The role of endothelin-1 in cardiometabolic and vascular function in a bi-ethnic population: The SABPA study

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Thesis submitted for the degree *Doctor Philosophiae* in Physiology at the Potchefstroom Campus of the North-West University

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November 2016

AKNOWLEDGEMENTS

I wish to thank various people for their contribution to this project:

- My supervisor, **Doctor Ruan Kruger**, for his valuable and constructive suggestions during the planning and development of this research work. His willingness to give his time so generously has been very much appreciated;
- My co-supervisors, **Professor Hugo W Huisman** and **Professor Carina MC Mels**, for their patient guidance, enthusiastic encouragement and useful critiques of this research work;
- The SABPA **participants**, **staff**, **postgraduate students** and the **Department of Education (North-West province, South Africa)** for participating, starting and running the project;
- The financial assistance of the **South African National Research Foundation** (UID 65607), the **South African National Research Foundation Thuthuka** (80643), the **North-West University** (Potchefstroom Campus, South Africa), **ROCHE Diagnostics** (South Africa) and the **Metabolic Syndrome Institute** (France);
- The financial assistance of the **South African National Research Foundation** (**NRF SARCHi Postgraduate bursary** and **DAAD-NRF Joint in-country scholarship** (UID 105119)) and the **North-West University doctoral bursary** (Potchefstroom Campus, South Africa) toward this research study is appreciated;
- My parents, **Magda and David du Plooy**, and my brother, **David du Plooy**, thank you for all your love, encouragement and support throughout my academic career;
- My fiancé, **Christo de Lange**, for all your understanding and support;
- My grandparents, for their love and understanding.

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PREFACE

This thesis is presented in the article format as approved, supported and defined by the North-West University guidelines for postgraduate PhD-level studies. The first chapter includes an introduction, motivation and literature overview of the applicable topics investigated in the separate research articles, followed by the overall aims and hypotheses. The SABPA study protocol, methods of data collection and analyses that were performed is discussed in detail in Chapter 2. Chapter 3, 4 and 5 contain the individual manuscripts in the form of original research articles submitted to peer-reviewed journals. The supervisor and co-supervisors were included as co-authors in each manuscript. The first author was responsible for the initiation and all parts of this thesis, including literature searches, statistical analyses and the interpretation of results, as well as writing the research articles. All co-authors have given their consent for the research articles to be submitted for publication and for inclusion in this thesis. The final chapter (Chapter 6) provides a summary of the main findings and includes the critical discussions of all the presented results, conclusions drawn and applicable recommendations made from the manuscripts.

This thesis consists of peer-reviewed published or submitted articles. The first article was published in the *Journal of Amino Acids,* the second article was published in *Hypertension Research* and the third and final paper submitted to the *Journal of Hypertension.* References are listed at the end of Chapter 1, 2 and 6 according to the Vancouver referencing style. The references of the respective research articles (Chapter 3, 4 and 5) are listed according to the instructions for authors as specified by the applicable journal.

SUMMARY

Motivation

The black population in South Africa has a high prevalence of hypertension and atherosclerosis. Endothelin-1 (ET-1), a potent vasoconstrictor peptide, has been implicated as an important biomarker in the development of vascular dysfunction and cardiovascular disease, including arteriosclerosis, atherosclerosis and hypertension. Resting ET-1 levels are higher in black Americans than in whites, and higher in men than in women under normal physiological conditions. Under pathophysiological conditions, the biosynthesis of ET-1 is stimulated by cardiovascular risk factors such as elevated levels of oxidised low-density lipoprotein cholesterol, hypertension and aging. Prolonged exposure to cardiovascular risk factors seems to disrupt the balance between vasodilation and vasoconstriction, leading to conditions such as increased blood pressure, increased inflammation, arterial stiffness, oxidative stress and vascular remodeling. In this regard it is important to determine the potential impact of ET-1 levels on the cardiovascular system over time, especially in the black population of South Africa. This study included markers of cardiovascular function (systolic blood pressure, pulse pressure and mean arterial pressure), inflammation (interleukin-6 and C-reactive protein), oxidative stress (reactive oxygen species (ROS)), anti-oxidant capacity (total glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPx), and glutathione reductase-to-glutathione peroxidase ratio (GR-to-GPx ratio)), and vascular remodeling (carotid intima media thickness (CIMT), carotid cross-sectional wall area (CSWA), and arterial compliance) to address the vascular changes that augment vascular damage. The study was motivated by the awareness of limited data in this regard, especially in South Africans.

Aim

The general aim of this study is to explore the possible associations of ET-1 levels with cardiometabolic and vascular function. Furthermore, to determine whether ET-1 levels differ among sex and race and if there is an association between ET-1 levels with markers of cardiovascular function, inflammation, oxidative stress, anti-oxidant capacity, and vascular remodeling in black and white South Africans.

Methodology

This study was embedded in the *Sympathetic Activity and Ambulatory Blood Pressure in Africans* (SABPA) study. The study was a prospective cohort study that included 409 black and white schoolteachers working in the Kenneth Kuanda Education District of the North West Province at baseline (2008/09). At follow-up (2011/12) the cohort totalled 359 participants. The total group was stratified by race and sex and in the third manuscript by an increase or a decrease in ET levels after three years. Cardiovascular measurements were performed and ET-1, interleukin-6, C-reactive protein, ROS, GSH, GPx, and GR levels were determined. T-tests were done to compare means between groups. Chi-square and crosstabs were used to compare proportions between baseline values or baseline and follow-up values, respectively. Pearson and partial correlations were performed to investigate the associations between various variables with adjustments for age, body mass index, C-reactive protein, total energy expenditure, anti-hypertension medication, gamma glutamyl transferase, race and sex in the relevant manuscripts. Multiple regression analyses were performed to investigate associations of ET-1 with cardiovascular and biochemical markers according to the specific focus of each research manuscript.

Results and conclusions of each manuscript

The objectives of the first manuscript (Chapter 3) were to compare ET-1 levels among sex and race and to explore the association of ET-1 with cardiovascular function and inflammation. The black men and white women had significantly higher ET-1 levels when compared to their counterparts after adjusting for C-reactive protein ($p<0.001$). Furthermore, partial and multivariate regression analyses showed an independent association of ET-1 with interleukin-6, systolic blood pressure and pulse pressure in black women only ($p<0.01$). These associations suggest that ET-1 and its link with subclinical arteriosclerosis are potentially driven by low-grade inflammation in the black female cohort.

The second manuscript (Chapter 4) investigated the associations of ET-1 with markers of oxidative stress and anti-oxidant capacity in black and white South Africans. Multiple regression analyses showed that ET-1 associated positively with GR activity ($\beta=0.232$; $p=0.020$) and tended to associate with GR-to-GPx ratio ($\beta=0.190$; $p=0.057$) in black men, while there was an inverse association between ET-1 and GSH ($\beta=–0.214$; $p=0.026$) in black women. There was a positive association with ROS ($\beta=0.260$; $p=0.010$) and

negative association with GPx activity (β=–0.233; p=0.020) in white men. The results suggest that ET-1 may contribute to GR up-regulation through increased ROS production in black men, while higher GSH levels may act as a counter-regulatory mechanism to protect against oxidative vascular damage attributed to ET-1 in black women. In white men, the negative association observed between ET-1 and GPx and positive association with ROS may describe the expected physiological relationship between ET-1 and ROS.

The third manuscript (Chapter 5) investigated the association of change in ET-1 levels and the change of markers implicated in vascular remodeling after three years in a black and white South African population. Multiple regression analysis, after splitting for race, indicated that the increase in ET-1 levels associated positively with the change in pulse pressure (β=0.278; p=0.036), while a borderline association exist between the extent of decrease in ET-1 levels and a lesser change in CSWA (β=–0.201; p=0.054) in the black population only. Anti-hypertension medication also played an important role in this study. After excluding patients using anti-hypertension medication the borderline inverse association between the decrease in ET-1 levels and a change in CSWA disappeared in the black participant, but became significant in the white participants (β=–0.127; p=0.046). The results suggest that in the black participants with increased ET-1 levels after three years, the positive association between ET-1 levels and pulse pressure suggest subclinical haemodynamic changes with potential premature onset of cardiovascular disease, while anti-hypertension treatment and statin usage seem to slow down adverse vascular remodeling caused by elevated ET-1 levels in the white population only.

General conclusion

Our study is the first to indicate a link between a marker of vascular function (ET-1) and inflammation, oxidative stress, anti-oxidant capacity and vascular remodeling in a bi-ethnic South African population. Our results persistently found that ET-1 contributed to a higher risk of early vascular deterioration (arteriosclerosis) and future comorbidities, potentially driven by low-grade inflammation (black women), ROS production (black men), decreased anti-oxidant capacity (black men) and vascular remodeling in the black population, whereas a decrease in ET-1 slow down adverse vascular remodeling in the white population.

Keywords: anti-oxidant capacity, arterial stiffness, blacks, carotid intima-media thickness, cross-sectional wall area, endothelin-1, inflammation, oxidative stress, vascular remodeling.

AUTHORS' CONTRIBUTION

The relative contributions of each researcher involved in this study are provided in the following table:

Name	**Contribution in the study**
Ms. CS du Plooy	Responsible for writing the complete thesis, preparation of blood samples for biochemical analyses, compiling an ethics application, proposal, and literature searches for individual chapters in this thesis. Other responsibilities included statistical analyses, the design and planning of the articles and the interpretation of findings.
Dr. R Kruger	Assisted with data collection, advice and guidance with regard to statistical procedures and analyses. Supervised the writing of the research articles and critical appraisal of the individual articles and thesis. Critical assessment of the complete thesis.
Prof. HW Huisman	Involved in data collection, provided advice and recommendations during the writing of the articles and ensured the proper evaluation of findings. Critical assessment of the complete thesis.
Prof. CMC Mels	Involved in the study design, biochemical data collection, reviewing statistical analyses of data and reviewing all literature as part of the thesis and manuscripts. Critical assessment of the complete thesis.

By signing this document, the co-authors verify their individual contributions and involvement in this study as stated above and grant their permission that the research articles may be published as part of this thesis:

Hereby, I declare that I approved the aforementioned manuscripts and that my contribution in this study, as stated above, is representative of my actual contribution. I also give my consent that these manuscripts may be published as part of the Ph.D. thesis of Christine Susara du Plooy.

Dr. R Kruger | Prof. HW Huisman | Prof. CMC Mels

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CHAPTER 4

ABBREVIATIONS

ABPM	-	Ambulatory blood pressure monitor
Adj.	-	Adjusted
AMS	-	Artery Measurement Systems
AT1	-	Angiotensin II receptor type 1
BH_4	-	tetrahydrobiopterin
Ca^{2+}	-	calcium ion
cGMP	-	cyclic guanosine monophosphate
CIMT	-	carotid intima-media thickness
cm	-	centimeter
CRP	-	C-reactive protein
CSWA	-	cross-sectional wall area
CuZN-SOD	-	copper-zinc superoxide dismutase
DAG	-	diacylglycerol
DOCA	-	deoxycorticosterone acetate
ECLIA	-	electrochemiluminescence immunoassay
EC SOD	-	extracellular form of superoxide dismutase
EDTA	-	ethylenediaminetetra acetic acid
eGFR	-	estimated glomerular filtration rate
ELISA	-	enzyme linked immunosorbent assay
eNOS	-	endothelial nitric oxide synthase
ET-1	-	endothelin-1
ET-2	-	endothelin-2
ET-3	-	endothelin-3
et al.	-	et alla “and other”
ET_AR	-	endothelin A receptors
ET_BR	-	endothelin B receptors
GPx	-	glutathione peroxidase

GR	-	glutathione reductase
GSH	-	reduced glutathione
GSSG	-	glutathione disulfide
H^+	-	hydrogen ion
H_2O	-	water
H_2O_2	-	hydrogen peroxide
HDLC	-	high-density lipoprotein cholesterol
iNOS	-	induced nitric oxide synthase
IL-6	-	interleukin-6
IP_3	-	inositol triphosphate
kg	-	kilogram
kg/m^2	-	kilogram per square meter
LDLC	-	low-density lipoprotein cholesterol
MDRD	-	modification of diet in renal disease
µmol/L	-	micromole per liter
mg/dL	-	milligram per deciliter
mg/mL	-	milligram per milliliter
mg/mmol/l	-	milligram per millimol per liter
ml	-	milliliter
ml/min	-	milliliter per minute
ml/mmHg	-	millilter per millimetre mercury
mm	-	millimeter
mmHg	-	millimeter mercury
mmol/l	-	millimol per liter
Mn SOD	-	manganses superoxide dismutase
mRNA	-	messenger RNA
m/s	-	meter per second
n	-	number of
ng/mL	-	nanogram per milliliter

$NADP^+$	-	nicotinamide adenine dinucleotide
N**ADPH**	-	nicotinamide adenine dinucleotide phosphate
NF-kβ	-	nuclear factor kappa beta
NO	-	nitric oxide
NWU	-	North-West University
O_2	-	oxygen
$O_2^{\cdot -}$	-	superoxide anions
$OH^{\cdot}$	-	hydroxyl radicals
$ONOO^-$	-	peroxinitrate
pg/mL	-	picogram per millilitier
PKC	-	protein kinase C
RAAS	-	renin-angiotesin aldosterone system
ROS	-	reactive oxygen species
SABPA	-	Sympathetic Activity and Ambulatory Blood Pressure in Africans
SOD	-	superoxide dismutase
TNF-α	-	tumor necrosis factor alpha
U/L	-	Units per liter
VSMC	-	Vascular smooth muscle cells

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

The cardiovascular health status of the African population is poor, with elevated blood pressure frequently reported in this population.[1,2] Vascular endothelial cell function is critical in maintaining cardiovascular health.[3] The inner linings of blood vessels are composed of the vascular endothelium, a vital autocrine/paracrine organ responsible for regulating vascular tone, vascular function and cell proliferation.[4] Dysfunction of the vascular endothelium causes a reduction in endothelial derived relaxing factors such as nitric oxide (NO)[5,6] and prostacyclin[6,7] and increased production of contracting factors such as endothelin[8,9] and angiotensin II.[9,10] These changes in vasoconstrictor and vasodilator substances leads to conditions such as increased blood pressure, arterial stiffness and oxidative stress.[11-16]

Endothelial dysfunction promotes both early and late mechanisms for the development of atherosclerosis and hypertension, including the disruption of the vasomotor tone of the endothelium.[12,17] Endothelin-1 (ET-1) is the most potent vasoconstrictor of the contracting factors and levels of ET-1 seem to be higher in the black than white population.[18-20] The black population is also at higher risk for the development of early vascular changes associated with the development of hypertension and atherosclerosis.[21-26] Increased ET-1 production can cause endothelial dysfunction,[27,28] inflammation,[6,29] oxidative stress[30-32] and vascular remodeling,[27,33-35] thereby contributing to these early vascular changes and consequently increasing the black population's susceptibility to cardiovascular disease. This chapter provides a brief literature review to provide the necessary background of the peptide ET-1 and its relationship with vascular function.

1.2. Endothelins

Endothelins are a family of three distinct 21-residue peptides named ET-1, ET-2 and ET-3.[8,18] ET-1 was identified in 1988 by Yanagisawa and colleagues[8] and showed a vasoconstricting effect on animal coronary, basilar, mesenteric, femoral and renal arteries, as well as the mesenteric and pulmonary artery branches of humans, acting directly on the smooth muscle cells of these arteries. The other two peptides, ET-2 and ET-3, differ from ET-1 by 2 and 6 amino-acids.[6] ET-2 is expressed in the ovaries and by intestinal epithelial cells and maintains ovulation and intestinal epithelial cell homeostasis.[36] ET-3 is

found in endothelial cells and shows a potent depressor response by releasing NO,[37] inducing phosphoinositide breakdown and inducing an increase in intracellular calcium ions in endothelial cells.[38] Beyond the functions of ET-2 and ET-3, ET-1 is primarily responsible for changes that occur in the vasculature; therefore the literature study of this thesis focuses on ET-1 only.

1.2.1. The ET-1 system

1.2.1.1. Production of ET-1

Under normal physiological conditions, ET-1 is produced mainly by vascular endothelial cells.[16,39,40] However, under pathophysiological conditions, ET-1 can also be produced by vascular smooth muscle cells (VSMC)[6,8] and inflammatory cells such as macrophages[41] and leukocytes.[42] The production of ET-1 is regulated predominantly at the level of messenger ribonucleic acid (mRNA) transcription and translation to form prepro-ET-1 (Figure 1).[8,36] The production of ET-1-mRNA can also be stimulated by other substances such as angiotensin II,[43,44] thrombin[45] and transforming growth factor beta.[46] Prepro-ET-1 then undergoes furin-like endopeptidase to form the biologically inactive intermediate big-ET-1 (Figure 1).[36] Endothelin-converting enzyme 1 or endothelin-converting enzyme 2, which is a family of membrane-bound zinc metalloproteases, mediate the processing of big-ET-1 into active mature ET-1 (Figure 1).[36] In addition to endothelin-converting enzymes, other enzymes such as non-endothelin-converting enzymes metalloproteinase[47] and chymase[36] can also contribute to the final step of processing (Figure 1).[48] ET-1 is released continuously from secretory vesicles by the constitutive secretory pathway or stored and released via regulated secretory pathways by endothelial cell-specific storage granules known as Weibel-Palade bodies in response to external physiological or pathophysiological stimuli to maintain the endogenous vascular tone.[49-52] ET-1 has a half-life of approximately one minute in healthy humans and is removed from the circulation by the lungs, kidneys and endothelin B receptors.[53,54]

1.2.1.2. Endothelin receptors and their functions

The active mature ET-1 can only perform its function after it binds to one of two 7-transmembrane domain G protein-coupled endothelin receptors, endothelin A receptors (ET_AR) or endothelin B receptors (ET_BR) (Figure 1).[36,55] The ET_AR have a higher affinity for ET-1 and ET-2, whereas ET_BR has an equal

affinity for all the endothelin peptides.[55] In humans, ET_AR is predominantly found on the VSMC mediating vasoconstriction and mitogenesis via phospholipase C and intracellular calcium ion mobilisation.[6,50,56-58] Additionally, less than 15% of ET_BR are present on the VSMC, where they contribute to vasoconstriction in diseased tissue.[59,60] A single layer of ET_BR found on endothelial cells of blood vessels is responsible for the release of endothelium-derived relaxing factors such as NO and prostacyclin.[7,55,60] ET_BR are also found in the kidney, localising to nonvascular tissues, where it functions as a clearing mechanism to remove ET-1 from the circulation.[50,55] The net effect of ET-1 is determined by the condition of the endothelium, the presence of receptor location and the balance between ET_AR and ET_BR.[6]

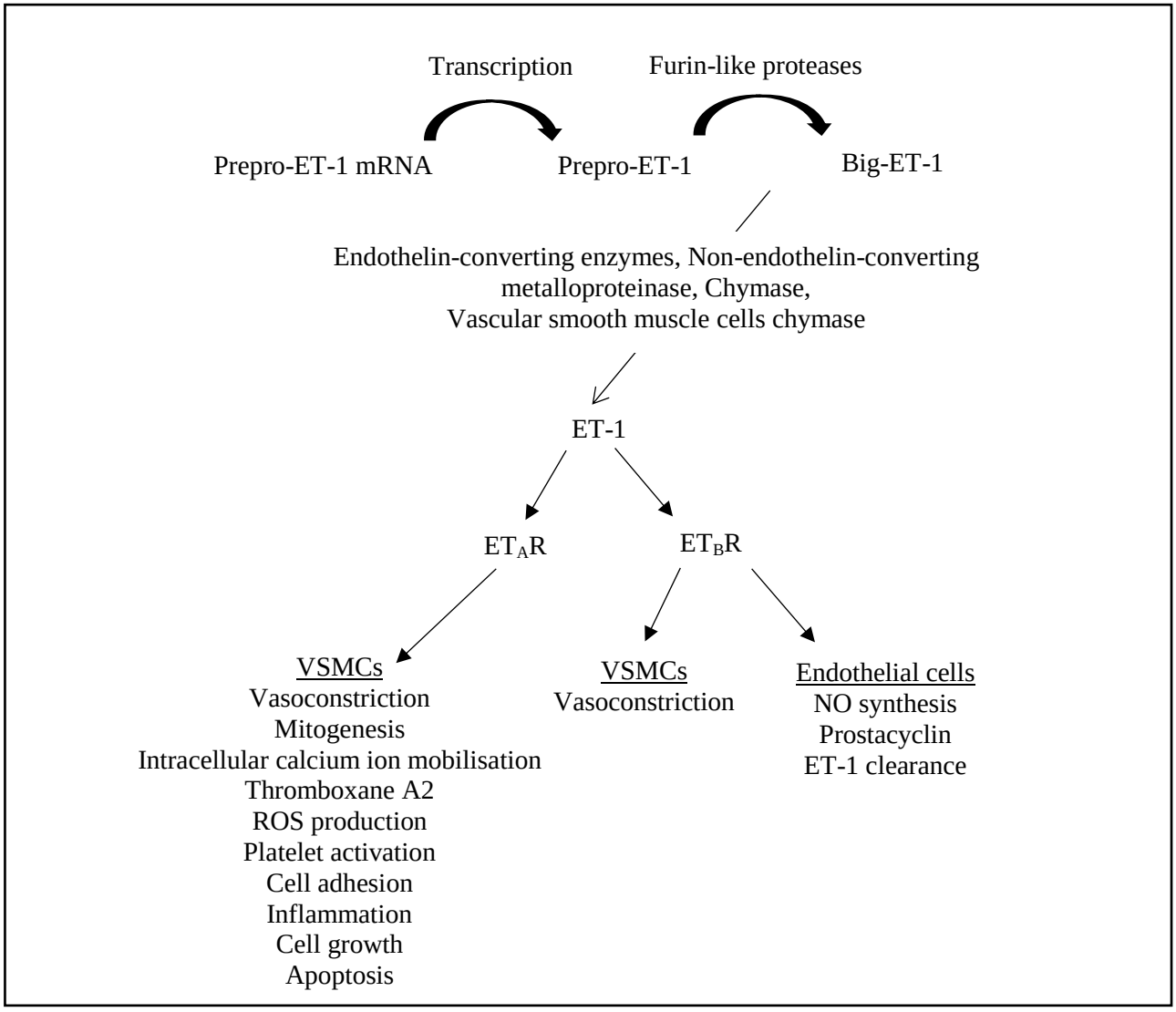

Figure 1: The production and function of endothelin-1. Abbreviations: endothelin-1 (ET-1); endothelin A/B receptor ($ET_{A/B}R$); messenger ribonucleic acid (mRNA); nitric oxide (NO) reactive oxygen species (ROS); vascular smooth muscle cells (VSMC). Adapted from Barton and Yanagisawa.[36]

1.2.2. Endothelial function and ET-1

During our lifetime, the endothelium is exposed to tearing and scarring, leading to endothelial dysfunction as the years progress.[34,61] The endothelium maintains homeostasis by actively regulating vascular tone and blood pressure through vasoconstricting and vasodilating substances.[12,15,62,63] Additionally, the endothelium can suppress inappropriate activation of the coagulation system through the production of antithrombotic factors and regulate cell proliferation and angiogenesis through secretion of various growth factors and vasoactive substances.[12,15,62,63] Vasoactive substances include endothelial-derived vasodilators such as NO, prostacyclin and endothelium-derived hyperpolarising factor[11-13] and endothelial-derived vasoconstrictors such as ET-1, angiotensin II and reactive oxygen species (ROS).[15,16,64]

ET-1 plays an important role in the health of the endothelium.[36,65] In a healthy endothelium, the endothelium responds to changes in turbulent blood flow and shear stress by producing a minimal amount of ET-1.[39] The ET-1 binds to an ET_AR or ET_BR coupled with a G-protein, activating the protein kinase C (PKC).[39,66-68] This triggers the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) (Figure 2).[39,66-68] Increased IP_3 releases calcium from the sarcoplasmic reticulum, which causes smooth muscle contraction,[69] whereas DAG is responsible for recruiting PKC to the membrane and activating it.[70] In order to maintain homeostasis, an intact endothelium has a high bio-availability of NO, inhibiting the action of ETs through increased signalling of cyclic guanosine monophosphate (cGMP), favouring vasodilation (Figure 2).[6] The production of NO is dependent on the rate at which shear stress changes.[71] Calcium ion (Ca^{2+}) dependent pathways (during initial shear stress) continuously produce low levels of nitric oxide accompanied by an up-regulation of endothelial nitric oxide synthase (eNOS) expression, the enzyme involved in the conversion of L-arginine to NO.[72,73] The up-regulation of eNOS leads to the inactivation of apoptosis-inducing exogenous oxygen radicals in endothelial cells, protecting the endothelium from the damage caused by shear stress.[73-75] The endothelial cells can also protect themselves by down-regulating ET-1 through the up-regulation of ET_BR binding sites on endothelial cells.[76] Activation of ET_BR causes eNOS expression, resulting in the clearance of circulating ET-1[76] and an increased production of prostacyclin, which possesses anti-platelet aggregation properties.[77]

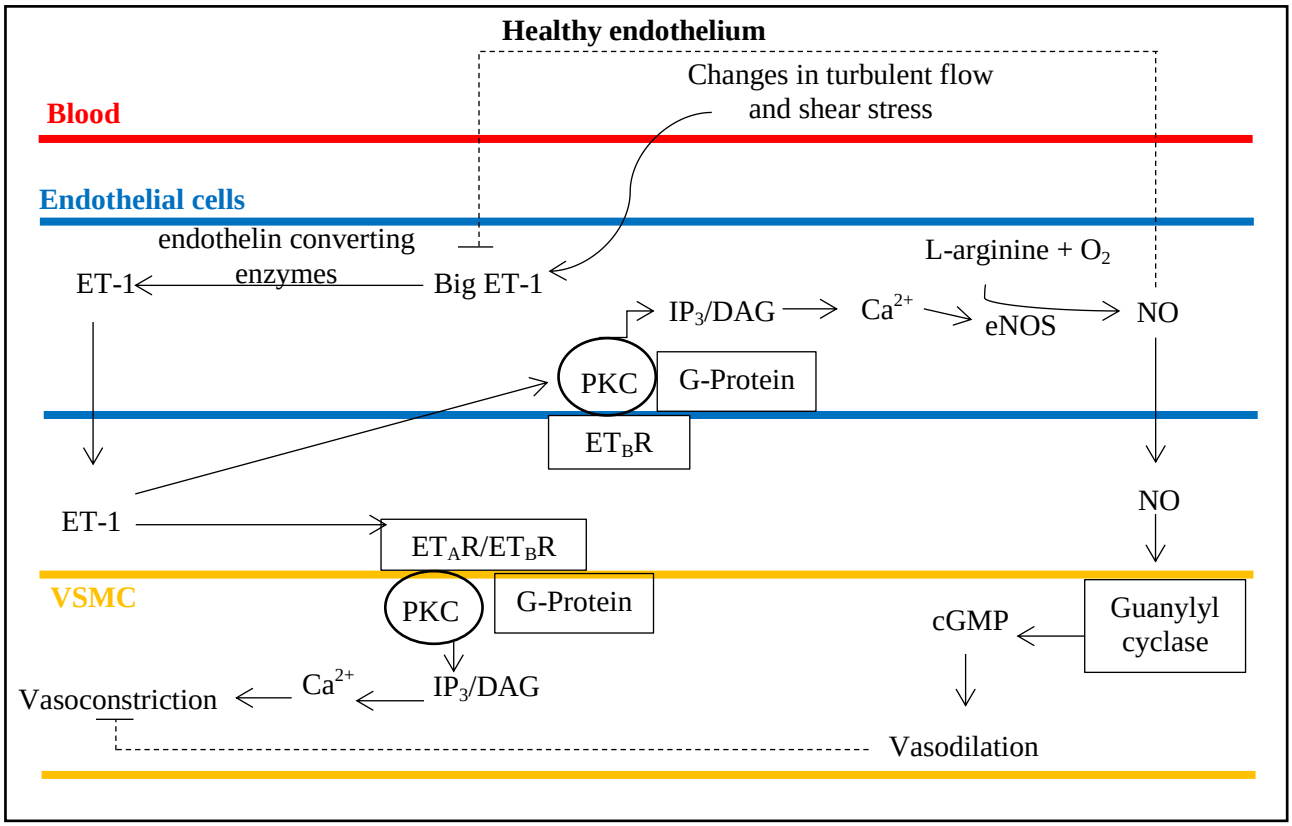

Figure 2: This figure illustrates the main concepts from the literature related to the role ET-1 plays in regulating vascular tone in a healthy endothelium. Abbreviations: calcium ions (Ca^{2+}); cyclic guanosine monophosphate (cGMP); endothelin-1 (ET-1); diacylglycerol (DAG); endothelin A/B receptors ($ET_{A/B}R$); endothelial nitric oxide synthase (eNOS); inositol triphospate (IP_3); nitric oxide (NO); oxygen (O_2); protein kinase C (PKC); vascular smooth muscle cells (VSMC). Solid lines represent stimulation and broken lines represent inhibition.

During pathological conditions such as hypertension and inflammation, ET-1 appears to contribute in the development of endothelial dysfunction.[36,78] Endothelial dysfunction is brought on by conditions such as abnormal regulation of ROS,[79,80] hyperlipidaemia[81-84] and environmental irritants such as tobacco smoke.[85-87] The endothelium will up-regulate adhesion molecules and permit the entry of lipids, monocytes and leukocytes into the arterial wall (Figure 3).[85,88,89] Active inflammatory cells result in the release of nuclear factor kappa beta (NF-kβ),[6,88] tumor necrosis factor alpha (TNF-α),[6,90] interleukin-6 (IL-6)[6,88,91] and C-reactive protein (CRP)[6,92,93] (Figure 3). IL-6 is the principle stimulus of the acute phase reaction during inflammation, in turn releasing CRP, leading to an increase in the production of ET-1 and the down-regulation of NO production by inhibiting eNOS (Figure 3).[6,94] Ca^{2+} independent pathways (during prolonged shear stress) produce large amounts of NO through the activation of the induceable form of NOS (iNOS), which can provoke free radical superoxide anion ($O_2^{\cdot-}$) formation and binding to the excess NO.[72,73] This yields a harmful and highly reactive species, namely peroxynitrite ($ONOO^-$)

(discussed later) (Figure 3).[72,73] Additionally, CRP can also cause an increased production of ET-1 through the expression of adhesion molecules, monocyte chemoattractant protein-1 and the production of other cytokines such as thrombin and angiotensin II (Figure 3).[94-96] Stimulation of human VSMC by ET-1 results in the increased accumulation of IL-6 mRNA.[88] The activation of these cytokine genes requires the induction of the NF-kβ, meaning that ET-1 release is regulated by the IL-6 through the NF-kβ mechanism (Figure 3).[97,98] Oxygen free radicals and vasoactive peptides like angiotensin II may also activate the NF-kβ system,[99-101] as well as the additional expression of cytokines through the generation of $O_2^{\cdot-}$.[102] This suggests that ET-1 production can occur in response to ROS (discussed later) (Figure 3).[102] ET-1 also causes the production of TNF-α when it activates the ET_AR or ET_BR, in turn activating tyrosine kinase.[103] This causes phosphorylation of intercellular proteins, resulting in transcription and translation of the TNF-α gene (Figure 3).[103] Previous studies found that TNF-α plays an important pro-inflammatory role in inducing endothelial dysfunction by reducing NO-dependent relaxation through the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, resulting in increased production of $O_2^{\cdot-}$ (Figure 3).[104,105]

Additionally, during inflammation, the endothelium also becomes more permeable to lipid particles and immune cells and native low-density lipoprotein cholesterol (LDLC) becomes oxidised, damaging surrounding endothelial cells that send out more chemotactic agents to attract more macrophages.[106] The activation of the oxidised LDLC receptor, the lectin-like oxidised receptor-1, generates ROS and activates the NF-kβ, resulting in the down-regulation of NO and the upregulation of ET-1 (Figure 3).[107] These macrophages become saturated and die off, forming foam cells.[85,106] The foam cells begin to accumulate at the site of injury, forming a fatty streak.[85] Platelets that are caught in the fatty streak begin to release platelet-derived growth factor, which in turn causes the growth of VSMC.[108] Macrophages are joined by VSMC from the *tunica media* where they multiply, depositing collagen and elastic fibers to form a fibrous cap over the core of dead foam cells.[85] Calcium is also drawn into the plaque, creating calcium crystals and hardening the endothelium.

Increased production of ET-1 leads to a prolonged vasoconstrictive effect in the arteries, increasing shear stress and vascular damage.[6,88,109] The inability of the endothelium to protect the underlying VSMC is

known as endothelial dysfunction.[110] Endothelial dysfunction precedes the development of local atherosclerosis and predisposes the vasculature to the development of structural vascular changes.[111]

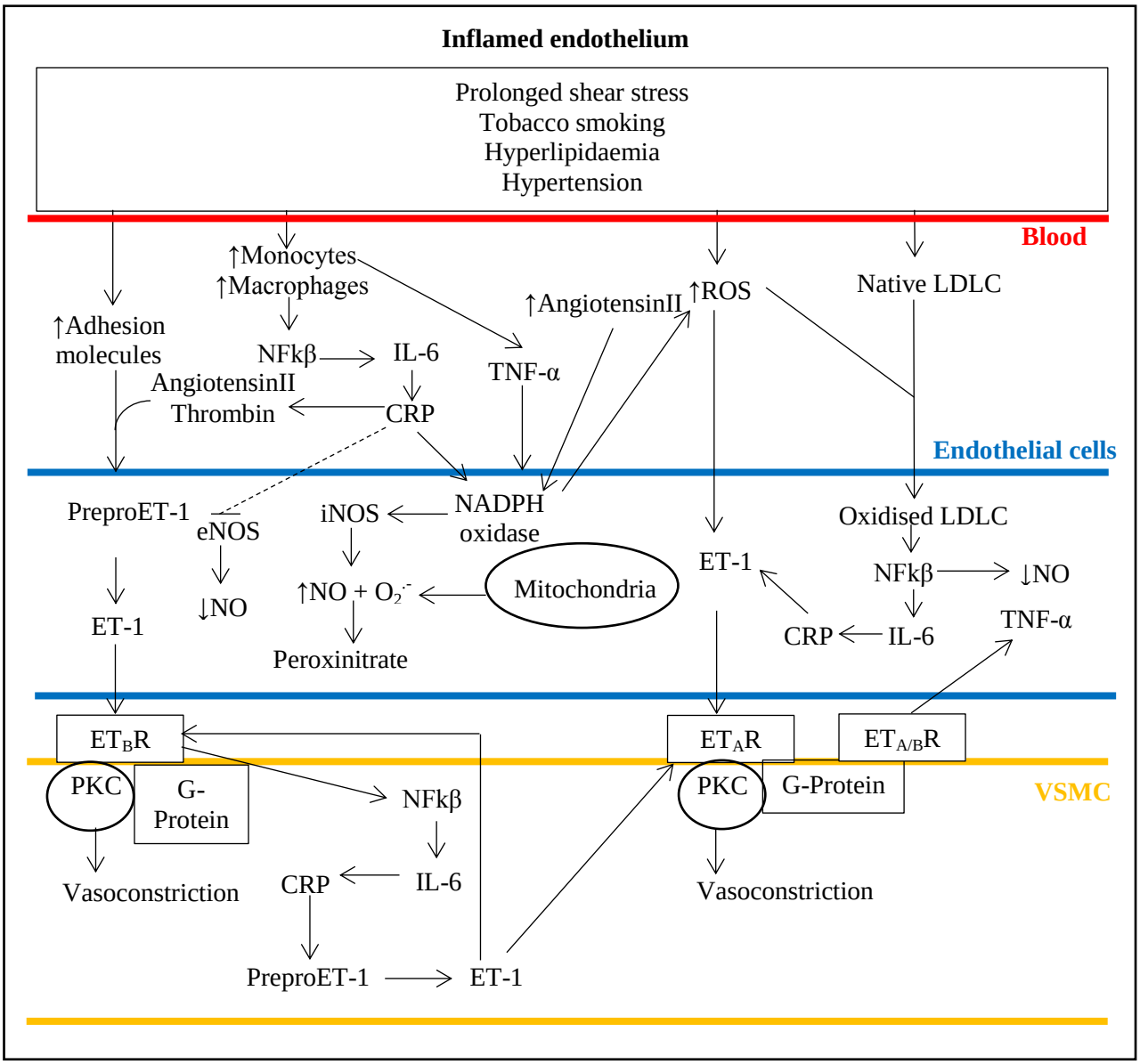

Figure 3: This figure illustrates the main concepts from the literature and represents the interrelated function of endothelin-1 with the inflammatory system during inflammation. Abbreviations: C-reactive protein (CRP); endothelial nitric oxide synthase (eNOS); endothelin-1 (ET-1); endothelin A/B receptors ($ET_{A/B}R$); interleukin-6 (IL-6); inducible nitric oxide synthase (iNOS), low-density lipoprotein cholesterol (LDLC); nicotinamide adenine dinucleotide phosphate (NADPH); nitric oxide (NO); nuclear factor kappa β (NF-kβ); superoxide anions ($O_2^{\cdot-}$); protein kinase C (PKC); reactive oxygen species (ROS); tumor necrosis factor-α (TNF-α); vascular smooth muscle cells (VSMC). Solid lines represent stimulation and broken lines represent inhibition.

1.2.4. Oxidative stress and ET-1

Molecular oxygen is a central molecule in cellular respiration, important for all living aerobic species.[112] Certain derivatives of oxygen are highly toxic to cells and oxygen-containing free radicals are responsible for toxic effects in aerobic organisms.[112,113] Approximately 5% of inhaled oxygen is converted to ROS such as $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$).[112] Angiotensin II[73,114] and inflammatory markers such as IL-6 and CRP[109,115,116] are three of the many substances responsible for activating the formation of $O_2^{\cdot-}$, H_2O_2, NO and $ONOO^-$. An imbalance between oxidants and anti-oxidants in favour of the oxidants results in the disregulation of cellular functions and disruption of redox signalling.[73,117]

NO is a gas that can easily diffuse between cells and tissues.[118] It is synthesised from L-arginine by a family of enzymes termed NOS.[118,119] The NOS enzyme contains three isoforms known as neuronal NOS, iNOS and eNOS.[118] eNOS are regulated by calcium-calmodulin-dependent enzymes that continuously produce low levels of NO to maintain vascular homeostasis, whereas iNOS are regulated by inflammatory cytokines and produce large numbers of NO to reverse endothelial damage.[73,119-122] Co-factors such as tetrahydrobiopterin (BH_4), haem, flavin adenine dinucleotide, flavin mononucleotide, calmodulin and NADPH are important for the production of NO.[118] If there is a dietary deficiency of these co-factors, it will lead to the formation of other products, for example water, H_2O_2 and $O_2^{\cdot-}$.[72,109,118] On the other hand, if NO reacts with the excess in $O_2^{\cdot-}$, it can form a harmful and higher reactive nitrogen species, $ONOO^-$, through a process known as eNOS uncoupling.[72,118,123] Additionally, $ONOO^-$ can also form during low availability of eNOS co-factors or during the oxidation of BH_4, encouraging NOS to become an $ONOO^-$ generator rather than an NO generator.[124] eNOS plays an extremely important role in cardiovascular health, since its main function is to regulate blood flow, regulate blood pressure and inhibit platelet activation.[118,125,126]

$O_2^{\cdot-}$ is one of the most important reactive oxygen species in the vasculature.[80] The main source of increased $O_2^{\cdot-}$ in the endothelial cells of the vascular wall is believed to be through xanthine oxidase, uncoupled eNOS, NADPH oxidase complexes and spill-over from the mitochondrial respiratory chain

(Figure 5).[80,127] Xanthine oxidase catalyse the catabolism of hypoxanthine to form xanthine and can in turn catabolise xanthine to form uric acid.[128] NADPH oxidase is considered one of the most important enzyme systems in vascular function because of its responsiveness to other agonists such as angiotensin II.[129] NADPH oxidase produces $O_2^{\cdot-}$ by oxidising BH_4 in conditions such as hypertension.[130] ET-1 mainly produces $O_2^{\cdot-}$ by stimulating NADPH or xanthine oxidase in the endothelium (Figure 4).[30,131] The exact mechanism for oxidative stress induced increase in ET-1 is not clear. However, a few studies have provided suggestions. Kahler et al.[30] suggest that oxygen-derived radicals interfere with intracellular calcium metabolism, in turn stimulating preproET-1 gene expression. Secondly, Loomis et al.[131] and An et al.[132] posit that NADPH oxidase mediates angiotensin II, which elicits its actions through the generation of ROS and ET-1. ET-1 expression is therefore achieved through an angiotensin-ROS-mediated mechanism. Other studies propose that ROS-mediated ET-1 expression is achieved by $O_2^{\cdot-}$ activating the preproET-1 promoter and subsequently increasing mRNA concentration in endothelial cells (Figure 4).[31,88,132,133-135] ET-1 expression can also be achieved by oxidative stress activating NF-kβ, which may stimulate preproET-1 gene expression (Figure 4);[132,136,137] or oxidative stress stimulating the generation of transforming growth factor beta in glomerular cells, which could markedly enhance ET-1 expression in VSMC and endothelial cells.[132]

In the vasculature, ET-1 promotes $O_2^{\cdot-}$ production through an ET_AR-NADPH oxidase-mediated-pathway.[133,134] An increase in LDLC and oxidised LDLC can affect the trafficking of eNOS to the caveolae, causing the uncoupling of eNOS, which results in increased $O_2^{\cdot-}$ production and NADPH oxidase induction (Figure 4).[73] An increase in ET-1 levels promotes oxidised LDL uptake into endothelial cells, accelerating the development of atherosclerosis.[107] ET-1 is believed to down-regulate eNOS expression through the dissociation of ET_BR activating eNOS phosphorylation, similar to its effect on ROS generation.[138,139] ROS and ET-1 are generated by inflammatory cells, which accumulate in inflammatory conditions such as endothelial damage.[140] Although ET_BR on endothelial cells causes the release of vasodilators and inhibits cell apoptosis, the thin layer of these receptors is broken down, which leads to an increased availability of ET_AR on smooth muscle cells. This in turn causes prolonged vasoconstriction, promotes cell growth and mediates cell mitogenesis in diseases associated with endothelial dysfunction (Figure 4).[103]

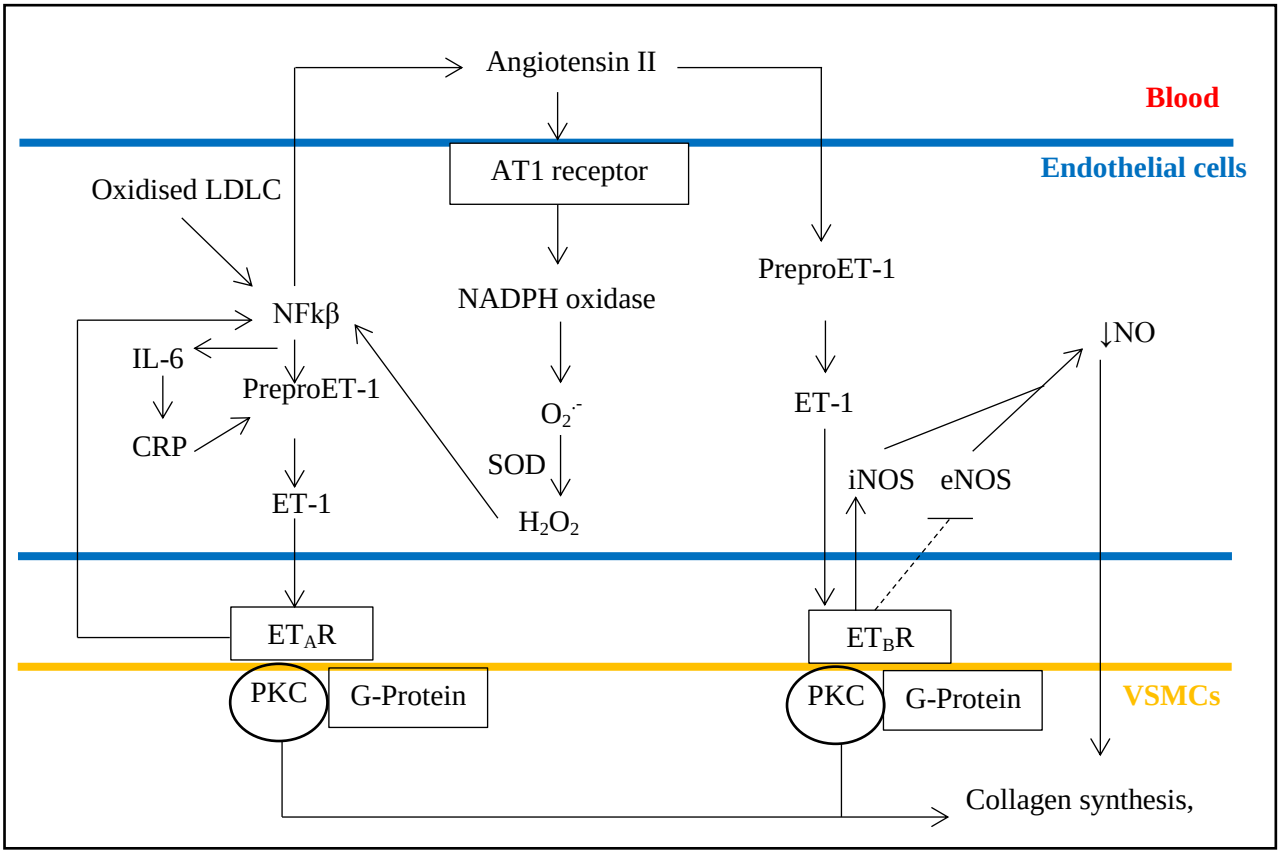

Figure 4: This figure illustrates the main concepts from the literature and represents the role ET-1 plays in oxidative stress. Abbreviations: Angiotensin II receptor type 1 (AT1); C-reactive protein (CRP); endothelial nitric oxide synthase (eNOS); endothelin-1 (ET-1); endothelin A/B receptors ($ET_{A/B}R$); induced nitric oxide synthase (iNOS); hydrogen peroxide (H_2O_2); interleukin-6 (IL-6); low-density lipoprotein cholesterol (LDLC); nicotinamide adenine dinucleotide phosphate (NADPH); nitric oxide (NO); nuclear factor kappa β (NF-kβ); superoxide ($O_2^{\cdot-}$); protein kinase C (PKC); superoxide dismutase (SOD); vascular smooth muscle cells (VSMC). Solid lines represent stimulation and broken lines represent inhibition.

1.2.5. Anti-oxidant capacity and ET-1

In healthy endothelium, the first line of defence against ROS production includes non-enzymatic and enzymatic antioxidants.[141] ROS overproduction has been implicated in numerous diseases as a result of increased presence of risk factors such as hypertension, increased LDLC, smoking, sedentary lifestyle, diabetes, obesity, hypercholesterolemia, inflammation, genetic predisposition and hyperglycaemia.[73,112,142,143] Non-enzymatic anti-oxidants include bilirubin, uric acid, vitamin C, vitamin A, β-carotene, coenzyme Q10 and glutathione (GSH) and enzymatic anti-oxidants include superoxide dismutase (SOD), catalase and the glutathione system (glutathione reductase (GR), glutathione peroxidase (GPx)).[141] Due to the scope of the study, we only focus on the literature that includes the antioxidants SOD, catalase, GSH, GR and GPx.

1.2.5.1. SOD

SOD is an antioxidant enzyme that catalyses the dismutation of two $O_2^{\cdot-}$ into H_2O_2 and molecular oxygen (Figure 5).[141,144,145] Three forms of SOD are present in humans, cytosolic or copper-zinc SOD (CuZn-SOD), mitochondrial manganese SOD (Mn-SOD) and extracellular form of SOD (EC-SOD).[144] The human arterial wall contains large amounts of secreted soluble EC-SOD and low concentrations of Mn-SOD and CuZn-SOD.[146] In healthy endothelium, the major source of soluble EC-SOD are from the VSMC, but in atherosclerotic and hypertensive vessels this enzyme is produced by both the VSMCs and macrophages.[147] This latter statement is supported by a study that found very high soluble EC-SOD activity, but not CuZnSOD activity effective in reducing vascular $O_2^{\cdot-}$ levels and mean arterial pressure.[148] Additionally, angiotensin II and hypertension modulate EC-SOD expression.[147,149] The direct link between ET-1 and SOD in human arteries is uncertain. However, in cultured human coronary artery smooth muscle cells, SOD significantly reduced ET-1 because of ET-1 primarily being secreted by $O_2^{\cdot-}$.[30] It may be possible that ET-1 levels can be modulated through the regulation of other factors such as angiotensin II, vascular cell adhesion molecule-1, NADPH oxidase and $O_2^{\cdot-}$ metabolism by EC-SOD,[30,150-152] but further investigation is warranted.

1.2.5.2. Catalase and the glutathione system

Catalase and GPx are important scavengers of H_2O_2, leading to the formation of water.[141,145] Catalase catalyses the formation of molecular oxygen and water from two H_2O_2 molecules (Figure 5).[153] It is suggested that catalase does not play such an important role as SOD in ET-1 metabolism, since the main source of ET-1 expression is through $O_2^{\cdot-}$.[30] GPx is a selenium-containing peroxidase that shares the substrate H_2O_2 with catalase and uses GSH as a substrate to be converted into glutathione disulfide (GSSG) and forms water and molecular oxygen (Figure 5).[145,153,154] GSH is synthesised in a two-step enzymatic process. Firstly, glutamate and cysteine form gamma glutamylcysteine by the activity of gamma glutamylcysteine synthase, and secondly, GSH is formed by the activity of GSH synthase by using glycine as a substrate.[155] GSH is freely distributed in the cytosol and compartmentalised to the mitochondria, endoplasmic reticulum and the nuclei matrix.[155] GSSG can be reduced to form GSH through the action of GR, using NADPH as an electron donor (Figure 5).[145,156] The GSH-to-GSSG or GR-to-GPx ratio is often used as a measure of cellular oxidative stress.[156]

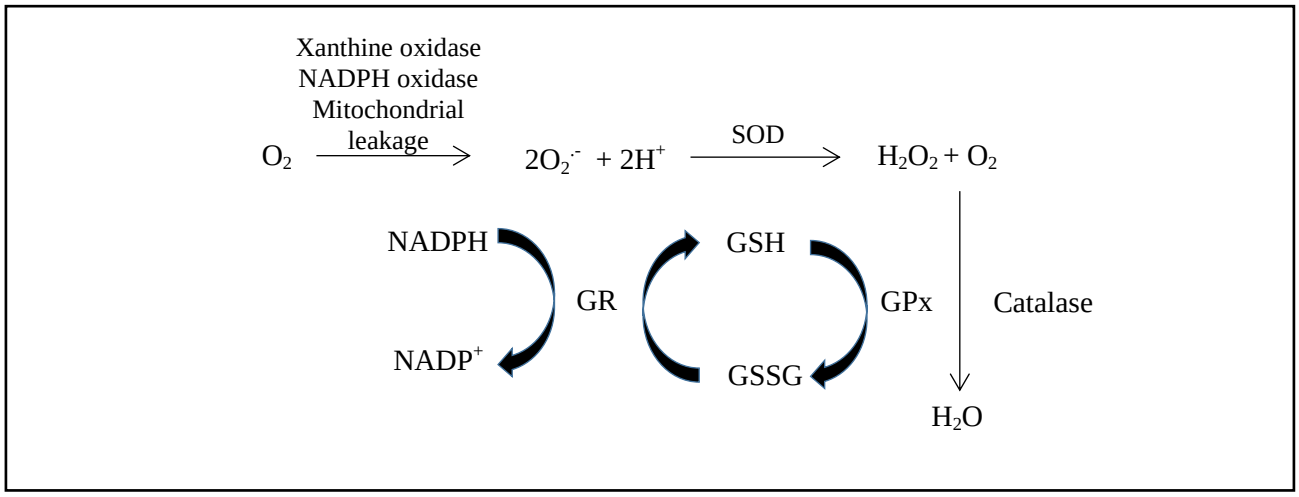

Figure 5: The anti-oxidant system. Abbreviations: glutathione peroxidase (GPx); glutathione reductase (GR); reduced glutathione (GSH); oxidised glutathione (GSSG); hydrogen ion (H^+); hydrogen peroxide (H_2O_2); nicotinamide adenine dinucleotide ($NADP^+$); nicotinamide adenine dinucleotide phosphate (NADPH); oxygen (O_2); superoxide anion ($O_2^{\cdot-}$); superoxide dismutase (SOD); water (H_2O). Adapted from Young and Woodside.[145]

A decrease in GPx activity is associated with conditions such as an hyperhomocysteinemia,[157] carotid atherosclerotic plaque[158] and coronary artery disease.[159] A previous study demonstrated that ET-1 may lead to oxidative stress in the heart tissue and pulmonary arterial endothelial cells in rats by reducing the GSH-to-GSSG ratio, stimulating lipid peroxidation and increasing TNF-α concentration via NADPH and glucose oxidase.[160] Scalera et al.[161] found that ET-1 increases the intracellular GSH levels and reduces the efflux of GSH out of the cell, which leads to the synthesis of more GSH, decreasing ET-1 and increasing prostacyclin. In women with preeclampsia there was an increase in oxidative stress and a decrease in the production of GSH in response to ET-1 increase.[162] ET-1 also increases the uptake of cysteine into the cell, leading to a decrease in GSH efflux.[163] Cysteine is an important determinant of GSH synthesis and decreased cysteine levels, and leads to inflammation in blood vessels, resulting in atherogenesis.[164,165] Low concentrations of cysteine were found to increase homocysteine oxidation dramatically, which leads to the formation of superoxide and H_2O_2.[166,167] Sethi et al.[168] found that homocysteine stimulates ET-1 synthesis through a ROS-dependent pathway. Homocysteine also impairs the production of vasodilators by decreasing the synthesis of prostacyclin and reducing NO bio-availability.[169] This could suggest that ET-1 increases blood pressure in hypertensives by means of a decrease in anti-oxidant activity and an increase in oxidative stress through a cysteine-related mechanism. Previous studies on the South African population found associations between the antioxidant system and cardiovascular variables.[170-171] Black South Africans have a lower GPx and a higher GR activity compared to their white counterparts.[170]

Higher GPx activity was independently associated with lower carotid pulse wave velocity in the white group of the South African population.[170] Additionally, a lower GPx activity in black women associated with higher blood pressure[172] and a greater carotid intima media thickness (CIMT) associated with lower total GSH in hypertensive black men.[171] The association with ET-1 and anti-oxidant capacity in this population is unclear and warrants further investigation.

1.2.6. ET-1 and its association with cardiovascular disease risk factors

The early identification of cardiovascular disease risk factors and endothelial dysfunction is important to combat the increased mortality rate as a result of cardiovascular disease in South Africans. Risk factors can be divided into two types: modifiable risk factors and non-modifiable risk factors.[173,174] Modifiable risk factors include tobacco smoking, a diet high in saturated fat and salt, physical inactivity, abuse of alcohol, obesity, high plasma cholesterol levels, high blood pressure, low high-density lipoprotein cholesterol (HDLC) levels, high LDLC levels and high triglycerides levels.[173] Non-modifiable risk factors includes age, sex and race.[174] Previous studies indicate that ET-1 is involved in the activity of most cardiovascular risk factors[6,175-178] and therefore the following section discusses the association of ET-1 with various cardiovascular risk factors.

1.2.6.1. Smoking and ET-1

Cigarette smoking in South Africa has decreased tremendously from 31% to 18.2% since 1994 because the country uses excise tax increase as a tobacco control measure.[179] However, the prevalence of tobacco smoking among men are still four times higher than in women,[180,181] and black South African adults are more likely to use tobacco products than whites.[180,181] Additionally, approximately 10% of deaths occur due to second-hand smoking.[92] Smoking affects endothelial function, oxidative stress, inflammation and vasomotor function,[182] eventually leading to atherosclerosis. Previous clinical and animal studies have demonstrated that cigarette smoking causes reduced NO bio-availability.[86,175,183-185] The reduced expression of eNOS and increased generation of oxidative stress through NADPH oxidase and xanthine oxidase leads to smoking-related endothelial dysfunction.[86,175,183-185] Haak et al.[186,187] report that ET-1 concentrations increase remarkably in short-term smokers and in chronic smokers with hyperlipidemia. It

is speculated that increased concentrations of plasma ET-1 may be because of reduced binding of ET-1 or enhanced release by either physiological or pathological stimuli.[188] However, the exact physiological role of ET in smoking is still unclear. It can be hypothesised that cigarette smoking contributes to elevated levels of ET-1 as well as enhanced vasoconstriction and an increase in the levels of ROS.[175,189] Increased levels of ROS increase the activity of NF-kβ following the induction of cytokines, chemokines and adhesion molecules,[185,190,191] decrease the protective activity of plasma HDLC[192-195] and enhance the oxidation of LDLC.[196]

1.2.6.2. Diet and ET-1

The South African population has a fast growing epidemic of obesity, especially in women.[197-199] Possible causes include over-nutrition and food high in cholesterol, saturated fat, trans fats and salt.[198,199] An excess in cholesterol, saturated fats, trans fats, and salt correlated with an increased risk of cardiovascular disease such as stroke and coronary artery disease as a result of atherosclerosis.[200]

Increased circulating ET-1 levels and oxidised LDLC have been found in patients with hyperlipidemia, hypercholesterolemia, and atherosclerosis.[176-178] Lipoproteins such as chylomicrons which transport exogenous or dietary cholesterol, and very low-density lipoproteins, which transport endogenous triglycerides and cholesterol, are important to control the lipid metabolism.[201] ET-1 augments the uptake of oxidised LDLC, whereas oxidised LDLC in turn stimulates the production of ET-1.[202,203] An over-synthesis of LDLC may lead to increased levels of LDLC in the blood, which in turn may lead to cholesterol being laid down as deposits in the arterial wall.[200] It was suggested that increased ET-1 levels in these patients are due to oxidised LDLC-mediated secretion of ET-1 in VSMCs and macrophages.[176-178] Oxidised LDLC can also up-regulate ET_BR expression in both VSMCs and monocyte-derived macrophages and ET_AR expression in coronary artery VSMC.[178] HDLC is responsible for removing the cholesterol deposits in the artery wall to maintain homeostasis.[200] If HDLC levels are low, LDLC remains in the arterial wall and become oxidised, leading to a series of steps that eventually cause atherosclerosis.[200] Additionally, HDLC can also trigger a variety of intracellular signalling pathways in many cell types, unrelated to cholesterol homeostasis.[204,205] Low concentrations of HDLC were found to stimulate the production of ET-1 through PKC activation.[204,205] On the other hand, cholesterol can also

directly diminish the contractile effect of ET-1 by influencing store-operated channels.[206] Pro-inflammatory stimuli such as a diet high in saturated fat, obesity and hypercholesterolemia can also activate the endothelium by increasing levels of cytokines such as IL-6 and TNF-α,[26,207] which increase ET-1 levels in the plasma.[207]

Treatment with ET blockers and statins was found to improve the damaging effects on the endothelium caused by ET-1 in hypercholesterolemia and atherosclerosis patients significantly. Studies investigating the role of ET-1 receptors in hypercholesterolemia found that an ET_AR blockade leads to a reduction of macrophage infiltration, reduces fatty streaks, increases stable NO metabolites and normalises NO-mediated endothelium-dependent relaxation.[111,208,209] Hypercholesterolemia patients are known for having a decrease in NO bio-availability,[210] and ET-1 interferes with NO synthesis. ET-1 contributes to a decrease in NO bio-availability by increased expression of eNOS protein and eNOS enzyme activity, stimulating free radical forming enzymes and promoting the interaction of caveolin and eNOS.[111,208-210] On the other hand, statin therapy improves the effect of ET blockers on NO-mediated vasodilation in hypercholesterolemia.[6,211] Statins decrease the expression of prepro-ET-1 mRNA in endothelial cells and the vasoconstrictor response to ET-1, attenuating the negative effect of ET-1 on endothelial function.[6,211-213] Black South Africans are known for having a favourable serum lipid profile of low cholesterol and high HDLC when compared to their white counterparts, although urban black South Africans are beginning to adapt to a more Westernised diet. This increases the body mass index and total serum cholesterol of men and women, making this population susceptible to an increased risk for cardiovascular disease.[214-217] It is clear that ET-1 levels increase in patients with high cholesterol levels, leaving the South African population at risk for the development of atherosclerosis and other lipid-related diseases.

1.2.6.3. Hypertension and ET-1

South Africa has one of the highest rates of hypertension worldwide. Currently, the main causes of hypertension in South Africans are lifestyle factors such as obesity and a diet high in salt.[218] It is estimated that between 65% and 78% of cases of hypertension could be attributed to obesity[219,220] and the prevalence of obesity is exceptionally high in urban black women of sub-Saharan Africa.[221] Previous

studies found that obesity, salt sensitivity and ET-1 contribute to the development of hypertension in the black American population.[18,222-225]

Adipose tissue plays an important role in obesity. Dysfunction of the adipose tissue causes endothelial dysfunction, vascular hypertrophy and impaired pressure natriuresis through the activation of the renin-angiotensin aldosterone system (RAAS), sympathetic nervous system, oxidative stress and inflammation, which all lead to hypertension.[226-230] Leptin is an important adipocyte-derived hormone that regulates food intake, body weight and increases in energy expenditure by activating the sympathetic nervous system.[231] Increasing leptin production can also affect blood pressure by renal sympathetic activation and NO synthesis.[225] Chronic administration of leptin decreases natriuresis, increases urinary excretion of NO metabolites and increases the level of systemic and intrarenal oxidative stress, leading to NO deficiency.[225] Increasing ET-1 levels also decrease NO bio-availability in obese patients.[222-224] This increase in ET-1 levels of obese individuals may be as a result of leptin, which has the ability to up-regulate ET-1 production in human endothelial cells.[232,233] Leptin can also stimulate the generation of ROS and through an ET-1-ET_AR-ROS pathway, exert its action through the inflammatory system.[233-235] These findings suggest that leptin-induced ET-1 can contribute to increased hypertension in obese individuals.[232,233]

Although leptin can release ET-1 through a ROS-dependent pathway, ET-1 can also exert its effects through non-endothelial pathways similar to those found in adipose tissue such as the RAAS.[19,236] The RAAS is a hormonal cascade that plays a critical role to control arterial pressure and extracellular volume in high-salt diets.[237] During low blood pressure, renin is responsible for converting angiotensinogen to angiotensin I and angiotensin I to angiotensin II via angiotensin-converting enzyme.[238] Angiotensin II then binds to angiotensin II type 1 receptors on the vascular endothelial and VSMC, impairing NO synthesis, leading to vasoconstriction.[238] The binding of angiotensin II to angiotensin II type 1 receptors also releases aldosterone, responsible for sodium retention, which together with vasoconstriction causes an increase in blood pressure.[238] After the RAAS have increased blood pressure, renin is decreased through a negative feedback system.[238] Non-renin pathways, or alternative pathways, do not make use of renin or angiotensin-converting enzymes to control blood pressure.[239] In these non-renin pathways, tonin

and cathepsin D release angiotensin I and tissue plasminogen activator and produce angiotensin II directly from angiotensinogen.[239] Chymase, endopeptidase and carboxypeptidase produce angiotesin II from angiotensin I via an angiotensin-convering enzyme-independent pathway.[239] Angiotensin-converting enzyme degrades bradykinin directly and the binding of angiotensin II to angiotensin II type 1 receptors decreases NO synthesis, and vasoconstriction, releasing aldosterone and increasing blood pressure.[239] In response to an increase in blood pressure through the direct or alternative pathways of RAAS, angiotensin II can activate free radical production and vascular cell adehesion molecule-1 expression, which leads to chronic inflammation and eventually endothelial dysfunction.[239]

Low plasma renin activity is much higher in blacks than whites.[240] This is possibly due to blacks retaining more sodium and blood pressure regulation being more salt-sensitive than in whites.[240] It can also be as a result of the role ET-1 has in the renovasculature.[18] A diet high in salt increases renal ET-1 production and eNOS expression.[241] ET-1 exerts a vasoconstrictor function on both the afferent and efferent arterioles and causes a decrease in renal blood flow and glomerular filtration rate, reducing sodium excretion.[18] The black population was found to have increased circulating ET-1 levels, which can exert the latter effect more profoundly than in the white population.[18] ET-1 exerts effects on the RAAS by inhibiting renin production and stimulating aldosterone production from the adrenocortical zona glomerulosa.[242-244] The binding of ET-1 to ET_AR or ET_BR increases aldosterone directly from the zona glomerulosa cells, increasing plasma volume and inhibiting juxtaglomerular cells from releasing renin.[242,244] A study assessing the effect of 24-hour salt restriction on plasma ET-1 levels in salt-sensitive patients, found that ET-1 levels are elevated together with catecholamines even with blunted renin activity in this group.[245] It was also suggested that the distribution of ET receptors could explain the racial differences found in the relation to the effect exerted by ET-1.[18,246] However, studies on receptor distribution in the arterioles of the kidneys are unclear.

1.2.6.4. Aging and ET-1

Aging causes structural and functional modification in the vasculature such as infiltration of VSMCs, atherosclerotic plaque, arterial thickening, arterial stiffness and reduced NO bio-availability.[247] Changes due to aging is an unavoidable fate. However, due to cardiovascular risk factors the effects of aging

through the inflammatory process seems to appear earlier. Changes often seen in older patients such as an increase in arterial stiffness, increase in the thickness of large arteries and endothelial dysfunction are more extensive in patients with hypertension or atherosclerosis at a younger age.[247] Previous studies have demonstrated that smoking, overproduction of ROS, inhibition of DNA repair, increased RAAS activity, endothelial cell senescence, decreased cell proliferation, increased sodium intake, increased calcium content and decreased NO bio-availability speed up the arterial aging process.[248-251] Experimental[28,252] and human studies[28,253] showed an increased expression of ET-1 with aging, possibly due to an increase in the deterioration of endothelial function.[28,253] The mechanism through which ET-1 causes early vascular aging is unclear. However, it is hypothesised that conditions associated with aging, such as increased levels of angiotensin II,[44,132] reduced bio-availability of NO and increased levels of oxygen-derived free radicals, [30,31,132,252] increase ET-1 levels with age.[252] A few studies demonstrated that the black population of South Africa is at risk for developing early vascular diseases normally associated with aging.[254-256] There is currently no other study investigating the association between aging and ET-1 levels in the South African population, and since this group is subjected to early vascular damage ET-1 may be an important risk factor for this group.

1.2.6.5. Race and sex differences of ET-1 levels and receptors

As in African Americans, blacks in South Africa have a higher prevalence of CVD.[257] Resting ET-1 levels was found to be higher in black Africans and black Americans than in whites.[258] Campia et al.[258] have demonstrated that young, healthy black African subjects have reduced NO-mediated vasodilation of forearm resistance vessels compared to their white counterparts. This reduced NO-mediated vasodilation indicates the presence of impaired vascular smooth muscle relaxation, which may lead to increased vascular tone and hypertension.[258] Plasma ET-1 levels were also found to be increased in hypertensive blacks compared to whites.[259] The smooth muscle cells of saphenous veins obtained from black Africans appeared to contain both receptor subtypes, whereas the VSMCs of saphenous veins obtained from white Africans possessed a higher density of the ET_A subtypes receptor.[19] These differences in ET-1 levels may be due to the different distribution of ET receptor subtypes (ET_A and ET_B) in black and white Africans.[19]

Sex also appears to have an effect on ET-1 levels. Adult white males have higher circulating levels of ET-1 than females.[19,259,260] The saphenous veins from black men contain fewer ET-binding sites than those of white men, and black women have twice as few ET receptors than white women.[260] Evidence indicates that the lower levels of ET-1 observed in women, may be due to the modulatory action of female gonadal hormones.[261] This statement is supported by previous studies that found healthy postmenopausal women have higher ET-1 and lower NO levels than younger women and that the ET-1 levels decreased and NO levels increased remarkably after hormone replacement therapy.[262-264]

1.2.7. ET-1, blood pressure and vascular remodeling

1.2.7.1. Blood pressure

Experimental studies indicated an enhanced production of ET-1 in some hypertensive models.[265,266] Blood pressure is an important determinant of vascular dysfunction.[267,268] Aging leads to a steady increase in systolic blood pressure, with a decline in diastolic blood pressure after the age of 55 years.[267,269] The increase in systolic blood pressure and decrease in diastolic blood pressure leads to an increase in pulse pressure due to an increase in the systolic-diastolic blood pressure difference.[267] As seen previously in this chapter, blood pressure changes play an important role in the homeostasis of the endothelium. Although systolic blood pressure effects have been investigated at length, previous studies found pulse pressure is a stronger independent predictor to endothelial-related changes and is closely associated with left ventricular hypertrophy and carotid atherosclerosis.[270-272] ET-1 also plays an important role in blood pressure and have a positive inotropic effect on the heart,[60,273] decreasing cardiac output, in turn decreasing heart rate and stroke volume.[274] High concentrations of ET-1, as seen in patients with heart failure and renal failure, accelerated pulse wave velocity, increasing systolic blood pressure and pulse pressure due to this decrease in cardiac output.[274] In South Africa, a large number of studies have found an increased risk for hypertension in black South Africans,[26,214,221,275,276] proposing that 24% of blacks that have high blood pressure will develop hypertension.[218] This association of ET-1 with markers of vascular function could shed some light on the increasing risk of the black South Africans to develop hypertension.

1.2.7.2. CIMT and arterial stiffness

The CIMT shows the thickness of the *tunica intima* and *tunica media* of the blood vessels and is used for evaluating the regression and/or progression of atherosclerotic cardiovascular disease.[277-279] The 2013 ESH/ESC hypertension guidelines confirmed a CIMT ≥ 0.9 mm as a marker of asymptomatic organ damage.[280] Between the ages of 20 and 90 years, the CIMT increases 2 to 3 times because of luminal dilation and increased wall stiffness associated with aging.[281,282] Although CIMT measures the extent of atherosclerosis,[277-279] Lakatta et al.[282] concluded that CIMT and endothelial dysfunction is not a result of atherosclerotic risk factors, but rather age-related remodeling of the arterial wall, increasing the sensitivity of the wall to atherosclerotic risk factors. Structural changes in aging arteries include an increase in the medial thickness through VSMC hypertrophy, an increase in collagen content and a decrease in elastin density. This leads to arterial stiffness due to decreased arterial compliance.[283] Aging in arteries is also accompanied by a decrease in the production of vasoactive substances such as NO, independent of hemodynamic factors.[284]

The decrease in arterial compliance and increase in vasoconstriction can result in an increase in systolic blood pressure and pulse pressure, increasing the risk for the development of hypertension and atherosclerosis in aging individuals.[285,286] These diseases are also associated with morphological and functional alterations of arteries similar to those found during aging.[287] Previous studies found an association between increased CIMT and left ventricular mass in response to the hypertrophy of the media layer and increased CIMT and adaptive thickening in response to changes in the transmural pressure, shear stress and lumen diameter.[278] Studies also found an association between CIMT and chronic heart disease such as stroke and myocardial infarction.[279,288,289] These studies indicate that with every 0.13 mm increase in the CIMT of the common carotid artery, the risk of coronary death or myocardial infarction increased by 40%.[279,288,289] Cardiovascular risk factors such as age,[290] sex,[291,292] smoking,[291] high cholesterol,[293] high systolic blood pressure,[294] increased ET-1 levels,[295-299] increased TNF-α activity,[291] increased NADPH-oxidase activity,[296] and increased homocysteine levels[300] also had an effect on CIMT. Hypercholesterolemic,[293,301] hypertensive,[294] diabetic,[295] and smoking[291] patients have a 5-12% increase in CIMT compared to patients without these conditions.[302] Studies investigating the link between ET-1 and CIMT suggest that the association of ET-1 with increasing CIMT may be due to an

increase in NADPH oxidase activity and $O_2^{\cdot-}$ increasing ET-1 expression.[296-299] ET-1 was found to also contribute to collagen synthesis and elastin degradation, leading to an increase in CIMT.[303] Statins[293,301] and regular aerobic exercise[295] were found to decrease the progression of CIMT in turn decreasing the risk for atherosclerosis and hypertension. Schutte et al.[25] investigated arterial stiffness in black and white South Africans and found that the change in arterial stiffness is age-related in whites and pressure-related in blacks, increasing their risk for the development of atherosclerosis in this population. It is possible that ET-1 can be the culprit responsible for age- and pressure-related changes in this population, but further research is needed to test this hypothesis.

1.2.7.3. Cross-sectional wall area (CSWA) and arterial stiffness

CSWA is the area encompassed by artery circumference minus the lumen area.[304] Changes in the CSWA occur in response to alterations in blood flow, blood pressure and atherosclerosis. [304] These changes are termed remodeling.[304] Remodeling can be characterised as inward or outward changes in vessel lumen diameter and increased change (hypertrophic), decreased change (atrophic) or no change (eutrophic) in the vessel wall thickness.[304,305] Increased blood flow and atherosclerosis cause the vascular wall to undergo eutrophic outward remodeling, whereas hypertrophic inward remodeling is associated with an increase in blood pressure.[304] Outward remodeling occurs mainly due to inhibiting production of NO with eNOS inhibitors,[306] whereas inward remodeling occurs due to the activation of autocrine mechanisms that stimulate VSMC growth and paracrine mechanisms that increase the production of angiontensin II in resistance arteries, changing the vessel wall matrix.[27] ET-1, a peptide that acts in an autocrine and paracrine fashion, can contribute to outward remodeling through its NADPH oxidase function by increasing oxidative stress and inward remodeling in response to the CRP-angiotensin II-induced ROS production. This results in the VSMC migration and proliferation.[27,307] Additionally, atherosclerotic arteries, which are associated with an abundant VSMC migration into the intima, were found to have increased ET_AR mRNA, contributing to outward remodeling.[308] In South Africans, CSWA is associated with leptin levels.[309] Leptin can contribute to vascular remodeling by increasing the release of vasoconstrictive substances, increasing the production of ROS and secreting inflammatory cytokines, all of which contribute to obesity-related hypertension and atherosclerosis.[309] Additionally, CSWA also correlated negatively with GSH levels in the same population.[172] Other studies found that leptin increases

ET-1 production by endothelial cells, and ET-1 in turn increases ROS production, decreasing GSH levels. This association between ET-1, leptin and ROS could provide a possible leptin-mediated-, ROS-mediated-ET-1 effect on vascular remodeling in the general South African population. However further investigation is needed.

1.3. Aims

General aim

The general aim of this study is to explore the link between ET-1 levels and cardiometabolic function and arterial stiffness.

The detailed aims are as follows:

Chapter 3 – The association of ET-1 with markers of arterial stiffness in black South African women: The SABPA study

Chapter 3 aims to:

- determine whether ET-1 levels differ among sex and race in black and white South Africans.
- investigate the association of ET-1 with cardiovascular function (systolic blood pressure, pulse pressure and mean arterial pressure) and inflammation (interleukin-6 and C-reactive protein) in a bi-ethnic South African population.

Chapter 4 – The association of ET-1 with markers of oxidative stress in a bi-ethnic South African cohort: The SABPA study

Chapter 4 aims to:

- investigate associations of ET-1 with markers of oxidative stress (serum peroxides) and anti-oxidant capacity (GPx, GR, catalase, GSH) in black and white South Africans.

Chapter 5 – Three-year change in endothelin-1 and markers of vascular remodeling in a bi-ethnic South African cohort: The SABPA study

Chapter 5 aims to:

- investigate the association of change in ET-1 levels and the association of these changes with markers of vascular remodeling such as CIMT, CSWA and arterial compliance after three years in black and white South Africans.

1.4. Hypotheses

With regard to the literature and the specific study population, the following hypotheses are tested in this research:

Chapter 3 - The association of ET-1 with markers of arterial stiffness in black South African women: The SABPA study

- ET-1 levels are higher in black men and women in comparison to their white counterparts.
- ET-1 associates with cardiovascular deterioration (systolic blood pressure, pulse pressure and mean arterial pressure) and inflammatory markers (interleukin-6 and C-reactive protein) in black men and women.

Chapter 4 – The association of ET-1 with markers of oxidative stress in a bi-ethnic South African cohort: The SABPA study

- ET-1 associates positively with markers of oxidative stress and negatively with markers of anti-oxidant capacity (GPx, GR, catalase, GSH).

Chapter 5 – Three-year change in endothelin-1 and markers of vascular remodeling in a bi-ethnic South African cohort: The SABPA study

- An increase in ET-1 levels associates positively with markers of vascular remodeling (CIMT, CSWA, and arterial compliance) after three years
- The black population has more adverse changes in vascular remodeling (such as CIMT, CSWA, and arterial compliance).

1.5. Structure of the thesis

The format of this thesis complies with the prescribed article format as approved by the North-West University. The thesis consists of three manuscripts accepted or submitted for publication in international peer-reviewed journals.

- Chapter 1 provides an introduction, literature overview, aims, objectives and the hypotheses related to the topic of the thesis.
- Chapter 2 discusses the study protocol along with the materials and methods used to obtain the data from the SABPA study.
- Chapter 3 (the first manuscript) determines whether ET-1 levels differ among sex and race and investigates the association of ET-1 with cardiovascular function and inflammation in a bi-ethnic South African population.
- Chapter 4 (the second manuscript) investigates the associations in ET-1 with markers of oxidative stress and anti-oxidant capacity (GPx, GR, SOD, catalase, GSH) in black and white South Africans.
- Chapter 5 (the third manuscript) investigates the association of change in ET-1 levels (after 3 years) and markers of vascular remodeling such as CIMT, CSWA and arterial compliance in black and white men and women.
- The final chapter (Chapter 6) provides a critical summary of the results, providing final conclusions and recommendations.

The relevant references are provided at the end of each chapter according to the style as described by the journal. The Vancouver referencing style reference style is used in all unpublished chapters.

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CHAPTER 2

STUDY PROTOCOL AND METHODOLOGY

2.1. Study design

The Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study was a prospective target population study that consisted of 200 black (101 men and 99 women) and 209 white (101 men and 108 women) South-African school teachers from the Dr. Kennith Kuanda Education District of the North-West Province. The reason for this selection was to obtain a homogenous sample from a similar socioeconomic class with income parity. The cultural background of the ethnic groups is different, and this may play a role in nutrient intake, alcohol intake and smoking habits. Our sub-study is embedded in the SABPA study and detailed layout of the experimental protocol and data collection procedures was previously described.[1] The methodology appropriate to this sub-study will be discussed. All participants were between 20 and 65 years of age. The baseline and follow-up data collection was conducted in two phases to minimize seasonal effects. Phase I commenced in 2008 and Phase II in 2011 for baseline data. For follow-up data collection, Phase I commenced in 2009 and Phase II in 2012. Exclusion criteria included participants that recently donated blood, had an elevated ear temperature (>37°C), were pregnant, were lactating, were users of alpha and beta blockers, had vaccinations 3 months or less prior to participation or were using psychotropic substances. Contact between baseline and 3-year follow-up was sustained via a mobile cellular messaging system and feedback workshops to explain results and make referrals.

The Ethics Committee of the North-West University, Potchefstroom Campus, approved the study (project no: NWU-00036-07-A6). All participants were informed of the procedures regarding the study prior to their recruitment and black field workers were available to relay information to the black participants in their home language. For the investigation of human subjects, the study protocol conforms to the ethical guidelines of the Declaration of Helsinki (as revised in 2008). All participants were coded with their personal information and data treated confidentially and also only linked to a numeric value that is not traceable by any outsider. Data was stored from all devices on external hard disks and DVD-ROM discs, recorded into Excel spreadsheets and password protected for security of the data.

For the purpose of this study, additional inclusion and exclusion criteria were implemented for each article as follows:

- In article 1, the study included 99 black men and 95 black women as well as 99 white men and 98 white women. Excluded from the study was outliers of endothelin-1 (ET-1) (n=10) by residual statistics as well as participants with missing ET-1 data (n=8).
- In article 2, the study included 99 black men and 96 black women as well as 99 white men and 99 white women. Excluded from the study was outliers of ET-1 (n=10) by residual statistics as well as participants with missing ET-1 data (n=8).
- In article 3, 48 participants did not participate in the follow-up study and were excluded from the study together with outliers of ET-1 by residual statistics (baseline: n=8; follow-up: n=0) and participants with missing ET-1 data for both baseline and follow-up (n=14). Included were 87 black men and 79 black women as well as 87 white men and 84 white women.

2.2. Materials and methods

2.2.1. Organisational procedures

An ambulatory blood pressure monitoring (ABPM) device (Meditech CE120® Cardiodens, Budapest, Hungary) was fitted to the non-dominant arm of each participant with an appropriately sized cuff between 07h00 and 08h00 from Mondays to Thursdays. The ABPM device was programmed to measure blood pressure in 30 minute intervals during the day (08h00 to 22h00) and 60 minute intervals during the night (22h00 to 06h00). The same day the ABPM device was fitted; participants spent the night at a Metabolic Unit facility on the Potchefstroom-campus of the North-West University where they were asked to complete questionnaires which included questions about their cardiovascular health history, contraceptive usage and chronic medication usage such as statins and anti-hypertensive drugs. Each participant also received human immunodeficiency virus pre-counselling. At 20h30 the participants received a standardized dinner and abstained from consuming alcohol and caffeine and refrained from smoking for the remainder of the night. Participants were encouraged to be in bed by approximately 22h00. The following morning participants were woken at 05h45. At 06h00 the ABPM apparatus were removed and a urine sample was collected.

2.2.2. Anthropometric and physical activity measurements

Qualified anthropometrists measured the body height, body weight as well as the waist circumference of each participant according to standard procedures.[2] Body composition of each participant was obtained in triplicate. Body height and weight were measured to the nearest 1.0 cm and 0.1 kg, respectively, with calibrated instruments (Invicta Stadiometer, IP 1465, London, UK and Precision Health Scale, A&D Company, Tokyo, Japan) while participants were in minimal clothing. Waist circumference was measured with a non-stretchable metal flexible measuring tape (Holtain Ltd., Dyfed, UK). Body mass index was calculated as weight in kilograms divided by height in meters squared and rounded to 1 decimal point.[3] At baseline total energy expenditure was calculated using the Actical® activity monitor (Mini Mitter Co., Inc, Bend, OR; Montreal, Quebec, Canada) over a 24 h period taking the resting metabolic rate into account.

2.2.3. Cardiovascular measurements

The cardiovascular measurements were tested by making use of the validated Finometer device (FMS, Finapres Measurement Systems, Amsterdam, Netherlands) [4-6]. The participant was requested to lie in the Fowler's position with their arm at heart level. A finger cuff was placed on the middle phalanx of the left hand, and a brachial cuff is connected to the upper arm. A 2-minute calibration is performed to provide an individual subject-level adjustment of the finger arterial pressure with the brachial arterial pressure [5]. The highest precision in cardiovascular measurement can be achieved only after this calibration. Measurements were recorded continuously for at least five minutes. Afterwards the Beatscope 1.1 software was used to calculate the systolic blood pressure, diastolic blood pressure, mean arterial pressure, pulse pressure, stroke volume and arterial compliance [7]. As part of the nonlinear three-element model which includes aortic characteristic impedance, arterial compliance and systemic vascular resistance, the Windkessel compliance or Buffer Compliance of the arterial system was computed from an age-dependent, aortic pressure-area relationship and represents the lumped compliance of the entire arterial system. [7]. To minimize inter- and intra individual variability of measurements, all measurements were performed in a temperature controlled room and only trained staff members performed the measurements.

The Complior SP Acquisition System (Artech-Medical, Pantin, France) was used to measure pulse wave velocity from the carotid to dorsalis-pedis pulse sites [8]. The carotid-femoral pulse wave velocity are determined non-invasively from an arterial segment that includes both elastic and muscular arteries by measuring the distance between the carotid artery and the femoral artery divided by the transit time (the time of travel of the foot of the wave over the distance) [9]. All measurements were taken by the same two observers on the left side of each participant while the participants were lying in the supine position. This method was regarded as the gold standard for measuring arterial stiffness, unfortunately the femoral measurement was a sensitive measure to perform in a population study outside of a clinical setting, therefore it was unavailable. At the time of the study, more advanced mechanical methods such as the SphygmoCor and the Doppler Ultrasound was not yet available at our research unit.

The Cardiotens (CE120, Meditech, Hungary) is an ABPM device that is a non-invasive, fully automated technique in which blood pressure is recorded over an extended period of time, usually 24 hours [10]. ABPM are superior to other clinical measurements such as office blood pressure measurements, since ABPM predicts blood pressure-related complications more accurately, whereas office blood pressure measurements are subjected to mechanical defects, physician error and white coat effects [10]. The Cardiotens (CE120, Meditech, Hungary) was used to record 24-hour ambulatory systolic and diastolic blood pressure, which were used to derive 24h pulse pressure. Participants with a systolic blood pressure $\geq$ 135 mmHg and/or diastolic blood pressure $\geq$ 85 mmHg were considered hypertensive [11]. The 24-hour blood pressure data were downloaded onto a database using the CardioVisions 1.9.0 Personal Edition software.

Carotid intima-media thickness (CIMT) are used as a surrogate marker for atherosclerosis [12, 13]. Ultrasound measurements of CIMT can be used to reclassify patients at intermediate risk, discriminate between patients with and without prevalent of cardiovascular disease and predict major adverse cardiovascular events [14]. The carotid ultrasound also has a potential advantage to the computed tomography coronary artery calcium testing method since it does not involve exposure to ionizing radiation and it is more accurate when measuring the intima-media thickness [14, 15]. In this study, CIMT was measured using a SonoSite Micromaxx ultrasound system (SonoSite, Bothell, WA, USA) and

a 6-13 MHz linear array transducer from at least two optimal angles of the left and right common carotid artery. Subsequently, the carotid cross-sectional wall area was calculated with the formula: cross-sectional wall area = $\pi(d/2 + \text{carotid intima media thickness})^2 - \pi(d/2)^2$. Interpretations were made for both carotid intima-media thickness and carotid cross-sectional wall area by a single reader with a semi-automated program, the Artery Measurement Systems (AMS) II v1.139 (Gothenburg, Sweden).

2.2.4. Biochemical analyses

A registered nurse collected fasting blood samples with a sterile winged infusion set from the participants' antebrachial vein branches. Serum samples were prepared according to standard procedures and frozen at -80°C until analysed. Procedures followed for quantitative analyses were the same for both baseline and follow-up unless stated otherwise.

ET-1 was determined by an ET-1 Quantikine enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). Serum interleukin-6 was determined with a high sensitivity interleukin-6 Quantikine ELISA (R&D Systems, Mineapolis, MN, USA). Serum cotinine was determined with a homogenous immunoassay on a Roche Modular system (Roche, Basil, Switzerland). Gamma glutamyl transferase was used as an indication of alcohol abuse and cotinine for smoking.[16, 17] At baseline fasting lipids (triglycerides, total and high-density lipoprotein cholesterol), glycated hemoglobin A1c, C-reactive protein, gamma glutamyl transferase and creatinine were determined using two sequential multiple analysers in serum samples (Konelab 20i, Thermo Scientific, Vantaa, Finland and Unicel DXC 800, Beckman and Coulter, Germany) and at follow-up, the variables were determined using the Integra 400 plus (Roche, Basel, Switzerland). Although HbA1c% is regarded a less sensitive variable in non-diabetic subjects than glucose, the majority of our total population was prediabetic or diabetic (53.7%) hence the use of HbA1c%. Low density lipoprotein cholesterol was calculated with the Friedewald formula: low density lipoprotein cholesterol = Total cholesterol – high density lipoprotein cholesterol – (triglycerides/2.2), provided that no value of triglycerides inserted is higher than 4000 mmol/L [18]. Urinary albumin and creatinine levels were determined (Konelab 20i, Thermo Scientific, Vantaa, Finland; Unicel DXC 800, Beckman and Coulter, Germany) and the albumin:creatinine ratio was calculated [19]. The modification of diet in renal disease (MDRD) formula was used to calculate

estimated glomerular filtration rate (eGFR) from serum creatinine levels, age, sex, and ethnicity: eGFR = 175 * (standardized serum creatinine (mg/dL))$^{-1.154}$ * (age (years))$^{-0.203}$ * 1.212 [if black] * 0.742 [if female] [20]. Estimated creatinine clearance was calculated by using the Cockcroft-Gault formula as follows: Creatinine clearance (ml/min) = (140-age) x weight (kg) x constant/serum creatinine (μmol/L), where the constant for females are 1.23 and 1.04 for males [21]. Serum estradiol and testosterone levels were determined using an electrochemiluminescence immunoassay (ECLIA) (Elecsys 2010, Roche, Basil, Switzerland). Human immunodeficiency virus status was measured, using the First Response Kit (Premier Medical Corporation, Daman, India) as well as the Pareekshak test (Bhat Biotech, Bangalore, India).

One of the measurable reactive oxygen species, total peroxides, was determined in serum samples [22]. Total glutathione levels were determined with the BIOXYTECH GSH/GSSG-412 supplied by OxisResearch (Forster City, CA, USA). GPx and GR (ethylenediaminetetra acetic acid (EDTA) plasma) and serum SOD activities were determined with assay kits (Cayman Chemical Company, Ann Arbor, MI, USA), whereas serum catalase activity was determined with a Oxiselect Fluorometric kit from Cell Biolabs (San Diego, CA, USA) with appropriate apparatus (Synergy H_4hybrid Microplate Reader; BioTek, Winooski, VT, USA). Antioxidant enzyme ratios were calculated to assess antioxidant defenses and included the GR-to-GPx ratio and the GPx-to-SOD.

2.2.5. Statistical analyses

G*Power version 3.1.9.2 software (University of Kiel, Kiel, Germany) was used to compute the achieved power in post hoc analysis [23]. Statistical analyses were done using IBM SPSS Statistics version 22 and version 23 (IBM Corp., Armonk, NY, USA, 2013 and 2016). Main effects of race, sex and increased/decreased ET-1 levels with cardiometabolic-, oxidative stress- and arterial stiffness-related markers were tested by means of multiple regression analyses. Variables with a non-Gaussian distribution were logarithmically transformed and the central tendency and spread were represented by the geometric mean and the 5th and 95th percentile intervals. *T*-tests were used to compare means and Chi-square tests to compare proportions between the race/sex groups and groups with increased/decreased ET-1 levels after 3 years. Unadjusted and adjusted correlation between ET-1 and variables associated with cardiometabolic-,

oxidative stress- and arterial stiffness-related variables were performed. Multivariable linear regression models for each group were performed with ET-1 as the main dependent variable.

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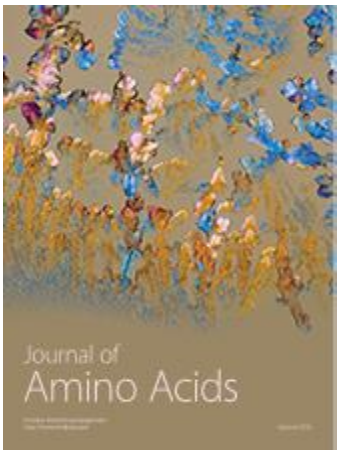
CHAPTER 3

The association of endothelin-1 with markers of arterial stiffness in black South African women: The SABPA study



This article is published in the *Journal of Amino Acid*

Hindawi Publishing Corporation
Journal of Amino Acids
Volume 2015, Article ID 481517, 8 pages
http://dx.doi.org/10.1155/2015/481517

Research Article

The Association of Endothelin-1 with Markers of Arterial Stiffness in Black South African Women: The SABPA Study

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Received 15 October 2015; Revised 26 November 2015; Accepted 1 December 2015

Academic Editor: Hieronim Jakubowski

Background. Limited data exist regarding endothelin-1 (ET-1), a vasoactive contributor in vascular tone, in a population subjected to early vascular deterioration. We compared ET-1 levels and explored its association with markers of arterial stiffness in black and white South Africans. *Methodology*. This cross-sectional substudy included 195 black (men: $n = 99$; women: $n = 95$) and 197 white (men: $n = 99$; women: $n = 98$) South Africans. Serum ET-1 levels were measured as well as markers of arterial stiffness (blood pressure, pulse wave velocity, and arterial compliance). ET-1 levels were higher in black men and white women compared to their counterparts after adjusting for C-reactive protein. In both single and partial (adjusting for body mass index and gamma glutamyl transferase) regression analyses ET-1 correlated with age, interleukin-6, high density lipoprotein cholesterol, systolic blood pressure, pulse pressure, and pulse wave velocity in black women. In multivariate regression analyses the independent association of ET-1 with systolic blood pressure (Adj. $R^2 = 0.13$; $\beta = 0.28$, $p < 0.01$) and pulse pressure (Adj. $R^2 = 0.11$; $\beta = 0.27$, $p < 0.01$) was confirmed in black women only. ET-1 additionally associated with interleukin-6 in black women ($p < 0.01$). *Conclusion*. Our result suggests that ET-1 and its link with subclinical arteriosclerosis are potentially driven by low-grade inflammation as depicted by the association with interleukin-6 in the black female cohort.

- This article is prepared according to the author's instructions from the *Journal of Amino Acids* (Annexure A). Please note that some of the format requirements were changed to ensure uniformity throughout the thesis.

Abstract

Background. Limited data exist regarding endothelin-1 (ET-1), a vasoactive contributor in vascular tone, in a population subjected to early vascular deterioration. We compared ET-1 levels and explored its association with markers of arterial stiffness in black and white South Africans.

Methodology. This cross-sectional sub-study included 195 black (men: n = 99; women: n = 95) and 197 white (men: n = 99; women: n = 98) South Africans. Serum ET-1 levels were measured as well as markers of arterial stiffness (blood pressure, pulse wave velocity and arterial compliance). ET-1 levels were higher in black men and white women compared to their counterparts after adjusting for C-reactive protein. In both single and partial (adjusting for body mass index and gamma glutamyl transferase) regression analyses, ET-1 correlated with age, interleukin-6, high density lipoprotein cholesterol, systolic blood pressure, pulse pressure and pulse wave velocity in black women. In multivariate regression analyses the independent association of ET-1 with systolic blood pressure (Adj. R^2 = 0.13; β = 0.28, p < 0.01) and pulse pressure (Adj. R^2 = 0.11; β = 0.27, p < 0.01) was confirmed in black women only. ET-1 additionally associated with interleukin-6 in black women (p < 0.01).

Conclusion. Our result suggests that ET-1 and its link with subclinical arteriosclerosis are potentially driven by low-grade inflammation as depicted by the association with interleukin-6 in the black female cohort.

Keywords: endothelin-1, arterial stiffness, black, white, South-African, inflammation

1. Introduction

The high incidence of hypertension and development of early vascular deterioration among the black population remains to be elucidated [1-3]. Vascular aging is characterised by vascular dysfunction manifested as thinning, fraying and fracturing of elastic laminae and increasing connective tissue and collagen fibers that will lead to stiffening of the arteries [4,5]. Apart from natural or biological ageing, it is also known that hypertension, atherosclerosis, type 2 diabetes mellitus, chronic renal disease, excessive salt use, and changes in neurohormonal regulation influence the onset and augmentation of arterial stiffness [6].

ET-1 is an important vasoactive biomarker due to its pivotal role in vascular tone and dysfunction [7,8]. Endothelial dysfunction is a precursor for vascular disease usually elicited by the release of a variety of paracrine factors such as endothelin-1 that interact with platelets, inflammatory cells and the vessel wall [9]. Experimental studies demonstrated that higher ET-1 levels associated with aging may contribute to vascular endothelial dysfunction [10-13]. ET-1 was also positively associated with large artery stiffness in patients with coronary artery disease [14,15]. The upregulation of ET-1 activates inflammatory cells and leads to nitric oxide synthase inhibition associated with arterial stiffness [7,9,16-18].

Ergul et al. [19-21] reported that the black population have higher ET-1 levels and disparities in renal and smooth muscle cell endothelin receptors and are also predisposed to early vascular alterations compared to white individuals. However, limited information is available on the link between ET-1 and arterial stiffness (or arteriosclerosis), especially in sub-Saharan Africa, and therefore we aimed to compare ET-1 levels and explore the association of ET-1 with markers of arterial stiffness along with its potential determinants in a black and white South African cohort.

2. Materials and Methods

2.1. Study Population and Protocol. Cross-sectional data from the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study was used including 194 black (men: n = 99; women: n = 95) and 197 white (men: n = 99; women: n = 98) South Africans. Detailed information regarding the

procedures of the SABPA study has been published previously [22]. Patients who were pregnant, were lactating, and were using alpha and beta blockers and patients with an ear temperature ≥37°C and who had a vaccination or donated blood 3 months prior to participation were excluded from the prospective cohort study. However, in the current analysis we excluded outliers of ET-1 (n = 10) by residual statistics with participants with missing ET-1 data (n = 6). A standard health survey was used for the collection of demographic information and anti-hypertension medication usage. The Health Research Ethics Committee of the North-West University, Potchefstroom Campus, granted ethical approval for this substudy (NWU-00036-07-A6). The study protocol conforms to the ethical guidelines of the Declaration of Helsinki (as revised in 2008) for the investigation on human subjects.

2.2. Anthropometric Measurements. Body composition of each participant was obtained in triplicate according to standard procedures [23]. The body height was measured to the nearest 1.0 cm (Invicta Plastics 1465, Leicester, UK) and body mass to the nearest 0.1 kg (Precision Health Scale, A&D Company, Tokyo, Japan). Waist circumference was measured with a nonstretchable metal flexible measuring tape (Holtain Ltd., Dyfed, UK) and body mass index rounded to 1 decimal point [24].

2.3. Cardiovascular Measurements. The cardiovascular measurements were taken in a semirecumbent position for each participant. Five-minute continuous measurements of cardiovascular variables were recorded using the validated Finometer (Finapres Medical Systems, Amsterdam, Netherlands), based on the vascular unloading technique of Peñáz, and were processed with the Beatscope 1.1 software to obtain systolic blood pressure, diastolic blood pressure, mean arterial pressure, pulse pressure, stroke volume and arterial compliance [25]. The Complior SP Acquisition System (Artech-Medical, Pantin, France) was used to measure pulse wave velocity from the carotid to dorsalis pedis.

2.4. Biochemical Analyses. A fasting blood sample was collected from each participant and serum prepared according to standard procedures. Serum samples were frozen at -80°C until analysed. ET-1 was determined by an ET-1 Quantikine enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). Intra- and interassay variability for ET-1 were 2.7% and 17.15%, respectively. Serum interleukin-6 was determined with a high sensitivity interleukin-6 Quantikine ELISA (R&D

Systems, Mineapolis, MN, USA). Intra- and interassay variation of interleukin-6 were 4.2% and 6.4%, respectively. Serum cotinine was determined with a homogenous immunoassay on a Roche Modular system (Roche, Basil, Switzerland). Fasting lipids (triglycerides, total and high density lipoprotein cholesterol), glycated hemoglobin A1c, C-reactive protein and gamma glutamyl transferase were determined using two sequential multiple analysers in serum samples (Konelab 20i, Thermo Scientific, Vantaa, Finland, and Unicel DXC 800, Beckman and Coulter, Germany). Intra- and interassay variability were less than 10%. Gamma glutamyl transferase was used as an indication of alcohol abuse and cotinine for smoking [26,27]. Low density lipoprotein cholesterol was calculated with the Friedewald formula: low density lipoprotein cholesterol = Total cholesterol – high density lipoprotein cholesterol – (triglycerides/2.2), provided that no value of triglycerides inserted is higher than 4000 mmol/L [28]. Urinary albumin and creatinine levels were determined (Konelab 20i, Thermo Scientific, Vantaa, Finland; Unicel DXC 800, Beckman and Coulter, Germany) and the albumin:creatinine ratio was calculated [29]. Estradiol levels were determined using an electrochemiluminescence immunoassay (ECLIA) (Elecsys 2010, Roche, Basil, Switzerland). Intra- and interassay variability were less than 10% for both albumin:creatinine ratio and estradiol.

2.5. Statistical Analyses. G*Power version 3.1.9.2 software was used to compute the achieved power in post hoc analysis to determine a fixed model, single regression coefficient for black women in linear regression analysis [30]. At α error probability of 0.05, effect size (f2) of 0.15, and one-tailed input method, the achieved power (1-β error probability) was estimated at 98.21%. Statistical analyses were done using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA, 2013). Main effects of race and sex were tested on the associations between ET-1 and cardiovascular components by means of multiple regression. *t*-tests were used to compare means and Chi-square tests to compare proportions between the groups. Bivariate and partial correlations were used to determine the correlation of ET-1 with cardiovascular and biochemical variables. Forward stepwise multiple regression analyses were performed to determine independent associations between ET-1 and cardiovascular measures. We applied a sensitivity analysis for estradiol and anti-hypertension medication as covariates in the same multiple regression models.

The following covariates were considered for entry into the models: age, waist circumference, gamma glutamyl transferase, glycated hemoglobin A1c, high density lipoprotein cholesterol, albumin:creatinine ratio, and interleukin-6. Each model included a main independent measure of vascular function or arterial stiffness, that is, model 1 with systolic blood pressure, model 2 with pulse pressure, model 3 with arterial compliance and model 4 with pulse wave velocity, whereas ET-1 was the designated dependent variable. Additionally in model 3 (pulse wave velocity) mean arterial pressure was added to the list of covariates. Graphpad v5.03 (GraphPad Software, Inc., San Diego, California, USA) was used to plot endothelin-1 against systolic blood pressure, pulse pressure, pulse wave velocity and arterial compliance in black women only (Figure 1).

3. Results

Basic descriptive characteristics of this study population are listed in Table 1. Due to significant interactions on the association of ET-1 with systolic blood pressure, the population was stratified according to race ($F(391) = 6.78$; $p < 0.001$) and sex ($F(391) = 2.39$; $p < 0.05$).

There was no significant difference in ET-1 levels between the black and white groups before adjusting for C-reactive protein. We additionally adjusted for C-reactive protein to investigate the effect of inflammation on ET-1 levels. After adjusting for C-reactive protein, the ET-1 levels were significantly higher in black men than white men, and higher in white women than black women (all $p < 0.001$). Black men and women had higher systolic blood pressure, diastolic blood pressure, mean arterial pressure and pulse wave velocity in comparison with their white counterparts (all $p < 0.05$). C-reactive protein, interleukin-6 and glycated hemoglobin A1c were higher in the black compared to the white groups (all $p < 0.05$). White participants had higher low density lipoprotein cholesterol and total cholesterol levels in comparison with black individuals ($p < 0.001$). Gamma glutamyl transferase levels were higher in black men and women ($p < 0.001$), with no significant difference in cotinine levels between the two groups.

In bivariate analysis (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/481517), no correlations were evident between ET-1 and cardiovascular or biochemical variables in men. A positive correlation was observed between ET-1 and systolic blood

pressure (r = 0.27; p = 0.008), pulse pressure (r = 0.25; p = 0.014) and pulse wave velocity (r = 0.23; p = 0.026) in black women only (Figure 1). In white women a positive correlation was observed between ET-1 and stoke volume (r = 0.23; p = 0.026). A positive correlation was observed between ET-1 and age (r = 0.26; p = 0.009), interleukin-6 (r = 0.27; p = 0.007), high density lipoprotein cholesterol (r = 0.23; p = 0.026), and a borderline positive correlation with mean arterial pressure (r = 0.20; p = 0.053) in black women.

After adjustments for body mass index and gamma glutamyl transferase (Table 2), the positive correlation remained between ET-1 and interleukin-6 (r = 0.22; p = 0.031), high density lipoprotein cholesterol (r = 0.25; p = 0.017), systolic blood pressure (r = 0.26; p =0.013), pulse pressure (r = 0.23; p = 0.025), pulse wave velocity (r = 0.20; p = 0.050) and a borderline positive correlation with mean arterial pressure (r = 0.19; p = 0.063) in the black women. An inverse correlation between ET-1 and arterial compliance (r = –0.24; p = 0.018) also emerged in black women. The correlation between ET-1 and stroke volume persisted in white women (r = 0.24; p = 0.021).

Since no significant correlations existed in men, the forward stepwise multiple regression analyses were only performed in women. The previous association between ET-1 and stroke volume in white women disappeared (Adj. R^2 = 0.025; β = 0.12; p = 0.55). In black women (Table 3), an independent association of ET-1 with systolic blood pressure (Adj. R^2 = 0.178; β = 0.269; p = 0.005), pulse pressure (Adj. R^2 = 0.159; β = 0.233; p = 0.017) and mean arterial pressure (Adj. R^2 = 0.149; β = 0.211; p = 0.031) was confirmed, but no association with pulse wave velocity (Adj. R^2 = 0.149; β = 0.197; p = 0.197) and arterial stiffness (Adj. R^2 = 0.142; β = –0.018; p = 0.880) was confirmed. ET-1 also associated with high density lipoprotein cholesterol (all models $p < 0.05$) and interleukin-6 (all models $p < 0.01$) in all four models and with age in model 4 (Adj.R^2 = 0.142; β = 0.199; p = 0.047).

3.1. Sensitivity Analysis. After performing the same multiple regression analyses and additionally correcting for estrogen and hypertension medication, no change was observed in the previous association of ET-1 with systolic blood pressure (Adj. R^2 = 0.178; β = 0.269; p = 0.005), pulse pressure (Adj. R^2 =

0.159; β = 0.233; p = 0.017), pulse wave velocity (Adj. R^2 = 0.149; β = 0.197; p = 0.197), and arterial compliance (Adj. R^2 = 0.142; β = –0.018; p = 0.880) in black women.

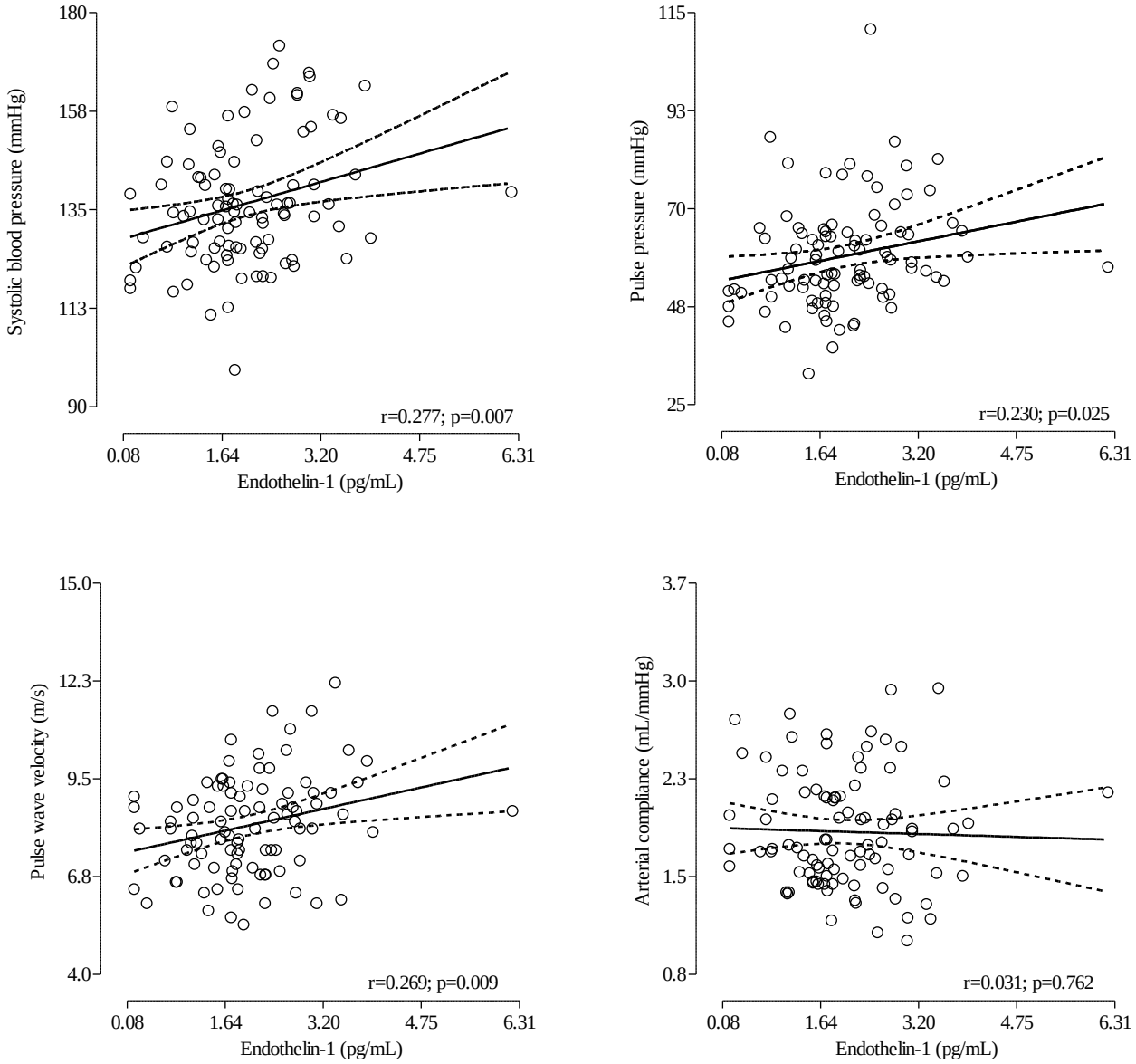

FIGURE 1**:** Endothelin-1 with systolic blood pressure, pulse pressure, pulse wave velocity and arterial compliance in black women only

TABLE 1**:** Population characteristics stratified by race and sex

Variable	Men (*n* = 198)			Women (*n* = 193)		
	Black (*n* = 99)	White (n = 99)	*p* value	Black (*n* = 95)	White (*n* = 98)	*p* value
Age (years)	43.1 ± 8.08	45.0 ± 11.1	0.18	45.6 ± 7.95	44.7 ± 10.7	0.52
Body mass index (kg/m^2)	27.6 ± 5.80	29.1 ± 5.23	0.061	32.9 ± 7.29	26.1 ± 5.62	<0.001
Waist circumference (mm)	93.6 ± 15.5	101.7 ± 14.5	<0.001	93.9 ± 15.6	87.8 ± 13.0	<0.001
Cardiovascular variables						
Systolic blood pressure (mmHg)	146.0 ± 21.0	137.0 ± 13.0	<0.001	136.0 ± 14.0	132.0 ± 15.0	0.042
Diastolic blood pressure (mmHg)	86.0 ± 11.0	80.0 ± 8.0	<0.001	77.0 ± 8.0	73.0 ± 7.00	<0.001
Pulse pressure (mmHg)	61.0 ± 8.0	56.0 ± 7.0	0.018	50.0 ± 10.0	46.0 ± 7.00	0.80
Mean arterial pressure (mmHg)	111.0 ± 14.0	102.0 ± 9.0	<0.001	101.0 ± 9.0	97.0 ± 9.00	<0.001
Stroke volume (mL)	101.0 ± 25.6	103.0 ± 20.4	0.48	102.0 ± 30.0	93.0 ± 24.0	0.014
Arterial compliance (mL/mmHg)	1.89 ± 0.42	2.33 ± 0.52	<0.001	1.86 ± 0.42	1.88 ± 0.40	0.65
Pulse wave velocity (m/s)	9.18 ± 2.29	8.62 ± 1.34	0.039	8.19 ± 1.39	7.47 ± 1.19	<0.001
Hypertension status, n (%)	64 (64.6)	85 (85.9)	<0.001	63 (66.3)	87 (88.8)	<0.001
Anti-hypertensive medication, *n* (%)	35 (17.7)	14 (7.1)	<0.001	33 (16.9)	12 (6.2)	0.019
Biochemical variables						
Endothelin-1 (pg/mL)*	2.06 ± 1.67	1.92 ± 1.69	<0.001	1.74 ± 1.87	1.90 ± 1.72	<0.001
C-Reactive Protein (mg/L)	2.73 (0.27 – 16.1)	1.82 (0.99 – 8.20)	0.003	7.09 (0.78 – 35.7)	2.22 (0.99 – 14.3)	<0.001
Interleukin-6 (pg/mL)	1.95 (0.33 – 3.57)	0.87 (0.27 – 2.90)	0.032	1.24 (0.41 – 3.06)	0.95 (0.29 – 3.63)	0.016
Glycated hemoglobin A1c (%)	6.16 (5.20 – 9.60)	5.65 (5.10 – 6.60)	0.032	5.79 (5.10 – 6.60)	5.36 (4.99 – 5.90)	<0.001
High density lipoprotein cholesterol (mmol/L)	1.04 ± 0.34	1.00 ± 0.27	0.36	1.20 ± 0.31	1.42 ± 0.42	<0.001
Low density lipoprotein cholesterol (mmol/L)	2.86 ± 0.95	3.91 ± 1.07	<0.001	3.05 ± 1.09	3.94 ± 1.11	<0.001
Total cholesterol (mmol/L)	4.72 ± 1.17	5.59 ± 1.21	<0.001	4.46 ± 1.21	5.54 ± 1.31	<0.001
Triglycerides (mmol/L)	1.46 (0.57 – 4.96)	1.30 (0.54 – 3.16)	0.16	1.11 (0.42 – 2.13)	0.81 (0.40 – 2.22)	0.13
Albumin:creatine ratio (mg/mmol/L)	1.46 ± 1.81	0.40 ± 0.99	<0.001	1.73 ± 3.54	0.80 ± 1.60	0.019
Cotinine (ng/mL)	62.9 (5.00 – 275.0)	77.3 (1.00 – 623.0)	0.50	48.4 (3.87 – 271.1)	91.7 (6.00 – 307.0)	0.21
Gamma glutamyl transferase(U/L)	63.0 (23.6 – 382.9)	27.5 (11.0 – 101.9)	<0.001	35.6 (16.7 – 116.6)	14.2 (6.00 – 41.0)	<0.001

Values are arithmetic mean ± standard deviation, geometric mean (5th and 95th confidence interval), or number of participants. *Analysis of covariance was performed by adjusting for C-reactive protein only.

TABLE 2: Partial correlations of endothelin-1 with cardiometabolic variables adjusted for body mass index and gamma glutamyl transferase.

	Endothelin-1 (pg/mL)			
	Men (n = 198)		Women (n = 193)	
	Black (n = 99)	White (n = 99)	Black (n = 95)	White (n = 98)
Cardiovascular variables				
Systolic blood pressure (mmHg)	r = 0.097; p = 0.35	r = 0.078; p = 0.45	r = 0.26; p = 0.013	r = 0.12; p = 0.23
Diastolic blood pressure (mmHg)	r = 0.028; p = 0.79	r = 0.15; p = 0.16	r = 0.11; p = 0.27	r = –0.047; p = 0.65
Pulse pressure (mmHg)	r = 0.11; p = 0.28	r = –0.022; p = 0.83	r = 0.23; p = 0.025	r = 0.18; p = 0.080
Heart rate (beats per minute)	r = –0.050; p = 0.64	r = –0.002; p = 0.99	r = 0.16; p = 0.13	r = –0.11; p = 0.29
Mean arterial pressure (mmHg)	r = 0.061; p = 0.56	r = 0.105; p = 0.31	r = 0.19; p = 0.063	r = 0.048; p = 0.64
Stroke volume (mL)	r = 0.12; p = 0.26	r = –0.103; p = 0.31	r = –0.001; p = 0.99	r = 0.24; p = 0.021
Arterial compliance (mL/mmHg)	r = 0.012; p = 0.91	r = –0.064; p = 0.53	r = –0.24; p = 0.018	r = 0.11; p = 0.29
Pulse wave velocity (m/s)	r = 0.13; p = 0.21	r = 0.15; p = 0.14	r = 0.20; p = 0.050	r = 0.11; p = 0.29
Biochemical variables				
Interleukin-6 (pg/mL)	r = –0.031; p = 0.76	r = –0.15; p = 0.14	r = 0.22; p = 0.031	r = –0.14; p = 0.16
Glycated hemoglobin A1c (%)	r = –0.047; p = 0.65	r = 0.004; p = 0.97	r = 0.12; p = 0.27	r = 0.007; p = 0.95
Total cholesterol (mmol/L)	r = 0.064; p = 0.53	r = 0.050; p = 0.62	r = 0.044; p = 0.67	r = –0.070; p = 0.50
High density lipoprotein cholesterol (mmol/L)	r = 0.050; p = 0.63	r = 0.16; p = 0.12	r = 0.25; p = 0.017	r = 0.060; p = 0.56
Triglycerides (mmol/L)	r = 0.077; p = 0.46	r = 0.081; p = 0.43	r = –0.020; p = 0.85	r = –0.023; p = 0.82
Albumin:creatinine ratio (mg/mmol/L)	r = 0.058; p = 0.57	r = –0.065; p = 0.53	r = 0.042; p = 0.68	r = 0.087; p = 0.40

TABLE 3: Forward stepwise regression analyses of endothelin-1 with measures of arterial stiffness in black women.

	Endothelin-1 (pg/mL) (n = 95)	
Model 1: systolic blood pressure	Adj. R^2 = 0.178	
	Std β (95% CI)	p value
Systolic blood pressure (mmHg)	0.269 (0.083 – 0.455)	0.005
Interleukin-6 (pg/mL)	0.290 (0.104 – 0.476)	0.003
High density lipoprotein cholesterol (mmol/L)	0.203 (0.047 – 0.419)	0.016
Model 2: pulse pressure	Adj. R^2 = 0.159	
	Std β (95% CI)	p value
Pulse pressure (mmHg)	0.233 (0.045 – 0.421)	0.017
Interleukin-6 (pg/mL)	0.278 (0.090 – 0.466)	0.005
High density lipoprotein cholesterol (mmol/L)	0.231 (0.043 – 0.419)	0.018
Model 3: pulse wave velocity	Adj. R^2 = 0.149	
	Std β (95% CI)	p value
Interleukin-6 (pg/mL)	0.296 (0.108 – 0.484)	0.003
High density lipoprotein cholesterol (mmol/L)	0.239 (0.051 – 0.427)	0.015
Mean arterial pressure	0.211 (0.023 – 0.399)	0.031
Model 4: arterial compliance	Adj. R^2 = 0.142	
	Std β (95% CI)	p value
Interleukin-6 (pg/mL)	0.258 (0.066 – 0.450)	0.010
Age (Years)	0.199 (0.005 – 0.393)	0.047
High density lipoprotein cholesterol (mmol/L)	0.209 (0.017 – 0.401)	0.036

Covariates considered for entry into the models included age, waist circumference, gamma glutamyl transferase, glycated hemoglobin A1c, high density lipoprotein, albumin:creatinine ratio, total cholesterol, interleukin-6 and in model 3 additionally mean arterial pressure.

4. Discussion

It was previously shown that the black population are predisposed to early vascular alterations compared to white individuals [19-21]; however, limited information is available on the link between ET-1 and arterial stiffness within a bi-ethnic South African population. Our results indicated an independent association of ET-1 with systolic blood pressure and pulse pressure in black women only. We also found an independent association between ET-1 and interleukin-6, suggesting that the link between ET-1 and subclinical vascular dysfunction may be mediated by pro-inflammation. Our study further contributes to the lack of information regarding ET-1 and cardiovascular function in black populations from sub-Saharan Africa, especially in black women prone to arterial stiffness and hypertensive heart disease.

Levels of ET-1 were not different between race groups in our study population, however after considering C-reactive protein as confounding variable through univariate analysis of covariance, there was a significant difference in ET-1 levels between race groups. White women had higher levels of ET-1 than black women in our population. This is in contradiction with other studies that found that black individuals have higher ET-1 levels than whites [20,31-33]. The link observed between ET-1 and pulse pressure and systolic blood pressure in black women with lower ET-1 levels compared to the other groups may suggest that even at this low ET-1 concentrations cardiovascular changes are present and potentially driven by an inflammatory condition as depicted by high C-reactive protein levels and higher prevalence of overweight and obesity. Black men have higher ET-1 levels in our study than white men. This coincides with other studies [20,31-33]. Since the black population is also subjected to the development of early vascular deterioration, especially in pulse pressure and systolic blood pressure in black women, our result may support this trend in black urbanized women [3]. The lack of association between ET-1 and more pronounced measures of arterial stiffness (pulse wave velocity and arterial compliance) may be due to the younger age of this cohort and these overt changes had not yet occur, apart from higher C-reactive protein levels and marked overweight.

Our study also found a positive association between systolic blood pressure and ET-1 levels, only in black women. Some studies found no difference in ET-1 levels between normotensive and hypertensive participants, whereas other studies suggested a possible link between elevated ET-1 and increasing

systolic blood pressure [34-36]. The activation of receptor-bound ET-1 is associated with growth and pro-inflammatory effects and the remodeling of resistance arteries via increased oxidative stress and consequent vascular endothelial dysfunction [11,34]. ET_A receptor activation causes vasoconstriction, enhancement of nerve-stimulated adrenal catecholamine release, and positive inotropy which in turn may increase blood pressure [20,35,37] while ET_B receptor produces vasodilation, increases sodium excretion, and inhibits growth and inflammation [38]. The black population have both ET_A and ET_B receptors on vascular smooth muscle cells, but the total number of ET_B receptors is lower than that in the white population. Although our study did not assess ET receptors, the decrease of ET_B receptor ratio on vascular smooth muscle cells previously indicated in black populations [20] could be favoring vasoconstriction-promoting receptors, providing a possible explanation for the association of ET-1 with increased systolic blood pressure [20,21,36]. Even though the black men have higher pulse pressure and pulse wave velocity levels than black women, the lack of association of these markers with ET-1 might suggest that black men of this population are already subjected to subclinical organ damage at macrovascular and cardiac level. It is possible that ET-1 could correlate with markers of myocardial damage instead; unfortunately we lack the data to investigate this hypothesis.

Prolonged elevated blood pressure was found to lead to increased ET-1 and interleukin-6 levels [16,17,39]. We found higher levels of interleukin-6 and C-reactive protein in the black population compared to the white population and only in black women. ET-1 related positively with interleukin-6. Previous studies have shown that the black population have higher levels of inflammatory markers, especially C-reactive protein and soluble urokinase plasminogen activator receptor compared to the white population [40,41]. The absence of these findings among white women were discussed by Schutte et al [41] suggesting that obesity can be associated with chronic activation of the immune system leading to very high levels of inflammatory markers in the black women due to the fact that obesity is an inflammatory condition. Perivascular fat tissue may interact in an autocrine/paracrine manner with the endothelial cells (where ET-1 is released) subsequently contributing to endothelial dysfunction, often associated with markers of inflammation. ET-1 has previously been found to stimulate the release of interleukin-6 and has been implicated in the development of atherosclerosis and vascular dysfunction [7]. ET-1 and interleukin-6 have also been suggested to be involved in the proinflammatory effect of C-

reactive protein [7]. Damage or infection to the endothelium causes ET-1 to bind to ET_B receptors on smooth muscle cells and control the macrophages and its release of inflammatory cytokines such as tumor necrosis factor-alpha, interleukin-6, and interleukin-1 [7,17,42,43]. The aim of our study was not to investigate the role of inflammation and ET-1 in this population group; however we did find a link between interleukin-6 and ET-1. The absence of C-reactive protein associated with ET-1 in the population may be because there is a discrepancy in the adjustments for body composition.

We also observed a positive association between ET-1 and high density lipoprotein cholesterol in our black female group. Previous studies have mentioned that black Africans are prone to hypertension and weight gain, which could lead to elevated high density lipoprotein cholesterol levels [41,44]. It is noteworthy to mention that the black women in this study population did not suffer from severe arterial stiffness and this may be explained by the protective nature of high density lipoprotein cholesterol having the ability to remove cholesterol from macrophage foam cells in the arterial wall and carry it to the liver for excretion into the bile, reducing the risk to atherosclerosis [45]. The positive association of ET-1 with high density lipoprotein cholesterol may counterregulate proinflammation until arterial stiffness manifests in this group. Although vascular inflammation can be limited by anti-inflammatory counterregulatory mechanisms that maintain the integrity and homeostasis of the vascular wall, chronic exposure to cardiovascular risk factors such as high blood pressure, tobacco overuse, obesity, physical inactivity and raised blood glucose may render these counterregulatory mechanisms defenseless [18]. Further studies could shed light on the precise mechanism by which ET-1 and inflammation are involved in the increase of pulse pressure and systolic blood pressure and subsequent cardiovascular disease in black women.

The results of this study need to be interpreted within the context of its limitations and strengths. This was a cross-sectional study and we cannot pinpoint any cause or effect. Pulse wave velocity was measured in our group; unfortunately femoral pulse wave velocity could not be obtained. Although the results were consistent after multiple adjustments, we cannot exclude residual confounding. Further data on autonomic and endothelial function are needed to delineate possible physiological mechanisms at play. The strength of the study can be measured on the basis that it was a well-designed study under controlled conditions (two ethnic and homogenous socioeconomic groups).

In conclusion, ET-1 independently associated with systolic blood pressure, pulse pressure and interleukin-6 in black women. Our result suggests that ET-1 and its link with subclinical arteriosclerosis are potentially driven by low-grade inflammation as depicted by interleukin-6 in the black female cohort.

Disclosure

The funders played no role in the design and conduct of the data.

Disclaimer

The views expressed in this paper are those of authors and not necessarily of the funding bodies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors sincerely thank all SABPA participants, staff, postgraduate students and the Department of Education North-West Province (South Africa) who assisted in the data sampling. The SABPA study was supported by the South African National Research Foundation (UID 65607), the North-West University (Potchefstroom Campus, South Africa), ROCHE Diagnostics (South Africa) and the Metabolic Syndrome Institute (France).

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY TABLE 1: Single correlations of endothelin-1 with cardiovascular and metabolic markers in black and white men and women.

	Endothelin-1 (pg/mL)			
	Men (n = 198)		Women (n = 193)	
	Black (n = 99)	White (n = 99)	Black (n = 95)	White (n = 98)
Age (years)	r = 0.041; p = 0.69	r = –0.104; p = 0.30	r = 0.26; p = 0.009	r = –0.006; p = 0.96
Body mass index (kg/m^2)	r = 0.030; p = 0.77	r = 0.065; p = 0.52	r = 0.17; p = 0.093	r = 0.056; p = 0.58
Waist circumference (mm)	r = 0.055; p = 0.59	r = 0.096; p = 0.34	r = 0.14; p = 0.17	r = 0.051; p = 0.62
Cardiovascular variables				
Systolic blood pressure (mmHg)	r = 0.12; p = 0.24	r = 0.049; p = 0.63	r = 0.27; p = 0.008	r = 0.14; p = 0.18
Diastolic blood pressure (mmHg)	r = 0.048; p = 0.65	r = 0.153; p = 0.13	r = 0.12; p = 0.27	r = -0.024; p = 0.82
Pulse pressure (mmHg)	r = 0.14; p = 0.19	r = –0.069; p = 0.50	r = 0.25; p = 0.014	r = 0.19; p = 0.063
Mean arterial pressure (mmHg)	r = 0.087; p = 0.40	r = 0.089; p = 0.38	r = 0.20; p = 0.053	r = 0.065; p = 0.52
Stroke volume (mL)	r = 0.13; p = 0.22	r = –0.093; p = 0.36	r = 0.11; p = 0.30	r = 0.23; p = 0.026
Arterial compliance (mL/mmHg)	r = –0.008; p = 0.94	r = –0.011; p = 0.91	r = –0.078; p = 0.45	r = 0.13; p = 0.22
Pulse wave velocity (m/s)	r = 0.15; p = 0.15	r = 0.118; p = 0.25	r = 0.23; p = 0.026	r = 0.11; p = 0.27
Biochemical analyses				
C-Reactive Protein (mg/L)	r = 0.015; p = 0.88	r = 0.178; p = 0.078	r = 0.090; p = 0.38	r = –0.016; p = 0.87
Interleukin-6 (pg/mL)	r = –0.017; p = 0.87	r = –0.139; p = 0.17	r = 0.27; p = 0.007	r = –0.118; p = 0.25
Glycated hemoglobin A1c (%)	r = –0.024; p = 0.81	r = 0.020; p = 0.84	r = 0.16; p = 0.13	r = 0.010; p = 0.92
High density lipoprotein cholesterol (mmol/L)	r = 0.040; p = 0.69	r = 0.137; p = 0.18	r = 0.23; p = 0.026	r = 0.041; p = 0.69
Low density lipoprotein cholesterol (mmol/L)	r = 0.025; p = 0.81	r = –0.040; p = 0.69	r = 0.011; p = 0.91	r = –0.090; p = 0.38
Total cholesterol (mmol/L)	r = 0.100; p = 0.32	r = 0.004; p = 0.97	r = 0.082; p = 0.43	r = –0.059; p = 0.56
Triglycerides (mmol/L)	r = 0.155; p = 0.12	r = –0.010; p = 0.92	r = 0.030; p = 0.77	r = 0.002; p = 0.98
Albumin-to-creatinine ratio (mg/mmol/L)	r = 0.040; p = 0.70	r = –0.069; p = 0.50	r = 0.046; p = 0.66	r = 0.093; p = 0.36
Cotinine (ng/mL)	r = –0.17; p = 0.32	r = 0.044; p = 0.87	r = 0.28; p = 0.24	r = 0.65; p = 0.41
Gamma glutamyl transferase (U/L)	r = 0.18; p = 0.075	r = –0.170; p = 0.092	r = 0.15; p = 0.15	r = –0.018; p = 0.86

CHAPTER 4

The association of endothelin-1 with markers of oxidative stress in a bi-ethnic South African cohort: The SABPA study

This article is published in the journal *Hypertension Research*, doi:10.1038/hr.2016.128.

Hypertension Research (2016), 1–7

www.nature.com/hr

ORIGINAL ARTICLE

The association of endothelin-1 with markers of oxidative stress in a biethnic South African cohort: the SABPA study

Christine Susara du Plooy[1], Catharina Martha Cornelia Mels[1], Hugo Willem Huisman[1,2] and Ruan Kruger[1]

Both endothelin-1 and oxidative stress have important roles in the development of cardiovascular diseases such as hypertension and atherosclerosis. Limited information is available on the interaction between oxidative stress, the glutathione system and endothelin-1 in humans. We aimed to investigate the association of endothelin-1 with markers of oxidative stress and the antioxidant capacity in a biethnic South African cohort. This cross-sectional study included 195 black and 198 white South Africans. Serum endothelin-1 levels and oxidative stress-related markers such as reactive oxygen species (measured as serum peroxides), glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase were measured. In single, partial and multiple regression analyses endothelin-1 correlated positively with glutathione reductase activity (adj. $R^2 = 0.10$; $\beta = 0.232$; $P = 0.020$) and negatively with antihypertension medication ($P = 0.02$) and tended to correlate with glutathione reductase-to-glutathione peroxidase ratio (adj. $R^2 = 0.10$; $\beta = 0.19$; $P = 0.057$) in black men. In white men, endothelin-1 correlated positively with ROS (adj. $R^2 = 0.09$; $\beta = 0.26$; $P = 0.01$) and negatively with glutathione peroxidase activity (adj. $R^2 = 0.05$; $\beta = -0.23$; $P = 0.02$). In black women, endothelin-1 correlated negatively with total glutathione (adj. $R^2 = 0.22$; $\beta = -0.214$; $P = 0.026$). Endothelin-1 may contribute to glutathione reductase upregulation through increased reactive oxygen species production mediated via endothelin-1 in black men. In white men, we observed a negative association between glutathione peroxidase and endothelin-1, describing the expected physiological relationship between endothelin-1 and reactive oxygen species. Higher total glutathione levels may act as a counter-regulatory mechanism to protect against oxidative vascular damage attributed by endothelin-1 in black women.
Hypertension Research advance online publication, 29 September 2016; doi:10.1038/hr.2016.128

Keywords: antioxidant capacity; endothelin-1; ethnicity; oxidative stress; reactive oxygen species

- This article is prepared according to the author's instructions from the journal *Hypertension Research* (Annexure A). Please note that some of the format requirements were changed to ensure uniformity throughout the thesis.

ABSTRACT

Both endothelin-1 and oxidative stress have important roles in the development of cardiovascular diseases such as hypertension and atherosclerosis. Limited information is available on the interaction between oxidative stress, the glutathione system and endothelin-1 in humans. We aimed to investigate the association of endothelin-1 with markers of oxidative stress and the antioxidant capacity in a bi-ethnic South African cohort. This cross-sectional study included 195 black and 198 white South-Africans. Serum endothelin-1 levels and oxidative stress-related markers such as reactive oxygen species (measured as serum peroxides), glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase were measured. In single, partial and multiple regression analyses endothelin-1 correlated positively with glutathione reductase activity (adj. $R^2 = 0.10$; $\beta = 0.232$; $P = 0.020$) and negatively with antihypertension medication ($P = 0.02$) and tended to correlate with glutathione reductase-to-glutathione peroxidase ratio (adj. $R^2 = 0.10$; $\beta = 0.19$; $P = 0.057$) in black men. In white men, endothelin-1 correlated positively with reactive oxygen species (adj. $R^2 = 0.09$; $\beta = 0.26$; $P = 0.01$) and negatively with glutathione peroxidase activity (adj. $R^2 = 0.05$; $\beta = –0.23$; $P = 0.02$). In black women, endothelin-1 correlated negatively with total glutathione (adj. $R^2 = 0.22$; $\beta = –0.214$; $P = 0.026$). Endothelin-1 may contribute to glutathione reductase upregulation through increased reactive oxygen species production mediated via endothelin-1 in black men. In white men, we observed a negative association between glutathione peroxidase and endothelin-1, describing the expected physiological relationship between endothelin-1 and reactive oxygen species. Higher total glutathione levels may act as a counter-regulatory mechanism to protect against oxidative vascular damage attributed by endothelin-1 in black women.

Keywords: antioxidant capacity; endothelin-1; ethnicity; oxidative stress; reactive oxygen species

INTRODUCTION

Endothelin-1 has an important physiological role in the maintenance of vascular tone.[1] Under pathophysiological conditions, plasma endothelin-1 is elevated and causes enhanced vasoconstriction and endothelial dysfunction.[2-5] Endothelial dysfunction is described as a precursor in the development of cardiovascular diseases related to hypertension, atherosclerosis and arteriosclerosis.[6-9]

In addition to the role of endothelin-1 in the regulation of vascular tone, reactive oxygen species (ROS) are also important modulators in this regard.[1] In response to increased production of ROS, antioxidant enzymes such as superoxide dismutase, catalase and the glutathione system (glutathione peroxidase (GPx) and glutathione reductase (GR)) are activated to maintain the balance between oxidants and antioxidants.[10,11] However, when the production of ROS exceeds the availability of antioxidant defence mechanisms, it may have detrimental effects such as endothelial injury.[10,11] Oxidative stress may, therefore, also have an important role in the development and progression of cardiovascular disease such as atherosclerosis.[9-13]

The regulation of vascular tone via ROS is achieved through different mechanisms. The first involves the inactivation of the vasodilator, nitric oxide, by binding with superoxide.[14] ROS also regulates vascular tone by increasing intracellular Ca^{2+} uptake in vascular smooth muscle cells, thereby inducing smooth muscle contraction and proliferation.[14] Finally, experimental results indicated ROS can lead to increased production of endothelin-1,[12,15] which may result in vasoconstriction by binding to ET_A receptors.[16] Increased endothelin-1 may in turn lead to increased production of superoxide radicals.[12,15,17]

Previous results from the sympathetic activity and ambulatory blood pressure in Africans (SABPA) study indicated that higher ROS levels in black men and lower GPx activity in black women are associated with higher blood pressure.[9,18] It was also found that increased carotid intima-media thickness were associated with higher GR levels in black men and decreased total glutathione levels in hypertensive black men.[18,19] Additionally, increased endothelin-1 were also found to be independently associated with blood pressure and inflammatory markers in this population.[20] These findings suggest that endothelin-1- and oxidative stress-related markers may contribute to the development of hypertension and subclinical atherosclerosis

in the sub-Saharan population. Although the link between endothelin-1 and oxidative stress were demonstrated in experimental studies (*in vitro* and *in vivo*), limited information is available on humans, especially in a South African context. We therefore aimed to investigate the association of endothelin-1 with markers of oxidative stress and antioxidant capacity in a cohort of black and white individuals.

METHODS

Study population and protocol

The SABPA study was a cross-sectional study that included 202 black and 208 white teachers from the Dr. Kenneth Kuanda Education District of the North-West Province of South Africa. Detailed information regarding the procedure of the SABPA study were published previously.[21] Exclusion criteria for the SABPA study were pregnant or lactating women, individuals using α- and β-blockers, participants with an ear temperature ≥37°C and those who had a vaccination or donated blood 3 months before participation. In the substudy, we included 195 black (men: n = 99; women: n = 96) and 198 white (men: n = 99; women: n = 99) South Africans. Excluded from the substudy were outliers of endothelin-1 (n = 10) by residual statistics (3 x s.d.) as well as participants with missing endothelin-1 data (n = 6). A standard health survey was used for the collection of demographic information and anti-hypertension medication usage. The Health Research Ethics Committee of the North-West University, Potchefstroom campus, granted approval for this substudy. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki for the investigation on human subjects.

Anthropometric and physical activity measurements

Waist circumference was measured with a non-stretchable metal flexible measuring tape (Holtain, Dyfed, UK) and body mass index was determined.[22] The total energy expenditure was obtained in kcal per 24h by the Actical omnidirectional accelerometer (Mini Mitter, Bend, OR, USA and Montreal, QC, Canada) taking the resting metabolic rate into account.

Biochemical analyses

A fasting blood sample was collected from each participant and serum and plasma were prepared according to standard procedures. Serum and plasma samples were frozen at -80°C until analyzed.

Endothelin-1 was determined with a Quantikine enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). Intra- and interassay variability for endothelin-1 were 2.7% and 17.2%, respectively. Serum interleukin-6 was determined with a high-sensitivity Quantikine enzyme-linked immunosorbent assay (R&D Systems). Intra- and interassay variation of interleukin-6 were 4.2% and 6.4%, respectively. Serum cotinine was determined with a homogenous immunoassay on a Roche Modular system (Roche, Basil, Switzerland). Fasting lipids (total and high-density lipoprotein cholesterol), glycated hemoglobin A1c and γ-glutamyl transferase were determined using two sequential multiple analyzers in serum samples (Konelab 20i (Thermo Scientific, Vantaa, Finland) and Unicel DXC 800 (Beckman and Coulter, Germany)). Intra- and interassay variability were <10%. Low-density lipoprotein cholesterol was calculated with the Friedewald formula: low-density lipoprotein cholesterol = total cholesterol – high density lipoprotein cholesterol – (triglycerides/2.2) provided that no values of triglycerides inserted is higher than 4000 mmol l^{-1}.[23] Human immunodeficiency virus status was measured, using the First Response Kit (Premier Medical Corporation, Mumbai, India) as well as the Pareekshak test (Bhat Biotech, Bangalore, India). Estradiol levels were determined using an electrochemiluminescence immunoassay (Elecsys 2010; Roche, Basil, Switzerland). Intra- and interassay variability was <10%.

One of the measurable ROS, namely total peroxides, was determined in serum samples.[24] Total glutathione (GSH) levels were determined with the BIOXYTECH GSH/GSSG-412 supplied by OxisResearch, (Foster City, CA, USA). GPx and GR (EDTA plasma) and serum superoxide dismutase activities were determined with Assay Kits (Cayman Chemical Company, Ann Arbor, MI, USA), whereas serum catalase activity was determined with a Oxiselect Fluorometric Kit from Cell Biolabs (San Diego, CA, USA) with appropriate apparatus (Synergy H_4hybrid Microplate Reader; BioTek, Winooski, VT, USA). The intra- and interassay variability of these analyses were <10%. Antioxidant enzyme ratios were calculated to assess antioxidant defenses and included the glutathione reductase-to-glutathione peroxidase ratio (GR-to-GPx ratio) and the glutathione peroxidase-to-superoxide dismutase ratio (GPx-to-SOD ratio).

Cardiovascular measurements

The cardiovascular measurements were taken in a semi-recumbent position for each participant. Five minute continuous measurements of cardiovascular variables were recorded using the validated Finometer (Finapres Medical Systems, Amsterdam, The Netherlands), based on the vascular unloading technique of Peñáz and were processed with the Beatscope 1.1 software (Finapres Medical Systems, Amsterdam, The Nethderlands) to obtain systolic blood pressure and diastolic blood pressure.[25] The Complior SP Acquisition System (Artech-Medical, Pantin, France) was used to measure pulse wave velocity from the carotid to dorsalis-pedis pulse sites.[26]

Statistical analysis

G*Power version 3.1.9.2 software (University of Kiel, Kiel, Germany) was used to compute the achieved power in *post hoc* analysis.[27] At a probability of 0.05, effect size of 0.5 and one-tailed input method, the achieved power (1–β error probability) was estimated at 96.86% in men and 96.66% in women. Statistical analyses were carried out using IBM SPSS Statistics version 23 (IBM Corp., Armonk, NY, USA; 2016). Main effects of race and sex were tested based on the association between endothelin-1- and oxidative stress-related markers by means of multiple regression. *T*-tests were used to compare means and X^2 tests to compare proportions between the groups. Single and partial correlations were used to determine correlations of endothelin-1 with cardiovascular-, biochemical- and oxidative stress-related variables. Forward stepwise multiple regression analyses were performed to determine independent associations between endothelin-1- and oxidative stress-related variables. The main independent variables included GR and GPx in black men (Models 1 and 2, respectively), ROS and GPx in white men (Models 3 and 4, respectively) and GSH in black women (Model 5). Covariates considered for entry in the models included age, body mass index, total energy expenditure, interleukin-6, γ-glutamyl transferase, high-density lipoprotein cholesterol and antihypertension medication. We applied a sensitivity analysis for glycated hemoglobin A1c, human immunodeficiency virus infection status, estradiol, oral contraceptives and testosterone by adding these variables as covariates in applicable multiple regression models.

RESULTS

Basic descriptive characteristics of this study population are listed in Table 1. Owing to significant interactions of race ($F(391) = 2.33$; $P < 0.05$) and sex ($F(391) = 2.22$; $P < 0.05$) on the association of endothelin-1 with GR, we stratified the population accordingly.

There were no differences in endothelin-1 levels between the black and white groups. ROS was higher in black compared with that in white men ($P = 0.008$), but similar in women. GSH, GR, catalase, and GR-to-GPx ratio were higher in black men and women compared with that in their white counterparts (all $P \leq 0.009$). GPx was similar when comparing black and white men, whereas lower values were observed in black women ($P < 0.001$) compared with white women. GPx-to-SOD ratio were higher in black men than white men ($P < 0.003$), with no differences when comparing women. Interleukin-6, glycated hemoglobin A1c and γ-glutamyl transferase were higher in black men compared with women (all $P \leq 0.032$) than their white counterparts. Total cholesterol and low-density lipoprotein cholesterol were higher in white men and women (all $P < 0.001$) compared with their black counterparts. Systolic blood pressure, diastolic blood pressure and pulse wave velocity were higher in black men and women compared with the white groups (all $P \leq 0.042$). The prevalence of human immunodeficiency virus was higher among the black groups and both black men and women were more likely to use antihypertension medication than their white counterparts.

In both single (Supplementary Table 1) and partial regression analyses after adjusting for age, body mass index, total energy expenditure and antihypertension medication (Table 2), endothelin-1 correlated positively with GR ($P \leq 0.02$) and GR-to-GPx ratio ($P < 0.05$) in black men only. In white men, a positive correlation existed between endothelin-1 and ROS ($P \leq 0.03$) and an inverse correlation between endothelin-1 and GPx ($P \leq 0.03$). In black women, a borderline correlation were found between endothelin-1 and GSH ($P = 0.06$). No correlation existed in white women.

In forward stepwise multiple regression analysis, we performed a separate model for each group based on previous findings in single and partial regression analyses (Table 3). In black men, an independent positive association of endothelin-1 with GR (Model 1: adj. $R^2 = 0.10$; $\beta = 0.23$; $P = 0.02$) was confirmed.

Additionally, in black men a borderline association between endothelin-1 and GR-to-GPx ratio (Model 2: adj. R^2 = 0.05; β = 0.19; P = 0.057) was observed. A negative association of endothelin-1 with antihypertension medication (Model 1: adj. R^2 = 0.10; β = –0.24; P = 0.015; Model 2: adj. R^2 = 0.05; β = –0.24; P = 0.016) was also confirmed in black men. An independent positive association between endothelin-1 and ROS (Model 3: adj. R^2 = 0.09; β = 0.26; P = 0.010) and a negative association between endothelin-1 and interleukin-6 (Model 3: adj. R^2 = 0.09; β = –0.24; P = 0.016) were confirmed in white men. Additionally, a negative association between endothelin-1 and GPx (Model 4: adj. R^2 = 0.045; β = –0.23; P = 0.02), in white men, was confirmed. In Model 5, an independent negative association between endothelin-1 and GSH (adj. R^2 = 0.22; β = –0.21; P = 0.026), as well as a positive association between endothelin-1 and age (adj. R^2 = 0.22; β = 0.23; P = 0.016), body mass index (adj. R^2 = 0.22; β = 0.34; P = 0.001) and high-density lipoprotein cholesterol (adj. R^2 = 0.22; β = 0.23; P = 0.019) was found in black women. No significant correlations existed in white women.

Sensitivity analyses

After performing the same multiple regression analyses and additionally correcting for human immunodeficiency virus infection, glycated hemoglobin A1c, estradiol, hormonal contraceptive usage and testosterone, no change in the relationships between endothelin-1- and oxidative stress-related markers were found in men or women. An additional sensitivity analysis was performed by removing the participants using antihypertension medication and we found that a positive association of endothelin-1 with GR (Model 1: adj. R^2 = 0.141; β = 0.303; P = 0.014), GR-to-GPx ratio (Model 2: adj. R^2 = 0.150; β = 0.317; P = 0.010) and interleukin-6 (Model 1: adj. R^2 = 0.141; β = 0.288; P = 0.019; Model 2: adj. R^2 = 0.150; β = 0.300; P = 0.015) was observed in black men.

Table 1 Population characteristics stratified by sex and race.

	Men (n = *198)*			*Women* (n = *195)*		
	Black (n = *99)*	*White* (n = *99)*	*P-value*	*Black* (n = *96)*	*White* (n = *99)*	*P-value*
Age (Years)	43.1 ± 8.08	45.0 ± 11.1	0.18	45.6 ± 7.95	44.7 ± 10.7	0.51
Body mass index (kg m^{-2})	27.6 ± 5.81	29.1 ± 5.23	0.061	32.9 ± 7.29	26.0 ± 5.62	<0.001
Waist circumference (cm)	93.6 ± 15.5	101.7 ± 14.5	<0.001	93.9 ± 15.6	84.8 ± 13.0	<0.001
Total energy expenditure (kcal per day)	2723.3 ± 805.8	3659.3 ± 2069.5	<0.001	2664.2 ± 800.1	2577.7 ± 620.0	0.40
Human immunodeficiency virus, n (%)	13 (13)	0 (0)	<0.001	5 (5.21)	0 (0)	0.021
Anti-hypertension medication, n (%)	35 (35.4)	14 (14.1)	<0.001	33 (34.4)	12 (12.1)	<0.001
Biochemical variables						
Endothelin-1 (pg ml^{-1})	2.26 ± 0.81	2.16 ± 1.02	0.41	2.02 ± 0.96	2.19 ± 1.22	0.27
Interleukin-6 (pg ml^{-1})	1.07 (0.93–1.23)	0.87 (0.76–0.99)	0.032	1.24 (1.06–1.46)	0.95 (0.82–1.10)	0.016
Glycated hemoglobin A1c (%)	6.25 ± 1.23	5.67 ± 0.48	<0.001	5.85 ± 1.00	5.37 ± 0.30	<0.001
Gamma glutamyl transferase (U l^{-1})	63.0 (54.7–72.4)	27.5 (24.2–31.3)	<0.001	35.6 (31.4–40.4)	14.2 (12.5–16.2)	<0.001
Cotinine (ng ml^{-1})	62.9 (43.4–91.2)	77.3 (31.1–192.3)	0.60	48.5 (26.8–87.4)	91.7 (36.1–233.4)	0.21
Total cholesterol (mmol l^{-1})	4.72 ± 1.17	5.59 ± 1.21	<0.001	4.46 ± 1.21	5.54 ± 1.31	<0.001
High density lipoprotein cholesterol (mmol l^{-1})	1.04 ± 0.34	1.00 ± 0.27	0.36	1.20 ± 0.31	1.41 ± 0.43	<0.001
Low density lipoprotein cholesterol (mmol l^{-1})	2.86 ± 0.95	3.91 ± 1.07	<0.001	2.80 ± 1.02	3.71 ± 1.07	<0.001
Oxidative stress related variables						
Reactive oxygen species (U[a])	81.9 (78.1–85.9)	75.2 (72.2–78.4)	0.008	104.1 (98.6–109.8)	98.3 (93.4–103.5)	0.13
Total Glutathione (μM)	929.5 ± 194.1	859.7 ± 180.4	0.009	868.8 ± 127.6	782.6 ± 163.0	<0.001
Glutathione peroxidase (U ml^{-1})	34.6 ± 14.0	34.9 ± 7.83	0.85	31.9 ± 14.0	37.4 ± 7.90	<0.001
Glutathione reductase (U ml^{-1})	7.71 (6.82–8.71)	2.19 (1.75–2.75)	<0.001	6.43 (5.62–7.34)	2.81 (2.34–3.40)	<0.001
Superoxide dismutase (U ml^{-1})	3.92 (3.16–4.86)	4.23 (3.92–4.57)	0.50	4.69 (3.83–5.74)	4.09 (3.67–4.55)	0.23
Catalase (U ml^{-1})	4.29 (3.92–4.57)	4.25 (4.23–4.28)	0.009	4.29 (4.28–4.30)	4.23 (4.20–4.25)	<0.001
GR-to-GPx ratio	0.34 ± 0.30	0.09 ± 0.07	<0.001	0.34 ± 0.46	0.10 ± 0.07	<0.001
GPx-to-SOD ratio	17.2 ± 27.1	8.87 ± 4.6	0.003	10.9 ± 12.5	10.6 ± 6.95	0.85
Cardiovascular variables						
Systolic blood pressure (mm Hg)	146.2 ± 20.6	136.5 ± 12.8	<0.001	136.4 ± 14.4	132.1 ± 15.2	0.042
Diastolic blood pressure (mm Hg)	85.8 ± 11.0	80.2 ± 8.36	<0.001	77.3 ± 7.69	73.4 ± 6.72	<0.001
Pulse wave velocity (m s^{-1})	9.18 ± 2.29	8.62 ± 1.34	0.039	8.19 ± 1.39	7.47 ± 1.20	<0.001

Abbreviations: GPx-to-SOD ratio, glutathione peroxidase-to-superoxide dismutase ratio; GR-to-GPx ratio, glutathione reductase-to-glutathione peroxide ratio; H_2O_2, hydrogen peroxide. Values are arithmetic mean plus/minus s.d., geometric mean (5th and 95th confidence interval). [a]1 U = 1mg l^{-1} H_2O_2.

Table 2 Partial correlations of endothelin-1 with oxidative stress related and inflammatory markers.

	Endothelin-1 (pg ml^{-1})			
	*Men (*n = *198)*		*Women (*n = *195)*	
	*Black (*n = *99)*	*White (*n = *99)*	*Black (*n = *96)*	*White (*n = *99)*
	Biochemical variables			
Interleukin-6 (pg ml^{-1})	*r*=0.043; *P*=0.68	*r*=–0.19; *P*=0.07	*r*=0.12; *P*=0.27	*r*=–0.10; *P*=0.32
Glycated hemoglobin A1c (%)	*r*=–0.080; *P*=0.44	*r*=–0.035; *P*=0.74	*r*=0.020; *P*=0.85	*r*=–0.071; *P*=0.50
Gamma glutamyl transferase (U l^{-1})	*r*=0.19; *P*=0.07	*r*=–0.13; *P*=0.21	*r*=0.22; *P*=0.04	*r*=–0.046; *P*=0.66
Total cholesterol (mmol l^{-1})	*r*=0.10; *P*=0.31	*r*=0.081; *P*=0.43	*r*=–0.070; *P*=0.51	*r*=–0.10; *P*=0.34
High density lipoprotein cholesterol (mmol l^{-1})	*r*=0.14; *P*=0.18	*r*=0.23; *P*=0.03	*r*=0.23; *P*=0.03	*r*=0.042; *P*=0.69
Low density lipoprotein cholesterol (mmol l^{-1})	*r*=0.025; *P*=0.81	*r*=0.061; *P*=0.56	*r*=–0.15; *P*=0.17	*r*=–0.14; *P*=0.19
Oxidative stress and anti-oxidant variables				
Reactive oxygen species (U[a])	*r*=–0.17; *P*=0.10	*r*=0.23; *P*=0.03	*r*=0.18; *P*=0.08	*r*=0.039; *P*=0.71
Total Glutathione (μM)	*r*=0.023; *P*=0.83	*r*=–0.007; *P*=0.95	*r*=–0.20; *P*=0.06	*r*=0.063; *P*=0.55
Glutathione peroxidase (U ml^{-1})	*r*=–0.020; *P*=0.85	*r*=–0.22; *P*=0.03	*r*=–0.009; *P*=0.93	*r*=–0.13; *P*=0.22
Glutathione reductase (U ml^{-1})	*r*=0.23; *P*=0.02	*r*=–0.13; *P*=0.23	*r*=0.024; *P*=0.82	*r*=–0.14; *P*=0.16
Superoxide dismutase (U ml^{-1})	*r*=–0.18; *P*=0.10	*r*=–0.16; *P*=0.13	*r*=0.073; *P*=0.50	*r*=0.060; *P*=0.56
Catalase	*r*=–0.10; *P*=0.34	*r*=–0.024; *P*=0.82	*r*=–0.035; *P*=0.74	*r*=–0.004; *P*=0.97
GR-to-GPx ratio	*r*=0.20; *P*=0.047	*r*=–0.13; *P*=0.23	*r*=0.001; *P*=0.99	*r*=–0.10; *P*=0.33
GPx-to-SOD ratio	*r*=0.19; *P*=0.07	*r*=0.055; *P*=0.60	*r*=–0.090; *P*=0.39	*r*=–0.13; *P*=0.23
Cardiovascular variables				
Systolic blood pressure (mm Hg)	*r*=0.10; *P*=0.35	*r*=0.013; *P*=0.90	*r*=0.13; *P*=0.21	*r*=0.083; *P*=0.43
Diastolic blood pressure (mm Hg)	*r*=0.022; *P*=0.84	*r*=0.11; *P*=0.29	*r*=0.11; *P*=0.32	*r*=–0.100; *P*=0.34
Pulse wave velocity (m s^{-1})	*r*=0.15; *P*=0.16	*r*=0.13; *P*=0.20	*r*=0.11; *P*=0.31	*r*=0.12; *P*=0.27

Abbreviations: GPx-to-SOD ratio, glutathione peroxidase-to-superoxide dismutase ratio; GR-to-GPx ratio, glutathione reductase-to-glutathione peroxide ratio, H_2O_2, hydrogen peroxide. Adjustments applied for age, body mass index, total energy expenditure and antihypertension medication.

[a]1 U = 1mg l^{-1} H_2O_2.

Table 3 Forward stepwise multiple regression analyses of endothelin-1 with measures of oxidative stress related markers

	Endothelin-1 (pg ml^{-1})	
Black men (n = 99)	*Adj.* R^2=0.10	
Model 1:	Std β (95% *CI*)	P-*value*
Glutathione reductase (U ml^{-1})	0.232 (0.039–0.424)	0.020
Antihypertension medication	–0.243 (–0.435 to –0.051)	0.015
Model 2:	*Adj.* R^2=0.051	
GR-to-GPx ratio	0.191 (0.003–0.384)	0.057
Anti-hypertension medication	–0.247 (–0.443 to –0.051)	0.016
White men (n = 99)		
Model 3	*Adj.* R^2=0.085	
Reactive oxygen species (U[a])	0.257 (0.065–0.449)	0.010
Interleukin-6 (pg ml^{-1})	–0.240 (–0.432 to –0.048)	0.016
Model 4	Adj. R^2=0.045	
Glutathione peroxidase (U ml^{-1})	–0.233 (–0.427 to –0.039)	0.020
Black women (n = 96)		
Model 5	*Adj.* R^2=0.22	
Total glutathione (μM)	–0.214 (–0.400 to –0.028)	0.026
Age (years)	0.232 (0.046–0.418)	0.016
Body mass index (kg m $^{-2}$)	0.342 (0.156–0.528)	0.001
High density liporprotein cholesterol (mmol l^{-1})	0.226 (0.040–0.412)	0.019

Abbreviations: CI, confidence interval; GR-to-GPx ratio, glutathione reductase-to-glutathione peroxidase ratio; H_2O_2, hydrogen peroxide; NS, not significant; Std β, standardized regression β-coefficients.

Main independent variables included glutathione reductase, GR-to-GPx ratio, reactive oxygen species, glutathione peroxidase, and total glutathione respectively, for Models 1–5. Covariates considered for entry: age, body mass index, total energy expenditure, interleukin-6, γ-glutamyl transferase, high density lipoprotein cholesterol, systolic blood pressure, and antihypertension medication.

[a]1 U = 1mg l^{-1} H_2O_2.

DISCUSSION

To our knowledge, we are the first to describe a link of endothelin-1 with markers of oxidative stress and antioxidant capacity in a black and white cohort. Our results indicated an independent positive association of endothelin-1 with GR and GR-to-GPx ratio in black men. A previous study in our cohort, linked higher GR levels with increased carotid intima-media thickness.[19] Increased endothelin-1 levels may lead to an increase in ROS production; however, increased ROS production can also lead to increased endothelin-1 level, which in turn leads to an increase in anti-oxidant enzyme activity, such as GR.[28] Therefore, our results suggests that endothelin-1 may have an indirect role in the upregulation of GR activity in this group and therefore contribute to the increased risk for the development of atherosclerosis often seen in the black population. Blood glutathione concentrations is an useful indicator of glutathione status in humans with cardiovascular diseases such as atherosclerosis and hypertension, and GR activity and GR-to-GPx ratio similarly gives an indication of glutathione regeneration.[19,29] Under normal physiological conditions, an increase in GR activity are indicative of increased regeneration potential to recycle GSSG to GSH and thereby make more GSH available for use in other enzyme reactions such as the inactivation of hydrogen peroxide by GPx.[29] However, under pathophysiological conditions, such as hypertension, when there is an increase in hydrogen peroxide production, more GSH is consumed by GPx, which may lead to an even further upregulation of GR in attempt to maintain the redox balance.[29] In this black male cohort, the positive association between endothelin-1 and GR activity may therefore be because of the upregulation of GR as a result of increased ROS production via endothelin-1-mediated stimulation of the NAD(P)H oxidase enzyme [28] Additionally, the increase in vascular ROS production may also impair endothelium-dependent NO-mediated relaxation by inactivating endogenous NO.[30,31] A previous study has suggested that increased oxidative stress may counteract NO bioavailability by increasing NO inactivation in black men.[32] This might be due to endothelin-1 also having the ability to counteract NO bioavailability,[33] which suggests an interconnected role between endothelin-1 and oxidative stress. Even though antihypertension medication can protect the vasculature against increased endothelin-1-mediated vasoconstriction, we still observed an association between endothelin-1 and GR and GR-to-GPx ratio, when taking anti-hypertension medication usage into account. Although the black men and women were more likely to take antihypertension medication, it is noteworthy to mention that black people do not get the correct hypertension treatment. Thus, antihypertension medication is effective enough to lower blood

pressure and still show a similar physiological association between endothelin-1 and antioxidant capacity to maintain homeostasis. Even after removing the participants using antihypertension medication, the results remained robust in the black male group. This might suggest that antihypertension medication protects the vasculature against inflammatory markers released in response to the presence of endothelin-1. Endothelial damage together with reduced NO bioavailability may alter the balance between vascular injury and repair, increasing the risk for atherosclerotic disease in black men of this population.

In white men, endothelin-1 associated positively with ROS and negatively with GPx. The GPx enzyme has a critical role in the reduction of lipid peroxides and hydrogen peroxide.[34,35] In this white male cohort, the positive association between endothelin-1 and ROS as well as the negative association between endothelin-1 and GPx activity may be as a result of nuclear factor-κB-mediated endothelin-1 synthesis. In turn, endothelin-1 then bind to ET_A receptors on nuclear factor-κB, it may lead to the activation of angiotensin II stimulation of NADPH oxidase leading to ROS production[36,37] as well as the induction of an inflammatory response in human vascular smooth muscle cells without the release of interleukin-6.[36] The negative association between endothelin-1 and interleukin-6 is contradictory to previous findings.[36,38,39] This could be because of a chance finding or that come confounding variable, which we are unaware of, might contribute to this findings. Although similar GPx activity were observed in the white and black men of this study, white men had lower ROS levels, suggesting that the black men may be at a disadvantage as they have to scavenge more ROS with similar GPx activity, which may exaggerate the effect of endothelin-1 in the black men. Therefore, the opposite associations of endothelin-1 with ROS and GPx activity may indicate the physiological relationship between these factors.

In black women, we found a negative association of endothelin-1 with GSH. GSH levels is determined by the synthesis of GSH in the cell *vs*. the efflux of GSH out of the cell.[40] The most important determinant of GSH synthesis is the availability of cysteine [41] while elevated cysteine levels inside endothelial cells may lead to injury, increasing inflammation in blood vessels and in turn leads to atherogenesis.[42,43] A previous study demonstrated that endothelin-1 increases the uptake of cysteine into cells, but reduce the efflux of GSH out of the cell[41] favoring the accumulation of cysteine in the cells. Furthermore it was demonstrated that black premenopausal women also had higher plasma total homocysteine levels than white women,

possibly due to lifestyle factors, and as homocysteine levels can also be converted to cysteine, it may further increase their risk for coronary artery disease such as atherosclerosis.[44] Previous studies demonstrated that age and body mass index are associated with enhanced endothelin-1-mediated vasoconstriction that contributes to endothelial vasodilator dysfunction and may have a role in the increased prevalence of hypertension often seen in black men and women.[45-47] On the other hand, black women also have elevated high-density lipoprotein cholesterol levels that provides a protective mechanism against oxidative stress, reducing the risk to cardiovascular diseases such as atherosclerosis.[44] This antiatheroprotective role might decrease the additional release of endothelin-1 and vasoconstriction in the smooth muscle cells in comparison with the black men in our cohort. Flagg *et al*. [48] found that men had higher levels of plasma glutathione compared with women and that the use of estrogen-containing oral contraceptives was associated with lower plasma glutathione levels. However, after we adjusted for estradiol and hormonal contraceptive usage, our results remained the same. Despite the negative role of endothelin-1 on GSH synthesis, this may suggest that the combined protective nature of GSH and high-density lipoprotein cholesterol are still sufficient to counter regulate proinflammation, protecting the black women against vascular damage. A schematic representation of the possible mechanisms found from our results can be seen in Supplementary Figure 1.

The results of this study need to be interpreted within the context of its limitations and strengths. This was a cross-sectional study and we cannot pinpoint any cause or effect. Although the results were consistent after multiple adjustments, we cannot exclude residual confounding. It is known that renin-angiotensin-system inhibitors, calcium antagonists, diuretics, β-blockers and aldosterone antagonists can also lower blood pressure;[49-53] however, the amount of participants who use different types of medication were statistically incomparable in this study, and larger samples are needed to test the effects of blood pressure treatment on oxidative stress and antioxidant capacity markers. Therefore, a collective variable of overall antihypertension medication usage were used as a covariate in sensitivity analysis. This study lacked dietary data to quantify amino-acid (such as cysteine) and antioxidant intake. The strength of this study can be measured on the basis of its design and implementation under controlled conditions (two ethnic and homogenous socioeconomic groups). The inclusion of various factors involved in oxidative stress

aided to elucidate the mechanistic relationships between endothelin-1- and oxidative stress-related markers within this population.

In conclusion, our study suggests that in black men, endothelin-1 may contribute to GR upregulation through increased ROS production mediated via endothelin-1, whereas the expected physiological tendency between endothelin-1 and ROS was observed in white men. In black women, higher total GSH levels may act as a counter-regulatory mechanism to protect against oxidative vascular damage attributed by endothelin-1.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank all SABPA participants, staff, postgraduate students and the Department of Education North-West Province (South Africa) who assisted in the data sampling. The SABPA study was supported by the South African National Research Foundation (UID 65607), the South African National Research Foundation Thuthuka (80643), the North-West University (Potchefstroom Campus, South Africa), Roche Products (Pty), South Africa and the Metabolic Syndrome Institute (France). The financial assistance of the National Research Foundation (NRF SARCHi Postgraduate bursary) toward this research study is hereby acknowledged. The funders played no role in the design and conduct of the data.

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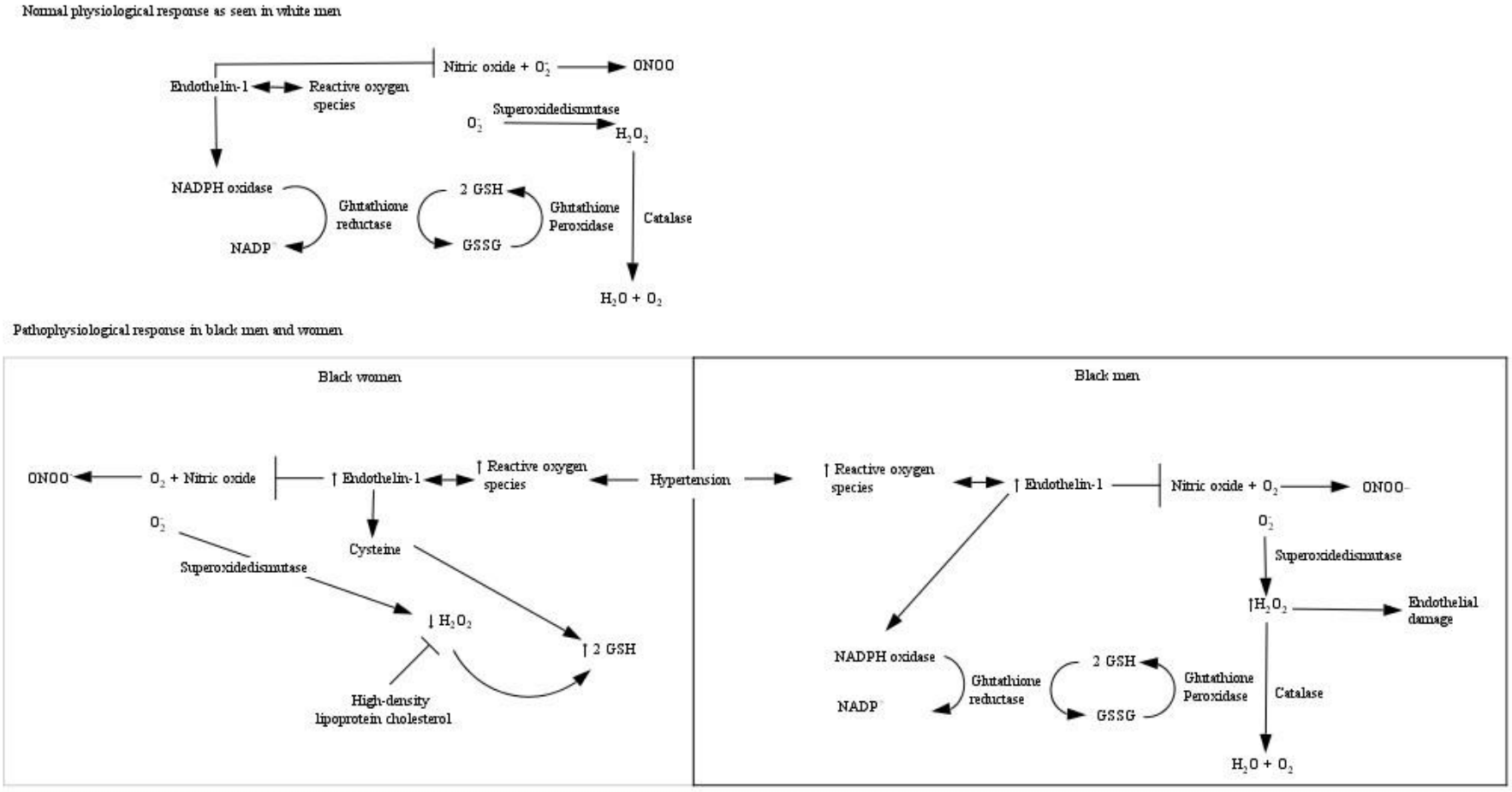
SUPPLEMENTARY INFORMATION

Supplementary Table 1 Single regression correlations of endothelin-1 with oxidative stress, antioxidant and inflammatory variables

	Endothelin-1 (pg ml^{-1})			
	Men (n = 198)		*Women (n = 195)*	
	Black (n = 99)	*White (n = 99)*	*Black (n = 96)*	*White (n = 99)*
Age (years)	r=0.064; P=0.53	r=–0.13; P=0.19	r=0.31; P=0.002	r=0.052; P=0.61
Body mass index (kg m^{-2})	r=0.13; P=0.19	r=0.020; P=0.85	r=0.28; P=0.006	r=0.074; P=0.47
Waist circumference (cm)	r=0.17; P=0.10	r=0.054; P=0.60	r=0.29; P=0.005	r=0.085; P=0.40
Total energy expenditure (kcal per day)	r=0.067; P=0.51	r=0.090; P=0.37	r=0.19; P=0.07	r=0.12; P=0.23
Biochemical variables				
Interleukin-6 (pg ml^{-1})	r=0.10; P=0.31	r=–0.20; P=0.05	r=0.21; P=0.04	r=–0.085; P=0.40
Glycated hemoglobin A1c (%)	r=–0.061; P=0.55	r=–0.030; P=0.77	r=0.18; P=0.08	r=–0.038; P=0.71
Gamma glutamyl transferase (U l^{-1})	r=0.22; P=0.03	r=–0.14; P=0.16	r=0.26; P=0.01	r=–0.11; P=0.91
Total cholesterol (mmol l^{-1})	r=0.099; P=0.33	r=0.043; P=0.67	r=0.049; P=0.64	r=–0.049; P=0.63
High density lipoprotein cholesterol (mmol l^{-1})	r=0.05; P=0.57	r=0.16; P=0.11	r=0.22; P=0.04	r=0.053; P=0.60
Low density lipoprotein cholesterol (mmol l^{-1})	r=0.030; P=0.77	r=0.025; P=0.81	r=–0.039; P=0.71	r=–0.088; P=0.39
Oxidative stress and anti-oxidant variables				
Reactive oxygen species (U[a])	r=–0.10; P=0.33	r=0.22; P=0.03	r=0.25; P=0.02	r=0.062; P=0.54
Total Glutathione (μM)	r=–0.034; P=0.74	r=0.003; P=0.98	r=–0.19; P=0.07	r=0.052; P=0.61
Glutathione peroxidase (U ml^{-1})	r=–0.065; P=0.52	r=–0.23; P=0.02	r=–0.058; P=0.58	r=–0.106; P=0.30
Glutathione reductase (U ml^{-1})	r=0.24; P=0.02	r=–0.15; P=0.14	r=0.10; P=0.32	r=–0.11; P=0.26
Superoxide dismutase (U ml^{-1})	r=–0.18; P=0.08	r=–0.15; P=0.15	r=–0.015; P=0.88	r=0.018; P=0.86
Catalase (U ml^{-1})	r=–0.11; P=0.26	r=–0.012; P=0.91	r=–0.085; P=0.42	r=0.003; P=0.98
GR-to-GPx ratio	r=0.20; P=0.04	r=–0.14; P=0.16	r=0.11; P=0.31	r=–0.084; P=0.41
GPx-to-SOD ratio	r=0.18; P=0.07	r=0.034; P=0.74	r=–0.027; P=0.80	r=–0.069; P=0.50
Cardiovascular variables				
Systolic blood pressure (mm Hg)	r=0.13; P=0.23	r=–0.006; P=0.95	r=0.27; P=0.007	r=0.13; P=0.19
Diastolic blood pressure (mm Hg)	r=0.062; P=0.55	r=0.12; P=0.25	r=0.16; P=0.13	r=–0.050; P=0.63
Pulse wave velocity (m s^{-1})	r=0.18; P=0.09	r=0.079; P=0.44	r=0.27; P=0.009	r=0.12; P=0.23

Abbreviations: GPx-to-SOD ratio, glutathione peroxidase-to-superoxide dismutase ratio; GR-to-GPx ratio, glutathione reductase-to-glutathione peroxide ratio; H_2O_2, hydrogen peroxide. [a]1 U = 1mg l^{-1} H_2O_2.



Supplementary Figure 1 The interrelated role between ET-1, oxidative stress and anti-oxidant capacity during physiological and pathological conditions. During normal physiological conditions, endothelin-1 secretion is low and the availability of nitric oxide is efficient in maintaining vascular tone. Endothelin-1 increase reactive oxygen species and vice versa, however, in some instances, such as stress, endothelin-1 receptors can mediate the formation of superoxide (O_2^-) that decrease the biological activity of nitric oxide, forming ONOO-, shifting the balance toward vasoconstriction. In healthy vasculature, the increase in O_2^- levels lead to the upregulation of glutathione reductase activity in an attempt to maintain the redox balance. Increased glutathione reduction activity can also activate endothelin-1 mediated NADPH oxidase to lower peroxidase levels though the activity of glutathione peroxidase to break down hydrogen peroxide (H_2O_2) into water and oxygen. During pathophysiological conditions, such as hypertension, increased endothelin-1 and increased reactive oxygen species levels leads to an increase in O_2^- and H_2O_2 production, and in turn result in an up-regulation of glutathione reductase activity. Prolonged oxidative stress could lead to increased nitric oxide inactivation in these patients, thus resulting in excess peroxidase, causing endothelial damage, and eventually lead to diseases such as arteriosclerosis. However, in black premenopausal women, it is possible that the association between high-density lipoprotein cholesterol and endothelin-1, might have a protective counter-regulatory mechanism against oxidative stress, that was confirmed by an upregulation of both endothelin-1 and glutathione levels in this study. Although the black men and women in our population study did use anti-hypertensive medication, their blood pressure remained elevated, suggesting a possible vascular phenotype difference between black and white patients in their ability to maintain homeostasis.[2, 10, 12, 16, 18, 29, 32, 34, 41, 49-53]

CHAPTER 5

Three-year change in endothelin-1 and markers of vascular remodeling in a bi-ethnic South African cohort: The SABPA study

Three-year change in endothelin-1 and markers of vascular remodeling in a bi-ethnic South African cohort: The SABPA study

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Running title: Endothelin-1 change and vascular remodeling

Word count: 3901

Number of tables: 4

Number of figures: 0

Number of supplementary tables: 2

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Conflicts of Interest and Source of Funding: The authors have no conflict of interest to declare. The SABPA study was supported by the South African National Research Foundation (UID 65607), the South African National Research Foundation Thuthuka (80643), the North-West University (Potchefstroom Campus, South Africa), Roche Products (Pty) Ltd, South Africa and the Metabolic Syndrome Institute (France). Christine S du Plooy received the financial assistance of the National Research Foundation (NRF SARCHi Postgraduate bursary and DAAD-NRF Joint in-country scholarship (UID 105119)) toward this research study and is hereby acknowledged. The funders played no role in the design and conduct of the study.

- This article is prepared according to the author's instructions from the *Journal of Hypertension* (Annexure A). Please note that some of the format requirements were changed to ensure uniformity throughout the thesis.

ABSTRACT

Background South Africans are at high risk for developing cardiovascular disease. Endothelin-1 is known for its vasoconstrictive properties and its ability to contribute to vascular structural changes. In this study we investigated the association of change in endothelin-1 levels and change in markers implicated in vascular remodeling after three years.

Methods: Serum endothelin-1 levels and markers of vascular remodeling such as carotid intima media thickness, carotid cross-sectional wall area (CSWA) and arterial compliance were measured. Participants were divided into two groups according to an increase (n=185) and a decrease (n=152) in plasma endothelin-1 levels after three years.

Results: In partial regression analysis, the extent of endothelin-1 increase correlated positively with a change in pulse pressure and inversely with the change in arterial compliance in the group with increased endothelin-1 levels after three years, whereas the extent of a decrease in endothelin-1 correlated inversely with a change in CSWA in the group with decreased endothelin-1 levels. In multiple regression analysis, after splitting for race, the increase in endothelin-1 levels associated positively with the change in pulse pressure (Adj. R^2=0.092; β=0.278; p=0.036). A borderline association exist between the extent of decrease in endothelin-1 and a lesser change in CSWA (Adj. R^2=0.076; β=–0.201; p=0.054) in black participants only. The latter result disappeared after removing those using anti-hypertension medication.

Conclusions: In black participants with increased endothelin-1 levels after three years, the positive association between endothelin-1 and pulse pressure suggest subclinical haemodynamic changes with potential premature onset of cardiovascular disease.

Keywords: arterial stiffness, cross-sectional wall area, pulse pressure, endothelin-1, vascular remodeling

INTRODUCTION

Vascular homeostasis is partially maintained by the endothelium through a variety of vasoconstrictive and vasodilator substances such as endothelin-1 (ET-1), nitric oxide and prostacyclin, acting locally in the blood vessel wall to protect the large arteries from adverse remodeling [1,2]. Although age is associated with an increase in luminal diameter and stiffening of the vessel wall, vascular remodeling may occur earlier in individuals predisposed to or at risk of premature hypertension, atherosclerosis and/or arteriosclerosis [3,4].

ET-1 is known for the role it plays in the development of cardiovascular diseases by inducing vascular hypertrophy and increasing blood pressure as shown in experimental studies [3,5,6]. ET-1 secretion occurs abluminally towards the smooth muscles in the vascular wall, and plasma ET-1 levels are the result of a spill over into the bloodstream [7]. It has also been shown that ET-1 levels are modulated via oxidative stress [8]. Studies indicated that pathophysiological conditions such as hypertension and atherosclerosis are associated with increased plasma ET-1 levels [5,9-11]; whereas others found that plasma ET-1 levels are similar in normotensive and hypertensive individuals [12-15]. ET-1 levels contribute to decreased arterial compliance in patients with hypertension through increased elastin degradation [16] associated with decreased large vessel elasticity and increased pulse pressure [17]. Experimental [6,18] and human [19,20] studies found that ET-1 also participate in hypertrophic remodeling in hypertensive human and rat models indicated by a change in carotid intima-media thickness (CIMT) [19] and carotid cross-sectional wall area (CSWA) [6,18,20].

In previous studies the association with early predictors of cardiovascular diseases suggests that ET-1 could, together with other markers contribute to the development of cardiovascular disease over time [3, 6]. To our knowledge, no study has investigated if an association of a change in ET-1 levels over time with markers of vascular remodeling exist. Therefore, we investigated the association of change in ET-1 levels with the change of markers implicated in vascular remodeling including CIMT, CSWA and arterial compliance after three years in a bi-ethnic South African cohort.

MATERIALS AND METHODS

Study population and protocol

This study formed part of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study. Detailed information regarding the procedures of the SABPA study was published previously [21]. Exclusion criteria for baseline data collection of the SABPA study were pregnant or lactating women, individuals using alpha and beta blockers, participants with an ear temperature ≥37° C and those who had a vaccination or donated blood 3 months or less prior to participation. At baseline, 409 black and white South Africans were included. The three year follow-up data collection had an 87.8% success rate and included 359 participants. In this study, we excluded participants who did not participate in the follow-up phase (n=48), those with missing data of main variables (n=14) and outliers of ET-1 levels by residual statistics (baseline: n=10; follow-up: n=0). The interactions between change in ET-1 levels, race and sex were determined (sex: $F(337)=6.68$, $p=0.313$; race: $F(337)=0.76$, $p=0.326$), but no significant interaction was observed. Additionally, a significant interaction of change in ET-1 levels on markers of vascular remodeling (systolic blood pressure: $F(337)= 1.42$; $p=0.095$; diastolic blood pressure: $F(337)=5.05$; $p=0.025$; pulse pressure: $F(337)=27.24$; $p<0.001$; arterial compliance: $F(337)=4.93$; $p=0.027$; CIMT: $F(337)=71.87$; $p<0.001$; CSWA: $F(337)=127.24$; $p<0.001$) was also tested, therefore the participants were stratified accordingly.The included participants were stratified according to an increase (n=185) or a decrease (n=152) in plasma ET-1 levels after three years. A standard health survey was used for the collection of demographic information, anti-hypertension medication and statin usage. All participants provided written informed consent. The Health Research Ethics Committee of the North-West University, Potchefstroom campus, granted approval for this study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki for the investigation of human subjects.

Anthropometric measurements

Body height and weight were measured with calibrated instruments (Invicta Stadiometer, IP 1465, London, UK; Precision Health Scale, A&D Company, Tokyo, Japan) while participants were in minimal clothing. Body mass index was calculated for each participant [22].

Biochemical analyses

Fasting blood samples were collected from each participant and serum and plasma were prepared according to standard procedures. Procedures followed for quantitative analyses were the same for both baseline and follow-up. Serum and plasma samples were frozen at –80° C until analysed. ET-1 was determined with a Quantikine enzyme linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). Intra assay variability for ET-1 was 2.7% and inter assay variability for ET-1 was less than 18% for both baseline and follow-up data.

Fasting lipids (total and high-density lipoprotein cholesterol), glycated haemoglobin A1c, C-reactive protein, gamma glutamyl transferase and creatinine were determined using two sequential multiple analysers at baseline (Konelab 20i, Thermo Scientific, Vantaa, Finland and Unicel DXC 800, Beckman and Coulter, Germany) and at follow-up the Integra 400 plus (Roche, Basel, Switzerland) was used. Serum cotinine was determined by using a homogenous immunoassay (Sarstedt®, Numbresht, Germany). Inter- and intra assay variability was less than 10%.

One of the measurable reactive oxygen species, namely total peroxides, was determined in serum samples [23]. The EDTA plasma levels of glutathione reductase and glutathione peroxidase was determined with assay kits (Cayman Chemical Company, Ann Arbor, MI, USA) using the Synergy H_4hybrid Microplate Reader BioTekWinooski, VT, USA). The intra and inter assay variability were less than 10% for baseline and follow-up data for these analyses. Glutathione reductase-to-glutathione peroxidase ratio (GR-to-GPx ratio) was calculated to assess antioxidant defenses. The modification of diet in renal disease (MDRD) formula was used to calculate estimated glomerular filtration rate (eGFR) from serum creatinine levels, age, sex, and ethnicity: eGFR = 175 * (standardized serum creatinine (mg/dL))$^{-1.154}$ * (age (years))$^{-0.203}$ * 1.212 [if black] * 0.742 [if female] [24]. Estimated creatinine clearance was calculated by using the Cockcroft-Gault formula as follows: creatinine clearance (ml/min) = (140-age) x weight (kg) x constant/serum creatinine (μmol/L), where the constant for females are 1.23 and 1.04 for males [25].

Cardiovascular measurements

The Cardiotens (CE120, Meditech, Hungary) was used to record 24-hour ambulatory systolic and diastolic blood pressure, which were used to derive 24-hour pulse pressure. Participants with a systolic blood pressure ≥ 135 mmHg and/or diastolic blood pressure ≥ 85 mmHg were considered hypertensive [26]. The 24-hour blood pressure data were downloaded onto a database using the CardioVisions 1.9.0 Personal Edition software.

Mean resting arterial compliance (Windkessel compliance) was determined by the Finometer device (Finapres Medical Systems, Amsterdam, Netherlands). CIMT was measured using a SonoSite Micromaxx ultrasound system (SonoSite, Bothell, WA, USA) and a 6-13 MHz linear array transducer from at least two optimal angles of the left and right common carotid artery. Subsequently, the carotid CSWA was calculated with the formula: $CSWA = \pi(d/2 + CIMT)^2 - \pi(d/2)^2$. Interpretations were made for both CIMT and carotid CSWA with a semi-automated program, the Artery Measurement Systems (AMS) II v1.139 (Chalmers University of Technology, Gothenburg, Sweden).

Statistical analysis

Statistical analyses were done using IBM® SPSS® Statistics version 23 (IBM Corp., Armonk, NY, USA, 2016). Main effects of race and sex were tested for interactions on the association between ET-1 and vascular remodeling related markers by means of multiple regression analyses (sex: $F(337)=6.68$, $p=0.313$; race: $F(337)=0.76$, $p=0.326$). Participants with increased ET-1 levels at follow up were grouped together as "ET-1 increase after three years", whereas participants that had a decrease in ET-1 levels from baseline to follow up were grouped together as "ET-1 decrease after three years". The G*Power version 3.1.9.2 software was used to compute the achieved power in post hoc analysis [27]. At a probability of 0.05, medium effect size of 0.5 and two-tailed input method, the achieved power (1–β error probability) was estimated at 99.99% in both groups. Dependent T-tests were used to compare means for continuous variables and cross-tabulation to compare proportions of dichotomous variables between the groups. Partial correlations were used to determine correlations of change in ET-1 with vascular remodeling related variables. Multivariate regression analyses were performed with increased or decreased change in

ET-1 levels as the main dependent variable and vascular remodeling related markers as independent variables.

Sensitivity analyses

Sensitivity analyses were applied for reactive oxygen species, GR-to-GPx ratio, hypertension status, eGFR, creatinine clearance, statin usage, and anti-hypertension usage, respectively by using multiple regression analyses. Due to an association between race and ET-1 in models, an additional multiple regression analysis was performed to determine the race in which the associations occurred.

RESULTS

Basic descriptive characteristics of this study population are listed in Table 1. In both groups (ET-1 increase and ET-1 decrease after three years) body mass index, statin usage, pulse pressure, CIMT, and CSWA increased over time (all $p<0.05$), whereas reactive oxygen species, total cholesterol, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol, decreased over time (all $p<0.05$). In the group with an increase in ET-1 levels, there was also a decrease in glutathione peroxidase activity ($p=0.022$), while in the group with a decrease in ET-1 levels, there was a decrease in gamma glutamyl transferase levels and arterial compliance (all $p<0.05$).

In partial regression analysis with adjustments for age, body mass index, race and sex (Table 2), the extent of an increase in ET-1 correlated positively with a change in pulse pressure ($r=0.168$; $p=0.041$) and negatively with a change in arterial compliance ($r=–0.201$; $p=0.015$) in the increased ET-1 level group. The extent of a decrease in ET-1 correlated negatively with a change in CSWA ($r=–0.171$; $p=0.022$) in the decreased ET-1 levels group.

In multiple regression analysis, we performed a separate model for each group (ET-1 increase and ET-1 decrease after three years). Firstly, we investigated the association of increased ET-1 with a change in pulse pressure and a change in arterial compliance and established that the extent of an increase in ET-1 levels associated positively with a change in pulse pressure ($\beta=0.171$; $p=0.033$) and inversely with a change in arterial compliance ($\beta=–0.170$; $p=0.045$) in the increased ET-1 group (Supplementary Table 1).

Due to the significant association observed with race (pulse pressure: β=0.200; p=0.022; arterial compliance: β=0.230; p=0.009) (Supplementary Table 1), we divided the group according to black and white groups and performed the same multivariate regressions for pulse pressure (Table 3) and arterial compliance (Table 4) in the increased ET-1 group. The extent of an increase in ET-1 associated positively with a change in pulse pressure (β=0.278; p=0.036) in the black participants only, while in the white participants the extent of increased ET-1 associated inversely with GR-to-GPx ratio (Table 4: β=–0.297; p=0.007; Table 5: β=–0.322; p=0.004) only. There was no association between increased ET-1 and change in arterial compliance in white or black participants (black: Adj. R^2=0.074; β=–0.155; p=0.270; white: Adj. R^2=0.120; β=–0.189; p=0.092). Secondly, we investigated the association of the decrease in ET-1 with a change in CSWA and found that the extent of decrease in ET-1 associated positively with a change in CSWA (β=0.183; p=0.017), a change in high-density lipoprotein cholesterol (β=0.147; p=0.049) and with race (β=–0.178; p=0.024) in the decreased ET-1 group (Supplementary Table 1). Since there was an association with race, we divided the decreased ET-1 group into black and white participants (Supplementary Table 2) and found that the extent of a decrease in ET-1 associated inversely with GR-to-GPx ratio (Adj. R^2=0.076; β=–0.234; p=0.022) in the black group only.

Sensitivity analyses

In sensitivity analysis, our results remained unchanged after sensitivity analyses were performed for hypertension status, statin usage and anti-hypertension medication (*data not shown*). However, when excluding participants on anti-hypertension medication (blacks: n=43 (43.4%); whites: n=16 (18.6%)), an inverse association existed between a decrease in ET-1 and CSWA (Adj. R^2=0.054; β=–0.127; p=0.046) in the white group only. The same trend was observed after excluding those using statins (blacks: n=3 (3.03%); whites: n=21 (24.4%)), with an inverse association between a decrease in ET-1 and CSWA (Adj. R^2=0.049; β=–0.104; p=0.030) in the white group only. Additionally, sensitivity for change in eGFR and change in creatinine clearance was also tested to investigate the association between ET-1 with markers of renal function, but the results remained unchanged (*data not shown*) even after excluding participants using anti-hypertension medication and statins.

Table 1: Population characteristics of baseline and three years follow-up stratified by an increase and decrease in endothelin-1 levels

	ET-1 decrease after three years (n=185)			ET-1 increase after three years (n=152)		
	Baseline	Follow-up	p-value	Baseline	Follow-up	p-value
Age (years)	45.0 ± 0.66	48.0 ± 0.66	–	46.2 ± 0.69	49.2 ± 0.69	–
Race, black, n (%)	99 (53.5)	99 (53.5)	–	67(44.1)	67(44.1)	–
white, n (%)	86 (46.5)	86 (46.5)	–	85 (55.9)	85 (55.9)	–
Sex, men, n (%)	105 (56.8)	105 (56.8)	–	69 (45.4)	69 (45.4)	–
women, n (%)	80 (43.2)	80 (43.2)	–	83 (54.6)	83 (54.6)	–
Body mass index (kg/m^2)	28.7 ± 0.47	29.6 ± 0.49	<0.001	28.9 ± 0.45	29.8 ± 0.47	<0.001
Hypertension status, n (%)	117 (63.2)	111 (60.0)	0.274	92 (60.5)	88 (57.9)	0.656
Anti-hypertension drug usage, n (%)	54 (29.2)	55 (29.7)	0.862	30 (19.7)	37 (24.3)	0.238
Statins usage, n (%)	7 (3.8)	17 (9.2)	0.012	4 (2.6)	17 (11.2)	<0.001
Biochemical variables						
Endothelin-1 (pg/mL)	2.49 ± 0.06	1.66 ± 0.04	<0.001	1.61 ± 0.06	2.52 ± 0.07	<0.001
Reactive oxygen species (Units*)	91.7 ± 23.5	84.1 ± 24.7	<0.001	91.7 ± 27.5	75.9 ± 22.2	<0.001
C-reactive protein (mg/L)	6.00 ± 0.67	4.62 ± 0.62	0.071	4.82 ± 0.50	4.19 ± 0.71	0.396
Gamma glutamyl transferase (U/L)	53.4 ± 5.66	46.3 ± 4.97	0.028	41.2 ± 4.48	41.6 ± 4.87	0.934
Glutathione reductase (U/mL)	6.53 ± 0.52	6.37 ± 0.77	0.853	5.40 ± 0.27	6.24 ± 0.98	0.427
Glutathione peroxidase (U/mL)	34.1 ± 0.89	35.3 ± 0.92	0.389	36.2 ± 0.87	33.0 ± 0.85	0.022
GR-to-GPx ratio	0.25 ± 0.03	0.19 ± 0.02	0.073	0.17 ± 0.01	0.19 ± 0.03	0.479
Cotinine (ng/mL)	29.9 ± 6.21	32.2 ± 7.00	0.577	19.0 ± 4.29	18.1 ± 4.40	0.754
Total cholesterol (mmol/L)	5.16 ± 0.10	4.40 ± 0.08	<0.001	5.12 ± 0.11	4.49 ± 0.08	<0.001
High-density lipoprotein cholesterol (mmol/L)	1.17 ± 0.03	0.98 ± 0.02	<0.001	1.17 ± 0.03	1.09 ± 0.03	<0.001
Low-density lipoprotein cholesterol (mmol/L)	3.35 ± 1.17	2.80 ± 0.89	<0.001	3.37 ± 1.10	2.87 ± 0.89	<0.001
Cardiovascular variables						
Systolic blood pressure (mmHg)	129.8 ± 1.10	130.6 ± 1.25	0.301	127.5 ± 1.18	127.5 ± 1.19	0.988
Diastolic blood pressure (mmHg)	81.7 ± 0.75	80.9 ± 0.82	0.171	79.3 ± 0.81	78.2 ± 0.78	0.063
Pulse pressure (mmHg)	48.1 ± 0.56	49.7 ± 0.65	0.001	48.2 ± 0.65	49.3 ± 0.68	0.030
Arterial compliance (mL/mmHg)	1.98 ± 0.04	1.93 ± 0.04	0.042	1.94 ± 0.04	1.95 ± 0.04	0.871
Carotid intima media thickness (mm)	0.67 ± 0.01	0.69 ± 0.01	0.004	0.67 ± 0.01	0.70 ± 0.01	<0.001
Cross-sectional wall area (mm^2)	13.9 ± 0.28	14.6 ± 0.27	<0.001	13.2 ± 0.37	14.8 ± 0.32	<0.001

Values are arithmetic mean ± standard deviation. Abbreviations: ET-1 – endothelin-1; GR-to-GPx ratio – Glutathione reductase-to-glutathione peroxidase ratio. *1 Unit=1mg/L H_2O_2.

Table 2: Partial correlations of the change in endothelin-1 levels and change in main independent variables after three years

	ET-1 change (pg/mL)	
	ET-1 decrease after three years (n=185)	ET-1 increase after three years (n=152)
Ambulatory systolic blood pressure (mmHg)	r=–0.029; p=0.702	r=0.153; p=0.063
Ambulatory diastolic blood pressure (mmHg)	r=–0.055; p=0.464	r=0.099; p=0.234
Ambulatory pulse pressure (mmHg)	r=0.004; p=0.955	*r=0.168; p=0.041*
Arterial compliance (mL/mmHg)	r=–0.006; p=0.941	*r=–0.201; p=0.015*
Carotid intima media thickness (mm)	r=–0.108; p=0.153	r=–0.062; p=0.463
Cross-sectional wall area (mm^2)	*r=–0.171; p=0.022*	r=0.006; p=0.945

Adjustments applied for age, body mass index, race and sex. Abbreviations: ET-1 – endothelin-1.

Table 3: Forward stepwise multiple regression analysis of change in endothelin-1 levels and change in markers of vascular remodeling (pulse pressure) with an increase in endothelin-1 after three years

	ET-1 increase after three years			
	ET-1 change (pg/mL)			
	Black participants (n = 65)		White participants (n = 83)	
Adjusted R^2	0.092		0.072	
	Beta (95% CI)	**p-value**	**Beta (95% CI)**	**p-value**
Pulse pressure change (mmHg)	0.278 (0.020 to 0.536)	0.036	0.151 (–0.066 to 0.368)	0.170
GR-to-GPx ratio	–0.140 (–1.549 to 0.425)	0.259	–0.297 (–0.908 to –0.147)	0.007

Adjusted for race, sex, follow-up data for age, body mass index, C-reactive protein, glutathione reductase to glutathione peroxidase ratio and change in high-density lipoprotein cholesterol after three years. **Abbreviations**: ET-1 – endothelin-1; GR-to-GPx ratio – Glutathione reductase-to-glutathione peroxidase ratio.

Table 4: Forward stepwise multiple regression analysis of change in endothelin-1 levels and change in markers of vascular remodeling (arterial compliance and systolic blood pressure) with an increase in endothelin-1 after three years

	ET-1 increase after three years			
	ET-1 change (pg/mL)			
	Black participants (n = 63)		White participants (n = 82)	
Adjusted R^2	0.074		0.120	
	Beta (95% CI)	**p-value**	**Beta (95% CI)**	**p-value**
Arterial compliance change (mL/mmHg)	–0.155 (–0.842 to 0.240)	0.270	–0.189 (–1.026 to 0.080)	0.092
GR-to-GPx ratio	–0.184 (–1.741 to 0.268)	0.147	*–0.322 (–0.955 to –0.191)*	*0.004*

Adjusted for race, sex, follow-up data for age, body mass index, C-reactive protein, glutathione reductase to glutathione peroxidase ratio and change in high-density lipoprotein cholesterol after three years. Abbreviations: ET-1 – endothelin-1; GR-to-GPx ratio – Glutathione reductase-to-glutathione peroxidase ratio.

DISCUSSION

Our most prominent result indicated that an increase in ET-1 levels after three years are positively associated with a change in pulse pressure in the black group only. In addition, anti-hypertension treatment and statin usage seem to slow down adverse vascular remodeling in the white population only.

The association we found of an increase in ET-1 levels with a change in pulse pressure over time in the black participants, are similar to results found in previous studies in which it were indicated that an increase in ET-1 levels contribute to an increased risk of developing hypertension by decreasing large vessel elasticity and increasing pulse pressure [10,28,29]. Changes in smooth muscle tone by means of vasoactive peptides such as ET-1 and angiotensin II [29] affect the stiffness of arteries. ET-1 overexpression is a common phenomenon in the black population [10,30] and could lead to altered vascular structure and function by directly inducing significant hypertrophic remodeling in resistance vessels, thereby increasing pulse pressure [3]. A previous study in our population found that the black group showed increased reactivity of vascular resistance during stressors and that an urban Westernized lifestyle could contribute to the changes in autocrine and paracrine factors [31], such as ET-1. The absence of decreased arterial compliance are supported by a study that found that even during sustained hypertension, normal distensibility of medium sized arteries are maintained, despite hypertrophy of the arterial wall [32].

Our second result showed that a decrease in ET-1 levels is inversely associated with a change in CSWA in the black population. This suggests that at lower ET-1 levels, vascular hypertrophy or vascular remodeling of the CSWA occur at a slower rate, only if anti-hypertension treatment is used, in comparison to the increased vascular remodeling that occur at increasing ET-1 levels. Anti-hypertension medication studies associated hypertension treatments with an improvement in endothelial function and protection against the increased endothelin-mediated vasoconstriction [11,33]. After excluding participants on anti-hypertension medication and statins in our study population, we found that the inverse association between a decrease in ET-1 levels and CSWA shifted towards the white population. This shift might suggest that anti-hypertension medication protects the vasculature against vascular changes in white individuals by indirectly leading to decreasing ET-1 levels. Without anti-hypertensive

treatment the balance between vascular injury and repair may be altered, thereby increasing the risk for atherosclerotic disease in the white population. This response supports other studies that found black participants to exert pressure-related arterial stiffness which leads to early deterioration in this group, possibly due to the activity of ET-1; whereas white individuals exert age-related arterial stiffness not yet present in this population [20,34,35].

We also investigated the role of ET-1 with renal function, oxidative stress and anti-oxidant capacity. After performing a sensitivity analysis for markers of renal function (a change in estimated glomerular filtration rate and a change in creatinine clearance) the results in our study remained the same. Previously our group found an association between ET-1 and anti-oxidant capacity [36]. This latter study showed that ET-1 contributes to a larger GR-to-GPx ratio through increased reactive oxygen species production and in the present study this was confirmed prospectively.

The results of this study need to be interpreted within the context of its limitations and strengths. A major strength of the study is that this was a cohort study with three years follow-up and the first to describe ET-1 could be one of the many risk markers for hypertension and atherosclerosis observed in the South African population. An additional strength of the study can be measured on the basis of its longitudinal design and implementation under controlled conditions (two ethnic and homogenous socioeconomic groups). Although the results were consistent after multiple adjustments, we cannot exclude residual confounding. Another limitation is that the study consisted of a relatively small sample size and larger cohort population studies is needed to confirm these results in other South African populations.

In conclusion, our study found that with increased endothelin-1 levels after three years, the positive association between endothelin-1 and pulse pressure may suggest subclinical haemodynamic changes with potential premature onset of cardiovascular disease in the black population.

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SUPPLEMENTARY INFORMATION

Supplementary table 1: Forward stepwise multiple regression analysis of the change in endothelin-1 levels and the change in markers of vascular remodeling (cross-sectional wall area, arterial compliance and pulse pressure) with an increase and decrease in endothelin-1 after 3 years

	ET-1 change (pg/mL)			
	ET-1 decrease after 3 years (n=180)		ET-1 increase after 3 years (n=149)	
	ET-1 and CSWA			
Adjusted R^2	0.073		0.086	
	Beta (95% CI)	**p-value**	**Beta (95% CI)**	**p-value**
Race	–0.178 (–0.449 to –0.032)	0.024	0.175 (0.001 to 0.534)	0.049
CSWA change (mm^2)	0.183 (0.033 to 0.333)	0.017	–0.001 (–0.038 to 0.038)	0.993
HDLC change (mmol/L)	0.147 (0.002 to 0.657)	0.049	–0.059 (–0.592 to 0.271)	0.464
GR-to-GPx ratio	0.055 (–0.209 to 0.472)	0.447	–0.242 (–0.846 to –0.178)	0.003
	ET-1 and pulse pressure			
Adjusted R^2	0.042		0.116	
	Beta (95% CI)	**p-value**	**Beta (95% CI)**	**p-value**
Race	–0.222 (–0.506 to –0.093)	0.005	0.200 (0.044 to 0.568)	0.022
Pulse pressure	–0.009 (–0.018 to 0.015)	0.900	0.171 (0.151 to 0.191)	0.033
GR-to-GPx ratio	0.055 (–0.215 to 0.477)	0.457	–0.245 (–0.847 to –0.190)	0.002
	ET-1 and arterial compliance			
Adjusted R^2	0.036		0.138	
	Beta (95% CI)	**p-value**	**Beta (95% CI)**	**p-value**
Race	–0.226 (–0.515 to –0.095)	0.005	0.230 (0.066 to 0.594)	0.009
Arterial compliance change (mL/mmHg)	–0.003 (–0.305 to 0.291)	0.964	–0.170 (–0.754 to –0.009)	0.045
GR-to-GPx ratio	0.058 (–0.212 to 0.490)	0.435	–0.257 (–0.873 to –0.214)	0.001

Adjusted for race, sex, follow-up data for age, body mass index, C-reactive protein, glutathione reductase to glutathione peroxidase ratio and change in high-density lipoprotein cholesterol after three years. Abbreviations: CSWA – Cross-sectional wall area; ET-1 – endothelin-1; GR-to-GPx ratio – Glutathione reductase-to-glutathione peroxidase ratio; HDLC .

Supplementary Table 2: Forward stepwise multiple regression analysis of the change in endothelin-1 levels and the change in markers of vascular remodeling (cross-sectional wall area) with a decrease in endothelin-1 after three years

	ET-1 decrease after three years			
	ET-1 change (pg/mL)			
	Black participants (n = 96)		White participants (n = 83)	
Adjusted R^2	0.076		0.008	
	Beta (95% CI)	**p-value**	**Beta (95% CI)**	**p-value**
Cross-sectional wall area change (mm^2)	–0.201 (–0.406 to 0.004)	0.054	–0.155 (–0.382 to 0.072)	0.179
GR-to-GPx ratio	*–0.234 (– 1.308 to –0.102)*	*0.022*	0.144 (–0.164 to 0.776)	0.199
Age (years)	*–0.251 (–0.470 to –0.032)*	*0.025*	–0.073 (–0.298 to 0.152)	0.521

Adjusted for race, sex, follow-up data for age, body mass index, C-reactive protein, glutathione reductase to glutathione peroxidase ratio and change in high-density lipoprotein cholesterol after three years. Abbreviations: ET-1 – endothelin-1; GR-to-GPx ratio – Glutathione reductase-to-glutathione peroxidase ratio.

CHAPTER 6

GENERAL FINDINGS AND CONCLUSIONS

6.1. Introduction

This chapter summarises the main findings of the three research articles included in the thesis. The results are discussed, interpreted, explained and compared to relevant literature. Conclusions are drawn and recommendations are made to researchers investigating the association of endothelin-1 (ET-1) with cardiometabolic and vascular function in a prospective cohort study.

6.2. Summary of the main findings

The primary aim of this study was to assess the influence of the levels of ET-1 with markers of cardiometabolic and vascular function in black and white South-Africans. This study also aimed to explore the association of three year-changes in ET-1 levels and markers of vascular remodeling in a bi-ethnic South African cohort. The relevant findings of each article were:

6.2.1. The association of endothelin-1 with markers of arterial stiffness in black South African women: The SABPA study (Chapter 3)

The aims of this study were to determine if ET-1 differs among sex and race in a South-African cohort and whether there is an association of ET-1 with cardiovascular function and inflammation in this population.

The first hypothesis stated that ET-1 levels will be higher in black men and women in comparison with their white counterparts. In our study, no significant difference was found in ET-1 levels between the black and white groups before investigating the effect of inflammation on ET-1 levels. After the appropriate adjustments for inflammation were made, our study found higher levels of ET-1 in black men in comparison to white men, while white women had higher levels of ET-1 than black women. Therefore, the hypothesis is only partially accepted since higher levels of ET-1 were anticipated in the total black population in comparison to the white population.

The second hypothesis proposed that ET-1 will associate with cardiovascular deterioration and inflammatory markers (C-reactive protein (CRP) and interleukin-6 (IL-6)) in black men and

women. The results of this study indicate an independent association of ET-1 with systolic blood pressure, pulse pressure and IL-6, especially in black women. This suggests a link between ET-1 and subclinical vascular dysfunction, which may be mediated by pro-inflammation. Also, even after adjustments were made for significant covariates, the positive significant association between ET-1, systolic blood pressure, pulse pressure and IL-6 remained in the black women, but not in black men, therefore the second hypothesis is partially accepted.

6.2.2. The association of endothelin-1 with markers of oxidative stress in a bi-ethnic South African cohort: The SABPA study (Chapter 4)

The objective of this study was to investigate the associations of ET-1 with markers of oxidative stress (serum peroxides) and anti-oxidant capacity (glutathione peroxidase (GPx), glutathione reductase (GR), catalase, and total glutathione (GSH)) in black and white South Africans.

The hypothesis was that ET-1 associates positively with markers of oxidative stress and negatively with markers of anti-oxidant capacity in both black and white individuals. In our study, ET-1 associated positively with GR and glutathione reductase-to-glutathione peroxidase ratio (GR-to-GPx ratio) in black men and negatively with GSH in black women. Additionally, ET-1 associated positively with reactive oxygen species and negatively with GPx in white men only. Contradictory to the hypothesis, ET-1 positively associated with anti-oxidant capacity (GR and GR-to-GPx ratio) in black men, therefore the hypothesis is only partially accepted.

6.2.3. Three-year change in endothelin-1 and markers of vascular remodeling in a bi-ethnic South African cohort: The SABPA study (Chapter 5)

The aim of the last study was to investigate the changes in ET-1 levels and markers of vascular remodeling (carotid intima media thickness (CIMT), carotid cross-sectional wall area (CSWA) and arterial compliance) after three years in black and white South Africans.

The hypothesis indicated that an increase in ET-1 levels after three years will associate positively with markers of vascular remodeling and that the black population will have more adverse changes

to these vascular remodeling than their white counterparts. The results of the study were that an increase in ET-1 levels after three years contributes to a change in pulse pressure in the black population. Additionally, a decrease in ET-1 levels associated inversely with a change CSWA in the white population. There was no link between changes in ET-1 levels, CIMT and arterial compliance in any of the groups. Therefore, our hypothesis is only partially accepted.

6.3. Discussion of main findings

The black population is at high risk for developing cardiovascular disease such as hypertension attributable to early changes within the vasculature, due to inherent and lifestyle risk factors.[1,2] These risk factors can alter the endothelial function, leading to pathological conditions such as pro-inflammation, impaired modulation of vascular growth and dysregulation of vascular remodeling often seen during hypertension and atherosclerosis.[3-5] The exact mechanism responsible for the development of hypertension in the black population of South Africa is not yet known, but it is suggested that ET-1 may play an important role in cardiovascular disease development.[6]

ET-1 levels between black and white South Africans

It is known that an increase in ET-1 levels is associated with pathological conditions such as hypertension and is an early marker of coronary atherosclerosis and coronary endothelial dysfunction.[7] Since the black population is at high risk for developing hypertension attributable to early changes within the vasculature, Evans et al.[8] and Ergul et al.[9] studied the differences in ET-1 levels in healthy and hypertensive black and white Americans. Evans et al.[8] showed that black men have significantly higher levels of ET-1 than white men, with no differences in ET-1 levels between black and white women; whereas Ergul et al.[9] found no significant difference between race and sex in normotensive black and white participants and higher ET-1 levels in black hypertensives compared to white hypertensives and black normotensives. To our knowledge, we are the first to investigate the differences in ET-1 levels in an apparently healthy bi-ethnic South African population. There was no significant difference between ET-1 levels, race and sex before adjustments were made, while Evans et al.[8] reported that ET-1 levels are higher in black than white healthy men with no significant difference between women. After adjustments were made for

inflammation, ET-1 levels in men were in line with results found in Evans et al.[8] and Ergul et al.[9], while black women had lower levels than white women, a finding that contradicts these earlier findings.

ET-1, cardiovascular function and inflammation

Inflammation and high blood pressure have both been implemented in vascular endothelial dysfunction and *vice versa*,[10,11] and studies found that conditions such as elevated blood pressure increase inflammatory cell activity, releasing IL-6 and CRP and in turn increasing the production of ET-1.[12-14] Previous studies have indicated that ET-1, CRP and IL-6 exerts pro-inflammatory and pro-atherosclerotic effects and increase the risk for development of atherosclerotic vascular disease.[13,15-18] Our results indicated an interrelated role between ET-1, inflammation (IL-6 and CRP), blood pressure (systolic and pulse pressure) and high-density lipoprotein cholesterol (HDLC) in the black population exist, especially black women. This interrelated role between ET-1 and blood pressure (pulse pressure in the prospective study) may explain the difference in ET-1 levels in the black men compared with white men and supports previous findings that ET-1 levels may lead to increased pulse pressure in black hypertensive patients.[19,20]

The association with systolic blood pressure (cross-sectional study), pulse pressure (cross-sectional and prospective study), inflammation and HDLC may explain the contradictory results in ET-1 levels from this study found in the black women compared to white women. HDLC is known for the role it plays in protecting against the development of vascular diseases and eventually corrects endothelial dysfunction in the pathogenesis of athersoclerosis.[21,22] Additionally, studies found that HDLC inhibits the secretion of ET-1 as observed in the ET-1 levels of black women from our study.[23,24] In the black population of South Africa, the black women are prone to obesity, which leads to increased levels of CRP and IL-6.[25] It is believed that increased weight gain and hypertension could lead to elevated HDLC in response to high levels of CRP.[25-27] In our study we found a positive association between ET-1, body mass index, IL-6 and HDLC in the black women, which suggests that HDLC indirectly protects the black women against the pathological properties of ET-1 until arterial stiffness manifests in this group. In the black men, there is the possibility that endothelial dysfunction already exists through the vasoconstrictive properties of ET-1.

ET-1, oxidative stress and anti-oxidant capacity

During inflammation, the endothelium becomes more permeable to lipid particles and immune cells.[12] Damaged endothelial cells generate reactive oxygen species, in turn upregulating ET-1 and *vice versa*.[12,28] Once ET-1 release increases, nitric oxide bio-availability is reduced and the vascular tone shifts toward vasoconstriction, causing endothelial damage and necrotic cell death, which contribute to arterial hypertension and athersoclerosis.[29-31] Additionally, the anti-oxidant activity of GR, GPx, GSH and catalase increase in response to an increase in reactive oxygen species generation.[32] Prolonged oxidative stress gradually overshadows the function of anti-oxidant activity, resulting in inflammation.[33]

To our knowledge, this study is the first to report a link between ET-1, oxidative stress and anti-oxidant capacity in a black and white South African population. In the white men from our cohort, ET-1 associated positively with reactive oxygen species and negatively with GPx. These findings are in line with current knowledge that a prolonged increase in reactive oxygen species will lead to a decrease in GPx activity,[34] a phenomenon found in patients with coronary artery disease,[34] acute myocardial infarction,[35] hypertension[36,37] and atherosclerosis.[38] In the white men this association is considered a normal physiological reaction in order to maintain vascular tone, since reactive oxygen species levels in this group are lower than in the black men.

In the vasculature, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the main generator of superoxide in a mechanism that is angiotensin II dependent.[39] Previous experimental studies indicated that ET-1 binds to endothelin A receptors on nuclear factor kappa β and leads to the activation of angiotensin II stimulation of NADPH oxidase, increasing vascular superoxide.[40-42] Reactive oxygen species together with high blood pressure was proposed through the enhanced inactivation of nitric oxide by superoxide radical.[43,44] Since ET-1 is known for generating superoxide radicals through a NADPH oxidase mechanism, [40-42] our study adds to the current knowledge that ET-1 may be one of the markers responsible for increased reactive oxygen species and a dysfunctional redox system seen in the black population. Elevated levels of reactive oxygen species is also an important feature of cardiovascular disease contributing to endothelial dysfunction and vascular inflammation in hypertensives.[37,39] In our study the reactive oxygen species, inflammation and blood pressure levels in the black men and women

are higher and an increased GR activity is present. Additionally, in the black women the GPx levels are lower than in the white women. These characteristics indicate a pathological condition, possibly hypertension or early atherosclerosis, at work in the black population. Reactive oxygen species oxidises GSH to glutathione disulfide (GSSG), leading to a decrease in GSH and an increase in GSSG concentration, which in the presence of prolonged reactive oxygen species conditions can overwhelm anti-oxidants and lead to the inhibition of GPx.[36] Reactive oxygen species can also decrease GSH levels through the accumulation of GSH in the cells through an ET-1-cysteine action[45] and cause an increase in GSSG concentration, in turn increasing GR activity in an attempt to restore homeostasis.[46] We did not distinguish between the GSH and GSSG, thus we could not indicate the amount of substrate available for each enzyme. However, we did find a positive association between GR-to-GPx ratio in the same group, indicating higher GR to GPx activity. The negative association between ET-1 and GSH in our group supports the ability of ET-1 to increase the uptake of cysteine into the cells, which leads to a decrease in GSH out of the cell.[45,47,48] A previous study found that premenopausal black women have higher plasma homocysteine levels than white women.[49] Homocysteine can be converted to cysteine, which in the presence of ET-1 could lead to an increase uptake of cysteine into the cell,[48,49] in turn decreasing GSH levels. An accumulation of cysteine in the cells can also lead to inflammation in the blood vessels, which yet again supports the increased levels of inflammatory markers we found in the black women.[50,51] Our result therefore suggest that a prolonged increase in ET-1 levels can lead to a chronic increase in oxidative stress, which will overshadow the function of anti-oxidant enzymes and increase the risk for coronary artery disease in the black men and women from our population.

ET-1 and markers of vascular remodeling

Vascular remodeling is characterised by changes that occur in the arterial wall structure and functions such as wall thickening and increased CSWA.[52,53] Long-term pressure and/or flow overload and the interaction between these mechanical stimuli and growth factors and vasoactive substances are usually the cause of structural alterations in the cardiovascular system.[54] Previous studies indicated that ET-1 stimulates cardiac growth and smooth muscle cell proliferation due to its mitogenic properties in the vascular smooth muscle cells in hypertensive humans.[55-57] Additionally, ET-1 was found to also directly induce vascular hypertrophy independently of blood pressure elevation in transgenically expressed human

proproET-1-restricted mice.[58] This suggests that even if hypertension is absent, elevated ET-1 levels can contribute to vascular remodeling. To our knowledge, our study is the first to investigate a change in ET-1 levels with the change of markers implicated in vascular remodeling after three years in a bi-ethnic South African population. There was only one other study that investigated the relationship between ET-1 levels and markers of vascular remodeling (arterial compliance and pulse wave velocity) in a black American hypertensive population.[20] Our study additionally added CSWA and CIMT as markers of vascular remodeling. In accordance with the mentioned study, we found a positive association between increased ET-1 levels and pulse pressure, but not with arterial compliance or CIMT. Our study also found an inverse association between a decrease in ET-1 after three years with CSWA in the white population. The previous study was a very small cross-sectional study of 13 black hypertensive patients and these participants did not receive anti-hypertension treatment. The study also did not include white participants.

Anti-hypertension medication plays an important role in reversing remodeling of resistance arteries in hypertensive patients. Studies investigating the effect of common anti-hypertensive treatment on hypertensive patients showed a decrease in ET-1 levels, systolic and diastolic blood pressure decreases after 10 months of treatment[59] and additionally normalised or reversed structural and functional abnormalities in vascular smooth muscle and endothelial cells.[60] It is, however, important to receive the correct anti-hypertensive treatment as hypertensive patients who received atenolol (a selective β_1 receptor antagonist) showed no change in small artery abnormalities, whereas patients who received an ACE inhibitor or calcium channel antagonists showed reverse changes in structural abnormalities.[60] Schiffrin et al.[60,61] propose that is because ACE inhibitors and calcium channel antagonists block the actions of angiotensin II (one of the substances that releases ET-1 and makes use of calcium to exert its vasoconstrictive function) whereas beta blockers induce a vasoconstrictor effect. Additionally, some calcium blockers also have anti-oxidant properties[60] that could lower ET-1 release by decreasing ROS. ET_AR antagonism also decreased oxidative stress and normalised remodeling by decreasing collagen and fibronectin deposition in blood vessels in hypertensive rat models.[61] These proposed actions of ACE inhibitors, calcium channel and ET_A receptor antagonists provides a future research opportunity for investigating the effect of anti-hypertension medication on ET-1 levels and vascular remodeling over time. In our cross-sectional (with oxidative stress markers) we found that anti-hypertension medication

affects ET-1 in the black population and in the prospective cohort (with markers of vascular remodeling) the white population. This indicates the importance of the correct anti-hypertension treatment in this population to reduce their risk for early vascular changes. Our study adds to current knowledge as we found that a decrease in ET-1 levels after three years will attenuate CSWA changes in the white and black population (only in the presence of anti-hypertensive treatment). Lower ET-1 levels together with lower blood pressure and lower oxidative stress are therefore important to improve the outcome in hypertensive patients.

6.4. Chance and confounding

It is important to indicate possible determinants that may have affected the results of this study.

With regard to methodology, two of the three studies were cross-sectional studies, therefore these manuscripts cannot offer conclusions about cause and effect or sequence of events. The sample size of the groups were of valued size (manuscript 1: men: n=198; women: n=193; manuscript 2: men: n=198; women: n=195; manuscript 3: increased ET-1 levels: n=185; decreased ET-1 levels: n=152), but longitudinal studies and larger epidemiological studies are encouraged to confirm our findings. The results obtained from this study revealed the general health of an availability sample from the population, so the entire South African population cannot be represented by this study group.

Additionally, the possibility of chance should also be considered. By adjusting for confounders such as age, race, sex, body mass index, CRP, total energy expenditure, gamma glutamyl transferase, GR-to-GPx ratio, HDLC, CSWA, pulse pressure, systolic blood pressure, arterial compliance and anti-hypertension medication for co-morbidities through partial correlation or forward stepwise analyses, it is possible that these confounders may have influenced the results by causing an over- or underestimation of the associations between ET-1 and markers of vascular function, oxidative stress and anti-oxidant capacity. Statistics indicated that by using partial correlation and forward stepwise regression analysis, significant correlation may be one out of twenty due to chance. The number of covariates that could influence the relationship was kept to a minimum of one covariate for every ten participants. Additionally, potential

cofounders such as dietary data (anti-oxidant intake and data to quantify amino-acid such as cysteine) and genetic predisposition could have affected the outcome of the research but were not available for consideration in the statistical analyses.

It is also important to mention that the statistical results were interpreted from a physiological perspective, which means that all statistical significance does not necessarily indicate physiological significance and *vice versa*.

6.5. Conclusion

Our study is the first to indicate a link between a marker of vascular function (ET-1) with inflammation, oxidative stress, anti-oxidant capacity and vascular remodeling in a bi-ethnic South African population. Our results persistently found that ET-1 contributed to a higher risk of early vascular deterioration (arteriosclerosis) and future comorbidities, potentially driven by low-grade inflammation (black women), reactive oxygen species production (black men), decreased anti-oxidant capacity (black men) and vascular remodeling in the black population, whereas a decrease in ET-1 slow down adverse vascular remodeling in the white population.

6.6. Recommendations

The following recommendations are proposed for future studies:

- Larger epidemiologic studies are encouraged to investigate a cause–effect relationship between ET-1, inflammation, oxidative stress, anti-oxidant capacity and vascular remodeling in other populations.
- The association between ET-1 and inflammation in our group is of importance and prospective studies could shed some light on the precise mechanism involved in the development of arterial stiffness and subsequent cardiovascular disease in the black population.
- Endothelin A/B receptor or mixed endothelin receptor blockers, renin-angiotensin-system inhibitors, calcium antagonists, diuretics, β-blockers and aldosterone antagonists can all lower blood pressure and further investigation is needed to determine the effect of these blood pressure

lowering substances on ET-1 levels, oxidative stress and anti-oxidant capacity markers in the South African cohort.

- Previous studies found that the distribution of endothelin A/B receptors and abnormal hemodynamic reactivity are important determinants of the function of ET-1 in the black population. Similar studies in a South African population could shed some light in this regard.
- The interconnection between angiotensin II and ET-1 is important in hypertension and should be further investigated to establish the results observed in our black South African population.

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ANNEXURE A

INSTRUCTIONS FOR AUTHORS

INSTRUCTIONS FOR AUTHORS: *Journal of Amino Acids*

Journal of Amino Acids is a peer-reviewed, open access journal that publishes original research articles, review articles and clinical studies in all areas of amino acids. Paper must be submitted on the understanding that they have not been published elsewhere and are not currently under consideration by another journal published by Hindawi or any other publisher.

Preparation of manuscript

Abstract: The manuscript should contain an abstract. The abstract should be self-contained and citation-free and should not exceed 200 words.

Introduction: This section should be succinct, with no subheadings.

Materials and Methods: This part should contain sufficient detail so that all procedures can be repeated. It can be divided into subsections if several methods are described.

Results and Discussion: This section may each be divided by subheadings or may be combined.

Conclusions: This should clearly explain the main conclusions of the work highlighting its importance and relevance.

References: Authors are responsible for ensuring that the information in each reference is complete and accurate. All references must be numbered consecutively and citations of references in text should be identified using numbers in square brackets (e.g., "as discussed by Smith [9]"; "as discussed elsewhere [9,10]"). All references should be cited within the text; otherwise, these references will be automatically removed.

Acknowledgments: All acknowledgments (if any) should be included at the very end of the paper before the references and may include supporting grants, presentations, and so forth.

Preparation of Figures and Tables: Tables should be cited consecutively in the text. Every table must have a descriptive title and if numerical measurements are given, the units should be included in the column heading. Vertical rules should not be used.

Disclosure Policy: If there is no conflict of interest, authors should state that "The authors declare that there is no conflict of interest regarding the publication of this paper."

Ethical guidelines

In any studies that involve experiments on human or animal subjects, the following ethical guidelines must be observed. For any experiments on humans, all work must be conducted in accordance with the Declaration of Helsinki (1964). Paper describing experimental work which carries a risk of harm to human subjects must include a statement that the experiment was conducted with the human subjects' understanding and consent, as well as a statement that the responsible Ethical Committee has approved the experiments.

INSTRUCTIONS FOR AUTHORS: *Hypertension Research*

The *Hypertension Research* journal publishes papers reporting original clinical and experimental research that contribute to the advancement of knowledge in the field of hypertension and related cardiovascular diseases.

Title page: The title page should give a concise but informative title, the first and last names and other initials of all authors, as well as their affiliations (but not degrees). Names of grants covering the research described should also be included on this page. Full contact details should be provided for the corresponding author. Provide a running title of no more than 50 characters including spaces.

Abstract: An abstract of not more than 250 words. The abstract should be comprehensible to readers before they have read the paper, and abbreviations and reference citations within the abstract should be avoided. It should outline the purpose of the study, the basic procedures and the most important conclusions. Three to five keywords, which may or may not appear in the title, should be given in alphabetical order below the abstract, each separated by a comma. Whenever possible, the terms should be from the Medical Subject Headings list of *Index Medicus*.

Body of manuscript

Introduction: This should give a short, clear account of the background and reasons for undertaking the study. It should not be a review of the literature.

Methods: The methods section should contain sufficient detail so that all experimental procedures can be repeated by others in conjunction with cited references. This section may be divided into subheadings to assist the reader. Use of standard abbreviations and SI units of measurement (according to the *Systeme International d'Unites*) is encouraged. Abbreviations, if used, should be defined on their first appearance in the text.

Results: The description of results should not simply reiterate data that appear in tables and figures and, likewise, the same data should not be displayed in both tables and figures. The results section should be concise and follow a logical sequence.

Discussion: Do not recapitulate the results, but discuss their significance against the background of existing knowledge, and identify clearly those aspects that are novel. The final paragraph should highlight the main conclusion(s), and provide some indication of the direction future research should take.

Acknowledgments: These should be brief, and should include sources of financial support, material (e.g. novel compounds, strains, etc.) not available commercially, personal assistance, advice from colleagues and gifts. Acknowledgments should be made only to those who have made a significant contribution to the study.

References: Authors are responsible for the accuracy of the references. All authors should be quoted. In the text of the manuscript, references to the literature should be numbered consecutively and indicated by a superscript. Each reference should be numbered individually and listed at the end of the manuscript.

Tables: These should be labelled sequentially as Table 1, Table 2, etc. Each table should be saved in a separate file, numbered and titled, and cited in the text. Reference to table footnotes should be made by means of Arabic numerals. Tables should have a brief footnote that identifies all abbreviations used. Tables should be supplied as separate electronic files (as Word or Excel file formats).

Text formatting: All papers should be written in concise English but should contain sufficient detail to illustrate how the results were obtained. Manuscripts should be double-spaced with wide margins.

Ethical standards: When reporting experiments on human subjects, indicate whether the procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) or with the Helsinki Declaration of 1978 (as revised in 1983). Include Institutional Review Board or Animal Care and Use Committee approvals. Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study It should also be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. These statements should be added in a separate section before the reference list. The editors reserve the right to reject manuscripts that do not comply with the above-mentioned requirements. The author will be held responsible for false statements or failure to fulfill the above-mentioned requirements

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The *Journal of Hypertension* publishes papers reporting original clinical and experimental research which are of a high standard and which contribute to the advancement of knowledge in the field of hypertension.

Title page

The title page should carry the

- full title of the paper, consisting of no more than 20 words (only common abbreviations should be used if absolutely necessary); titles should be clear and brief, conveying the message of the paper
- a brief short title, which will be used as running head (consisting of not more than 40 characters, including spaces)
- all authors' names: the full first name, middle initial(s) and last (family name) name of each author should appear; if the work is to be attributed to a department or institution, its full name and location should be included. The last (family name) must appear in CAPITAL letters. Persons listed as authors should be those who substantially contributed to the study's conception, design, and performance
- the affiliations of all the authors; when authors are affiliated to more than one institution, their names should be connected using a,b,c, etc. These letters should follow the surname but precede the address; they should be used for all addresses
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Preparing the manuscript

Margins should be not less than 3 cm. Double spacing should be used throughout the manuscript, which should include the following sections, each starting on a separate page: title page, abstract and keywords, text, acknowledgements, references, individual tables and captions. Pages should be numbered consecutively, beginning with the title page, and the page number should be placed in the top right hand corner of each page. Abbreviations should be defined on their first appearance in the text; those not accepted by international bodies should be avoided.

Abstracts: The second page should carry a structured abstract of no more than 250 words. The abstract should state the Objective(s) of the study or investigation, basic Methods (selection of study subjects or laboratory animals; observational and analytical methods), main Results (giving specific data and their statistical significance, if possible), and the principal Conclusions. It should emphasise new and important aspects of the study or observations.

Condensed Abstracts: A condensed abstract will be published in the 'forthcoming contents' section of the issue preceding the published article. This should be supplied with the submission, and should consist of no more that 100 words, this abstract should briefly summarise the main findings of your study.

Key Words: The abstract should be followed by a list of 3–10 keywords or short phrases which will assist the cross-indexing of the article and which may be published. When possible, the terms used should be from the Medical Subject Headings list of the Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Abbreviations and symbols: Use only standard abbreviations. Avoid abbreviations in the title and abstract.

Text: Full papers of an experimental or observational nature may be divided into sections headed Introduction, Methods (including ethical and statistical information), Results and Discussion (including a conclusion), although reviews may require a different format.

Acknowledgements: Acknowledgements should be made only to those who have made a substantial contribution to the study. Authors are responsible for obtaining written permission from people acknowledged by name in case readers infer their endorsement of data and conclusions.

References: References should be numbered consecutively in the order in which they first appear in the text. They should be assigned Arabic numerals, which should be given in brackets, e.g. [17]. References should include the names of all authors when seven or fewer; when eight or more, list only the first six names and add et al. References should also include full title and source information. Journal names should be abbreviated as MEDLINE (www.nlm.nih.gov/tsd/serials/lji.html).

Tables: Each table should be typed on a separate page in double spacing. Tables should not be submitted as photographs. Each table should be assigned an Arabic numeral, e.g. (Table 3) and a brief title. Vertical rules should not be used. Place explanatory matter in footnotes, not in the heading. Explain in footnotes all non-standard abbreviations that are used in each table. Identify statistical measures of variations, such as standard deviation and standard error of the mean. Be sure that each table is cited in the text. If you use data from another published or unpublished source, obtain permission and acknowledge the source fully.

Units of measurement: Measurements of length, height, weight, and volume should be reported in metric units (metre, kilogram, or litre) or their decimal multiples. Temperatures should be given in degrees Celsius. Blood pressures should be given in millimetres of mercury. All haematologic and clinical chemistry measurements should be reported in the metric system in terms of the International System of Units (SI). Editors may request that alternative or non-SI units be added by the authors before publication.

ANNEXURE B

TURN IT IN ORIGINALITY REPORTS

Turnitin Originality Reports

Chapter 1

TI (19c4d674-2dba-48a9-bac6-fac00abf8790)

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- ID: 739648918
- Word Count: 7475

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Chapter 6

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ANNEXURE C

DECLARATION OF LANGUAGE EDITING

cumlaude
languagepractitioners

Director: CME Terblanche - BA (Pol Sc), BA Hons (Eng), MA (Eng), TEFL

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DECLARATION OF LANGUAGE EDITING

I, Christina Maria Etrecia Terblanche, hereby declare that I edited the research study titled:

The role of endothelin-1 in cardiometabolic and vascular function in a bi-ethnic population: The SABPA study

for CS du Plooy for the purpose of submission as a thesis for examination. Changes were suggested and implementation was left to the discretion of the author.

Regards,

CME Terblanche

Cum Laude Language Practitioners (CC)

SATI accr nr: 1001066

Registered with PEG

ANNEXURE D

ADDITIONAL STATISTICS

Table 1: Population characteristics of baseline and three years follow-up on haemodynamic variables stratified by race

	Black population (n=166)			White population (n=171)		
	Baseline	Follow-up	p-value	Baseline	Follow-up	p-value
Endothelin-1 (pg/mL)	2.12 ± 0.83	2.03 ± 0.65	0.225	2.07 ± 0.94	2.07 ± 0.94	0.982
Systolic blood pressure (mmHg)	132.8 ± 16.2	135.2 ± 17.7	0.026	124.7 ± 12.1	123.4 ± 11.6	0.037
Diastolic blood pressure (mmHg)	83.7 ± 11.0	83.7 ± 11.0	0.985	77.5 ± 8.27	75.8 ± 8.30	<0.001
Pulse pressure (mmHg)	49.1 ± 8.46	51.5 ± 9.93	<0.001	47.2 ± 6.96	47.6 ± 6.70	0.376

Values are arithmetic mean ± standard deviation.

Table 2: Partial correlations of the change in endothelin-1 levels and main independent variables after three years

	Endothelin-1 change (pg/mL)	
	ET-1 decrease after three years (n=185)	ET-1 increase after three years (n=152)
Ambulatory systolic blood pressure (mmHg)	r=0.055; p=0.462	r=0.162; p=0.051
Ambulatory diastolic blood pressure (mmHg)	r=0.029; p=0.704	r=0.117; p=0.158
Ambulatory pulse pressure (mmHg)	r=0.055; p=0.466	*r=0.160; p=0.050*
Arterial compliance (mL/mmHg)	r=–0.044; p=0.562	*r=–0.167; p=0.047*
Carotid intima media thickness (mm)	r=–0.129; p=0.087	r=–0.048; p=0.576
Cross-sectional wall area (mm^2)	r=–0.125; p=0.095	r=–0.010; p=0.908

Adjustments applied for age, body mass index, race, sex and baseline ET-1. Abbreviations: ET-1 – endothelin-1.

Table 3: Population characteristics of the total population after three years

	Baseline (n=337)	Follow-up (n=337)	p-value
Age (years)	45.6 ± 8.81	48.6 ± 8.81	–
Race, black, n (%)	166 (49.3)	166 (49.3)	–
white, n (%)	171 (50.7)	171 (50.7)	–
Sex, men, n (%)	174 (51.6)	174 (51.6)	–
women, n (%)	163 (48.4)	163 (48.4)	–
Body mass index (kg/m^2)	28.8 ± 6.05	29.7 ± 6.32	<0.001
Hypertension status, n (%)	79 (23.4)	77 (22.8)	0.786
Anti-hypertension drug usage, n (%)	84 (24.9)	92 (27.3)	0.333
Statins usage, n (%)	11 (3.3)	34 (10.1)	<0.001
Biochemical variables			
Endothelin-1 (pg/mL)	2.10 ± 0.89	2.05 ± 0.81	0.486
Reactive oxygen species (Units*)	91.7 ± 25.3	80.3 ± 23.9	<0.001
C-reactive protein (mg/L)	5.47 ± 7.93	4.42 ± 8.59	0.052
Gamma glutamyl transferase (U/L)	47.9 ± 3.71	44.2 ± 3.50	0.145
Glutathione reductase (U/mL)	6.02 ± 5.61	6.31 ± 11.1	0.672
Glutathione peroxidase (U/mL)	35.0 ± 11.5	34.3 ± 11.6	0.438
GR-to-GPx ratio	0.21 ± 0.31	0.19 ± 0.32	0.350
Cotinine (ng/mL)	24.9 ± 3.92	25.8 ± 4.33	0.731
Total cholesterol (mmol/L)	5.14 ± 1.34	4.43 ± 1.03	<0.001
High-density lipoprotein cholesterol (mmol/L)	1.17 ± 0.39	1.03 ± 0.35	<0.001
Low-density lipoprotein cholesterol (mmol/L)	3.36 ± 1.14	2.83 ± 0.89	<0.001
Cardiovascular variables			
Systolic blood pressure (mmHg)	128.7 ± 14.8	129.2 ± 16.0	0.432
Diastolic blood pressure (mmHg)	80.6 ± 10.1	79.7 ± 10.6	0.024
Pulse pressure (mmHg)	48.2 ± 7.78	49.5 ± 8.66	<0.001
Arterial compliance (mL/mmHg)	1.96 ± 0.47	1.94 ± 0.50	0.156
Carotid intima media thickness (mm)	0.67 ± 0.12	0.69 ± 0.11	<0.001
Cross-sectional wall area (mm^2)	13.6 ± 4.13	14.7 ± 3.75	<0.001

Values are arithmetic mean ± standard deviation. Abbreviations: GR-to-GPx ratio – Glutathione reductase-to-glutathione peroxidase ratio. *1 Unit=1mg/L H_2O_2.

Table 4: Partial correlations of the change in endothelin-1 levels and main independent variables after three years in the total population

	Endothelin-1 change (pg/mL) (n=337)
Ambulatory systolic blood pressure (mmHg)	r=0.006; p=0.909
Ambulatory diastolic blood pressure (mmHg)	r=0.002; p=0.969
Ambulatory pulse pressure (mmHg)	r=0.013; p=0.815
Arterial compliance (mL/mmHg)	r=0.020; p=0.723
Carotid intima media thickness (mm)	r=–0.009; p=0.869
Cross-sectional wall area (mm^2)	r=0.045; p=0.413

Adjustments applied for age, body mass index, race and sex.

Table 5: Partial correlations of the percentage change in systolic blood pressure and change in main independent variable (percentage change endothelin-1) after three years in the total population

	Percentage change in cardiovascular variables (n=337)
Endothelin-1 percentage change (%)	Systolic blood pressure (%) r=0.087; p=0.115
	Change in diastolic blood pressure (%) r=0.074; p=0.182
	Change in pulse pressure (%) r=0.066; p=0.229
	Change in arterial compliance (%) r=0.011; p=0.849
	Change in carotid intima media thickness (%) r=–0.002; p=0.977
	Change in cross-sectional wall area (%) r=0.038; p=0.494

Adjustments applied for age, body mass index, race, sex and baseline levels of independent variable and separately for the main dependent variables (systolic blood pressure, diastolic blood pressure, pulse pressure,, arterial compliance, carotid intima media thickness and cross-sectional wall area) and independent variables.