [42]. In both the

NHLBI-FHS and BHS, telomere length was significantly higher in the black population compared to the white population [42]. Additionally, in the NHLBI-FHS, it was found that the women had significantly longer telomere lengths than men, but the same was not found in the BHS [42]. The BHS had a younger study population. Because of this younger age, telomere length difference in sex might not be as significant as in older people [42].

In contrast, a study by Weber et al. could not find a significant difference in telomere length between black and white participants in the United States of America, but found that black population smoked more and were less active than their white counterparts, which are both risk factors for telomere shortening [43]. Weber et al. also found that the men who smoked had shorter telomeres than those who did not smoke [43]. A previous report on the SAPBA study from South Africa, reported shorter telomere lengths in black participants than white participants [44]. When compared to another study in the United States of America on black, Hispanics and white participants, it was found that white participants had the longest telomeres at the same chronological age, while Hispanics had the shortest telomere length. It was almost the same length as those of the black population [45]. These findings by Roux et al. may be as a result of higher exposure to oxidative stress resulting from a poor diet, smoking and inactivity [45].

The results of telomere length and ethnicity therefore differ between the different studies. The genetic pools and living circumstances of individuals of the same ethnicity could be different. The black populations residing in America came from a genetic pool in North Africa. The genetic pool thus differs from black South Africans [46]. As with the NHLBI-FHS study, other studies also found differences in telomere length between sexes [47-50].

Bentos et al. studied men and women of the same age and found that the women had longer telomeres than their male counterparts [47]. A study on rats indicated that telomere shortening happens at a higher rate in male rats [48]. This was true for telomeres from the pancreas, liver, kidneys and in the lungs, but telomeres in the rat brains remained relative constant between sexes [48]. According to a Swedish study, telomere length is associated with an obesity-phenotype in women, but not in men [49]. They found significant negative correlations between telomere lengths of women and body mass index (BMI), weight and waist circumference (WC) [49]. Different results on telomere length and cardiovascular disease have been found in men and women [50]. Men with shorter telomere lengths have a higher mortality rate caused by cardiovascular disease [50]. Shorter telomeres are also associated with higher pulse pressure in European men [47]. An Australian study found an association between the telomere lengths and systolic blood pressure but not in diastolic blood pressure of middle-aged women [51]. For the most part, several studies that investigated the association between telomere length and blood pressure could not find an association in both men and women [42-54]. Bentos et al. also discovered that men had significantly higher pulse pressure (PP) and pulse wave velocities (PWV) than women [47].

2.2 Nitric oxide

2.2.1 Introduction

Nitric oxide (NO) acts as a signaling molecule for intra- and extracellular signaling [55]. The importance of NO in the cardiovascular system is the ability to dilate blood vessels, inhibit platelet aggregation and angiogenesis and it also influences the contractility of the heart [56]. In the cardiovascular system, NO is produced by the endothelial nitric oxide synthase (eNOS) in endothelial cells [57]. NO is relatively unstable and binds easily to superoxide [58]. The bioavailability of NO is determined by the rate of production and inactivation when NO binds to reactive oxygen species (ROS), such as superoxide [58].

2.2.2 Nitric oxide synthesis

Most NO *in vivo* are synthesized by nitric oxide synthase (NOS) [59]. This enzyme is responsible to convert L-arginine into L-citrulline and NO as in Figure 2.3 [60]. NOS requires oxygen (O_2) as well as four different co-factors for the synthesis of NO [61]. These co-factors include tetrahydrobiopterin (BH4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and nicotinamide-adenine-dinucleotide phosphate (NADPH) [61]. All the NOS enzymes contain haem and all the co-factors have the potential, in its reduced form, to become oxidized and form superoxide ($O_2^{\cdot-}$) [61]. Superoxide is also formed when L-arginine and BH4 levels are insufficient [61].

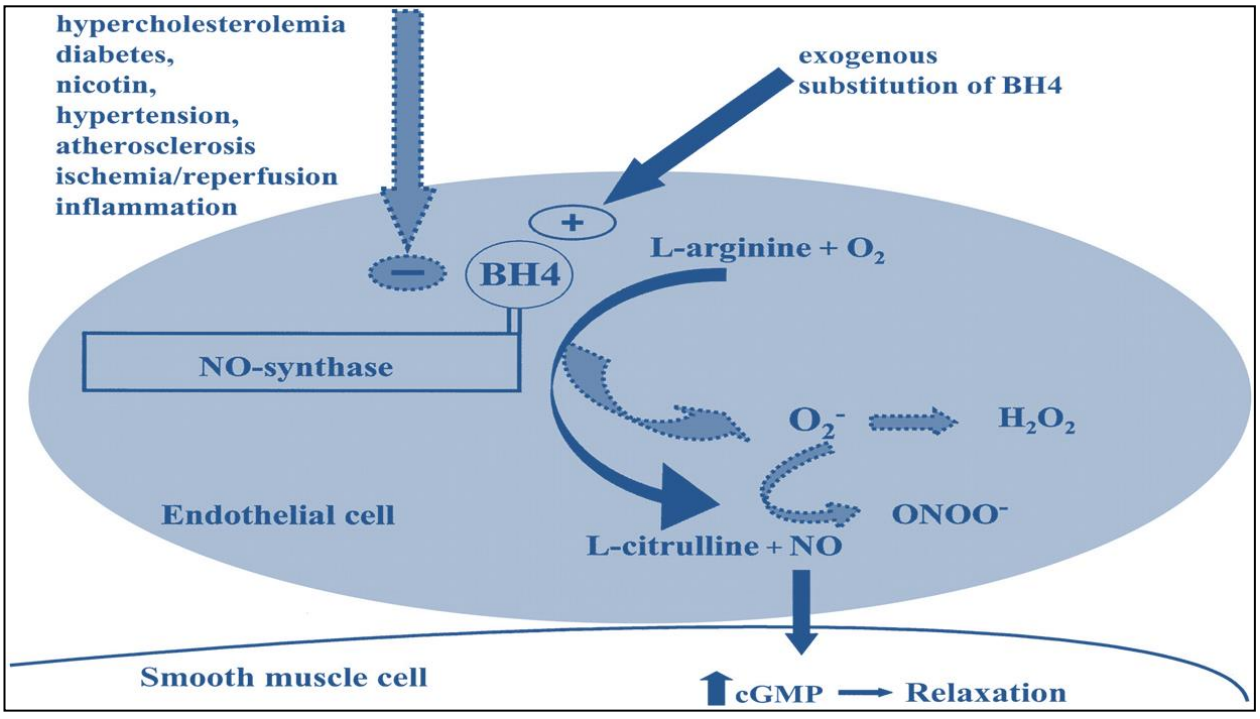

Fig 2.3 Nitric oxide synthesis [62]. Adapted from Tiefenbacher CP.

There are three varieties of NOS: iNOS, nNOS and eNOS. Inducible NOS (iNOS), also known as NOS2, is found at locations where inflammation occurs and vasopermeability and vasodilatation are necessary [57, 61]. Neural NOS (nNOS, NOS1) is found in the nervous system and assists in smooth muscle relaxation and regulation of blood pressure and vasodilatation [57, 61]. The enzyme of importance in this study is endothelial NOS (eNOS, NOS3). This enzyme is mainly produced by the endothelial cells and synthesize NO to aid in the control of blood pressure [57, 61]. The eNOS and nNOS are dependent on the abundance of intracellular calcium to synthesize NO, with lower levels of calcium (Ca^{2+}) leading to a decrease in synthesis [63]. Thus, Ca^{2+} activated calmodulin is imperative to regulate eNOS activity [62]. If intracellular Ca^{2+} increases, the binding of calmodulin to eNOS will also increase [63].

2.2.3 Nitric oxide inhibitors

Asymmetric dimethylarginine (ADMA) acts as an endogenous inhibitor of eNOS and increased levels of ADMA are associated with atherosclerosis and hypertension [64]. Symmetric dimethylarginine (SDMA) does not directly inhibit NO, but SDMA competes with the cellular uptake of arginine and potentially decreases availability of substrate for NOS [65].

2.2.4 Endothelial nitric oxide functions

NO synthesized from eNOS is the most prominent form of NO in the cardiovascular system [66]. In Fig 2.4, the vasodilator function of eNOS is portrayed.

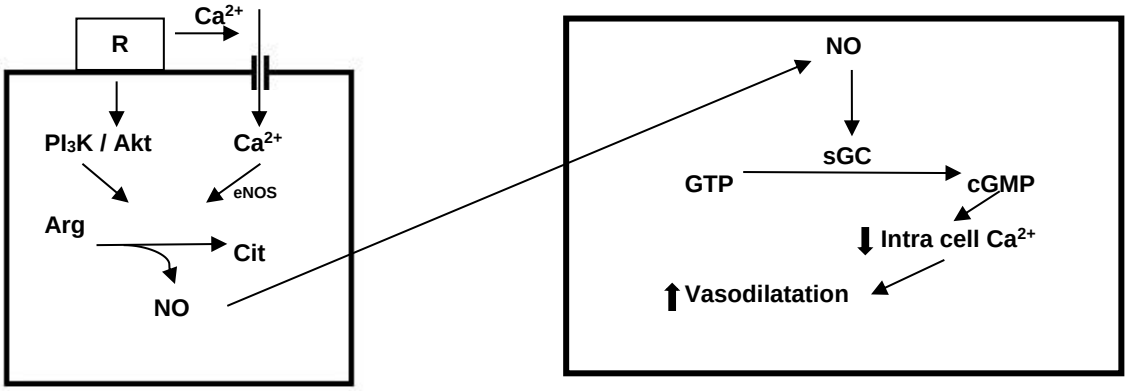

Fig 2.4 Vasodilatory function of eNOS in the cardiovascular system.

All the different blood vessels experience dilation when NO stimulates the increase in cGMP in the smooth muscle cells by binding to the haem center of soluble guanylate cyclase (sGC) [67]. NO protects the vascular system against the formation of atherosclerosis by inhibiting platelet aggregation and prevents the release of platelet derived growth factors [68]. Not only does NO reduce chemoattractant protein-1, but it can also prevent leukocyte adhesion molecules to join the endothelial cell surface [68]. The prevention of leukocyte adhesion is made possible when NO interferes with CD11/CD18 to bind to endothelial cell or by the suppression of CD11/CD18 in leukocytes [69].

NO synthesized by eNOS protects erythrocytes against deformity and also protects the endothelial cells of the pulmonary and coronary blood vessels against toxic lipids [70]. NO inhibits various cytokines which include, but are not limited to, tumor necrosis factor-α (TNF-α) and Interleukin-6 (IL-6) [71].

2.2.5 Measurements of nitric oxide metabolites

After NO is released from the endothelial cells and acts on target cells, it rapidly gets oxidized by oxyhemoglobin from the erythrocytes to form nitrates or it undergoes autoxidation in another media than hemoglobin to nitrite [73]. Nitrate and nitrite concentrations are termed NO metabolites (NOx) and are circulating in the blood until excreted in the urine [74]. If an individual's diet does not include high levels of nitrates, reliable *in vivo* measurement of NOx can be done [74]. The nitrite measured is representative of eNOS activity whereas the nitrates represents the systemic NO synthesis [75].

2.2.6 Nitric oxide and age

With aging, the fractional synthesis rate of NO production will start to decline [76]. The physiological systems are intertwined and to be able to fully understand the phenomena, a broader view is required. In the aging human, progressive decline of nNOS in the muscular system contributes to a decrease in muscle mass [77]. In the muscular system, ROS increase with age and NO concentrations decrease [78]. It is also known that, with age, ROS increase in the vascular system and act as a NO scavenger, thereby decreasing the bioavailability of vascular NO [79].

2.2.7 Nitric oxide and sex

Estrogen influences the production of NO in the cardiovascular system through the binding of estradiol (E_2) with the estrogen receptor (ER) [80]. Women who underwent an ovariectomy have a decrease in estrogen and consequently have lower NO levels and higher incidences of hypertension and cardiovascular disease [81, 82]. It has been shown that the physiological levels of 17β-estradiol can increase NO secretion from endothelial cells by activating the Ca^{2+} dependent K^+ channels of the vascular smooth muscles [79-82]. The effect of 17-β-estradiol on NO release starts to fade in menopause [83]. After menopause, women will have less NO production than men and increased vasoconstriction that expands the risk of cardiovascular disease such as hypertension [84].

Testosterone injected into the vascular system of rats caused an influx of extracellular calcium and consequently also increased the production of eNOS [85]. This study on rats therefore proposed that testosterone could potentially modulate endothelial cell growth and platelet aggregation [85]. The same was found with mice, where testosterone increase was positively associated with the increase of eNOS [86]. In humans, it was also found that testosterone at physiological levels increase eNOS activity, promoting NO synthesis and thus also vasodilation [87]. Where physiological testosterone levels increase endothelial NO, supraphysiological levels of testosterone increase oxidative stress and down regulates

endothelial NO synthesis through suppression of eNOS [88]. Supraphysiological levels of testosterone thus has adverse effects on the cardiovascular system [88].

2.2.8 Nitric oxide and ethnicity

The difference in NO levels in different ethnicities can partially be explained through polymorphisms in the eNOS enzyme [89]. There are three eNOS polymorphisms, found to be clinically relevant [89, 90]. The first polymorphism is in the promotor region ($T_{i}786C$) and is known as the C^{-786} variants [90]. The second polymorphism is the Asp298 variant found in the exon 7 region [89, 90]. The third important polymorphism is intron 4 that has a variable number of tandem repeats (VNTR) [89, 90]. Significantly more Asp298- and C^{-786} variants are present in white participants than in their black or Asian counterparts [89, 91], whereas intron 4 (4a variant) is more commonly found in African Americans than their white or Asian counterparts [89, 91].

The C^{-786} variant polymorphism decreases the eNOS enzyme activity [89, 90]. Asp298 has controversial evidence with regards to its influence on enzymatic activity [89, 90]. To the best of our knowledge, no data exists that indicates that intron 4 polymorphism affects enzyme activity [89-91].

The association between these three polymorphisms with cardiovascular disease has been controversial [92]. Despite the controversy, the difference in eNOS polymorphisms when comparing different ethnic groups, could partly explain NO bioavailability, cardiovascular risk and reaction to hypertension medication between ethnicities [89-93]. One must also consider that these ethnic differences were not tested in South Africa and might not be a true representation of the South African population.

2.3 Telomere length, nitric oxide and oxidative stress

2.3.1 Introduction

Oxidative stress is when an imbalance occurs between oxidants and antioxidants, favoring the oxidants [94]. There is a variety of oxidants that are synthesized through aerobic metabolic processes as well as through pathological conditions [94]. Antioxidants defend against oxidants by neutralizing the oxidants to limit damage to tissue [94]. Different studies have found that oxidative stress, cardiovascular disease, telomere length and endothelial NO are all independently, directly or indirectly associated with each other [95-96].

Atherosclerosis is prominent in individuals with shorter telomeres and/or decreased NO levels [68]. The formation of atherosclerotic plaque is primarily through the oxidation of low-density lipoproteins (LDL) by macrophages which cause the formation of foam cells [97]. Studies have found that the activity of telomerase in endothelial cells decreases when oxidation of LDL increases [98]. This will then also lead to an increased rate of telomere shortening and cell senescence [98].

Reactive oxygen species, such as superoxide (O_2^-), bind to NO to decrease the availability of NO, thus increasing atherosclerosis and risk for potential cardiovascular events [18, 68]. Oxidative stress has also been directly associated with the shortening of telomeres [12].

The imbalance of oxidative stress promotes tissue damage [94]. Endothelial damage leads to both the formation of atherosclerosis and shortening of cardiovascular telomeres [95-98]. Thus, oxidative stress does not only have a negative effect on telomere length, but also on cardiovascular health and NO balance. Less oxidative stress is also associated with longer telomeres, and higher NO levels are associated with cardiovascular health [12, 18, 68, 97, 98].

2.3.2 Reactive oxygen species

ROS consist of a family of reactive molecules that include an oxygen molecule and its by-products in aerobic cells [99]. High ROS levels may lead to the oxidation of molecules such as proteins, DNA, carbohydrates and lipids [100, 101]. This oxidation process is linked to various cardiovascular diseases and dysfunction of the vascular endothelium [100, 101]. High levels of ROS can inundate antioxidant systems, leading to cell death [102]. ROS and reactive nitrogen species are both free radicals that can alter the cardiovascular system, such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$) and peroxynitrate ($ONOO^-$) [101, 102]. The increase in ROS production triggers the release of other inflammatory mediators [103]. When cytokines continue to oxidize NAD(P)H, inflammation will increase [103, 104]. High levels of endothelin-1, angiotensin II (Ang II), cholesterol or glucose can increase the oxidation of NAD(P)H and therefore also stimulate vascular inflammation and ROS production [104].

ROS are associated with DNA damage, aging and age related disease [7]. McCrann et al. found a link between aging and ROS [105]. They found that one of the seven isoforms of NADPH oxidase (NOX), namely NOX4, caused polyploidy in vascular smooth muscle cells [105]. Normal human cells are diploid and contain two sets of homologues chromosomes [106-108]. A polyploidy cell can contain up to 128 sets of chromosomes [106-108]. Polyploidy cells are found with an increase in age [107-108]. The over-expression of NOX4 causes polyploidy through the down regulation of surviving mRNA [108]. Surviving at high levels suppresses cell apoptosis but in low levels it will facilitate in polyploidy [108]. This over-expression of NOX4 is not only found in vascular smooth muscle cells but it is also apparent in endothelial cells [108]. NOX4 is different than other NOX as it has the ability to stimulate peroxide production [109].

2.3.3 TBARS

Another method to establish the presence of oxidative stress, is to measure oxidative stress indirectly by assessing the damage it has caused [110]. Thiobarbituric acid reactive substances (TBARS) use thiobarbituric acid as a reactant to measure lipid peroxidation [111]. TBARS thus measure a by-product of some decomposing primary and secondary lipid peroxidation, namely malondialdehyde (MDA) [112].

MDA is associated with damage to tissue caused by either hypertension or smoking [113, 114]. Inflammation and MDA are also closely associated [115]. Furthermore, MDA is also negatively associated with telomere length and it has also been shown that MDA is significantly higher in the elderly when compared to children [114, 116].

The measurement of TBARS therefore is a reliable way of establishing if low chronic inflammation or oxidative damage is present in tissue.

2.3.4 eNOS uncoupling

Preventing the uncoupling of eNOS can act as a protective mechanism to decrease oxidative stress in the cardiovascular system [117]. The oxidative stress that mostly accompanies cardiovascular disease is responsible for the reduction of NO by binding it to $O_2^{\cdot-}$ to form $ONOO^-$ [70]. Another mechanism is the uncoupling of eNOS, through the depletion of eNOS co-factors such as tetrahydrobiopterin (BH_4) and L-arginine [118, 119]. It has also been proposed that oxidative damage to the zinc-thiolate cluster can lead to the inability of BH_4 and L-arginine to bind [120]. $ONOO^-$ can oxidize BH_4 to inactive trihydrobiopterin ($BH_3^{\cdot}$) and dihydrobiopterin (BH_2) [120, 121]. It can clearly be observed that a disruption of eNOS through oxidative stress could cause a decrease in NO syntheses in the cardiovascular system [119-120].

The uncoupling of eNOS was also evident in patients that had endothelial dysfunction due to hypercholesterolemia, diabetes, essential hypertension and in chronic smokers [122-125]. NOX was found to be a crucial element in vascular disease and eNOS uncoupling [126].

2.3.5 Nitrosative stress

Nitrosative stress is caused through an imbalance in the production and elimination of oxidants such as reactive nitrogen species (RNS) [127]. Nitrosative stress causes damage to cells [127]. Nitrosative stress is more prone in populations at higher altitudes, due to higher concentrations of free radicals found in blood [128]. When $O_2^{\cdot -}$ increases, NO will bind to $O_2^{\cdot -}$ to form $ONOO^-$ [129]. Thus, it becomes clear that NO declines and nitrosative stress increases if free radicals increase.

Hypertension can also be an indirect result of nitrosative stress [130]. Hypertension is brought about by different factors, but vasoconstriction is one of the main culprits [130]. The main vasodilator is NO [131]. NO should be produced continuously because of its short half-life [60, 61, 66]. NO is synthesized through the oxidation of L-arginine and nitrosative stress can act as a L-arginine NOS inhibitor [60, 61]. In conclusion, nitrosative stress can reduce NO indirectly, leading to vasoconstriction and potentially to hypertension as well [60, 61].

2.3.6 The anti-oxidant enzymatic system

The anti-oxidant system is comprised of various pathways as seen in Fig 2.5. These different pathways are now briefly discussed.

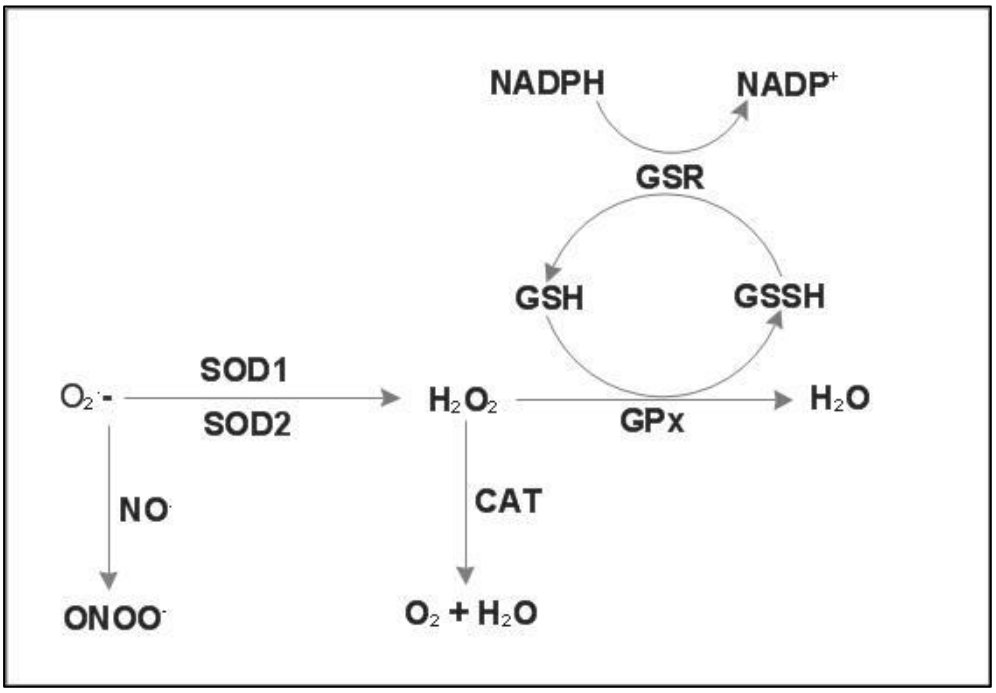

Fig 2.5 Anti-oxidant enzyme system pathways.

Initially $ONOO^-$ is formed when NO^- reacts with $O_2^{\cdot -}$ [129]. This is important in the cardiovascular system because $ONOO^-$ depletes anti-oxidants and therefore increases oxidative stress [122]. Large amounts of $O_2^{\cdot -}$ lead to vasoconstriction and are associated with hypertension [70, 120, 121].

Although NO reacts three times faster with $O_2^{\cdot-}$ than $O_2^{\cdot-}$ with SOD, SOD can still react with $O_2^{\cdot-}$ to form hydrogen peroxide (H_2O_2) [132]. This ability of SOD protects against NO depletion [132]. H_2O_2 is a less potent form of ROS than $ONOO^-$, thus, SOD protects the cardiovascular system by increasing the availability of NO signaling molecules and decreasing oxidative damage caused by $O_2^{\cdot-}$ [133]. Superoxide dismutase (SOD) has three different isoforms [154]. The three isoforms are respectively copper-zinc SOD (SOD-1 or CuZn-SOD), Manganese SOD (SOD-2 or Mn-SOD) and an extracellular copper-zinc SOD (SOD-3 or EC-SOD) [133]. In the cardiovascular system, SOD-1 is the most prominent [135].

The removal of H_2O_2 from the physiological system is aided with the enzyme glutathione peroxidase (GPx) [136]. The tempo at which H_2O_2 is removed by GPx depends on the concentration of reduced glutathione (GSH) [136]. The function of GPx is to act as an enzyme, assisting in the reduction of GSH to oxidized glutathione (GSSG) and thus transforms H_2O_2 to a water molecule [137]. Glutathione- reductase (GR) is an enzyme that reduces GSSG back to GSH, for GSH to function as an anti-oxidant again [138]. Catalase (CAT) is an enzyme that also removes H_2O_2 from the body by converting it into water and oxygen molecules [139].

Oxidative stress is one of the most important contributors to the reduction in telomere length in humans [15,140]. Increased SOD is not only related to decreased oxidative stress, but is also positively related to telomere length through these mechanisms [141]. SOD-3 has a stronger positive association with telomere length than either SOD-1 or SOD-2 [141].

A decrease in the extracellular SOD levels have directly been associated with cardiovascular pathology which includes endothelial dysfunction, hypertension and coronary artery disease [141-143]. NO will stimulate the mobilization of circulating endothelial progenitor cells (EPC) from the bone marrow [141-143]. EPC will then up-regulate SOD production if the vascular system experiences ischemia, injury, tumor formation or hypoxia [136-138]. The up-regulation of SOD through oxidative stress will neutralize $O_2^{\cdot-}$ [141-145].

2.3.7 Human immunodeficiency virus (HIV)

South Africa has the highest prevalence of HIV-infected people [146]. A South African study indicated an association between HIV-infection and acceleration of biological aging and thus shortened telomeres [147]. The pathophysiology of HIV-infection in the accelerated telomere shortening is through increased inflammation and oxidative stress [147, 148].

Oxidative stress is common in HIV-infected individuals due to an imbalance between the production of ROS and the expression of antioxidant enzymes [147, 148]. HIV-infected individuals who use highly active antiretroviral therapy (HAART) are also more prone to oxidative stress and at high risk of cardiovascular disease and increased biological aging [147-149].

People with HIV are shown to have an increase in genetic products that include the HIV-1 transactivator (Tat) [150]. This is directly linked to increased oxidative stress by decreasing both intra- and extracellular GSH and SOD [148, 150].

Infected HIV-cells are shown to have increased oxidative stress, which may lead to DNA damage [151]. The mechanism through which this is established is by inhibiting the telomerase activity and thus decreasing telomere length [152]. The inhibition of telomerase is not only evident in HIV-infected individuals but is also accelerated with the use of HAART [153, 154]. Due to the influence of HIV-infection and the use of HAART, it would be advisable to exclude participants that are infected to avoid confounding.

2.4 Lifestyle, telomere length and nitric oxide

2.4.1 Introduction

The shortening of telomeres and inhibition of telomerase are normal during the aging process [1]. There is no significant difference between male and female new-born babies which suggests that the difference between male and female telomere length is influenced by genetics, sex and environmental exposure after birth [155]. Aging is governed by genetics to a certain extent, but the influence of lifestyle may have significant detrimental effects in the acceleration of biological aging through further decreasing telomerase activity and increasing DNA damage and cardiovascular pathologies [156].

Under the next headings, the effect of several lifestyle behaviors will be discussed in more depth. These include the effects of alcohol and smoking, as well as factors such as dietary intake, physical activity and body composition. While lifestyle behavior can influence the oxidative stress profile, lifestyle factors are not solely responsible for oxidative stress. The effects of lifestyle factors, oxidative stress and NOx on telomere length are also discussed in more detail.

2.4.2 Alcohol intake

Habitual use of alcohol leads to increased oxidative stress and subsequently decreased availability of NO [157]. Chronic alcohol usage, even in small quantities is associated with shorter telomere lengths [158]. Decreased telomere length is associated with increased risk for cardiovascular events, but the use of alcohol is controversial in this regard [159]. Alcohol usage has a concordant J-shaped association with cardiovascular disease [159]. Low-dose daily consumption of alcohol showed to decrease cardiovascular risk more than abstaining from alcohol, binge drinking or drinking two drinks or more per day [159].

2.4.3 Tobacco use

The use of tobacco products, but more specifically cigarette smoking, has a tremendous contribution to the development of cardiovascular disease (CVD) [160]. The number of cigarettes smoked per day is positively associated with increased risk for CVD, but risk declines if smoking is stopped [160]. Incidences of smoking are increasing in developing countries such as South Africa and it is therefore an important factor to consider when investigating cardiovascular disease in this country [161].

Smoking causes telomeres to shorten through chronic exposure to oxidative stress and inflammation [162]. Mirabello et al. investigated telomere length and prostate cancer risk and found a negative association between telomere length and cigarette smoking [29]. They attributed the telomere/smoking association to increased oxidative stress [29].

NO production also decreases in smokers [63]. Barua et al. found that healthy smokers not only had a decrease in NO, they also had lower endothelial dependent vasodilatation [163]. The reason for the

decreased NO might be explained by the binding to $O_2^{\cdot-}$, but according to Barua et al., it was indicated that eNOS activity was decreased even with increased eNOS protein expression [163, 164]. BH4 deficiency is associated with chronic smoking and BH4 acts as co-factor for the formation of NO. It is therefore one of the potential mechanisms for the decreased serum NO in smokers [164]. After smoking a cigarette, the production of ROS increases which may lead to damage in the vascular wall [164-166]. Decreased antioxidant protection may lead to protein peroxidation and may finally activate phagocyte-platelet-endothelial processes that cause vascular wall pathology [165-166].

2.4.4 Body composition

Obesity is associated with impaired NO production by the endothelial cells [167]. The theorized mechanism is through increased free fatty acids found in overweight individuals [168]. These free fatty acids (FFA) increase oxidative stress and inflammation, which in turn can reduce NO levels and eNOS activity [168, 169]. Obese women have a higher rate of telomere shortening than their counterparts with normal body weight [170]. Higher abdominal adiposity is inversely associated with telomere length [171].

2.4.5 Physical activity

Moderate physical activity has a higher protective function on telomere length than either being inactive or having high energy expenditures [172]. Jakob et al. also found, in a study on twins, that being physically active in leisure time, is positively associated with telomere length when compared to their more sedentary counterparts [173]. Moderate exercise not only has a positive effect on maintaining telomere length, but it also increases NO production [173, 174]. Elderly individuals who are physically active restore NO levels, decrease oxidative stress and partially prevent age dependent endothelial dysfunction [174].

2.5 Hypotheses, aims, objectives

2.5.1 Hypotheses

Based on the literature, the hypotheses are:

Black individuals have shorter telomeres, lower NO metabolite levels, unfavorable oxidative stress, inflammatory profiles and higher ambulatory BP, than their white counterparts.

Leukocyte telomere length is positively associated with NO metabolites in both black and white participants.

2.5.2 Aim of this study

The aim of this study was to investigate the relation between leukocyte telomere length and NO metabolites of black and white individuals from the SABPA-study.

2.5.3 Detailed objectives

To determine whether ethnic differences exist in telomere length, NO metabolites, markers of oxidative stress (glutathione peroxidase, superoxide dismutase, thiobarbituric acid reactive substance, serum reactive oxygen species), inflammation (interleukin-6, tumor necrosis factor, c-reactive protein) and ambulatory BP in a bi-ethnic sample.

To investigate the relationship between telomere length and NO metabolites and whether ethnic differences exist.

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Chapter 3

Detailed methodology

Methodology of the SABPA study

Chapter 3: Detailed Methodology

3.1 Introduction

Our study on telomere length was done as a sub-study of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA). We used a cross-sectional design of the follow-up phase of the SABPA study.

Only measurements relating to this particular sub-study of the SABPA study will be discussed. A complete outlay of the methodology for the SABPA study is further explained in a recently published article [1].

3.2 Organizational procedures and ethical considerations

Interviews and meetings were held with, and permission granted by, the North-West Department of Education, the South African Democratic Teachers' Union, and school headmasters before commencing with recruitment of school teachers as study participants. From Mondays – Fridays every day for 10 – 12 weeks, a maximum of 5 participants per day participated in the study.

Participants were asked to wear an ambulatory blood pressure apparatus for 24 hours from the morning of day 1 (fitted at their place of work) until the morning of day 2. For the overnight stay at the Metabolic Unit, each participant had their own private room.

The details of the project were discussed with all voluntary participants in their home language, i.e. the exact objectives of the study, what procedures they would have been subjected to, what was expected from each of them (e.g. they were involved for 2 days). A standard participant information sheet was given to the participants at their recruitment/screening visit, and willing participants were requested to sign the informed consent form approved by the Research Ethics Committee of the North-West University, thus complying with the guidelines of the World Medical Association Declaration of Helsinki [3].

Pre- and post-counselling were performed for HIV-tests. Importantly, participants were informed about participant confidentiality and anonymity, prevailing during and after the execution of the SABPA project.

Possible discomfort or pain experienced by participants:

1. Minimal discomfort and, for some participants, disrupted sleep with the ambulatory blood pressure measurements.

2. Slight discomfort or pain during the collection of resting blood samples.

3.3 Facilities

The Metabolic Unit Research Facility of the North-West University is a research unit for human studies and equipped with 10 well-furnished bedrooms, a kitchen, two bathrooms and a television room.

3.4 Study design and participants

The SABPA study started in 2008/2009 and the 3-year follow up phase was conducted in February to May of 2011 and 2012. All participants taking part in the baseline phase were then re-invited to take part in the follow-up phase of the study. The study had a successful follow-up with 87.8% of participants returning. A total of 173 black and 186 white school teachers (aged 23 – 68 years) from the Dr Kenneth Kaunda Education district of the North West Province in South Africa, took part in this phase of the study.

Exclusion criteria for the initial study was the following: pregnancy, lactation, body temperature exceeding 37ºC, users of alpha- and beta blockers and blood donation and/or vaccination in the 3 months prior to commencement of the study. For our sub-study, HIV-infected participants were additionally excluded. After the exclusion, a total of 338 participants, 152 black (75 men and 77 women) and 186 white (90 men and 96 women) were considered eligible for our sub-study. All participants signed an informed consent form and were fully informed about the objectives and procedures of the study prior to the measurements. The study complied with all applicable requirements of the US and international regulations, in particular the Helsinki declaration of 1975 (as revised in 2008) for the investigation of human participants. Ethical clearance for the SABPA study was obtained from the Health Research Ethics Committee of the North-West University (NWU-00036-07-S6), and for this specific sub-study (NWU-00036-07-A6) [Appendix B].

The two-day procedure commenced on a normal working day between 07h00-08h00 where an ambulatory blood pressure monitoring (ABPM) device was attached to the participant's non-dominant arm. They then proceeded with their daily routine or work schedule.

At approximately 14h00 participants were transported to the North-West University. Between 17h00 and 18h00 participants were taken to the Metabolic Unit research facility of the North-West where they completed general health questionnaires. Before 20h30 they received a standardized dinner as well as a last beverage, coffee/tea and two biscuits. Thereafter they were asked to relax and to refrain from alcohol, smoking, caffeine or exercise. Participants were encouraged to go to sleep at 22h00. Participants were woken at 06h00 the following morning where the ABPM device was removed and further measurements were performed. A registered nurse obtained a blood sample.

3.5 Questionnaires

General health and demographic questionnaires were completed by participants.

3.6 Anthropometric measurements

Calibrated instruments were used to measure the participants' height (stature) (cm), weight (kg) and waist circumference (WC) (cm) whilst being in their underwear (Invicta Stadiometer, London; United Kingdom; IP 1465, Precision Health Scale, A&D Company, Tokyo, Japan; Holtain unstretchable flexible 7mm wide metal tape, Crosswell, Wales). WC was measured over the costal margin and the iliac crest. Body mass index (BMI) was calculated (kg/m^2). All measurements were performed by registered anthropomotrist and were done in triplicate per standard operating procedures.

3.7 Biochemical analyses

A registered nurse obtained fasting blood samples, with a sterile winged infusion set from the antebrachial vein branches. Fasting glucose, cholesterol, serum high density lipoprotein (HDL) cholesterol, high sensitivity C-reactive protein (CRP), gamma-glutamyl transferase (GGT), creatinine and triglyceride levels were determined with a sequential multiple analyzer (Cobas Integra 400 plus, Roche, Basel, Switzerland). A turbidimetric inhibition immunoassay was used to determine the percentage of glycated hemoglobin (HbA1c) in EDTA whole blood (Cobas Integra 400 plus, Roche, Basel, Switzerland). HIV-status was determined with the First response HIV card Test 1-2.0 (PMC medical, India Pvt Ltd) and confirmed by means of the Pareekshak HIV triline test (UCB Pharma, India). Serum cotinine levels were determined with a homogeneous immunoassay (Automated Modular, Roche, Basel, Switzerland) [2]. Interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) were analyzed using high sensitivity enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN USA).

NO metabolites (NOx) were estimated in plasma samples (R&D systems, MN, USA) on a microplate reader (Bio-Tek, Instruments, Inc., Highland Park, Winooski, VT, USA). This method is based on the enzymatic conversion of nitrate by nitrite reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Gries reaction. Inter-assay and intra-assay variability were 10.9% and 5.5%, respectively. L-homoarginine, asymmetric dimethyl arginine (ADMA), and symmetric dimethyl arginine (SDMA) were determined by a fully validated high-throughput liquid chromatography tandem mass spectrometry based method [3]. L-Citrulline was determined with an electrospray ionization tandem mass spectrometry method as previously described [3].

The following measurements form part of the oxidative stress profile. One of the measurable ROS, namely serum peroxides were determined, where 1,0 mg/L H_2O_2 represents one unit of ROS (Bio-Tek, Instruments, Inc., Highland Park, Winooski, VT, USA) [37]. Glutathione peroxidase (GPx), and superoxide dismutase (SOD) activities were determined with assay kits from Cayman Chemical Company (Ann Arbor, MI). Thiobarbituric acid reactive substances (TBARS) a marker of lipid peroxidation, was determined spectrophotometrically [4]. All these assays were performed on a Synergy multimode microplate reader (BioTek, Winooski, VT, USA).

3.8 Measurement of telomere length

The extraction of genomic DNA was done with the NucleoSpin 96 Blood Core kit (Machery Nagel, Duren, Germany) and stored at - 20^0C. Collected samples underwent dilution to a concentration of 10 ng/kl. In the preparation phase of the DNA samples, a combination of isolated DNA samples was combined in equivalent sizes, thus representing the average of all analyzed participants. Multiplex quantitative real-time polymerase chain reaction (Q-PCR) was used to assess leukocyte telomere length. Q-PCR have various advantages for measuring telomere length, it is a method with high screening capacity, is automated to a large degree, has the capacity to perform measurements on small amounts of DNA and are relatively quick [5]. A total of five concentrations of a reference DNA sample covering a 75-fold range of DNA concentrations were prepared by serial dilution and analyzed in triplicate. DNA samples were assayed three times with an average coefficient of 16.4%. The intrabatch coefficient of variation was 4.3% and the interbatch coefficient of variation was 4.8%. The CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) was used to perform all PCRs. PCR reactions contained a 20 Kl master mix (including 5 Kl of 5_ HOT FIREPol EvaGreen qPCR Mix Plus (no ROX; Solis BioDyne, Tartu, Estonia) and 5 Kl of each experimental DNA sample. For multiplex Q-PCR, the telomere primer pair telg was joined with primers for the single copy gene human A-globin (hbg) namely hbgu and telc was joined with primers for the single copy gene human A-globin (hbg) namely hbgd to give PCR products of 79 and 106 base pairs, respectively. The thermal cycling profile occurred as follows: Stage 1, 15 minutes at 95^0C; Stage 2, 2 cycles of 15 seconds at 94^0C and 15 seconds at 49^0C; and Stage 3, 32 cycles of 15 seconds at 94^0C, 10 seconds at 62^0C, 15 seconds at 73^0C with signal acquisition, 10 seconds at 84^0C, and 15 seconds at 87^0C with signal acquisition. The readings at 73^0C established Ct values that were used for amplification of the telomere template and the 87^0C readings gave values to Ct for the amplification of the hbg template. The relative leukocyte telomere length per cell was calculated by using the Relative Expression Software Tool (REST) software as the ratio (T/S) between relative content of telomere PCR product (T) and hbg PCR product (S) [6, 7].

3.9 Cardiovascular measurements

Ambulatory blood pressure measurements (Cardiotens CE120®, Meditech, Budapest, Hungary) were determined within 30 minute intervals during the day (08h00-22h00) and every hour during the night (22h00-06h00). The cuffs and Cardiotens CE120® device were attached to the participants' non-dominant arm and hip respectively. Successful inflation rate in a 24h period was 85% for the black and 92% for the white participants. It was expected of the participants to proceed with their daily routine and activities but to note any discomforts or abnormalities. The data was analyzed using Cardio Visions 1.9.0 Personal Edition software. Twenty-four-hour ambulatory blood pressure values exceeding 130/80 mmHg were classified as hypertensive [8].

3.10 Data analyses

Statistica version 13 (Statsoft Inc., Tulsa, OK, USA) was used to perform the statistical analyses of this study. The central tendency and spread were expressed as the arithmetic mean and standard deviation, for normal distributed data. Normal distribution of skew data was achieved by logarithmic transformation (NOx, SDMA, TBARS, SOD, CRP, HbA1c, TG, cotinine and γ-GT). The central tendency and spread of logarithmically transformed variables were expressed as the geometric mean and the 5th and 95th percentile intervals. Interaction of ethnicity and sex on the relationship between NOx and leukocyte telomere length were tested using multiple regression analyses. Comparisons of black women with white women and black men with white men were done with T-tests, while proportions were compared with Chi-square tests. Partial correlations were performed to evaluate the associations between telomere lengths and NOx levels while adjusting for age. Multiple regression analyses were performed in order to evaluate the independent associations of telomere length with NOx and oxidative stress markers in the bi-ethnic groups taking into account age, waist circumference, total cholesterol, HbA1c, γ-GT, TNF-α, SDMA, and systolic BP. We also considered the other variables presented in Table 1 for inclusion in the regression model, but the final list of variables included in the models indicated the strongest bivariate association with the dependent and main independent variables. Other variables such as anti-hypertensive drugs and other medicines did not have a statistical influence on results, and have therefore not be included.

3.11 My experience in the collection of data

As part of the Hypertension in African Research Team (HART) I gained clinical research experience and apply good clinical practice in a clinical research environment. I also received training on and gained experience with different measurement devices. I performed glucose, HbA1c, blood type, cholesterol and blood pressure measurements. I also assisted in gathering questionnaire data, and anthropometric measurements. Further I assisted with detailed cardiovascular assessments that were both included and excluded in my sub-study. Included measurements used in the sub-study was ambulatory blood pressure measurement. Measurements that I have been trained on and assisted with, but not used in my sub-study was the Finometer (Finapres Medical Systems, Amsterdam, the Netherlands) to monitor changes in blood pressure, heart rate and blood flow when exposing participants to stressors such as the cold pressor test and the color word conflict test. I was also trained and worked with the Sphygmocor device (AtCor Medical Pty. Ltd., Sydney Australia) to assess pulse wave velocity.

3.12 References

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Chapter 4

Research Article

Leukocyte telomere length and its relation to nitric oxide metabolites in a bi-ethnic sample: the SABPA study

Leukocyte telomere length and its relation to nitric oxide metabolites in a bi-ethnic sample: the SABPA study

Running title: Leukocyte telomere length, nitric oxide metabolites and oxidative stress

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Abstract:

Objectives: Shorter leukocyte telomere length is associated with increased cardiovascular risk and decreased nitric oxide (NO) bioavailability. These factors are also linked with increased oxidative stress and inflammation. Telomere length, NO metabolites (NOx), blood pressure, oxidative stress and inflammatory markers in a bi-ethnic population were compared. The associations of telomere length with NOx and markers of oxidative stress and inflammation were further investigated.

Methods: 152 black and 186 white teachers, aged between 23 and 68 years were included in this cross-sectional study and ambulatory blood pressure was measured. Leukocyte telomere length, NOx and glutathione peroxidase (GPx), as a marker of oxidative stress, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) as inflammatory markers were analyzed.

Results: Black men and women had higher blood pressure ($p < 0.001$), higher IL-6 ($p \leq 0.016$) and shorter telomeres ($p < 0.001$) but similar NOx levels than their white counterparts. GPx activity was higher in black compared to white groups ($p \leq 0.002$). Independent positive associations of telomere length with NOx (adj $R^2 = 0.21$; $\beta = 0.249$; $p = 0.03$) and GPx activity (adj $R^2 = 0.21$; $\beta = 0.225$; $p = 0.03$) were indicated in white men, while telomere length was independently associated with TNF-α (adj $R^2 = 0.33$; $\beta = 0.274$; $p = 0.033$) in white women. These associations were absent in the black groups.

Conclusion: Telomere length of black men and women was not associated with NOx or markers of oxidative stress, as observed in white men. The less favorable cardiovascular and inflammatory profiles of black participants were unrelated to shorter telomere lengths. Further investigations on early life exposures as potential contributor to shorter telomere length in the black population are proposed.

Keywords: Telomere length, nitric oxide, blood pressure, cardiovascular, aging, ethnicity, glutathione peroxidase, oxidative stress, black, African.

Introduction

It is a well-known phenomenon that aging accompanies cardiovascular disease (CVD) [1]. Recently, the emphasis has shifted towards early vascular aging and biological aging which are more indicative of cardiovascular risk [2]. Telomere length has emerged as a good indicator of biological age [3]. Telomeres are composed of nucleoproteins found on the terminals of eukaryotic chromosomes and have repetitive TTAGGG structures over thousands of base pairs [4, 5]. The function of telomeres is to protect chromosomes during the DNA replication process and with each cell division, telomeres are shortened [4, 5]. The shortening of telomeres is counteracted by increased telomerase activity which prevents loss of base pairs [6].

In different disease states as well as ethnicity and sex groups, telomere length has been differentially associated with CVD [7-12]. Various studies linked telomere shortening to CVD, cardiovascular events and CVD comorbidities such as heart failure, coronary heart disease, stroke, myocardial infarction and diabetes mellitus [7-10, 12]. A systematic review by Haycock et al. indicated an inverse association between telomere length and coronary heart disease, independent of the conventional cardiovascular risk factors [13]. Men with lower blood pressure have a slower rate of telomere shortening [14, 15]. Obese men have shorter telomeres which was associated with thickening of the carotid wall, although, in the general population telomere length was not associated with increased carotid intima media thickness or atherosclerosis [14-16].

Controversy exists regarding telomere length when comparing black and white populations. African Americans have longer telomeres than white Americans whereas other studies found contradictory results between black and white participants [16-18]. When comparing white women with their African American counterparts, coronary heart disease was significantly associated with shorter telomere length only in the white women [11]. Studies also indicated that certain black populations may have lifestyles favoring oxidative stress and accelerated telomere shortening through inhibition of telomerase activity [18-19].

Apart from the link between telomere length and cardiovascular health, the capacity of the vascular system to produce NO is also important to maintain cardiovascular health [20]. This is demonstrated by its effects on vasodilatation, inhibition of platelet aggregation, angiogenesis and contractility of the heart [21, 22]. Acceleration in telomere shortening and decreased telomerase activity are evident when NO bioavailability decreases [23]. Low telomerase activity exists in endothelial cells and when telomerase activity decreases with a decrease in NO bioavailability, it may lead to endothelial cell senescence [24, 25].

Studies such as the Transition and Health during Urbanisation of South Africans (THUSA), the INTERHEART African study, the Prospective Urban and Rural Epidemiology (PURE) study, the Heart of Soweto study and the African-PREDICT study yielded similar results with regards to the profound CVD vulnerability in the black South African population [26-31].

This was also shown in the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study, indicating higher ambulatory blood pressure, C-reactive protein (CRP) levels, alcohol abuse and shorter telomere lengths in black participants [32-34]. Results from the SABPA study also indicated an inverse association between a marker of endothelial damage, Von Willebrand factor (vWf) and shorter telomere length, in white, but not in black South Africans [34]. With shorter telomeres and less favorable cardiovascular profiles, these black individuals are clearly at higher risk for CVD, even though no vascular association with telomere length could thus far be established in the black participants of this study [32-34]. Additional investigations are therefore needed to discover the factors associated with shorter telomere length in the black population. The study aimed to investigate the relationship between telomere length and NOx in a black and white South African cohort, while factoring in other aspects that may affect telomere length such as oxidative stress and inflammation.

Methods

Study population

This study forms part of the follow-up phase of the SABPA study, a prospective cohort study. The initial phase of the study in 2008 and 2009 included a total of 409 participants. Initial exclusion criteria for the study was pregnancy, lactation and blood donation or vaccination three months prior to the commencement of the study. The follow-up phase of the SABPA study was conducted between February and May of 2011 and 2012. A total of 359 school teachers from the North West Province in South Africa participated in the follow-up phase of the study. Only 338 participants were included in this sub-study as additional exclusion criteria were applied, including human immunodeficiency virus (HIV) infection, since HIV-infection is associated with accelerated telomere shortening. Data of the follow-up phase was analyzed cross-sectionally for this sub-study as telomere lengths were not determined during the baseline phase. The detailed protocol of this study was described elsewhere [35].

Ethical considerations

Participants were fully informed about the objectives and procedures of the study prior to their inclusion. This study fulfilled all the necessary international regulations and the declaration of Helsinki for investigation on human participants. The study was approved by the Health Research Ethics Committee (HREC) of the North West University (Potchefstroom campus).

Questionnaire data collection

A general health questionnaire was completed by participants with the assistance of trained fieldworkers. This questionnaire included questions on lifestyle behaviors, medication usage and socio-economic status.

Anthropometric measurements

The height and weight of participants were measured while wearing only underwear. Measurements were taken in triplicate using standard methods with calibrated instruments (Precision Health Scale, A & D Company, Tokyo, Japan; Invicta Stadiometer, IP 1465, London, UK). Waist circumference (WC) was measured using a Holtain non-stretchable tape. All measurements were taken to the nearest 0.1cm or 0.1kg respectively. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Blood pressure measurements

An ambulatory blood pressure monitoring (ABPM) apparatus (Meditech CE120® Cardiotens; Meditech, Budapest, Hungary) was attached to the participants' non-dominant arm at their workplace. It was programmed to measure blood pressure at 30-minute intervals during the day (08:00–22:00) and every hour during night-time (22:00–06:00). Blood pressure data was downloaded onto a database using the CardioVisions 1.9.0 Personal Edition software (Meditech, Budapest, Hungary).

Blood sampling and biochemical analysis

A registered nurse obtained a blood sample from fasting participants with a sterile winged infusion set from the ante-brachial vein branches. Serum and plasma were prepared according to standard procedures.

NOx wes estimated in plasma samples (R&D systems, MN, USA) on a microplate reader (Bio-Tek, Instruments, Inc., Highland Park, Winooski, VT, USA). This method is based on the enzymatic conversion of nitrate by nitrite reductase. The reaction is followed by colourometric detection of nitrite as an azo dye product of the Gries reaction. Inter-assay and intra-assay variability were 10.9% and 5.5%, respectively. L-homoarginine, asymmetric dimethyl arginine (ADMA) and symmetric dimethyl arginine (SDMA) were determined by a fully validated high-throughput liquid chromatography tandem mass spectrometry based method [36]. L-Citrulline was determined with an electrospray ionization tandem mass spectrometry method as previously described [36].

Extraction of leukocyte genomic DNA was done with the NucleoSpin 96 Blood Core Kit (Machery Nagel, Düren, Germany) and stored at a temperature of -20°C. DNA samples were assayed in triplicate. A detailed description of telomere length measurement in this study was described elsewhere [34].

A sequential multiple analyzer (Integra 400; Roche, Basel, Switzerland) was used to analyses glycated hemoglobin (HbA1c) (EDTA whole blood), glucose (sodium fluoride plasma) and serum γ-glutamultransferase (γ-GT), high sensitivity C-reactive protein (CRP), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglycerides (TG). Interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) were analyzed using high sensitivity enzyme linked immunosorbent assays (R&D Systems, Minneapolis, MN USA).

The following measurements formed part of the oxidative stress profile. One of the measurable ROS, namely serum peroxides were determined, where 1,0 mg/L H_2O_2 represents one unit of ROS (Bio-Tek, Instruments, Inc., Highland Park,Winooski, VT, USA) [37]. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were determined with assay kits from Cayman Chemical Company (Ann Arbor, MI). Thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation, was determined spectrophotometrically [38]. All these assays were performed on a Synergy multimode microplate reader (BioTek, Winooski, VT, USA).

Statistical Analysis

Statistica version 13 (Statsoft Inc., Tulsa, OK, USA) was used to perform the statistical analyses of this study. The central tendency and spread were expressed as the arithmetic mean and standard deviation for normal distributed data. Normal distribution of skew data was achieved by logarithmic transformation (NOx, SDMA, TBARS, SOD, CRP, HbA1c, TG, cotinine and γ-GT). The central tendency and spread of logarithmically transformed variables were expressed as the geometric mean and the 5^{th} and 95^{th} percentile intervals. Interaction of ethnicity and sex on the relationship between NOx and leukocyte telomere length were tested using multiple regression analyses. Comparisons of black women with white women and black men with white men were done with T-tests, while proportions were compared with Chi-square tests. Partial correlations were performed to evaluate the associations between telomere lengths and NO metabolite levels while adjusting for age. Multiple regression analyses were performed in order to evaluate the independent associations of telomere length with NOx and oxidative stress markers in the bi-ethnic groups taking into account age, waist circumference, total cholesterol, HbA1c, γ-GT, TNF-α, SDMA and systolic BP. The other variables presented in Table 1 were also considered for inclusion in the regression model, but the final list of variables included in the models indicated the strongest bivariate association with the dependent and main independent variables.

Results

A significant interaction of ethnicity ($p = 0.042$) and a borderline interaction of sex ($p = 0.052$) were indicated for the relationship between NOx and leukocyte telomere length. The groups were divided in accordance to these interactions into different sexes stratified by ethnicity.

The characteristics of the participants in this study are shown in Table 1. The mean age of black men was approximately 3 years younger than the white men ($p = 0.042$), but telomere length was shorter in black men and women when compared to their white counterparts ($p < 0.001$). With regards to variables related to NO metabolism, both black men and women had respectively higher L-homoarginine ($p < 0.001$; $p = 0.046$), but lower L-citrulline ($p < 0.001$; $p < 0.001$) than their white counterparts. With regards to the endogenous NO inhibitors, SDMA and ADMA, black men had lower SDMA than white men ($p = 0.004$) while black women had higher ADMA than white women ($p = 0.009$). The black groups had higher systolic

and diastolic blood pressure as well as higher pulse pressure for both sexes compared to the white groups ($p \leq 0.014$). Despite the higher GPx activity ($p = 0.001$) and SOD activity ($p = 0.047$), black men also had higher TBARS ($p = 0.035$), a marker of lipid peroxidation, when compared to white men. Black women also had higher GPx activity when compared to the white women ($p = 0.002$). The inflammatory markers, CRP and IL-6 levels were higher in black men and women when compared to their white counterparts ($p \leq 0.016$), while TNF-α was lower in black women than white women ($p = 0.003$).

In partial regression analyses, positive correlations of telomere length with NOx were found only in the white participants (white men: $r = 0.33$; $p = 0.002$ and white women: $r = 0.25$; $p = 0.02$). In the white participants, the telomere length was also positively associated with SDMA (white men: $r = 0.24$; $p = 0.02$ and white women: $r = 0.26$; $p = 0.02$) and GPx activity (white men: $r = 0.39$; $p = 0.007$ and white women: $r = 0.23$; $p = 0.02$) (Tabel 2). In the same groups, telomere length was negatively associated with TNF-α (white men: $r = -0.21$; $p = 0.05$ and white women: $r = -0.34$; $p = 0.001$). No associations were found in black men, while in the black women, telomere length associated with SDMA ($r = 0.29$, $p = 0.02$) was found.

In the multiple regression analyses (Table 3), independent associations of telomere length with NOx ($R^2 = 0.21$; $\beta = 0.249$; $p = 0.030$), age ($\beta = -0.306$; $p = 0.004$), GPx ($\beta = 0.229$; $p = 0.030$) and SDMA ($\beta = 0.225$; $p = 0.046$) were indicated in white men. For white women, telomere length associated independently with age ($R^2 = 0.33$; $\beta = -0.433$; $p < 0.001$), waist circumference ($\beta = -0.306$; $p = 0.016$), TNF-α ($\beta = -0.249$; $p = 0.010$) and SBP ($\beta = 0.274$; $p = 0.033$). Black women had an unexpected positive association between telomere length and HbA1c ($R^2 = 0.13$; $\beta = 0.348$, $p = 0.017$). Additionally, we investigated, NOx, TNF-α, GPx and SDMA independently by adding it to separately to the MR models. Similar findings were found in black women (Tabel S1), as in white women, regarding the association of telomere length with age ($\beta = -0.279$; $p = 0.127$.) and waist circumference ($\beta = -0.329$; $p = 0.021$). With regards to this independent variable no other significant differences were found with the above reported results although the association of telomere length and TNF became borderline significant. No other consistent associations with telomere length were evident. Various multiple regression models were tested, considering different combinations of covariates, stratified with and without sex for both the bi-ethnic populations and the results remained robust. The researchers were unable to find significant associations between telomere length and any of the covariates (including age) tested in the black men.

Table 1 Characteristics of men and women stratified by ethnicities

	Men			Women		
	Black	**White**	**P**	**Black**	**White**	**P**
Number of participants	75	90		77	96	
Age (years)	46. 3 ± 7.92	49.2±10.2	**0.042**	49.0 ± 8.17	49.9 ± 9.62	0.498
Anthropometric measurements						
Body mass (kg)	84.1 ± 17.7	99.7 ± 17.5	**<0.001**	82.7 ± 18.3	75.5 ±19.7	**0.014**
Body mass index (kg/m^2)	28.6 ± 5.64	30.2 ± 5.14	0.058	33.3 ± 6.62	27.4 ± 6.85	**<0.001**
Waist circumference (cm)	99.1 ± 15.4	106 ± 13.1	**0.002**	97.8 ± 16.2	87.0 ± 14.4	**<0.001**
Cardiovascular measurements						
Ambulatory systolic BP (mmHg)	139 ± 18.1	128 ± 10.7	**<0.001**	132 ± 17.9	119 ± 11.0	**<0.001**
Ambulatory diastolic BP (mmHg)	86.9 ± 10.7	79.4 ± 7.63	**<0.001**	79.6 ± 9.79	72.0 ± 7.17	**<0.001**
Ambulatory pulse pressure (mmHg)	51.7 ± 10.1	48.5 ± 6.33	**0.014**	52.8 ± 10.7	47.1 ± 7.70	**<0.001**
Ambulatory heart rate (bpm)	79.1 ± 10.7	72.0 ± 10.5	**<0.001**	78.6 ± 9.85	74.4 ± 9.18	**0.003**
Telomere length (T/S)	0.84 ± 0.19	1.01 ± 0.23	**<0.001**	0. 83 ± 0.17	1.11 ± 0.29	**<0.001**
Nitric oxide metabolism						
Nitric oxide metabolites (µmol/L)	8.54 [1.23; 36.9]	10.7 [2.08; 129]	0.281	12.8 [2.18; 59.7]	12.9 [2.08; 97.5]	0.955
L-Homoarginine (µmol/L)	4.15 ± 1.74	3.31 ± 1.01	**<0.001**	3.60 ±1.37	3.17 ± 1.35	**0.046**
L-Citrulline (µmol/L)	27.6 ± 8.02	51.0 ± 14.1	**<0.001**	24.9 ±8.99	49.5 ± 12.9	**<0.001**
Asymmetric dimethyl arginine (µmol/L)	0.60 ± 0.12	0.61 ± 0.10	0.803	0.64 ± 0.14	0.58 ± 0.14	**0.009**
Symmetric dimethyl arginine (µmol/L)	0.33 [0.24; 0.52]	0.36 [0.26; 0.51]	**0.004**	0.32 [0.23; 0.49]	0.33 [0.22; 0.44]	0.557
Oxidative stress markers						
TBARS (mg/g)	0.11 [0.02; 0.45]	0.08 [0.03; 0.18]	**0.035**	0.11 [0.02; 0.44]	0.10 [0.03; 0.23]	0.305
Serum reactive oxygen species (units)*	73.3 ± 22.2	75.1 ± 17.4	0.552	87.6 ± 30.9	85.4 ± 22.0	0.590
Glutathione peroxidase (nmol/min/mL)	36.8 ± 16.3	30.5 ± 6.72	**0.001**	36.7 ± 10.7	32.5 ± 6.78	**0.002**
Superoxide dismutase (U/mL)	2.74 [0.08; 8.17]	2.09 [0.41; 5.67]	**0.047**	1.85 [0.37; 5.80]	2.42 [0.35; 9.13]	0.608
Inflammation markers						
Interleukin–6 (pg/mL)	1.53 [0.50; 7.12]	1.04 [0.36; 4.77]	**0.016**	1.62 [0.59; 3.54]	0.89 [0.23; 3.76]	**<0.001**
Tumor necrosis factor-α (pg/mL)	2.74 ± 2.70	2.58 ± 1.77	0.637	2.13 ± 1.74	2.90 ± 1.59	**0.003**
C-reactive protein (mg/L)	2.75 [0.29; 28.4]	1.16 [0.17; 28.4]	**<0.001**	4.97 [0.78; 17.7]	1.15 [0.14; 10.2]	**<0.001**
Metabolism markers						
Glucose	6.76 ± .81	6.04 ± 1.34	**0.033**	6.04 ± 1.28	5.60 ± 0.512	**0.002**
Glycated hemoglobin, %	6.18 [5.24; 10.6]	5.65 [5.01; 7.33]	**<0.001**	5.97 [5.23; 7.89]	5.47 [5.09; 6.04]	**<0.001**
Total cholesterol (mmol/L)	4.74 ± 1.05	4.20 ± 1.00	**0.001**	4.52 ± 1.01	44.4 ± 1.08	0.590
HDL-cholesterol (mmol/L)	0.96 ± 0.35	0.85 ± 0.23	**0.018**	1.08 ± 0.29	1.26 ± 0.23	**<0.001**
Triglycerides (mmol/L)	1.43 [0.60; 3.94]	1.22 [0.62; 2.96]	**0.048**	0.95 [0.46; 2.10]1	0.84 [0.38; 2.22]	0.106
Lifestyle factors						
Self-reported current smoking, n (%)	17 [22.7]	14 [15.6]	0.244	5 [6.50]	23 [9.38]	0.490
Self-reported current alcohol use n, (%)	51 [68.0]	56 [62.2]	0.489	23 [29.9]	28 [29.2]	0.920
Cotinine (ng/mL)	161 [68.0; 450]	193 [52; 532]	0.474	171 [88.0; 463]	161 [82.6; 25 3]	0.811
y-glutamyl transferase (U/L)	55.6 [19.3; 241]	26.8 [9.90; 97.3]	**<0.001**	26.4 [10.8; 98.4]	14.5 [6.00; 41.8]	**<0.001**
Medication						
Anti-hypertensive medication n (%)	24 [32.0]	29 [32.2]	0.976	29 [38.2]	24 [25.0]	0.063
Anti-inflammatory medication n (%)	11 [14.7]	10 [11.1]	0.495	15 [19.5]	13 [13.5]	0.292
Statins (%)	**1 [0.12]**	**21 [0.42]**	**<0.001**	**2 [0.16]**	**12 [0.33]**	**0.017**

Data are arithmetic mean ± SD or geometric mean (5th and 95th percentile intervals) for logarithmically transformed variables. N, number of participants; HDL-cholesterol, high-density lipo-protein cholesterol; T/S, telomere/single-copy gene ratio; TBARS, Thiobarbituric acid reactive substances; * 1 Unit = 1mg/L H_2O_2

Table 2
Partial correlation analyses of leukocyte telomere length with various biological markers stratified by sex and ethnicity. Adjusted for age.

	Leukocyte telomere length			
	Men		Women	
	Black (n=71)	**White (n=89)**	**Black (n=70)**	**White (n=92)**
Body mass index (Kg/m^2)	r = 0.12	r = - 0.09	r = - 0.15	r = - 0.14
	p = 0.34	p = 0.41	p = 0.20	p = 0.18
Ambulatory systolic BP (mmHg)	r = 0.07	r = - 0.04	r = 0.18	r = 0.13
	p = 0.57	p = 0.75	p = 0.14	p = 0.24
Nitric oxide metabolites (µmol/L)	r = - 0.20	**r = 0.33**	r = - 0.05	**r = 0.25**
	p = 0.13	**p < 0.01**	p = 0.70	**p = 0.02**
L-Citrulline (µmol/L)	r = 0.15	r = 0.06	r < 0.01	**r = 0.23**
	p = 0.23	p = 0.56	p = 0.98	**p = 0.03**
L-Homoarginine (µmol/L)	r = - 0.13	r = 0.03	r = 0.01	r = 0.09
	p = 0.28	p = 0.80	p = 0.97	p = 0.40
Asymmetric dimethyl arginine (µmol/L)	r = 0.11	r = - 0.02	r = 0.18	r = - 0.08
	p = 0.37	p = 0.85	p = 0.13	p = 0.45
Symmetric dimethyl arginine (µmol/L)	r = 0.12	**r = 0.24**	**r = 0.29**	**r = 0.26**
	p = 0.33	**p = 0.02**	**p = 0.02**	**p = 0.02**
Glutathione peroxidase (nmol/min/mL)	r = - 0.20	**r = 0.29**	r = - 0.01	**r = 0.23**
	p = 0.10	**p < 0.01**	p = 0.95	**p = 0.03**
TBARS (mg/g)	r = 0.23	r = - 0.09	r = - 0.01	r = - 0.16
	p = 0.06	p = 0.42	p = 0.91	p = 0.13
Reactive oxygen species (mg/L H_2O_2)	r = 0.10	r = - 0.04	r = 0.10	r = 0.06
	p = 0.42	p = 0.71	p = 0.41	p = 0.55
Superoxide dismutase (U/mL)	r = - 0.06	r = 0.13	r = 0.12	r = - 0.03
	p = 0.61	p = 0.26	p = 0.33	p = 0.81
Tumor necrosis factor-α (pg/mL)	r = 0.03	**r = - 0.21**	r < - 0.001	**r = - 0.34**
	p = 0.83	**p = 0.05**	p = 1.00	**p = 0.001**
Interleukin-6 (pg/mL)	r = 0.04	r = 0.02	r = - 0.14	r = - 0.16
	p = 0.73	p = 0.87	p = 0.24	p = 0.12
suPAR (ng/mL)	r = - 0.14	r = 0.09	r = - 0.06	r = - 0.05
	p = 0.24	p = 0.39	p = 0.62	p = 0.67
C-reactive protein (mg/L)	r = 0.10	r = 0.01	r = - 0.02	**r = - 0.25**
	p = 0.39	p = 0.90	p = 0.89	**p = 0.02**
Endothelin-1 (pg/mL)	r = - 0.11	r = - 0.41	r = 0.02	r = - 0.14
	p = 0.29	p = 0.72	p = 0.87	p = 0.17
Glycated hemoglobin, %	r = 0.13	r = 0.40	**r = 0.28**	r = - 0.01
	p = 0.29	p = 0.72	**p = 0.02**	p = 0.96
HDL-cholesterol (mmol/L)	r = - 0.09	r = 0.04	r = 0.14	r = - 0.03
	p = 0.45	p = 0.22	p = 0.24	p = 0.77
Cotinine (ng/mL)	r = - 0.11	r = 0.47	r = 0.21	r = - 0.39
	p = 0.69	p = 0.17	p = 0.65	p = 0.38
y-glutamyl transferase (U/L)	r = - 0.09	r = - 0.09	r = - 0.20	r = - 0.18
	p = 0.48	p = 0.04	p = 0.11	p = 0.08
Statins, %	r = 0.16	r = - 0.07	r = - 0.002	r = 0.15
	p = 0.18	p = 0.51	p = 0.99	p = 0.17
Anti-Inflammatory medication, %	r = - 0.02	r < 0.001	r = - 0.16	r = - 0.03
	p = 0.16	p = 1.00	p = 0.20	p = 0.78
Hypertensive medication, %	r = 0.05	r = 0.18	r = 0.02	r = 0.10
	p = 0.09	p = 0.09	p = 0.89	p = 0.32

Ambulatory systolic BP, Ambulatory systolic blood pressure.

Table 3 Multiple regression analyses of telomere length stratified by sex and ethnicity

	Telomere length											
	Black Men Adjusted R^2: 0.02			**White Men Adjusted R^2: 0.21**			**Black Women Adjusted R^2: 0.13**			**White Women Adjusted R^2: 0.33**		
Independent variables	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**
NO metabolites (µmol/L)	-0.274	(-0.55; 0.002)	0.058	**0.249**	**(0.03; 0.47)**	**0.030**	-0.132	(-0.39; 0.12)	0.312	0.066	(-0.13; 0.26)	0.500
Age (years)	-0.125	(-0.43; 0.18)	0.427	**-0.306**	**(-0.51; -0.10)**	**0.004**	-0.259	(-0.53; 0.01)	0.063	**-0.433**	**(-0.63; -0.24)**	**<0.001**
Waist (cm)	0.086	(-0.23; 0.40)	0.595	-0.068	(-0.29; 0.15)	0.546	-0.291	(-0.59; 0.01)	0.061	**-0.306**	**(-0.55; 0.06)**	**0.016**
GPx (nmol/min/mL)	-0.231	(-0.51; 0.05)	0.109	**0.229**	**(0.03; 0.43)**	**0.030**	0.089	(-0.17; 0.35)	0.503	0.171	(0.02; 0.36)	0.079
Cholesterol (mmol/L)	-0.221	(-0.51; 0.07)	0.140	-0.108	(-0.32; 0.10)	0.311	-0.035	(-0.30; 0.23)	0.793	-0.032	(-0.22; 0.16)	0.741
Glycated hemoglobin	0.162	(-0.13; 0.45)	0.276	0.048	(-0.18; 0.28)	0.686	**0.348**	**(0.07; 0.63)**	**0.017**	0.104	(-0.09; 0.30)	0.306
GGT (U/L)	-0.104	(-0.39; 0.18)	0.476	0.049	(-0.16; 0.25)	0.638	-0.151	(-0.40; 0.10)	0.240	-0.098	(-0.29; 0.09)	0.310
SDMA (µmol/L)	0.026	(-0.27; 0.32)	0.865	**0.225**	**(0.008; 0.44)**	**0.046**	0.129	(-0.14; 0.39)	0.346	0.089	(-0.11; 0.29)	0.374
TNF-α (pg/mL)	0.084	(-0.22; 0.39)	0.587	-0.125	(-0.33; 0.08)	0.230	-0.011	(-0.25; 0.23)	0.929	**-0.249**	**(-0.43; -0.06)**	**0.010**
Ambulatory SBP (mmHg)	0.150	(-0.16; 0.46)	0.343	-0.056	(-0.29; 0.18)	0.640	0.229	(-0.07; 0.53)	0.140	**0.274**	**(0.03; 0.52)**	**0.033**

GPX, Glutathione peroxidase; NO metabolites, Nitric oxide metabolites; SDMA, Symmetric dimethyl arginine; TNF-α, Tumor necrosis factor-α; GGT, y-glutamyl transferase; Ambulatory SBP, Ambulatory Systolic blood pressure.

Discussion

Cardiovascular aging can differ between ethnicities. These differences can be investigated through telomere length, cardiovascular health and other oxidative and inflammatory markers. According to the main findings with regards to the white population, negative associations between telomere length and NOx, between telomere length and age for men and between telomere length and TNF-α for women were found [39, 40]. The independent positive association between telomere length and NOx confirms previous reports and conforms with the hypothesis [21, 41]. However, no independent relationship between telomere lengths and NOx in the black participants or with age in black men were found.

The results of the white population were as expected, since aging are associated with increased cell division, telomere shortening and increased inflammation leading to cell damage, decreased NO bioavailability and consequently also decreased cardiovascular health [21, 39, 40].

The lack of an association between telomere length and NOx in the black participants is an unexpected finding as the literature indicates clearly that telomere length is strongly associated with aging [39]. One would expect to see higher levels of NOx which are indicative of a healthier cardiovascular system in younger individuals [23, 24]. In the United States, it was found that African Americans had longer telomere lengths at birth, but they also had a much faster rate of telomere shortening than their white counterparts [18]. It is therefore possible that the black participants from the study had undergone rapid telomere shortening in early life, possibly explaining the absence of an association between telomere length and age. This phenomenon warrants further investigation, especially due to factors contributing towards the rapid early life telomere shortening.

The link between telomere length and NO can be masked if the bioavailability of NO is affected. NO bioavailability is influenced by the synthesis capacity of NO or the binding of superoxide to NO, leading to the inactivation of NO and increased production of peroxynitrite ($ONOO^-$) [42]. High levels of $ONOO^-$ have the potential to damage DNA which include telomeres [49]. During the bio-synthesis of NO, L-citrulline is also formed as a by-product [43]. The black population of this study had lower L-citrulline in both sexes compared to the white population, which indicated an interruption in NO synthesis [43]. This may suggest that, even though the NO synthesis capacity is favorable in black men and women (higher levels of the substrate L-homoarginine and lower levels of the endogenous inhibitors, ADMA and SDMA), NO synthesis may still be suppressed. Additionally, it was previously suggested that the favorable NO synthesis capacity profile of the black population may be counteracted by increased NO inactivation [44]. The uncoupling of eNOS also plays a major part in the reduction of NO bioavailability [45]. The prevention of uncoupling of eNOS can act as a protective mechanism to decrease oxidative stress in the cardiovascular

system [46]. The mechanism of uncoupling of eNOS, is through the depletion of eNOS co-factors and substrates such as tetrahydrobiopterin (BH_4) and L-arginine [47, 48]. Oxidative stress not only decreases NO bioavailability, but also shortens telomere length [49]. No significant difference was found in NO metabolite levels between the different ethnicities but black men had a somewhat less favorable oxidative stress profile. As NO synthesis is increased, so will the rate of $ONOO^-$ production [50]. Another study has found NO production to be higher in black participants, but there was a decrease in sensitivity of vascular smooth muscles to dilate when compared to white counterparts [51]. Thus, high NOx is regarded as a marker of either a healthy vascular system or a desensitized one when oxidative stress is increased [52].

Oxidative stress is associated with shorter telomere length [53-57]. These associations have several causes, such as higher exposure to zinc, lead, high white bread intake, low antioxidant intake and increased cortisol levels through chronic stressful situations [54-56, 59, 59]. Oxidative stress is directly associated with cardiovascular disease [60]. Studies have found that the activity of telomerase in endothelial cells decreases when oxidation of LDL increases with consequent increased rate of telomere shortening and cell senescence [61]. Both the black men and women had higher GPx levels with concomitant increased TBARS levels in the black men, when compared to their white counterparts. These higher oxidative stress markers in the black population may suggest up-regulation of GPx activity in these groups. Up-regulation of antioxidant enzymes such as GPx is a protective mechanism against increased production of reactive oxygen species. However, in black men, these protective mechanisms seem to be inadequate to fully protect against oxidative damage (higher TBARS). GPx reduces free hydrogen to water and lipid hydroperoxides, but is unable to decrease $ONOO^-$, as $ONOO^-$ inactivates GPx [62, 63].

The black group does not only have a less favorable oxidative stress profile but also a less favorable inflammatory profile, with higher IL-6 and CRP levels. Previous results demonstrated increased inflammation in participants with chronic stress [64]. Furthermore, telomerase activity in the T-cells of these participants were also higher when compared to a control group, suggesting compensatory mechanisms to prevent telomere shortening [64]. This finding highlights the possibility that the black population experiences a more rapid decline in telomere length, accompanied by higher levels of oxidative stress and inflammation.

According to the findings, it is proposed that Africans may have a higher rate of telomere shortening. This may be due to several reasons such as early life exposures, diet, stress or psychological coping styles used when encountering stressful scenarios [65-69]. Furthermore, oxidative stress, psychological stress and telomere length are all independently associated with immune function [64, 69-70].

One of the limitation of this study was its cross-sectional nature. Cause-effect could thus not be studied, because telomere length data was only available in the 2010 – 2011 phase of the study. The study furthermore included a certain segment of socio-economic status, namely school teachers and the results may thus not be generalized to the broader population. Notwithstanding these limitations, this study is a first in Africa to investigate telomere length and cardiovascular health in bi-ethnic groups from South Africa.

To conclude, the study found that the shorter telomere length of black men and women was not associated with NOx, as was the case with white men. Furthermore, the less favorable cardiovascular and inflammatory profiles of the black population were unrelated to shorter telomere lengths. Further investigations on early life exposures as potential contributors to leukocyte telomere length in the black population are proposed, due to the lack of associations with telomere length.in the black population group.

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Supplementary statistics

Table S1 Multiple regression analyses of telomere length stratified by sex and ethnicity

	Telomere length											
	Black Men Adjusted R^2: 0.04			**White Men Adjusted R^2: 0.12**			**Black Women Adjusted R^2: 0.16**			**White Women Adjusted R^2: 0.26**		
Independent variables	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**
NO metabolites (µmol/L)	-0.259	(0.139)	0.068	**0.320**	**(0.114)**	**0.006**	-0.099	(0.123)	0.427	0.160	(0.099)	0.108
Age (years)	-0.121	(0.148)	0.419	**-0.287**	**(0.105)**	**0.008**	-0.255	(0.134)	0.062	**-0.418**	**(0.097)**	**<0.001**
Waist (cm)	0.106	(0.150)	0.485	-0.125	(0.114)	0.273	**-0.313**	**(0.146)**	**0.037**	**-0.357**	**(0.127)**	**0.006**
Cholesterol (mmol/L)	-0.196	(0.145)	0.183	-0.103	(0.109)	0.349	-0.002	(0.126)	0.989	-0.003	(0.100)	0.979
Glycated hemoglobin	0.170	(0.144)	0.243	-0.053	(0.117)	0.651	**0.383**	**(0.134)**	**0.006**	0.021	(0.102)	0.839
GGT (U/L)	-0.074	(0.142)	0.607	0.029	(0.107)	0.788	-0.166	(0.123)	0.182	-0.147	(0.101)	0.148
Ambulatory SBP (mmHg)	0.080	(0.153)	0.604	0.016	(0.118)	0.894	0.239	(0.136)	0.086	**0.361**	**(0.130)**	**0.007**

NO metabolites, Nitric oxide metabolites; GGT, y-glutamyl transferase; Ambulatory SBP, Ambulatory Systolic blood pressure

Table S2 Multiple regression analyses of telomere length stratified by sex and ethnicity

	Telomere length											
	Black Men Adjusted R^2: 0.01			**White Men Adjusted R^2: 0.10**			**Black Women Adjusted R^2: 0.16**			**White Women Adjusted R^2: 0.26**		
Independent variables	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**
GPx (nmol/min/mL)	-0.239	(0.128)	0.068	**0.265**	**(0.105)**	**0.001**	0.068	(0.120)	0.574	0.168	(0.097)	0.085
Age (years)	-0.098	(0.137)	0.476	**-0.231**	**(0.103)**	**0.028**	**-0.279**	**(0.127)**	**0.033**	**-0.433**	**(0.095)**	**<0.001**
Waist (cm)	0.103	(0.138)	0.460	-0.062	(0.114)	0.589	**-0.329**	**(0.139)**	**0.021**	**-0.358**	**(0.124)**	**0.005**
Cholesterol (mmol/L)	-0.167	(0.132)	0.210	-0.184	(0.105)	0.082	-0.012	(0.124)	0.925	-0.059	(0.097)	0.544
Glycated hemoglobin	0.115	(0.131)	0.386	0.001	(0.118)	0.994	**0.369**	**(0.127)**	**0.005**	0.083	(0.102)	0.416
GGT (U/L)	-0.124	(0.132)	0.352	0.001	(0.106)	0.995	-0.170	(0.117)	0.151	-0.150	(0.098)	0.130
Ambulatory SBP (mmHg)	0.092	(0.142)	0.520	-0.048	(0.121)	0.692	0.249	(0.134)	0.069	**0.371**	**(0.125)**	**0.004**

GPX, Glutathione peroxidase; GGT, y-glutamyl transferase; Ambulatory SBP, Ambulatory Systolic blood pressure

Table S3 Multiple regression analyses of telomere length stratified by sex and ethnicity

	Telomere length											
	Black Men Adjusted R^2:---			**White Men Adjusted R^2: 0.10**			**Black Women Adjusted R^2: 0.16**			**White Women Adjusted R^2: 0.26**		
Independent variables	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**
SDMA (µmol/L)	0.073	(0.135)	0.592	**0.268**	**(0.108)**	**0.015**	0.084	(0.126)	0.508	0.164	(0.098)	0.096
Age (years)	-0.143	(0.143)	0.325	**-0.273**	**(0.105)**	**0.011**	**-0.265**	**(0.126)**	**0.040**	**-0.473**	**(0.098)**	**<0.001**
Waist (cm)	0.115	(0.144)	0.428	-0.042	(0.114)	0.717	**-0.309**	**(0.140)**	**0.031**	**-0.339**	**(0.126)**	**0.009**
Cholesterol (mmol/L)	-0.149	(0.135)	0.272	**-0.212**	**(0.104)**	**0.045**	-0.006	(0.121)	0.961	-0.027	(0.096)	0.774
Glycated hemoglobin	0.130	(0.135)	0.339	0.041	(0.121)	0.737	**0.344**	**(0.133)**	**0.012**	0.049	(0.099)	0.620
GGT (U/L)	-0.070	(0.135)	0.604	-0.019	(0.105)	0.854	-0.148	(0.120)	0.220	-0.149	(0.098)	0.132
Ambulatory SBP (mmHg)	0.047	(0.143)	0.745	-0.029	(0.120)	0.810	0.204	(0.134)	0.133	**0.371**	**(0.125)**	**0.004**

SDMA, Symmetric dimethyl arginine; GGT, y-glutamyl transferase; Ambulatory SBP, Ambulatory Systolic blood pressure

Table S4 Multiple regression analyses of telomere length stratified by sex and ethnicity

	Telomere length											
	Black Men Adjusted R^2:---			**White Men Adjusted R^2: 0.07**			**Black Women Adjusted R^2: 0.16**			**White Women Adjusted R^2: 0.32**		
Independent variables	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**	**β (95% CI)**		**P**
TNF-α (pg/mL)	0.095	(0.134)	0.483	-0.204	(0.105)	0.054	0.014	(0.113)	0.901	**-0.287**	**(0.088)**	**0.002**
Age (years)	-0.151	(0.144)	0.300	-0.191	(0.105)	0.734	**-0.270**	**(0.127)**	**0.037**	**-0.421**	**(0.090)**	**<0.001**
Waist (cm)	0.154	(0.143)	0.287	-0.100	(0.115)	0.387	**-0.323**	**(0.139)**	**0.024**	**-0.362**	**(0.118)**	**0.003**
Cholesterol (mmol/L)	-0.162	(0.135)	0.235	-0.179	(0.106)	0.095	0.004	(0.121)	0.973	-0.015	(0.092)	0.869
Glycated hemoglobin	0.148	(0.135)	0.278	-0.032	(0.119)	0.787	**0.369**	**(0.128)**	**0.005**	0.065	(0.095)	0.495
GGT (U/L)	-0.074	(0.134)	0.582	-0.060	(0.106)	0.872	-0.166	(0.117)	0.162	-0.161	(0.093)	0.088
Ambulatory SBP (mmHg)	0.049	(0.143)	0.731	-0.043	(0.121)	0.726	0.230	(0.131)	0.084	**0.363**	**(0.119)**	**0.003**

TNF-α, Tumor necrosis factor-α; GGT, y-glutamyl transferase; Ambulatory SBP, Ambulatory Systolic blood pressure

Chapter 5

Concluding remarks and findings

Chapter 5 Concluding remarks and findings

5.1 Introduction

This is the concluding chapter where the main findings are summarized and compared to the relevant literature. The hypotheses that were set in Chapter 2 are also addressed.

5.2 Summary of main findings

This is the summary of the manuscript in Chapter 4.

Leukocyte telomere length and its relation to nitric oxide metabolites in a bi-ethnic sample: the SABPA study

In the manuscript, telomere length and nitric oxide metabolite (NOx) profiles of black and white teachers of South Africa were compared. Ethnic- and gender-specific associations between telomere length and nitric oxide were also investigated. The hypotheses that were put forward in the second chapter stated that black individuals have shorter telomeres, lower NOx levels, higher blood pressure and higher oxidative and inflammation markers than their white counterparts and that leukocyte telomere length is positively associated with NOx in both black and white participants.

The study found that the black group had shorter telomere lengths than their white counterparts, but no association between telomere length and NOx in the black group was found. Leukocyte telomere lengths were reported as shorter in the black participants, which is in contrast with parts of the literature [1]. The lack of an association between telomere length and NOx in certain ethnic groups contradicts current literature, while positive association of others is in line with current knowledge [2, 3]. To the knowledge of the researchers, this is the first study to investigate telomere/NO associations in a bi-ethnic South African population. This shorter telomeres and lack of association of telomere length with NOx in the black population, add to the literature. The reason why the black group had such unorthodox results is unknown. It is the suggestion of the researchers that oxidative stress and/or early life exposure to psychological/physical stress could potentially have aided in these results [4]. Oxidative stress could cause damage to cells [5, 6]. When cell damage occurs, the rate of telomere shortening increases and nitric oxide (NO) availability and cardiovascular health decrease [6-9]. The black group had higher blood pressure than the white group when stratified by sex, which is consistent with the literature [10]. Except for white women, blood pressure was not associated with telomere length in both ethnic or sex groups, which some literature indicate to be the case [11-12]. There was also no difference between NOx from different ethnicities, with literature showing that different ethnicities could have

different NO production rates due to polymorphisms in eNOS [13]. Therefore, the first hypothesis is partially accepted, because the black participants were found to have shorter telomeres, less favorable oxidative and inflammatory profiles, the same NOx levels but higher blood pressure than their white counterparts. The second hypothesis is also partially accepted, because a positive association was found between leukocyte telomere length and NOx, but only in the white population.

The results of the study confirm and contradict current literature. The study thus increases knowledge with regards to the NOx and telomere length association in different ethnic groups.

5.3 Discussion

It is important to compare the main findings of this study to those in the literature and to interpret the results of these findings. Some of the results contradict previous findings and thus contribute to the existing academic knowledge in the aging and cardiovascular fields.

The findings are unique as it is the first study that has attempted to establish an association between leukocyte telomere length and NOx in African populations. It was found that the white population had associations between telomere length and age and that white males had an association between telomere length and NOx. These findings are in accordance with literature [1-3, 12]. However, the black men had no associations between telomere length and age or NOx, while black women had no association between telomere length and NOx, which is contradictory with the current body of knowledge [2, 3, 12]. Since research indicates that a relation between telomere length and oxidative stress exists, as well as oxidative stress and cardiovascular disease, telomere length may indeed be associated with cardiovascular health [4, 14]. No associations between telomere length and blood pressure were evident in both ethnicities, except for the unexpected positive association in white women. Further investigation into associations between blood pressure and other cardiovascular markers are recommended.

The association between telomere length and NOx can be affected by the bioavailability of NO [15]. L-citrulline is synthesized as by-product in the production of NO [16]. In the current study, it was found that the black participants had significantly lower L-citrulline levels than their white counterparts. This may indicate that, even if the black population has a favorable NO synthesis profile, the bioavailability of NOx can be decreased through other suppression mechanisms, such as eNOS polymorphisms or oxidative stress.

A plausible explanation for the counteraction of the favorable NO synthesis profile in the black population is the decrease in NO bioavailability through NO inactivation [17]. Another deactivation mechanism is eNOS uncoupling, which decreases NO synthesis [18]. eNOS uncoupling is the

suppression of synthesis, through the depletion of substrates and co-factors such as L-arginine or tetrahydrobiopterin [19, 20]. Inactivation of NO occurs if oxidative stress increases [2, 3]. In this study, no significant differences were found in NOx profiles between black and white, but black men had higher inflammatory and oxidative stress profiles. Superoxide has a high affinity for NO and the combination results in the formation of $ONOO^-$ and consequently a decrease in NO bioavailability [21]. Increased levels of $ONOO^-$ have the ability to damage cells and thus accelerate telomere shortening [3, 21]. Another explanation comes from a study where it was found that the black population had a higher NO production compared to their white counterparts, but that they started to lose sensitivity to NO in vascular smooth muscles [22]. High levels of NOx usually indicate a healthy cardiovascular system, but could then also be an indication of a desensitized cardiovascular system.

Investigation into the bioavailability of NO and the factors causing the lack of association between telomere length and age in black African men is recommended for future studies.

5.4 Confounders and chance

It is important to consider the confounding factors that could influence the results.

The study population is only representative of people living in the North West Province, South Africa. The population is thus for only one of nine provinces and not representative of the rest of South Africa. It is only one country, thus also not representative of the rest of Africa or other continents. The study population was also part of the 'middle income' group, working as teachers. The results are therefore not representative of other socio-economic groups.

Markers such as telomerase were also not included nor was a longitudinal study design used, therefore the effect of oxidative stress on the rate of telomere shortening could not be established.

It is also important to consider that not all statistical significant results can be translated to physiological significant results.

5.5 Strengths and limitations

It is essential to look back on the strengths and limitations of the study.

5.5.1 Strengths

A controlled environment for measurements was set-up to acquire reliable samples. The participants stayed in the Metabolic Research Unit with climate control, while dietary intake and activity were monitored.

Seasonal change was anticipated, so measurements were only done during February and May.

The ambulatory blood pressure devices were worn during the normal working day to get the closest blood pressure valued to normal values. Thus, the data gathered is representative of a real-world setting and reliable for comparison to the general public.

Blood sample integrity was maintained at all times. A professional nurse drew blood from the participants early in the morning. Blood samples were instantly sent to onsite laboratory to be processed and were stored at -80ºC.

5.5.2 Limitations

To determine cause and effect would be beneficial. As this study was cross-sectional, future studies are needed to determine cause and effect.

Telomerase activity was not determined during this study.

5.6 Recommendations

To improve future studies that include South African participants, the following can be considered:

Firstly, a population group of other ethnicities can be included to see if the lack of association between telomere length and NOx is localized to a specific ethnic group or specific population group. Furthermore, a longitudinal study design that includes telomerase activity marker will shed light on the progress of telomere shortening. The rate of telomere shortening could be beneficial in concluding results.

Also of importance is to gather a full history to identify any events that could have led to potential increased telomere shortening in different phases of life. Though not always realistic, the ideal would be to have a baseline telomere length marker, for at birth.

The nitrate intake of the participants was not monitored, which could potentially have an influence on the measurement of NOx.

5.7 Final conclusion and perspectives

This study showed for the first time that black adults participating in the SABPA study, indicated no association between leukocyte telomere length and NOx. Furthermore, no associations in the black populations were evident between telomere length and ambulatory blood pressure, and telomere length and age in black men. Oxidative stress markers and blood pressure were significantly higher in the black than white population. The black population seemed to be more prone to cardiovascular disease, but no association with NOx could be established, which prompts further investigation on other potential factors that might be present.

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Appendix A Instructions and guidelines to authors

Research articles generally contain the following sections in this order: Title, Authors, Affiliations, Contact Information, Running title, Key words (6-7), Abstract, Introduction, Results, Discussion, Methods, Acknowledgements, References, Figure and Table Legends, Figures and Tables, Supplemental Data. The text (Title through Legends) should be provided as one document, which may also contain the Tables. Figures should be provided separately. Supplemental Data should be provided separately.

There is no word count limit for research articles. An article may contain up to 15 figures and/or tables. Gene symbols should be italicized; protein products of the loci are not italicized. Nonstandard abbreviations should be defined when first used in the text. Use of abbreviations should be kept at a minimum. Manuscript file types that we can accept for submission include Word and RTF.

The text should be double spaced and pages should be numbered. Although summaries need to be entered as text files separate from the body of the manuscript during the online submission process, they should also be included within the manuscript file as usual.

Manuscripts that do not conform to the format guidelines may be returned to the authors for reformatting.

Preparation of Specific Sections

Title

The title should convey the conceptual significance of the paper to a broad readership.

Authors/Affiliations

Author names should be spelled out rather than set in initials. Authors should be footnoted to corresponding affiliations. Affiliations should contain the following core information: department(s)/subunit(s); institution; city, state/region, postal code; country.

Contact

The contact line should include the email address and phone/fax numbers of the corresponding author. The published corresponding author is responsible for ensuring adherence to all editorial and submission policies and for any communications that may result post-publication.

Additional Footnotes

Footnotes are only allowed on page 1 of the text (and in tables). They may include a present address or statement of equal contribution to the manuscript.

Abstract

The Abstract consists of a single paragraph of fewer than 200 words. It should clearly convey the conceptual advance and significance of the work to a broad readership. References should not be cited in the Summary.

Introduction

The Introduction should be succinct, with no subheadings, and should present the background information necessary to provide a biological context for the results.

Results

This section should be divided with subheadings.

Discussion

The Discussion should explain the significance of the results and place them into a broader context. This section may in some cases be combined with the Results section.

Methods

The Methods section needs to include sufficient detail so that readers can understand how the experiments were done, and so that all procedures can be repeated, in conjunction with cited references. This section should also include a description of any statistical methods employed in the study.

Acknowledgments

This section may acknowledge contributions from non-authors, list funding sources, and should include a statement of any conflict of interests.

References

The list of references should be numbered consecutively according to the first time mentioned within the article. Cite only the number assigned to the reference: For example, according to Campisi [1]. Use [ ] not ( ). References should include only articles that are published or in press.

Unpublished data, submitted manuscripts, abstracts, and personal communications should be cited within the text only. Personal communication should be documented by a letter of permission.

Please use the following **style for references**:

Papers (except Editorials):

Please use the following style for references in all types of papers except Editorials:

1) Article in a periodical (strictly, no variation is allowed):

1. Bhaumik D, Scott GK, Schokrpur S, Patil CK, Orjalo AV, Rodier F, Lithgow GJ, and Campisi J. MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. Aging. 2009; 1:402-411.

Note: "et al." should only be used after 13 authors.

2) Article in a books and book chapters: any Style is acceptable.

Editorials:

Please use a special reference format: 1 author et al, no titles.

1. Patel AS, Allen JE, Dicker DT, et al. Oncotarget. 2011; 2:752-760.

Authors are strongly encouraged to use an automated reference manager such as Thomson Reuters EndNote. An output style for Aging journal is available to download on the following link: ftp://support.isiresearchsoft.com/pub/pc/styles/endnote4/Aging.ens

Appendix B Ethics Approval Letter

NORTH-WEST UNIVERSITY
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Email: Minrie.Greeff@nwu.ac.za

30 July 2015

Prof HW Huisman
HART

Dear Prof Huisman

APPROVAL: ETHICS APPLICATION: NWU-00036-07-A6 (HW HUISMAN-J COMBRINK) "SABPA STUDY"

Thank you for amending your sub-study "Leukocyte telomere length and its relation to nitric oxide metabolites in a bi-ethnic sample: the SABPA study" application. All ethical concerns have now been addressed and ethical approval is granted until 01/11/2016.

Please note that any changes to the approved application must be submitted to the Health Research Ethics Committee for approval before implementation.

Yours sincerely

Prof Minrie Greeff
HREC Chairperson

Current details: (13210572) C:\Users\13210572\Documents\HREC\HREC - Applications\2015 Applications\Applications 04 - 14 May 2015\NWU-00036-07-A6 (HW Huisman-J Combrink)\NWU-00036-07-A6 (HW Huisman-J Combrink) - AL\NWU-00036-07-A6 (HW Huisman-J Combrink) - AL.docm
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16 January 2017

Dear Prof Huisman

FEEDBACK ON HREC ANNUAL MONITORING REPORT: NWU-00036-07-A6

We would like to thank you for submitting the annual monitoring report for your project entitled, ***"Leukocyte telomere length and its relation to nitric oxide metabolites in a bi-ethnic sample: the SABPA study"*** to the Health Research Ethics Committee (HREC) in a timely manner. Please find below the decision of the HREC regarding the continuation of your project.

Classification	Mark with X	Comment
Clarification		
Completion (Final report)		
Suspended		
Continuation	X	**Next date : 30 November 2017**
Termination		

Should you have any further queries, please feel free to contact Ms Hildah Kilani at your earliest convenience (E-mail: Ethics-Monitoring@nwu.ac.za ; Tel: 018 299 1208). We wish you well in your future endeavours.

Yours sincerely

Prof Minrie Greeff
Head of Health Sciences Ethics
Office for Research, Training and Support

Dr Wayne Towers
Chairperson: HREC

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Email Ethics@nwu.ac.za

Dr L Malan

Dear Dr Malan

6 February 2008

ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title:.SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans)

***Ethics* number**: N W U - 0 0 0 3 6 - 0 7 - S 6
Institution | Project Number | Year | Status
Status: S = Submission, R = Re-Submission, P = Provisional Authorisation, A = Authorisation

Approval date: 12 November 2007 **Expiry date**: 11 November 2012

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-EC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-EC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof M M J Lowes
(chair NWU Ethics Committee)

Appendix C Language review report

DECLARATION

I, C Vorster (ID: 710924 0034 084), Language editor and Translator, and member of the South African Translators' Institute (SATI member number 1003172), herewith declare that I did the language editing of a dissertation written by J Combrink from the North-West University (student number: 21074615).

Title of the dissertation: Leukocyte telomere length and its relation to nitric oxide metabolites in a bi-ethnic sample: the SABPA study

C Vorster 16 October 2017

C Vorster Date

Appendix D Turn-it-in report

J Combrink Telomere and NO association

ORIGINALITY REPORT

14%	11%	9%	8%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

MATCH ALL SOURCES (ONLY SELECTED SOURCE PRINTED)

3%

★ von Känel, Roland, Nico T. Malan, Mark Hamer, and Leoné Malan. "Comparison of Telomere Length in Black and White Teachers From South Africa : The Sympathetic Activity and Ambulatory Blood Pressure in Africans Study", Psychosomatic Medicine, 2015.

Publication

Exclude quotes	On	Exclude matches	< 7 words
Exclude bibliography	On		

“Success means using your knowledge and experience to satisfy yourself.

Significance means using your knowledge and experience to change the lives of others.”

Bob Buford