

# The association of antioxidant enzyme activity with cardiovascular variables in a bi-ethnic population: the SABPA study

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Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Physiology at the Potchefstroom Campus of the North-West University

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November 2014

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# ACKNOWLEDGEMENTS

I would like to acknowledge and thank the following people with the deepest gratitude:

- This dissertation would not have been possible without the help of my Heavenly Father! I could not have done it without Him!
- I would like to thank my study supervisors for all the help and professional input throughout this process. Thank you for all the motivation and encouragement; it is greatly appreciated.
- A special thank you to all my loved ones for believing in me and for their ongoing support.

## AFFIRMATION BY AUTHORS

Each researcher contributed to the study in the following manner:

**Ms C van Zyl** (BSc Hons Physiology) was responsible for the collection of cardiovascular data and preparation of blood samples for biochemical analyses, performing the research involved in this study, compiling an ethics application regarding this study, performance of statistical analyses of the data, processing of data obtained, interpretation of results obtained in statistical analyses of the data, and the overall design, planning, writing and execution of the dissertation and manuscript.

**Dr CMC Mels** (PhD Biochemistry), the supervisor, was responsible for the study design, collection of data, reviewing statistical analyses of the data, and reviewing all literature as part of the dissertation and manuscript. She was also responsible for obtaining funding for the project.

**Prof HW Huisman** (PhD Physiology), the co-supervisor, was responsible for making valuable recommendations for all aspects of the dissertation and manuscript.

I, Caitlynd van Zyl, declare that the statements above are true representations of my contribution to the study, as well as those of my supervisor and co-supervisor. Therefore, I give my permission for this manuscript to be published as part of the dissertation for the Magister Scientiae degree in Physiology.

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Ms C van Zyl

The co-authors of his study confirm the roles of each individual, and they give their permission to publish this manuscript as part of the dissertation for the degree Magister Scientiae in Physiology.

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Dr CMC Mels

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# **SUMMARY**

**Title: The association of antioxidant enzyme activity with cardiovascular variables in a bi-ethnic population: the SABPA study**

## **Motivation**

Hypertension is an escalating problem in our South African population, especially among urban black Africans. The development of hypertension may be facilitated by various factors, including oxidative stress. Oxidative stress can be caused by a decrease in antioxidant capacity or an increase in the production of reactive oxygen species (ROS). Previous studies by our research team support the theory of increased oxidative stress in our black population, but it is unknown whether it is the result of decreased antioxidant enzyme activity, or an increase in ROS production.

## **Aims**

We aimed to determine whether black men and women have lower antioxidant enzyme activity than white men and women. We further aimed to determine if any relationships exist between cardiovascular variables, such as blood pressure and carotid intima media thickness (CIMT), with antioxidant enzyme activity such as glutathione reductase (GR) and glutathione peroxidase (GPx) among others.

## Methods

This study is part of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study, which was conducted between February 2008 and May 2009. The SABPA study is a cross-sectional target population study and included 101 black and 101 white male, and 99 black and 108 white female teachers from the Dr Kenneth Kaunda Education District in the North West Province of South Africa. Anthropometric and physical activity measurements were performed according to standardized procedures. Cardiovascular measurements included systolic blood pressure (SBP), diastolic blood pressure (DBP) and mean arterial pressure (MAP), as measured with the validated Finometer device. The high resolution SonoSite Micromaxx ultrasound system was used to measure CIMT. Cross-sectional wall area (CSWA) was calculated using the formula  $CSWA = \pi(d/2 + CIMT)^2 - \pi(d/2)^2$ . Fasting blood samples were obtained for the analyses of glycated haemoglobin (HbA1c), total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides,  $\gamma$ -glutamyl transferase (GGT), and C-reactive protein (CRP). The total cholesterol to high-density lipoprotein cholesterol ratio was calculated. The activity of GPx, GR and superoxide dismutase (SOD) were measured using assay kits (Cayman Chemical Company, Ann Arbor, MI, USA) and a Synergy H4 hybrid microplate reader. Catalase (CAT) activity was measured using a fluorometric OxiSelect catalase activity assay kit (Cell Biolabs Inc., San Diego, CA, USA). Total glutathione (GSH) was measured with the BIOXYTECH GSH/GSSG-412 kit on a Bio-Tek FL600 Microplate Fluorescence Reader. ROS (measured as serum peroxides) was measured using the method described by Hayashi *et al.* Tumor necrosis factor alpha (TNF $\alpha$ ) and cotinine were measured using a high sensitivity ELISA kit.

## **Results**

When comparing oxidative stress markers between black and white men it was found that the activity of GR was significantly higher in the black men and women when compared to their white counterparts. Results show that ROS was significantly higher in the black men when compared to white men, and both the black men and the black women had significantly higher total glutathione (GSH) levels than their white counterparts. In black women, GPx activity was significantly lower when compared to the white women. It was found that an independent positive association exists between GR activity and CIMT in black men. Meanwhile in black women an independent negative association exists between GPx activity and SBP, as well as between GPx and MAP. No associations were found in the white participants.

## **Conclusion**

The comparison of antioxidant enzyme activity in the black and white men revealed that the black men had significantly higher GR activity, total GSH and ROS. Meanwhile in the women it was found that the black women had significantly higher GR activity and total GSH, but significantly lower GPx activity when compared to their white counterparts. Increased GR activity and GSH levels have been linked to oxidative stress. Up-regulation of GR is suspected in the black men and women in order to combat increased depletion of GSH levels during oxidative stress. GPx enzymes are suspected to be inactivated by ROS, therefore allowing further accumulation of ROS and aggravating the state of oxidative stress in these participants. Thus we suggest that both the black men and black women have higher oxidative stress than the white participants.

Analyses of associations between cardiovascular variables and antioxidant enzyme activity in the black and white men showed an independent positive association between CIMT and GR in the black men. This may suggest that an increase in oxidative stress, as indicated by increased GR activity and ROS levels, could promote thickening of the carotid intima media, and eventually stimulate the development of atherosclerosis in these participants. Furthermore, a significant negative association between GPx activity and blood pressure measurements was found in the black women. The suspected oxidative stress in these participants, as indicated by increased GR activity and decreased GPx activity, is suspected to facilitate hypertension development in these participants.

# **OPSOMMING**

**Titel: Die assosiasie van anti-oksidadant ensiemaktiwiteit met kardiovaskulêre veranderlikes in 'n bi-etniese populasie: die SABPA studie**

## **Motivering**

Hipertensie is 'n toenemende probleem in ons Suid-Afrikaanse bevolking, veral onder stedelike swart Afrikane. Die ontwikkeling van hoë bloeddruk kan gefasiliteer word deur verskeie faktore, insluitend oksidatiewe stres. Oksidatiewe stres kan veroorsaak word deur 'n afname in anti-oksidadant kapasiteit of deur 'n toename in die produksie van reaktiewe suurstof spesies (RSS). Vorige studies deur ons navorsingspan ondersteun die teorie van verhoogde oksidatiewe stres in ons swart bevolking, maar dit is onbekend of dit die gevolg is van verminderde anti-oksidadant ensiemaktiwiteit, of weens 'n toename in RSS-produksie.

## **Doel**

Ons doel was om te bepaal of swart mans en vrouens 'n laer anti-oksidadant ensiemaktiwiteit as wit mans en vrouens het. Ons wou verder bepaal of enige verbande bestaan tussen kardiovaskulêre veranderlikes, soos bloeddruk en karotis intima media dikte (KIMD), met anti-oksidadant ensiemaktiwiteit soos onder andere glutatioon reduktase (GR) and glutatioon peroksidase (GPx).

## Metodes

Hierdie studie is deel van die “Sympathetic activity and Ambulatory Blood Pressure in Africans” (SABPA) studie, wat gedoen is tussen Februarie 2008 en Mei 2009. Die SABPA studie is 'n deursnee teikenpopulasie studie en sluit in 101 swart en 101 wit mans, en 99 swart en 108 wit vroulike onderwysers van die Dr Kenneth Kaunda Onderwys Distrik in die Noordwes-provinsie van Suid-Afrika. Antropometriese en fisiese aktiwiteit metings is uitgevoer volgens gestandaardiseerde prosedures. Kardiovaskulêre metings sluit in sistoliese bloeddruk (SBP), diastoliese bloeddruk (DBP), en gemiddelde arteriële druk (GAD), soos gemeet deur die gevalideerde Finometer apparaat. Die hoë resolusie “SonoSite Micromaxx ultrasound system” is gebruik om KIMD te meet. Die dwarsdeursnit oppervlak (DDO) van die bloedvat wand is bereken met behulp van die formule  $DMA = \pi(d/2 + CIMT)^2 - \pi(d/2)^2$ . Vastende bloedmonsters is verkry vir die analise van geglikosileerde hemoglobien (HbA1c), totale cholesterol, hoë-digtheid lipoproteïen (HDL) cholesterol, trigliseriede,  $\gamma$ -glutamieltransferase (GGT) en C-reaktiewe proteïen (CRP). Die totale cholesterol tot hoë-digtheid lipoproteïen cholesterol verhouding is bereken. Die aktiwiteit van GPx, GR en superoksied dismutase (SOD) is gemeet met behulp van toets stelle "(Cayman Chemical Company, Ann Arbor, MI, USA)" en 'n “Synergy H4 hybrid microplate reader”. Katalase (KAT) is gemeet deur 'n “fluorometric OxiSelect catalase activity assay kit” (Cell Biolabs Inc., San Diego, CA, USA). Totale glutatioon (GSH) is gemeet met die “BIOXYTECH GSH/GSSG-412 kit” en 'n “Bio-Tek FL600 Microplate Fluorescence Reader” in EDTA heel bloedmonsters. Serum ROS (gemeet as serum peroksiede) is gemeet deur die metode beskryf deur Hayashi *et al.* Tumor nekrose faktor alfa (TNF $\alpha$ ) en kotinien is gemeet deur 'n hoë sensitiwiteit ELISA stel.

## **Resultate**

Die aktiwiteit van GR was aansienlik hoër in die swart mans en vroue in vergelyking met hul wit eweknieë. Resultate toon dat RSS aansienlik hoër is in die swart mans in vergelyking met wit mans, en beide die swart mans en die swart vrouens het beduidend hoër GSH vlakke as hul wit eweknieë. In swart vroue was GPx aktiwiteit aansienlik laer as in die wit vroue. Daar is gevind dat 'n onafhanklike positiewe assosiasie tussen GR en KIMD in swart mans bestaan. Intussen is daar in swart vroue 'n onafhanklike negatiewe assosiasie tussen GPx aktiwiteit en SBP gevind, asook tussen GPx en GAD. Geen assosiasies is in die wit deelnemers gevind nie.

## **Gevolgtrekking**

Die vergelyking van anti-oksidant ensiemaktiwiteit in die swart en wit mense het aan die lig gebring dat die swart mans aansienlik hoër GR aktiwiteit en totale GSH en RSS het. Intussen is in die vroue gevind dat die swart vroue beduidend hoër GR aktiwiteit en totale GSH, maar aansienlik laer GPx aktiwiteit, in vergelyking met hul wit eweknieë het. Verhoogde GR aktiwiteit en GSH vlakke is gekoppel aan oksidatiewe stres. Op-regulering van GR word vermoed in die swart mans en vrouens om sodoende verhoogde uitputting van GSH vlakke te bestry tydens oksidatiewe stres. GPx ensieme word vermoedelik geïnaktiveer deur ROS, dus word verdere opeenhoping van ROS veroorsaak en vererger die toestand van oksidatiewe stres in hierdie deelnemers. Ons doen dus aan die hand dat beide die swart mans en swart vrouens hoër oksidatiewe stres as die wit deelnemers het.

Ontledings van die assosiasies tussen kardiovaskulêre veranderlikes en anti-oksident ensiemaktiwiteit in die swart en wit mans het 'n onafhanklike positiewe assosiasie tussen KIMD en GR in die swart mans aangetoon. Dit kan daarop dui dat 'n toename in oksidatiewe stres, soos aangedui deur die verhoogde GR aktiwiteit en ROS vlakke, verdikking van die karotis intima media kan bevorder, en uiteindelik aterosklerose ontwikkeling in hierdie deelnemers stimuleer. Verder is 'n negatiewe verband tussen GPx aktiwiteit en bloeddrukmetings gevind in die swart vrouens. Die veronderstelde oksidatiewe stres in hierdie deelnemers, soos aangedui deur die verhoogde GR aktiwiteit en verlaagde GPx aktiwiteit, word vermoed om hipertensie ontwikkeling in hierdie deelnemers te fasiliteer.

## PREFACE

This study forms part of the SABPA study and the dissertation is submitted in fulfilment of the Magister Scientiae degree in Physiology. The article format was used for this dissertation, which consists of a manuscript which is ready for submission. The peer reviewed journal, *Free Radical Research*, is considered for submission of the manuscript in Chapter 3. The structured format of this dissertation is as follows: Chapter 1 includes a background and motivation, and a reference list. Chapter 2 contains a literature overview of the topic along with the aims and hypotheses. Chapter 3 is the manuscript, titled: The association of antioxidant enzyme activity with cardiovascular variables in a bi-ethnic population: the SABPA study. It contains the keywords, abstract, introduction, materials and methods, results, discussion, conclusion, acknowledgements, conflicts of interest, and references according to the *Free Radical Research* guidelines. Chapter 4 is a concluding chapter which contains questions arising from the literature, final conclusions, strengths and limitations of the study, and future research in the relevant field of science.

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## LIST OF ABBREVIATIONS

AP-1	- Activator protein 1
AT1	- Angiotensin II receptor, type 1
BH <sub>2</sub>	- Dihydrobiopterin
BH <sub>4</sub>	- Tetrahydrobiopterin
BMI	- Body mass index
CIMT	- Carotid intima media thickness
CRP	- C-reactive protein
DBP	- Diastolic blood pressure
DNA	- Deoxyribonucleic acid
FAD	- Flavin adenine dinucleotide
G-6-PDH	- Glucose-6-phosphate dehydrogenase
GGT	- Gamma glutamyltransferase
GPx-1	- Cytosolic glutathione peroxidase
GPx-2	- Gastrointestinal glutathione peroxidase
GPx-3	- Plasma glutathione peroxidase
GPx-4	- Phospholipid hydroperoxide glutathione peroxidase
GPx-5	- Epididymal secretory glutathione peroxidase
GSH	- Reduced glutathione
GSSG	- Oxidized glutathione
H <sub>2</sub> O	- Water
H <sub>2</sub> O <sub>2</sub>	- Hydrogen peroxide
HbA1c	- Glycated haemoglobin
HIF-1	- Hypoxia-inducible factor 1
IL-6	- Interleukin-6
LDL	- Low-density lipoprotein

MAP	- Mean arterial pressure
MCP-1	- Monocyte chemotactic protein-1
NADPH	- Nicotinamide adenine dinucleotide phosphate
NF- $\kappa$ B	- Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	- Nitric oxide
NOS	- Nitric oxide synthase
O <sub>2</sub>	- Oxygen
O <sub>2</sub> <sup>-</sup>	- Superoxide anion
OH <sup>·</sup>	- Hydroxyl radical
ONOO <sup>-</sup>	- Peroxynitrite
RNS	- Reactive nitrogen species
ROS	- Reactive oxygen species
SABPA	- Sympathetic activity and Ambulatory Blood Pressure in Africans
SAfrEIC	- South African study on the influence of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function
SBP	- Systolic blood pressure

# CHAPTER 1:

# INTRODUCTION

It all starts here™



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## BACKGROUND AND MOTIVATION

Hypertension is an escalating worldwide epidemic [1], and is one of the leading contributors to morbidity and mortality [2, 3]. Hypertension is also a common problem in South Africa, especially in urban black Africans [4], and is noted as an important risk factor for the development of cardiovascular disease [5]. Various factors contribute to the development of cardiovascular disease, including increased oxidative stress, which is defined as an increase in the generation of reactive oxygen species (ROS), or a decrease in either the scavenging of the ROS, or the metabolism thereof [6, 7].

All types of cells in each layer of the vascular wall (endothelial cells, smooth muscle cells and the adventitial cells) have the ability to produce ROS via the action of various enzyme systems including xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and uncoupled nitric oxide synthase (NOS), among others [6]. Of the various ROS that are produced in the vascular cells, superoxide ( $O_2^{\cdot -}$ ) and hydrogen peroxide ( $H_2O_2$ ) appear to be especially important [8].

Under normal conditions, a balance exists between ROS levels and antioxidant capacity, and any disturbance in this equilibrium results in oxidative stress [7]. The body possesses various defence mechanisms in the fight against increased oxidative stress [7].

The antioxidant defence mechanism is composed of enzymatic and non-enzymatic antioxidants. Non-enzymatic antioxidants include glutathione, bilirubin, Ascorbate (vitamin C), tocopherols (vitamin E) and uric acid, while enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), thioredoxin and peroxiredoxin [8].

Oxidative stress is suggested to be involved in the development of various cardiovascular pathologies such as hypertension, atherosclerosis, heart failure, stroke and cardiac hypertrophy [6].

Two sub-studies, forming part of the SABPA study, have evaluated oxidative stress in our South African population. Firstly, it was found that the women had higher ROS levels than the men, and that hypertensive men had higher ROS levels than normotensive men [9]. Additionally it was found that positive associations existed between ROS and systolic blood pressure (SBP), and ROS and pulse pressure (PP) in the black men. They suggested that increased ROS levels may contribute to hypertension development and increased arterial stiffness in these black men [9]. In a second study, it was found that the hypertensive subjects had a decrease in total glutathione (GHS) levels, which was suggested to enhance atherosclerosis development in these participants [10].

The precise cause of excessive ROS levels is still unknown, as it could be due to up-regulation of enzymes responsible for ROS production, a decrease in the activity of antioxidant enzymes, or both.

The motivation for our study is to evaluate the possibility of a decrease in the activity of antioxidant enzymes in the African participants, and how it correlates with cardiovascular measures.

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# **CHAPTER 2:**

# **LITERATURE REVIEW**



## 1. INTRODUCTION

Hypertension is one of the leading contributors to morbidity and mortality worldwide [1, 2], and the prevalence of hypertension is increasing [3]. Hypertension is also a common problem in South Africa, especially in urban black Africans [4]. Hypertension is furthermore noted as an important risk factor for the development of various other cardiovascular diseases [5]. Various factors contribute to the development of hypertension, including increased oxidative stress [6, 7].

Oxidative stress occurs when there is a disruption in the equilibrium between the production of reactive oxygen species (ROS) and the breakdown of ROS by the antioxidant defence mechanisms [7-9]. All cell types in the vascular wall (endothelial cells, smooth muscle cells and adventitial cells) have the ability to produce ROS [9], and it has been shown that ROS is an important signalling molecule in various biological responses [7]. Although ROS has various roles in normal physiological processes, increased ROS and reactive nitrogen species (RNS) are both involved in the development of cardiovascular diseases, such as hypertension, atherosclerosis, cardiac hypertrophy, stroke and heart failure. In addition to its role in cardiovascular diseases, oxidative stress is also involved in the development of diabetes [6].

## 2. TYPES OF ROS

ROS are types of free radical molecules which contain an unpaired electron, thus making them inherently unstable and highly reactive. When ROS levels become excessive, it may lead to tissue damage [8].

Various types of ROS include, among others, superoxide anion ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) and the hydroxyl radical ( $OH^{\cdot}$ ), while RNS include nitric oxide ( $NO^{\cdot}$ ) and peroxynitrite ( $ONOO^-$ ) [6, 8].

### 3. METABOLISM OF ROS

As previously mentioned, all cell types in every layer of the vasculature have the ability to produce ROS [6]. This can occur systemically or via the action of various enzymes.

The systemic source of ROS is the result of various biochemical processes occurring in the body, including catecholamine oxidation, activation of the arachidonic acid cascade, activation of phagocytes, nitric oxide (NO) production and aerobic respiration [8].

The mitochondrial electron transport chain in cells is responsible for 95% of oxygen ( $O_2$ ) conversion to water ( $H_2O$ ) in cells [7]. During this process, two molecules of  $H_2O$  are formed by reducing the  $O_2$  by four electrons, along with a small amount of electron leakage [7].

This electron leakage leads to the formation of  $O_2^{\cdot-}$  under normal conditions. This  $O_2^{\cdot-}$  is then rapidly scavenged by the antioxidant defence mechanisms. Abnormalities in the functioning of the mitochondria leads to excessive  $O_2^{\cdot-}$  production and therefore oxidative stress [7].  $O_2^{\cdot-}$  is a highly reactive molecule with a short life span and the ability to attack and damage other molecules such as proteins, lipids and nucleic acids [10]. One such molecule which is often attacked by  $O_2^{\cdot-}$  is NO, thus leading to the inactivation of the NO molecule and consequent formation of  $ONOO^-$  [10]. Due to

the extremely reactive nature of this RNS, it too has the ability to attack and damage proteins, lipids and nucleic acids [10].

Enzyme systems which produce ROS include xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and uncoupled nitric oxide synthase (NOS).

### **3.1 XANTHINE OXIDASE**

Xanthine oxidase is an enzyme found in the vascular endothelium and is responsible for purine degradation, in which hypoxanthine and xanthine are converted into uric acid, consequently leading to  $O_2^-$  and  $H_2O_2$  formation [11, 12]. Increased xanthine oxidase activity has been implicated in endothelial dysfunction and hypertension [6].

### **3.2 NADPH OXIDASE**

NADPH oxidase is widely recognized as a major source of ROS production in the vasculature [7, 12]. NADPH oxidase is a functional enzyme which contains both cytosolic and membrane-bound subunits in order to form one transmembrane protein. NADPH oxidase is involved in the transport of electrons across the cell membrane in order to reduce  $O_2$  to form  $O_2^-$  [6, 7, 12]. It is thus suggested that NADPH oxidase derived ROS plays an important role in endothelial damage and hypertension [12].

### 3.3 UNCOUPLED NOS

Under normal conditions, the endothelial NOS enzyme is responsible for the production of the powerful vasodilator NO [6, 12]. This enzyme converts the substrate L-arginine and O<sub>2</sub> into L-citrulline and NO with the use of NADPH and the cofactor tetrahydrobiopterin (BH<sub>4</sub>) [6]. In the event of a deficiency in either the substrate (L-arginine) or the cofactor (BH<sub>4</sub>), NOS uncoupling occurs in which the NOS enzyme becomes structurally unstable, therefore generating O<sub>2</sub><sup>•-</sup> instead of NO [6]. ROS derived from uncoupled endothelial NOS have been implicated in the development of various pathological states such as atherosclerosis, diabetes and hypertension [6, 12].

## 4. ACTIONS OF ROS

Under normal physiological conditions, ROS play an important role as messengers in various biological pathways such as signal transduction, apoptosis, monitoring of cell growth, and fetal development [10]. Additional to these, ROS also acts as signalling molecules in the activation of host defence genes, the activation of ion transport systems, and the monitoring of extracellular matrix metabolism [6, 7].

Superoxide is also shown to play an important role in controlling vascular tone by the production of ONOO<sup>-</sup>, which leads to decreased NO bio-availability, both of which lead to vasoconstriction [13]. It has also been shown that ROS is involved in muscle contraction by increasing the intracellular calcium in the vascular smooth muscle cells [13].

Furthermore,  $O_2^{\cdot-}$  is also involved with the activation of various transcription factors such as nuclear factor kappa- B (NF- $\kappa$ B), activator protein 1 (AP-1) and hypoxia-inducible factor 1 (HIF-1) [13, 14]. Various other proteins involved in signal transduction are activated by  $O_2^{\cdot-}$ , such as mitogen-activated protein kinases and tyrosine kinases [13].

## 5. ANTIOXIDANT DEFENCE MECHANISMS

As previously mentioned, there is a normal balance between the production and catabolism of ROS, and any disturbance in this balance would have oxidative stress as effect [7-9]. The body possesses both enzymatic and non-enzymatic antioxidant defence mechanisms in the fight against oxidative stress [7, 15].

### 5.1 NON-ENZYMATIC ANTIOXIDANTS

Non-enzymatic antioxidant defences include glutathione (GSH), bilirubin, uric acid, ascorbate (vitamin C),  $\alpha$ -tocopherol (vitamin E),  $\beta$ -carotene and coenzyme Q<sub>10</sub> [7, 8, 15]. A detailed discussion of GSH follows hereafter, but the other abovementioned non-enzymatic antioxidant defences are beyond the scope of this dissertation.

#### 5.1.1 GLUTATHIONE

Glutathione (GSH) is one of the major non-enzymatic antioxidants, which is present in all cell types [16]. GSH is naturally formed in the cytosol of the cells, after which it

is transported into the endoplasmic reticulum, mitochondria and the nucleus where it acts as a scavenger of ROS [16, 17]. GSH is an especially powerful antioxidant in aqueous compartments such as the cytosol and the plasma [17].

This peptide exists in either a reduced (GSH) or oxidized (GSSG) form, and the reversible oxidation of these forms is an important part of the redox homeostasis in the cells [16].

Thus GSH has the ability to directly and indirectly scavenge ROS, since GSH is a co-substrate along with  $H_2O_2$  for the enzyme glutathione peroxidase (GPx) [16]. Furthermore, when oxidative stress increases, GSH levels decrease with a concomitant increase in the levels of GSSG due to ROS scavenging [16].

## **5.2 ENZYMATIC ANTIOXIDANTS**

The main enzymatic antioxidant defence mechanisms include superoxide dismutase (SOD), GPx, and catalase (CAT), while secondary enzymes include glutathione reductase (GR) and Glucose-6-phosphate dehydrogenase (G-6-PDH). Their actions are summarized in Figure 1. Other antioxidant enzymes include thioredoxin and peroxiredoxin, but these will not be further discussed [7, 15, 18].

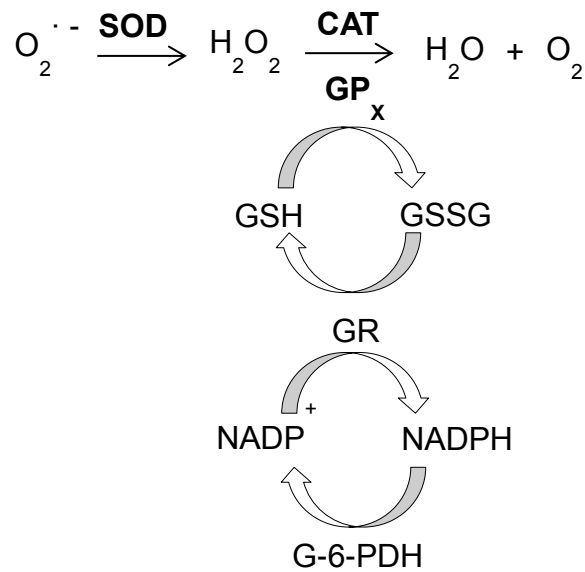


Figure 1: The major enzymatic antioxidant defence mechanisms. Image adapted from Li *et al.* [18].

### 5.2.1 SUPEROXIDE DISMUTASE

Under normal conditions,  $\text{O}_2^{\cdot -}$  is rapidly converted to  $\text{H}_2\text{O}_2$  and oxygen by SOD enzymes, of which three isoforms are present in mammals, namely cytoplasmic SOD, mitochondrial SOD and extracellular SOD [7, 10]. The product formed in this SOD catalysed reaction, namely  $\text{H}_2\text{O}_2$ , is a stable oxidant with the ability to diffuse across cell membranes and elicit various effects [7].

## 5.2.2 GLUTATHIONE PEROXIDASE

GPx is one of two enzymes involved in the catabolism of  $H_2O_2$  in the body [8]. The catabolism of  $H_2O_2$  is achieved through the oxidation of GSH to GSSG, converting  $H_2O_2$  to water [8, 10, 19]. GPx is a seleno-protein, and therefore possesses selenium in the form of selenocysteine at the active site [20]. The active form of the selenocysteine molecule, namely selenol, is responsible for the reduction of  $H_2O_2$  and is subsequently oxidized to form a selenic acid derivative [20]. This derivative has the ability to react with GSH to form a complex containing selenium and GSH [20]. This complex is then reduced back to the active selenol form by a second GSH molecule, yielding GSSG as an end product [20]. There are five subtypes of GPx enzymes in the body, namely GPx-1, GPx-2, GPx-3, GPx-4 and GPx-5:

*GPx-1:* Also known as cytosolic GPx, is present in various parts of the cell (such as the cytosol and mitochondria) [20]. This subtype of GPx is responsible for the protection against oxidative damage, as well as playing an important role in various aspects of inflammatory pathways [20].

*GPx-2:* Also known as gastrointestinal GPx, is predominantly found in the gastrointestinal tract, and function to protect the gastrointestinal tract against hydroperoxides consumed in the diet [20].

*GPx-3:* Also known as plasma glutathione peroxidase, and is found in blood plasma where it is able to reduce  $H_2O_2$ , alkyl hydroperoxides and phospholipid peroxides [20]. It is formed in various tissues (some of which include the heart, brain, liver and lungs) whereafter it is secreted into the extracellular fluid [20].

*GPx-4*: Also known as phospholipid hydroperoxide glutathione peroxidase, and is able to inhibit lipid peroxidation of various membranes in the presence of sufficient vitamin E, and is also capable of metabolizing cholesterol [20].

*GPx-5*: Also known as epididymal secretory glutathione peroxidase, and is found in the epididymis where it is responsible for the protection of spermatozoa from oxidative damage [20].

### 5.2.3 CATALASE

The second enzyme responsible for the breakdown of  $H_2O_2$  is catalase [10]. Catalase enzymes are present in the peroxisomes of various tissues where they act to convert  $H_2O_2$  to water and oxygen [8].

This enzyme is a tetrameric molecule with four identical subunits, able to protect cells from  $H_2O_2$  induced oxidative damage [21]. Catalase is one of the most efficient enzymes in the human body, with the exceptional ability to withstand saturation at any  $H_2O_2$  concentration [22].

### 5.2.4 GLUTATHIONE REDUCTASE

GR is an oxidoreductase flavoenzyme (an enzyme which utilizes either flavin adenine dinucleotide (FAD) or flavin mononucleotide as co-factor) [23], present in the cytosol and mitochondria [24].

GR acts to reduce GSSG to GSH while using NADPH, GSSG and H<sup>+</sup> as substrates and FAD as prosthetic group [23-26]. In the first part of this reaction, the binding of the various substrates leads to the reduction of the GR enzyme itself. Thereafter, electron transfer occurs between the GR enzyme and GSSG, yielding two GSH molecules and restoring the oxidized state of the enzyme [24].

### 5.2.5 GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Although G-6-PDH does not directly participate as an antioxidant enzyme against ROS, it is responsible for the production of the very valuable molecule NADPH, which is vital for the functioning of all the major antioxidant enzymes in one way or another [27]. GR requires NADPH as one of its substrates in the recycling of GSH (which further goes on to become a substrate for GPx), while CAT requires NADPH to protect it from hydrogen peroxide damage, and also to keep the enzyme in its most active form [27]. Though NADPH does not directly participate in the functioning of SOD, in the absence of NADPH there may be an increase in H<sub>2</sub>O<sub>2</sub> accumulation as a result of decreased GPx and CAT activity, which in turn inhibits SOD, thus indicating that SOD is indirectly dependent on NADPH [27].

## 6. EFFECTS OF INCREASED OXIDATIVE STRESS

Due to its short half-life, most ROS cannot be measured directly; therefore the products of oxidative modification to lipid, DNA and proteins are frequently used as

markers of oxidative stress [8]. Lipid peroxidation may occur in the lipoproteins, such as low-density lipoprotein (LDL) cholesterol, or in the phospholipid layers of cellular membranes (where damage leads to a change in signal transduction) [8, 28]. Furthermore, DNA damage is thought to play a key role in tumor formation, while protein damage is thought to alter the activities of various enzymes and transcription factors [28].

Oxidative damage in the human body has various detrimental effects on the vascular system (as depicted in Figure 2).

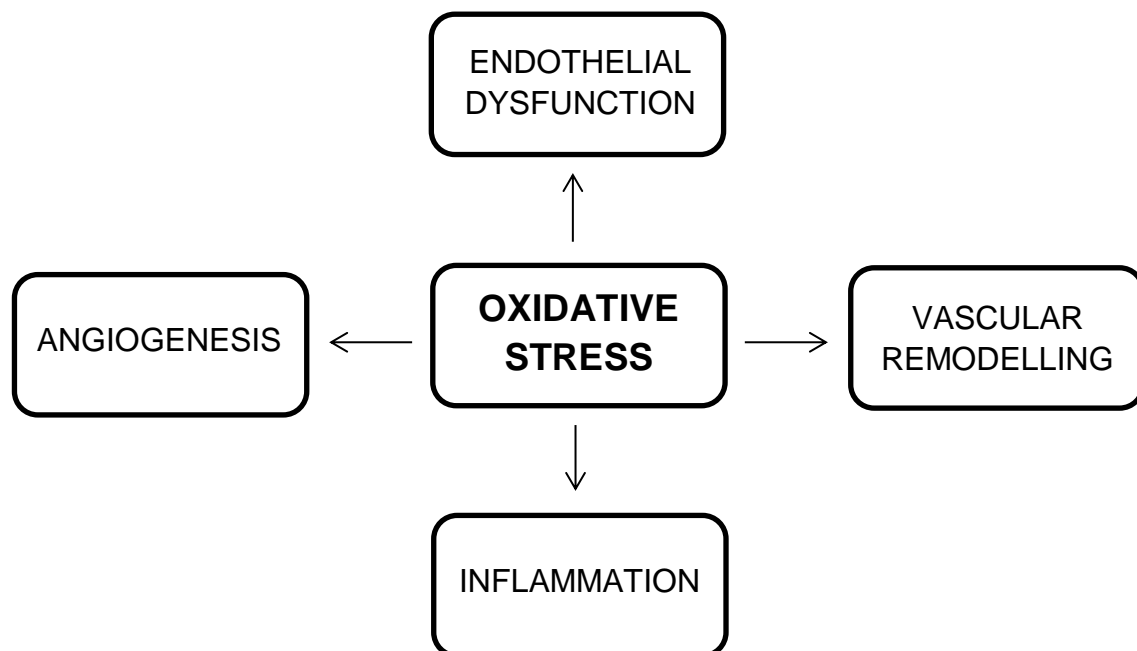


Figure 2: The vascular effects of oxidative stress in the human body.

## 6.1 ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction occurs, in part, due to a decrease in NO bio-availability brought about by excessive ROS. This is achieved in various ways. Firstly,  $O_2^{\cdot-}$

reacts with NO to form the powerful RNS ONOO<sup>-</sup> [7, 29]. Secondly, and as previously mentioned, ROS is able to uncouple the NOS enzyme by depleting its cofactor BH<sub>4</sub> [10]. This is achieved by oxidizing the cofactor BH<sub>4</sub> to dihydrobiopterin (BH<sub>2</sub>), which leads to endothelial NOS uncoupling and thus O<sub>2</sub><sup>-</sup> formation instead of NO formation [28]. The subsequent decrease in NO bio-availability leads to a decrease in endothelium dependent vasodilation, which plays a vital role in promoting endothelial dysfunction [29, 30].

## **6.2 VASCULAR REMODELLING**

Vascular remodelling consists of various mechanisms, including apoptosis, vascular smooth muscle cell growth, vascular smooth muscle cell migration and extracellular matrix metabolism.

### **6.2.1 APOPTOSIS**

Endothelial cells undergo apoptosis when exposed to excessive ROS, subsequently leading to a decrease in the number of endothelial cells, which has been implicated in altered haemostasis and the development of atherosclerosis [30, 31].

The loss in the number of endothelial cells facilitates the permeation of lipids, monocytes and smooth muscle cells into the intima layer of the vascular wall, which leads to further vascular damage and promotes the development of atherosclerosis [31].

### 6.2.2 VASCULAR SMOOTH MUSCLE CELL GROWTH

Exposure to excessive ROS leads to the growth of vascular smooth muscle cells via both mechanisms of hypertrophy and proliferation [30].

Hypertrophy is predominantly initiated by angiotensin-II induced ROS formation, in which angiotensin-II produces both  $O_2^{\cdot-}$  and  $H_2O_2$  that stimulates the hypertrophic response in vascular smooth muscle cells [30, 32, 33].

ROS also plays an important role in vascular smooth muscle cell proliferation by mediating the response to various ligands such as platelet-derived growth factor and thrombin [30]. Platelet-derived growth factor is well known for its effect on vascular smooth muscle cell proliferation [33]. This is achieved by the ability of platelet-derived growth factor to induce  $H_2O_2$  production in the smooth muscle cells, which thus leads to the proliferation of the vascular smooth muscle cells [32].

### 6.2.3 VASCULAR SMOOTH MUSCLE CELL MIGRATION

Smooth muscle cell migration is known to play a vital role in various vascular disorders, such as atherosclerosis [34]. Although the exact pathways leading to vascular smooth muscle cell migration are not clear, exposure to ROS has clearly been implicated in the migration process of vascular smooth muscle cells [30].

This migration occurs largely in reaction to platelet-derived growth factor, as this growth factor has the ability to increase the production of ROS in the vascular smooth muscle cells, which thus promotes the migration process [34].

#### 6.2.4 EXTRACELLULAR MATRIX METABOLISM

Matrix metalloproteinases are responsible for the breakdown and rearrangement of the extracellular matrix [30]. Both the activation and gene expression of various types of matrix metalloproteinases are regulated by ROS, thus indicating that ROS plays a crucial role in pathological vascular remodelling [30].

### 6.3 INFLAMMATION

As previously mentioned, one of the functions of ROS is to activate various transcription factors, such as NF- $\kappa$ B [13, 14].

Once activated, NF- $\kappa$ B leads to an increase in the production of various inflammatory molecules such as cytokines, chemokines and adhesion molecules [10]. Furthermore, NF- $\kappa$ B also leads to the activation and subsequent proliferation of lymphocytes [10]. These processes consequently lead to the activation, adhesion, and infiltration of immune cells, which further contribute to the state of oxidative stress due to their ability to produce ROS [10].

Additionally, NF- $\kappa$ B leads to the expression of various inflammatory genes, thus increasing the production of various inflammatory compounds such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6), which further aggravate the inflammatory state inside the body [30].

## 6.4 ANGIOGENESIS

ROS is noted to be involved in all the major processes of angiogenesis, namely endothelial cell migration, endothelial cell proliferation and tube formation [30]. This is achieved via two mechanisms. Firstly, each of the processes involved in angiogenesis is directly induced by  $H_2O_2$  in the human body [30]. Secondly, ROS is involved in mediating the activity of various growth factors involved in angiogenesis (such as vascular endothelial growth factor) [30].

The various processes by which oxidative stress influences vascular function may lead to the development of various cardiovascular diseases such as atherosclerosis and hypertension [6, 7, 10, 14, 28, 30].

## 7. ROLE OF OXIDATIVE STRESS IN ATHEROSCLEROSIS

Atherosclerosis is an inflammatory condition in which various cytokines induce the development of vascular lesions [30]. Inflammatory gene expression and vascular remodelling are vital processes in the development of atherogenesis, and are both aggravated by oxidative stress [30]. It has also been indicated that oxidative stress stimulates the oxidation of LDL cholesterol, which is an important process in the development of atherosclerosis [8, 14]. Oxidized LDL cholesterol can be absorbed by macrophages, which subsequently leads to the formation of foam cells [8]. These cells then accumulate in the sub-endothelial space to form fatty streaks associated with atherosclerosis [8].

This may lead to damage of the surrounding endothelium, which then promotes the aggregation of platelets, further stimulating lesion formation [8]. Furthermore, oxidized LDL cholesterol is able to inhibit nitric oxide formation via nitric oxide synthase enzymes, and is also able to induce inflammation, vasoconstriction, cytokine activation, smooth muscle cell proliferation and the expression of vascular endothelial growth factor, which are important in the progression of atherosclerosis [35].

Carotid intima media thickness is a non-invasive measure of atherosclerosis which is an independent risk factor for cardiovascular disease, and is associated with cardiovascular events [36, 37]. Carotid intima media thickness is a measurement of the carotid artery wall and is measured from the lumen-intima surface to the media-adventitia surface [37].

This measurement makes use of an ultrasound device in order to create an image of the carotid artery wall, and is able to show structural changes related to atherosclerosis [36, 37]. An increase in the CIMT is indicative of arterial wall remodelling which may be associated with atherosclerosis [37].

Previous studies suggested a link between altered antioxidant capacity and carotid intima media thickness [51, 52]. In one of these studies performed on patients in various stages of the development of atherosclerosis, it was found that as the carotid intima media thickened, the GSH concentration and the GHS/GSSG ratio decreased, while GSSG concentration increased [38]. In another study it was indicated that the activity of antioxidant enzymes, such as GPx and GR, was significantly lower in atheromatous plaques than in normal arteries [39].

## 8. ROLE OF OXIDATIVE STRESS IN HYPERTENSION

The relationship between hypertension and ROS probably develops at the level of the vasculature, as oxidative stress has multiple vascular effects as previously mentioned [6, 7].

An important mechanism in the development of hypertension is the ability of ROS to decrease the bio-availability of nitric oxide, contributing to endothelial dysfunction through a decrease in endothelium-dependent vasodilation [7, 29]. Additionally, it is known that the activation of the renin-angiotensin-aldosterone system plays an important role in the development of hypertension [7].

The binding of angiotensin-II to the type 1 angiotensin II receptors (AT1) leads to the activation of NADPH oxidase enzymes in the vascular wall, which is responsible for the production of ROS [10, 29, 40].

Evidence has also shown that the shear stretch associated with hypertension may also activate the NADPH oxidase enzyme in vascular smooth muscle cells [40]. Furthermore, it has been shown that ROS is not only able to increase the vascular smooth muscle tone (by increasing the calcium concentration in the cytoplasm), but ROS is also able to generate vasoconstrictive inflammatory substances (such as isoprostanes) which further contribute to hypertension development [10].

As previously mentioned, ROS becomes harmful when the antioxidant capacity is decreased, or when the production of ROS becomes excessive [6, 8]. It was found that pre-hypertensive participants presented with elevated oxidative stress (as indicated by increased malondialdehyde, 8-isoprostanes and

hydroxyoctadecadienoic acid levels) which may be associated with decreased antioxidant capacity [6, 41]. Furthermore, the activities of antioxidant enzymes including SOD, GPx and CAT were decreased in hypertensive subjects when compared to normotensive subjects [42].

Several studies displayed increases in  $O_2^{\cdot-}$  and  $H_2O_2$  levels, as well as increased angiotensin II in hypertensive patients [43, 44]. Further evidence has shown that Myeloperoxidase (an enzyme which produces hypochlorous acid and which is related to oxidative stress and inflammation) is positively associated with blood pressure in elderly subjects [45]. Furthermore, it has been suggested that the progression from pre-hypertension to hypertension may also be due to oxidative stress [46].

## 9. FACTORS INFLUENCING OXIDATIVE STRESS AND HYPERTENSION

### 9.1 AGE

Oxidative stress is thought to be associated with increased age as both ROS production and the oxidative damage to proteins, lipids and DNA are increased during aging [47].

There is much debate around the effect of age on antioxidant enzyme activity. One study indicated that SOD, GPx and CAT activity are all shown to decrease with increasing age [48, 49], whereas in another study no age-related change in SOD activity was shown, while CAT and GPx activity increased, and GR activity

decreased with increasing age [50]. Adding to the controversy, another study displayed decreases in SOD and GR activities with no change in GPx and CAT activities with increasing age [48, 51], whereas in yet another study increased SOD and GPx activity were indicated in the elderly [48, 52].

In contradiction to the controversy regarding antioxidant enzyme activities and aging, it is known that an increase in age is associated with an increased risk for the development of hypertension [53]. This is achieved through the promotion of various structural and functional changes in the vasculature. These changes are suggested to be brought about by increased oxidative stress, which may subsequently lead to endothelial dysfunction, and thus hypertension [53].

## 9.2 OBESITY

It has been shown that obesity contributes to oxidative stress by increasing ROS production via NADPH oxidase [54, 55]. The potential mechanism in which obesity increases the activity of this enzyme involves leptin [54]. Leptin has the ability to activate the NADPH oxidase enzyme, and because leptin levels are increased in obese patients, this may play a vital role in the development of oxidative stress [54].

Obesity is known to be an independent risk factor for the development of hypertension [56, 57]. It has also been shown that regular physical activity has a positive effect on blood pressure and hypertension risk [56, 58].

### 9.3 EXERCISE

The process of muscle contraction during exercise leads to the production of ROS, and excessive ROS production may further lead to exercise-induced DNA, protein and lipid oxidation [59].

Increased ROS production stems from various sources during exercise, such as the leakage of ROS from the respiratory chain in the mitochondria, NADPH oxidase enzymes which are stimulated during muscle contraction, and exercise-induced tissue damage in the form of shear stress [59, 60]. Shear stress created by exercise may further lead to vascular dysfunction and inflammation, thus increasing ROS and leading to oxidative stress [60].

In contrast to the possible excessive production of ROS during exercise, exercise has been shown to have a positive effect on blood pressure, and can reduce the risk of developing hypertension [58, 61]. This is done by reducing the systemic vascular resistance, and it is thought to be an important mechanism by which exercise lowers blood pressure [62].

### 9.4 ALCOHOL USAGE

Alcohol intake has been shown to facilitate oxidative stress by both increasing the production of ROS, and decreasing the antioxidant capacity when used on a moderate or prolonged basis [63, 64]. Alcohol intake has been shown to decrease the antioxidant capacity by decreasing the availability of the antioxidant GSH, thus decreasing the body's defence against oxidative damage [65]. Furthermore it has

also been found that markers of oxidative stress are increased in patients with alcoholic liver disease [65].

In addition to the effect of increased alcohol intake on oxidative stress it is also linked to an increased risk for the development of hypertension [63, 64]. In a review article it was suggested that one possible mechanism involved in the elevation in blood pressure due to long-term alcohol usage could be the development of a state of withdrawal which leads to increases in blood pressure [66].

## 9.5 SMOKING

Smoking has been implicated in the development of oxidative stress, and is shown to induce tissue damage in humans [55, 67]. Smoking has also been linked to hypertension development [68]. Nicotine found in cigarette smoke has the ability to stimulate catecholamine and vasopressin release, and is able to act as an adrenergic agonist, possibly being important mechanisms in hypertension development among smokers [68]. A second possible mechanism behind smoking-induced hypertension development could be due to oxidative stress, in which smoking enhances the inactivation of NO by ROS, which subsequently leads to endothelial dysfunction [69].

## 9.6 DIABETES

Oxidative stress plays an essential role in the development of diabetes and diabetic complications [8, 70, 71]. Elevated ROS production occurs via various mechanisms.

Firstly, hyperglycaemia can lead to glucose oxidation and non-enzymatic protein glycation which directly produces ROS in the body [70, 71]. Secondly, hyperglycaemia and elevated levels of free fatty acids lead to  $O_2^{\cdot-}$  leakage from the respiratory chain in the mitochondria, as well as activation of the NADPH oxidase enzyme responsible for ROS production [30]. Thirdly, it has been found that diabetic patients exhibit increased endothelial NOS uncoupling, which may lead to further ROS production [30].

Lastly, an important pathway for increased ROS production in diabetes is the formation of advanced glycation end-products [8, 70]. These substances bind with their receptors and lead to enzyme deactivation, ROS formation and inhibit the action of nitric oxide [70].

In addition to increased ROS production in diabetes, the antioxidant capacity is also notably decreased in diabetic patients as it has been shown that both antioxidant enzyme activity (including SOD and CAT) and non-enzymatic antioxidants (such as GSH, vitamin C and vitamin E) are decreased [8, 72].

Apart from the link between oxidative stress and diabetes, it has been shown that diabetes increases the risk for the development of hypertension, especially among patients with type II diabetes [73]. Pre-diabetic patients with insulin resistance are thought to have dysfunctional endothelium-dependent vaso-relaxation, which is thought to play an important role in hypertension development in these patients [73]. Two important mechanisms for hypertension development in both type I and type II diabetes are an increase in vascular resistance and an increase in vascular smooth muscle contraction [74]. Hyperglycemia has been found to increase sodium retention, promote vascular changes involved in arterial stiffness, promote

endothelial dysfunction and stimulate the sympathetic nervous system, thus increasing blood pressure [74].

## 10. RELATIONSHIP BETWEEN ETHNICITY AND ROS

There are minimal studies which examine the relationship between ethnicity and oxidative stress. One such study compared lipid peroxidation (malondialdehyde and F<sub>2</sub>-isoprostanes) between African Americans and white patients. It was shown that the African Americans had significantly lower F<sub>2</sub>-isoprostanes levels, but higher levels of malondialdehyde when compared to white patients [75].

In another study, the association between ethnicity and oxidative stress was investigated and it was found that white subjects had greater production of H<sub>2</sub>O<sub>2</sub> than African American subjects [44]. This may be due to a disturbance in the activities of various enzyme pathways which contribute to H<sub>2</sub>O<sub>2</sub> production (xanthine oxidase, NADPH oxidase, SOD,) or reduced H<sub>2</sub>O<sub>2</sub> breakdown (decreased GPx activity) [44].

In contrast we have previously indicated that serum peroxide levels were significantly higher in blacks than in whites [76, 77]. Further studies evaluating oxidative stress in our South African population have been performed as part of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study.

Firstly, upon investigation of 101 African men and 99 African women, it was found that ROS levels (in the form of serum peroxides) were higher in the women. When comparing hypertensive and normotensive men, ROS was higher in men with

hypertension. Furthermore, it was hypothesized that increased ROS may be implicated in the development of arterial stiffness and hypertension [77].

Secondly, in a population of 63 hypertensive and 34 normotensive African men, it was concluded that a decrease in glutathione in the hypertensive participants may contribute to thickening of the carotid intima media. This may lead to the development of atherosclerosis in this population [78].

This is of vital importance to our study as the disturbance in antioxidant enzyme activities and its association with cardiovascular variables is yet to be examined in our South African population.

Although these studies evaluated the relationship of ROS and glutathione with cardiovascular variables, it is still unknown whether the increase in ROS is due to an up-regulation of ROS-producing enzymes, a decrease in the functioning of antioxidant enzymes, or both. We intend to evaluate the possibility of decreased antioxidant enzyme activity in the black population, and how it correlates with cardiovascular variables.

Problem statement:

There is a lack of knowledge surrounding oxidative stress in the black South African population, and it is unknown whether antioxidant enzyme activity is decreased in these participants and whether this may have implications on various cardiovascular measurements.

The aims of this study are:

- 1) To compare antioxidant enzyme activity in black and white men, as well as black and white women.
- 2) To determine if any inverted relationship exists between cardiovascular variables and antioxidant enzyme activity in these groups.

We hypothesize that:

- 1) Antioxidant enzyme activity will be lower in black participants than in white participants.
- 2) Cardiovascular variables will exhibit an inverse relationship with antioxidant enzyme activity in black participants.

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# **CHAPTER 3:**

# **MANUSCRIPT**



# INSTRUCTIONS FOR AUTHORS: Free Radical Research

## MANUSCRIPT PREPARATION

### **File preparation and types**

Manuscripts are preferred in Microsoft Word format (.doc or .docx files). Documents must be double-spaced, with margins of one inch on all sides. Specific instructions for their submission are given below. References should follow the Council of Biology Editors style (see References section for example).

Manuscripts should be compiled in the following order: title page; abstract; main text; acknowledgments; declaration of interest statement; appendices (as appropriate); references.

### **Title Page**

A title page should be provided comprising the manuscript title plus the full names and affiliations of all authors involved in the preparation of the manuscript. One author should be clearly designated as the corresponding author and full contact information, including phone number and email address, provided for this person.

Five key terms that are not in the title should also be included on the title page. The keywords will assist indexers in cross indexing your article.

### **Abstract**

All articles should start with an abstract of less than 250 words, summarising the central core of knowledge that is the focus of the paper. It should be written in an

informative style permitting its use, without revision, by abstracting services, give essential details of research findings without further reference to the text, and avoid generalisations and nonessential information.

## **Main Text**

The body of the article should include the following distinct sections: introduction; methods; results; discussion; conclusion.

*Introduction:* This section should state the relevance and background to the study, and its rationale and purpose.

*Methods:* This section should include only information that was available at the time the plan or protocol for the study was being written. Please identify the methods, apparatus and procedures in sufficient detail to allow others to reproduce the results, and describe statistical methods with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. ***Free Radical Research*** requires that studies involving humans and animals be approved by an institutional review board, in accordance with approved published guidelines, prior to actually performing the research and publishing the data. This approval should be explicitly stated in the introduction and/or the methods section, together with the name of the appropriate board and the approved study number.

*Results:* Present your results in logical sequence in the text, tables, and illustrations.

*Discussion:* This should include implications of the findings and their limitations, with reference to all other relevant studies and the possibilities these suggest for future research.

*Conclusions:* This is often combined with the Discussion and must summarize the main paper. Ensure that extrapolations are reasonable and that conclusions are

justified by the data presented, and indicate if the study design can be generalized to a broader study population.

### **Acknowledgments**

The Acknowledgments section details special thanks, personal assistance, and dedications. Contributions from individuals who do not qualify for authorship should also be acknowledged here. Acknowledgments should be included in a separate headed section at the end of the manuscript preceding any appendices, and before the Declaration of Interest Section. Please do not incorporate acknowledgments into notes or biographical notes. Some authors may elect not to include any acknowledgments.

### **Declaration of Interest**

The Declarations of Interest Section should disclose any financial, consulting, and personal relationships with other people or organizations that could influence (bias) the author's work. Within this section also belongs disclosure of scientific writing assistance (use of an agency or freelance writer), grant support and numbers (including NIH/Wellcome-funded papers), and statements of employment.

### **References**

References should follow the Council of Biology Editors (CBE) style. Only works actually cited in the text should be included in the references. In-text references should be Arabic numbers inside square brackets. Spelling in the reference list should follow the original. References should then be listed in numerical order at the end of the article as follows:

Journal article: [1] Steiner U, Klein J, Eiser E, Budkowski A, Fetters LJ. Complete wetting from polymer mixtures. *Science* 1992;258:1122–1129.

## **Tables**

Tables should be used only when they can present information more efficiently than running text. Care should be taken to avoid any arrangement that unduly increases the depth of a table, and the column heads should be made as brief as possible, using abbreviations liberally. Lines of data should not be numbered nor run numbers given unless those numbers are needed for reference in the text. Columns should not contain only one or two entries, nor should the same entry be repeated numerous times consecutively.

# The association of antioxidant enzyme activity with cardiovascular variables in a bi-ethnic population: the SABPA study

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**Running head:** Antioxidant enzyme activity and cardiovascular variables.

**Word count:** 6613

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**Key words:** Antioxidant capacity; blood pressure; carotid intima media thickness; ethnicity; oxidative stress.

## Abstract

Oxidative stress may play a crucial role in the development of hypertension which is a growing problem in South Africa. Since oxidative stress may result from impaired antioxidant capacity, we aimed to compare antioxidant enzyme activity between black and white men, and black and white women. Furthermore, we aimed to investigate associations of cardiovascular variables with antioxidant enzyme activity. Our study consisted of black (n=101) and white men (n=101), and black (n=99) and white women (n=108). We measured carotid intima media thickness (CIMT) and blood pressure and determined glutathione peroxidase (GPx) and glutathione reductase (GR) activity.

GR activity was significantly higher in black men and women ( $p < 0.001$ ) when compared to their white counterparts. In black women, GPx activity was significantly lower ( $p < 0.001$ ) when compared to white women. An independent positive association exists between GR activity and CIMT ( $R^2 = 0.275$ ;  $\beta = 0.196$ ;  $p = 0.037$ ) in black men. In black women independent negative associations of GPx activity and systolic blood pressure (SBP) ( $R^2 = 0.184$ ;  $\beta = -0.251$ ;  $p = 0.009$ ), and GPx activity and mean arterial pressure (MAP) ( $R^2 = 0.131$ ;  $\beta = -0.303$ ;  $p = 0.003$ ) were indicated. No associations were found in the white groups.

Increased GR activity and ROS levels in black men, and increased GR activity and decreased GPx activity in black women may suggest increased oxidative stress in the black participants.

The positive association between GR activity and CIMT in black men and the inverse association between GPx activity and blood pressure in black women may propose a

role for oxidative stress in arterial remodelling and hypertension development in this population.

## **Introduction**

Hypertension is a severe problem in our society and the prevalence thereof is increasing [1]. Sub-Saharan Africa is no exception, with an increased incidence in hypertension, especially among urban blacks [2]. The development of hypertension may be stimulated by various factors including oxidative stress [3, 4].

Oxidative stress occurs as a result of an increase in the generation of reactive oxygen species (ROS) or a decrease in the antioxidant defence mechanisms, or a combination thereof [3, 5]. ROS is produced in all vascular cell types by the actions of various enzymes which are balanced by the counteractions of various antioxidant enzymes to prevent oxidative stress [5]. These antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), while secondary antioxidants include glutathione (GSH) and the enzyme glutathione reductase (GR), which play an important role in redox balance [4, 6, 7].

Oxidative stress is involved in the development of various cardiovascular diseases such as hypertension, atherosclerosis, cardiac hypertrophy, stroke and heart failure [3].

Our research group has previously indicated higher ROS levels in black women than in black men, and higher ROS levels in hypertensive men than in normotensive men. In the same study it was found that ROS was positively associated with both systolic blood pressure (SBP) and pulse pressure (PP) in the black men, suggesting that

elevated ROS levels may contribute to hypertension development and increased arterial stiffness in these groups [8]. It was also found that decreased GSH levels were linked with carotid intima media thickness (CIMT) in hypertensive black men [9].

From these studies it is unknown if excessive ROS levels in this population is the result of decreased antioxidant enzyme activity, and we therefore aimed to determine whether black participants have lower antioxidant enzyme activity than white participants. We further aimed to determine if any inverted relationships exist between cardiovascular variables, such as blood pressure and CIMT, with antioxidant enzyme activity.

## **Materials and methods**

### **Study population**

This study is embedded in the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study, which was conducted between February 2008 and May 2009 [10].

The SABPA study is a cross-sectional target population study and included 101 black and 101 white male, and 99 black and 108 white female teachers from the Dr Kenneth Kaunda Education District in the North West Province of South Africa. This selection was chosen to ensure a homogenous sample with similar socio-economic status. The cultural backgrounds of the two ethnic groups differ, and this may play a role in nutrient intake and lifestyle choices. The participants were between the ages of 20 and 65 years. Exclusion criteria included pregnancy, lactation, and an ear

temperature  $>37^{\circ}\text{C}$ . Participants were also excluded if they had donated blood or had been vaccinated 3 months prior to the commencement of the study.

Participants were fully informed about the objectives and procedures of the study prior to their recruitment. Assistance was provided for any participant who requested conveyance of information in their home language. The selected participants signed an informed consent form explaining the procedures and objectives before commencement of the study. Data was safely stored and protected by a password, and participants received feedback after the completion of the various measurements. The study complied with the Helsinki Declaration of 1975 (revised in 2008) and regulations regarding investigations among human participants, and was further approved by the Ethics Review Board of the North-West University (Potchefstroom campus) (NWU-00036-07-S6). This sub-study was also approved by the Health Research Ethics Committee of the North-West University (Potchefstroom campus) (NWU-00036-07-S6).

### **Study protocol**

The participants were admitted at 16:30 to the Metabolic Unit Research Facility of the North-West University. The facility contained a living room, a kitchen, two bathrooms and ten bedrooms. Each participant completed a lifestyle questionnaire, including questions about their cardiovascular health history and chronic medication use. At dinner time they received a standardized meal and had their last beverages (tea/coffee) and two biscuits at 20:30. Thereafter they relaxed by reading, watching television, or social interaction. Participants were encouraged to go to sleep at 22:00.

At 06:00 the next morning, the participants were woken and subsequent measurements commenced.

### **Anthropometric and physical activity measurements**

Participants' height and weight were measured while wearing minimal clothing using calibrated instruments (Precision Health Scale, A & D Company, Tokyo, Japan and Invicta Stadiometer, IP 1465, Invicta, London, UK). Measurements were taken in triplicate using standard methods (International Society for the Advancement of Kinanthropometry). Participants' body mass index (BMI) was calculated as  $\text{kg/m}^2$ . Total energy expenditure was calculated over a 24 hour period using the Actical® activity monitor (Mini Mitter Co., Inc., Bend, OR; Montreal, Quebec, Canada).

### **Cardiovascular measurements**

The validated Finometer device was used to measure SBP, diastolic blood pressure (DBP) and mean arterial pressure (MAP) (FMS, Finapres Medical Systems, Amsterdam, The Netherlands) [11, 12].

The Finometer device was connected, and after a 10 minute resting period, a 5 minute continuous measurement of resting cardiovascular parameters was taken. During the recording, after 2 minutes, a return-to-flow systolic calibration was performed to provide an individual subject-level adjustment of the finger arterial pressure with the brachial artery pressure. The highest precision in cardiovascular measurements is obtained only after this calibration. We obtained the average of the last 2 minutes of the recordings.

The high resolution SonoSite Micromaxx ultrasound system (SonoSite Inc., Bothell, WA, USA) was used to measure CIMT, and results were interpreted with a semi-

automated program (Artery Measurement Systems (AMS) II v1.139 (Gothenburg, Sweden)). Cross-sectional wall area (CSWA) was calculated using the formula  $CSWA = \pi(d/2 + CIMT)^2 - \pi(d/2)^2$ .

### **Biochemical measurements**

A fasting blood sample (for serum and EDTA plasma) was obtained from each participant by a registered nurse from the antebachial vein branches. All blood samples were stored at -80°C, as the various antioxidant enzymes have been shown to remain stable at this temperature for long periods of time [13]. Glycated haemoglobin (HbA1c) was measured in EDTA whole blood via a turbidometric inhibition immunoassay (Integra 400, Roche, Switzerland).

Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides,  $\gamma$ -glutamyl transferase (GGT) and C-reactive protein (CRP) were analysed in serum (Unicel DXC 800 device - Beckman and Coulter, Germany and Konelab™ 201 Sequential Multiple Analyser Computer). The total cholesterol to HDL cholesterol ratio was calculated by dividing the high-density lipoprotein cholesterol concentration by the total cholesterol concentration.

Assay kits from Cayman Chemical Company (Ann Arbor, MI, USA) were used to measure GPx, GR and SOD, while CAT was measured using a fluorometric OxiSelect catalase activity assay kit (Cell Biolabs Inc., San Diego, CA, USA). All the antioxidant enzyme activities were measured on a Synergy H4 hybrid microplate reader (BioTek, Winooski, VT, USA). Total glutathione (GSH) was measured with the BIOXYTECH GSH/GSSG-412 kit on a Bio-Tek FL600 microplate reader in EDTA whole blood samples. Serum ROS (measured as serum peroxides) was measured using the method described by Hayashi *et al* [14].

Tumor necrosis factor alpha (TNF $\alpha$ ) and cotinine were measured using a high sensitivity ELISA kit (R&D Systems, Minneapolis, MN USA).

### **Statistical analyses**

In agreement with our aims we divided our population into four groups, namely black men, white men, black women and white women. Statistica version 12 (Statsoft inc., Tulsa, OK, USA) was used to perform the statistical analyses of this study. The central tendency and spread for normally distributed data were expressed as arithmetic mean and standard deviation. Variables not normally distributed were logarithmically transformed (CRP, GR, SOD, CAT and GGT) and expressed as the geometric mean and the 5th and 95th percentile intervals.

Mean values were compared between groups using independent t-tests, while proportions were compared between groups using Chi-square tests. Differences in cardiovascular variables and antioxidant enzyme activity between the groups were confirmed with analysis of covariance (ANCOVA) while adjusting for age and BMI.

Single and partial correlations were performed to evaluate associations between cardiovascular variables and antioxidant enzyme activity. In partial correlations the associations between cardiovascular variables and antioxidant enzyme activity were adjusted for age and BMI. Multiple regression analyses were performed in order to evaluate independent associations of cardiovascular variables with antioxidant enzyme activity in each of the groups. Covariates were used as determined by a forward stepwise procedure and included age, BMI, total energy expenditure, GGT, cotinine, HbA1c, CRP, and the total cholesterol to high-density lipoprotein ratio. Models with CIMT and CSWA as dependent variables were additionally adjusted for MAP. In sensitivity analyses, HIV infected men (n=13) and women (n=6), and

women using hormonal contraception (n=24) were excluded and multiple regression analyses repeated. Sensitivity analyses were attempted while excluding normotensive participants (n=272) and participants not using hypertension medication (n=313).

## **Results**

### **Characteristics of the study population**

Black men had significantly lower body mass ( $p<0.001$ ), and borderline significantly lower BMI ( $p=0.060$ ) than those of the white men (Table 1A), while black women were shown to have significantly higher body mass and BMI than the white women (both  $p<0.001$ ) (Table 1B).

Both black men and black women displayed an unfavourable cardiovascular profile compared to their white counterparts, with SBP ( $p<0.001$ ), DBP ( $p<0.001$ ) and MAP being significantly higher (men:  $p<0.001$ ; women:  $p=0.004$ ) than those of the white participants. Additionally, black women showed increased CIMT ( $p<0.001$ ) and CSWA ( $p=0.008$ ) when compared to their white counterparts.

Oxidative stress markers revealed significantly higher ROS levels ( $p=0.002$ ) but also higher total GSH ( $p=0.013$ ) and GR activity ( $p<0.001$ ) in the black men when compared to the white men. In the women it was noted that total GSH, GR activity and CAT activity were all significantly higher (all  $p<0.001$ ), while GPx activity was significantly lower ( $p<0.001$ ) in the black women when compared to the white women.

Significantly higher HbA1c levels were indicated in black men and women ( $p<0.001$ ), while the total cholesterol to high-density lipoprotein ratio was significantly lower

( $p=0.005$ ) in the black men when compared to their white counterparts. No differences were found in the women.

Inflammatory markers such as CRP (men:  $p=0.002$ ; women:  $p<0.001$ ) and TNF $\alpha$  ( $p<0.001$ ) were higher in black men and women than in their white counterparts.

Measures of lifestyle indicated that black men and women had significantly higher GGT levels ( $p<0.001$ ) than their white counterparts. Additionally, black men were found to have significantly lower total energy expenditure than the white men ( $p=0.001$ ).

Characteristics of the study populations remained significant even after adjustments were made for age and BMI, with the exception of SBP ( $p=0.123$ ) and MAP ( $p=0.236$ ) in the women. Additionally, CIMT became significant in the men after adjusting for age and BMI ( $p=0.013$ ).

**Table 1A: Characteristics of black and white men.**

Variables	Black men	White men	P-values
N	101	101	
Age (years)	43.2 ± 8.17	45.1 ± 11.0	0.17
<b>Anthropometric measurements</b>			
Body Mass (kg)	80.3 ± 17.9	95.4 ± 17.6	<0.001
Body Mass Index (kg/m <sup>2</sup> )	27.6 ± 5.77	29.0 ± 5.20	0.06
<b>Cardiovascular measurements</b>			
Carotid intima media thickness (mm)	0.70 ± 0.14	0.68 ± 0.12	0.29
Cross-sectional wall area (mm <sup>2</sup> )	14.9 ± 5.26	14.7 ± 4.14	0.72
Systolic blood pressure (mmHg)	146 ± 20.4	133 ± 11.1	<0.001
Diastolic blood pressure (mmHg)	86 ± 10.9	80 ± 8.41	<0.001
Mean arterial pressure (mmHg)	111 ± 13.5	102 ± 9.34	<0.001
<b>Biochemical analyses</b>			
Glycated haemoglobin (%)	6.24 ± 1.23	5.66 ± 0.48	<0.001
Triglycerides (mmol/l)	1.81 ± 1.59	1.51 ± 0.87	0.10
Total cholesterol to high-density lipoprotein ratio (mg/dL)	5.06 ± 2.48	5.88 ± 1.49	0.005
<b>Inflammatory markers</b>			
C-reactive protein (log mg/l)	2.75 (0.27; 14.3)	1.80 (0.99; 8.00)	0.002
Tumor necrosis factor alpha (pg/ml)	3.69 ± 3.63	2.31 ± 1.91	<0.001
<b>Oxidative stress markers</b>			
Reactive oxygen species (mg/l)	84.4 ± 18.9	76.7 ± 15.6	0.002
Total glutathione (µM)	925 ± 197	859 ± 180	0.01
Glutathione peroxidase (nmol/min/ml)	34.6 ± 13.9	35.1 ± 8.03	0.75
Glutathione reductase (log nmol/min/ml)	7.71 (3.31; 18.8)	2.23 (0.22; 7.38)	<0.001
Superoxide dismutase (log U/ml)	3.94 (0.62; 23.3)	4.23 (1.90; 7.30)	0.52
Catalase (log U/ml)	4.29 (4.20; 18.8)	4.16 (4.00; 4.36)	0.18
<b>Lifestyle</b>			
Cotinine (ng/ml)	35.5 ± 65.0	30.9 ± 96.7	0.69
Gamma glutamyl transferase (log U/l)	62.7 (23.7; 281)	27.3 (11.0; 90.0)	<0.001
Total energy expenditure (kcal/day)	2715 ± 800	3674 ± 2059	0.001
Anti-hypertensive drugs (n)	36	14	<0.001
Multivitamin intake (n)	1	7	0.03
Anti-oxidant intake (n)	0	1	0.32
HIV infected (n)	13	0	<0.001

Data expressed as arithmetic mean ± standard deviation, geometric mean (5th-95th percentiles) or n.

**Table 1B: Characteristics of black and white women.**

Variables	Black women	White women	P-values
N	99	108	
Age (years)	45.6 ± 7.90	45.0 ± 10.7	0.65
<b>Anthropometric measurements</b>			
Body Mass (kg)	82.5 ± 19.0	73.1 ± 18.4	<0.001
Body Mass Index (kg/m <sup>2</sup> )	32.7 ± 7.22	26.3 ± 6.29	<0.001
<b>Cardiovascular measurements</b>			
Carotid intima media thickness (mm)	0.67 ± 0.11	0.61 ± 0.11	<0.001
Cross-sectional wall area (mm <sup>2</sup> )	12.9 ± 3.50	11.5 ± 3.51	0.008
Systolic blood pressure (mmHg)	136 ± 14.3	129 ± 15.8	<0.001
Diastolic blood pressure (mmHg)	77 ± 7.68	74 ± 6.90	<0.001
Mean arterial pressure (mmHg)	101 ± 9.20	98 ± 9.45	0.004
<b>Biochemical analyses</b>			
Glycated haemoglobin (%)	5.91 ± 1.14	5.37 ± 0.29	<0.001
Triglycerides (mmol/l)	1.04 ± 0.63	0.90 ± 0.48	0.08
Total cholesterol to high-density lipoprotein ratio (mg/dL)	3.87 ± 1.23	4.16 ± 1.26	0.10
<b>Inflammatory markers</b>			
C-reactive protein (log mg/l)	7.13 (0.78; 35.6)	2.27 (0.99; 14.3)	<0.001
Tumor necrosis factor alpha (pg/ml)	2.80 ± 2.41	1.50 ± 2.00	<0.001
<b>Oxidative stress markers</b>			
Reactive oxygen species (mg/l)	108 ± 27.9	103 ± 32.7	0.30
Total glutathione (µM)	863 ± 176	784 ± 159	<0.001
Glutathione peroxidase (nmol/min/ml)	31.9 ± 13.9	37.1 ± 7.82	<0.001
Glutathione reductase (log nmol/min/ml)	6.46 (2.04; 15.3)	2.86 (0.51; 7.64)	<0.001
Superoxide dismutase (log U/ml)	4.59 (0.86; 22.1)	4.05 (1.50; 10.8)	0.26
Catalase (log U/ml)	4.30 (4.20; 4.38)	4.24 (4.01; 4.34)	<0.001
<b>Lifestyle</b>			
Cotinine (ng/ml)	18.7 ± 55.4	15.1 ± 53.0	0.63
Gamma glutamyl transferase (log U/l)	35.5 (16.6; 117)	13.9 (6.00; 39.0)	<0.001
Total energy expenditure (kcal/day)	2649 ± 793	2587 ± 645	0.54
Contraception (n)	17	7	0.02
Anti-hypertensive drugs (n)	33	13	<0.001
Multivitamin intake (n)	0	14	<0.001
Anti-oxidant intake (n)	0	4	0.05
HIV infected (n)	6	0	0.01

Data expressed as arithmetic mean ± standard deviation, geometric mean (5th-95th percentiles) or n.

## Single regression analyses

In the group of black men it was found that GPx activity correlated negatively with SBP ( $r=-0.213$ ;  $p=0.037$ ) (Table 2A), while in the black women, GPx correlated negatively with SBP ( $r=-0.273$ ;  $p=0.007$ ), DBP ( $r=-0.231$ ;  $p=0.024$ ) and MAP ( $r=-0.335$ ;  $p=0.001$ ) (Table 2B). Furthermore, GR activity correlated positively with CIMT ( $r=0.245$ ;  $p=0.016$ ) and CSWA ( $r=0.228$ ;  $p=0.023$ ) in black men, while SOD correlated negatively with CIMT ( $r=-0.265$ ;  $p=0.009$ ) and CSWA ( $r=-0.285$ ;  $p=0.005$ ) in black women. In the white women it was found that GPx activity correlated positively with CIMT ( $r=0.198$ ;  $p=0.041$ ), whereas no correlations were found in the white men.

## Partial regression analyses

In the black men it was found that the positive correlation between GR activity and CIMT ( $r=0.221$ ;  $p=0.038$ ) remained significant after adjusting for age and BMI. All other correlations indicated in single regression analyses were no longer significant (Table 2A). In the black women, the negative correlations between GPx activity and SBP ( $r=-0.245$ ;  $p=0.016$ ), GPx activity and DBP ( $r=-0.220$ ;  $p=0.033$ ) and GPx activity and MAP ( $r=-0.319$ ;  $p=0.002$ ) remained significant after adjusting for age and BMI (Table 2B). In the same group, the negative correlation between SOD activity and CSWA remained significant ( $r=-0.237$ ;  $p=0.023$ ) once adjustments were made, while the correlation between SOD activity and CIMT was no longer significant. In the white women, the correlations of GPx activity with CIMT also lost significance after adjusting for age and BMI.

**Table 2A: Single and partial regression analyses of antioxidant enzymes and cardiovascular variables in black and white men.**

	CIMT (mm)		CSWA (mm <sup>2</sup> )		SBP (mmHg)		DBP (mmHg)		MAP (mmHg)	
<b>BLACK MEN (n=101)</b>										
	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>
GPx (nmol/min/ml)	r= -0.126 P= 0.220	r= 0.043 P= 0.689	r= -0.162 P= 0.107	r= -0.062 P= 0.567	<b>r= -0.213</b> <b>P= 0.037</b>	r= -0.130 P= 0.212	r= -0.035 P= 0.732	r= -0.004 P= 0.973	r= -0.135 P= 0.191	r= -0.072 P= 0.490
GR (log nmol/min/ml)	<b>r= 0.245</b> <b>P= 0.016</b>	<b>r= 0.221</b> <b>P= 0.038</b>	<b>r= 0.228</b> <b>P= 0.023</b>	r= 0.185 P= 0.083	r= 0.072 P= 0.485	r= -0.002 P= 0.983	r= -0.035 P= 0.738	r= -0.035 P= 0.740	r= 0.025 P= 0.812	r= -0.014 P= 0.897
SOD (log U/ml)	r= -0.013 P= 0.901	r= -0.068 P= 0.530	r= -0.055 P= 0.593	r= -0.104 P= 0.334	r= -0.050 P= 0.632	r= -0.113 P= 0.287	r= -0.066 P= 0.531	r= -0.064 P= 0.547	r= -0.062 P= 0.557	r= -0.091 P= 0.393
CAT (log U/ml)	r= -0.096 P= 0.349	r= -0.199 P= 0.061	r= -0.132 P= 0.189	r= -0.160 P= 0.134	r= -0.044 P= 0.667	r= -0.070 P= 0.502	r= -0.056 P= 0.588	r= -0.021 P= 0.841	r= -0.039 P= 0.707	r= -0.031 P= 0.766
<b>WHITE MEN (n=101)</b>										
	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>
GPx (nmol/min/ml)	r= 0.090 P= 0.373	r= 0.149 P= 0.146	r= -0.015 P= 0.880	r= -0.000 P= 0.999	r= -0.164 P= 0.102	r= -0.152 P= 0.134	r= -0.129 P= 0.200	r= -0.101 P= 0.320	r= -0.121 P= 0.230	r= -0.107 P= 0.291
GR (log nmol/min/ml)	r= 0.036 P= 0.726	r= -0.040 P= 0.701	r= -0.004 P= 0.970	r= -0.082 P= 0.429	r= -0.008 P= 0.940	r= -0.009 P= 0.934	r= -0.132 P= 0.192	r= -0.101 P= 0.326	r= -0.090 P= 0.374	r= -0.093 P= 0.366
SOD (log U/ml)	r= 0.042 P= 0.681	r= 0.010 P= 0.920	r= 0.032 P= 0.749	r= 0.066 P= 0.519	r= -0.073 P= 0.474	r= -0.076 P= 0.458	r= -0.069 P= 0.498	r= -0.080 P= 0.433	r= -0.026 P= 0.795	r= -0.028 P= 0.785
CAT (log U/ml)	r= -0.100 P= 0.322	r= -0.055 P= 0.594	r= -0.064 P= 0.523	r= 0.006 P= 0.951	r= -0.038 P= 0.709	r= -0.030 P= 0.772	r= 0.023 P= 0.817	r= -0.000 P= 1.000	r= 0.037 P= 0.711	r= 0.045 P= 0.658

n, number of participants; CIMT, carotid intima media thickness; CSWA, cross-sectional wall area; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase, CAT, catalase.

**Table 2B: Single and partial regression analyses of antioxidant enzymes and cardiovascular variables in black and white women.**

	CIMT (mm)		CSWA (mm <sup>2</sup> )		SBP (mmHg)		DBP (mmHg)		MAP (mmHg)	
<b>BLACK WOMEN (n=99)</b>										
	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>
GPx (nmol/min/ml)	r= -0.100 P= 0.333	r= -0.020 P= 0.853	r= 0.006 P= 0.955	r= 0.045 P= 0.670	<b>r= -0.273</b> <b>P= 0.007</b>	<b>r= -0.245</b> <b>P= 0.016</b>	<b>r= -0.231</b> <b>P= 0.024</b>	<b>r= -0.220</b> <b>P= 0.033</b>	<b>r= -0.335</b> <b>P= 0.001</b>	<b>r= -0.319</b> <b>P= 0.002</b>
GR (log nmol/min/ml)	r= 0.150 P= 0.145	r= 0.116 P= 0.271	r= -0.029 P= 0.778	r= -0.113 P= 0.285	r= 0.155 P= 0.132	r= 0.095 P= 0.361	r= 0.069 P= 0.502	r= 0.054 P= 0.606	r= 0.118 P= 0.252	r= 0.088 P= 0.399
SOD (log U/ml)	<b>r= -0.265</b> <b>P= 0.009</b>	r= -0.075 P= 0.477	<b>r= -0.285</b> <b>P= 0.005</b>	<b>r= -0.237</b> <b>P= 0.023</b>	r= -0.010 P= 0.921	r= 0.049 P= 0.638	r= -0.044 P= 0.667	r= -0.035 P= 0.742	r= 0.013 P= 0.904	r= 0.038 P= 0.714
CAT (log U/ml)	r= -0.160 P= 0.119	r= -0.000 P= 0.999	r= -0.110 P= 0.282	r= -0.044 P= 0.674	r= 0.048 P= 0.644	r= 0.102 P= 0.328	r= 0.014 P= 0.894	r= 0.020 P= 0.845	r= 0.060 P= 0.559	r= 0.081 P= 0.437
<b>WHITE WOMEN (n=108)</b>										
	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>	<i>Single</i>	<i>Partial</i>
GPx (nmol/min/ml)	<b>r= 0.198</b> <b>P= 0.041</b>	r= 0.087 P= 0.383	r= 0.148 P= 0.126	r= 0.059 P= 0.554	r= 0.080 P= 0.408	r= -0.038 P= 0.699	r= 0.070 P= 0.472	r= 0.005 P= 0.960	r= 0.085 P= 0.383	r= -0.008 P= 0.938
GR (log nmol/min/ml)	r= 0.069 P= 0.479	r= 0.014 P= 0.887	r= 0.026 P= 0.790	r= -0.040 P= 0.692	r= -0.047 P= 0.632	r= -0.171 P= 0.080	r= 0.037 P= 0.708	r= -0.034 P= 0.728	r= 0.002 P= 0.987	r= -0.098 P= 0.322
SOD (log U/ml)	r= -0.015 P= 0.878	r= 0.025 P= 0.804	r= -0.026 P= 0.793	r= 0.004 P= 0.969	r= -0.031 P= 0.754	r= 0.039 P= 0.695	r= -0.008 P= 0.935	r= 0.072 P= 0.466	r= -0.002 P= 0.985	r= 0.085 P= 0.391
CAT (log U/ml)	r= 0.064 P= 0.513	r= -0.041 P= 0.682	r= 0.036 P= 0.715	r= -0.027 P= 0.788	r= 0.025 P= 0.798	r= -0.111 P= 0.258	r= -0.038 P= 0.701	r= -0.136 P= 0.167	r= -0.032 P= 0.741	r= -0.162 P= 0.099

n, number of participants; CIMT, carotid intima media thickness; CSWA, cross-sectional wall area; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase, CAT, catalase.

## Multivariate analyses

In multiple regression analyses the positive association between GR activity and CIMT ( $R^2=0.275$ ;  $\beta=0.196$ ;  $p=0.037$ ) in the black men was confirmed to be independent of various confounding factors (Table 3A). In the black women, the negative associations between GPx activity and SBP ( $R^2=0.184$ ;  $\beta=-0.251$ ;  $p=0.009$ ), and between GPx activity and MAP ( $R^2=0.131$ ;  $\beta=-0.303$ ;  $p=0.003$ ) were also confirmed to be independent of various confounding factors (Table 3B).

## Sensitivity analyses

Sensitivity analyses were performed in which the multiple regression analyses were repeated after excluding HIV infected men ( $n=13$ ) and women ( $n=6$ ), and women using hormonal contraception ( $n=24$ ). After excluding HIV infected participants it was found that the association between CIMT and GR displayed borderline significance in the black men ( $R^2=0.264$ ;  $\beta=0.187$ ;  $p=0.064$ ), while in the black women it was found that the associations between GPx and SBP, GPx and DBP as well as GPx and MAP all remained significant (SBP:  $R^2=0.174$ ;  $\beta=-0.247$ ;  $p=0.013$ ; DBP:  $R^2=0.057$ ;  $\beta=-0.217$ ;  $p=0.040$ ; MAP:  $R^2=0.123$ ;  $\beta=-0.305$ ;  $p=0.004$ ) After excluding women using contraception, the association between GPx and SBP ( $R^2=0.179$ ;  $\beta=-0.154$ ;  $p=0.030$ ), as well as the association between GPx and MAP ( $R^2=0.150$ ;  $\beta=-0.156$ ;  $p=0.030$ ) remained significant.

**Table 3A: Multiple regression analyses of antioxidant enzymes and cardiovascular variables in black and white men.**

	Model 1: CSWA (mm <sup>2</sup> )		Model 2: CIMT (mm)	
<b>BLACK MEN (n=101)</b>				
	<i>Adj R<sup>2</sup>: 0.236</i>		<i>Adj R<sup>2</sup>: 0.275</i>	
<b>Independent variables</b>	<b>β (95% CI)</b>	<b>P</b>	<b>β (95% CI)</b>	<b>P</b>
GR (log nmol/min/ml)	0.114 (-0.070; 0.299)	0.227	0.196 (0.014; 0.378)	0.037
Age (years)	0.281 (0.072; 0.489)	0.010	0.446 (0.265; 0.628)	<0.001
BMI (kg/m <sup>2</sup> )	-	-	0.187 (0.007; 0.366)	0.045
TEE (kcal/day)	-	-	-	-
GGT (log U/l)	-	-	-0.102 (-0.286; 0.083)	0.282
Cotinine (ng/ml)	-	-	-	-
Glycated heamoglobin (%)	-	-	-	-
CRP (log mg/l)	0.195 (-0.011; 0.401)	0.067	-	-
Total cholesterol to HDL ratio (mg/dL)	-	-	-	-
MAP (mmHg)	0.177 (-0.007; 0.361)	0.063	-	-
<b>WHITE MEN (n=101)</b>				
	<i>Adj R<sup>2</sup>: 0.405</i>		<i>Adj R<sup>2</sup>: 0.461</i>	
<b>Independent variables</b>	<b>β (95% CI)</b>	<b>P</b>	<b>β (95% CI)</b>	<b>P</b>
GR (log nmol/min/ml)	-0.112 (-0.272; 0.047)	0.171	-0.100 (-0.253; 0.053)	0.204
Age (years)	0.536 (0.377; 0.695)	<0.001	0.620 (0.468; 0.773)	<0.001
BMI (kg/m <sup>2</sup> )	0.164 (0.006; 0.323)	0.045	0.095 (-0.057; 0.247)	0.223
TEE (kcal/day)	-	-	-	-
GGT (log U/l)	0.196 (0.036; 0.356)	0.018	0.203 (0.050; 0.355)	0.011
Cotinine(ng/ml)	0.205 (0.049; 0.360)	0.011	0.164 (0.016; 0.313)	0.033
Glycated heamoglobin (%)	-	-	-	-
CRP(log mg/l)	-	-	-	-
Total cholesterol to HDL_ratio (mg/dL)	-	-	-	-
MAP (mmHg)	0.135 (-0.025; 0.295)	0.101	0.105 (-0.048; 0.259)	0.180

Variables included in the models were age, body mass index, total energy expenditure, γ-glutamyl transferase, cotinine, glycated heamoglobin, C-reactive protein, antioxidant enzymes and the total cholesterol to high-density lipoprotein\_ratio (CIMT and CSWA were additionally adjusted for mean arterial pressure). n, number of participants; CSWA, cross-sectional wall area; CIMT, carotid intima media thickness; GR, glutathione reductase; BMI, body mass index; TEE, total energy expenditure; GGT, γ-glutamyl transferase; CRP, C-reactive protein; HDL, high-density lipoprotein, MAP, mean arterial pressure.

**Table 3B: Multiple regression analyses of oxidative stress enzymes and cardiovascular variables in black and white women.**

	Model 1: SBP (mmHg)		Model 2: DBP (mmHg)		Model 3: MAP (mmHg)	
<b>BLACK WOMEN (n=99)</b>						
	<i>Adj R<sup>2</sup>: 0.184</i>		<i>Adj R<sup>2</sup>: 0.074</i>		<i>Adj R<sup>2</sup>: 0.131</i>	
<i>Independent variables</i>	<i>β (95% CI)</i>	<i>P</i>	<i>β (95% CI)</i>	<i>P</i>	<i>β (95% CI)</i>	<i>P</i>
GPx (nmol/min/ml)	-0.251 (-0.435; -0.066)	0.009	-0.200 (-0.399; -0.001)	0.051	-0.303 (-0.496; -0.109)	0.003
Age (years)	0.266 (0.076; 0.457)	0.007	-	-	0.118 (-0.080; 0.316)	0.247
BMI (kg/m <sup>2</sup> )	-	-	-	-	-	-
TEE (kcal/day)	0.165 (-0.025; 0.355)	0.091	0.173 (-0.036; 0.381)	0.108	0.177 (-0.028; 0.383)	0.094
GGT (log U/l)	-0.098 (-0.285; 0.090)	0.310	-	-	-0.099 (-0.295; 0.096)	0.322
Cotinine (ng/ml)	-	-	-0.128 (-0.331; 0.075)	0.220	-0.134 (-0.331; 0.063)	0.186
Glycated heamoglobin (%)	0.168 (-0.024; 0.360)	0.090	0.137 (-0.065; 0.339)	0.187	0.135 (-0.066; 0.337)	0.192
CRP (log mg/l)	-	-	-0.172 (-0.381; 0.037)	0.110	-0.107 (-0.312; 0.099)	0.313
Total cholesterol to HDL_ratio (mg/dL)	-	-	-	-	-	-
<b>WHITE WOMEN (n=108)</b>						
	<i>Adj R<sup>2</sup>: 0.291</i>		<i>Adj R<sup>2</sup>: 0.142</i>		<i>Adj R<sup>2</sup>: 0.225</i>	
<i>Independent variables</i>	<i>β (95% CI)</i>	<i>P</i>	<i>β (95% CI)</i>	<i>P</i>	<i>β (95% CI)</i>	<i>P</i>
GPx (nmol/min/ml)	-	-	-	-	-	-
Age (years)	0.464 (0.304; 0.624)	<0.001	0.231 (0.054; 0.409)	0.012	0.348 (0.180; 0.517)	<0.001
BMI (kg/m <sup>2</sup> )	-	-	0.259 (0.063; 0.456)	0.011	0.288 (0.101; 0.475)	0.003
TEE (kcal/day)	0.314 (0.153; 0.474)	<0.001	-	-	-	-
GGT (log U/l)	-	-	-	-	-	-
Cotinine (ng/ml)	-	-	-	-	-	-
Glycated heamoglobin (%)	-	-	-	-	-	-
CRP (log mg/l)	-	-	-	-	-	-
Total cholesterol to HDL_ratio (mg/dL)	-	-	0.125 (-0.072; 0.322)	0.217	0.110 (-0.077; 0.297)	0.254

Variables included in the models were age, body mass index, total energy expenditure, γ-glutamyl transferase, cotinine, glycated heamoglobin, C-reactive protein, antioxidant enzymes and the total cholesterol to high-density lipoprotein ratio. n, number of participants; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; GPx, glutathione peroxidase; BMI, body mass index; TEE, total energy expenditure; GGT, γ-glutamyl transferase; CRP, C-reactive protein; HDL, high-density lipoprotein.

## Discussion

In this study we aimed to compare antioxidant enzyme activity between black and white men, and black and white women. In the black men it was found that GR activity, total GSH and ROS levels were all significantly higher, with no difference in GPx activity when compared to the white men. Our results confirm previous results obtained in a study performed on elderly normotensive and treated hypertensive patients [15]. In this study it was found that GSH levels and GR activity were significantly higher in the hypertensive group, with no difference in GPx activity between the groups [15]. Although their hypertensive patients were older than the black men in our study, their average blood pressure was lower than in our black men, possibly as a result of successful anti-hypertensive treatment.

As a result of an increase in GR activity, it is assumed that GSH will also increase [15], as this enzyme is responsible for the recycling of GSSG to GSH [16, 17]. Although evidence from the literature often indicates that lower GSH levels may be indicative of oxidative stress [18], it has previously been suggested that elevated GR activity and total GSH levels, as also seen in the black men of our study, can also be indicative of increased oxidative stress [15]. This notion is further strengthened in our study as the black men also presented with elevated ROS levels. Thus the higher GR activity in the black men of our study may be the result of a compensatory up-regulation of GR activity to restore GSH levels depleted during oxidative stress.

A similar profile was seen in the black women of our study, with black women having increased GR activity and total GSH, although there was no significant increase in ROS when compared to white women. Additionally, black women also had significantly lower GPx activity than their white counterparts.

Decreased GPx activity has been linked to oxidative stress [15, 19]. Superoxide anion ( $O_2^{\cdot-}$ ) which may accumulate as a result of decreased GPx activity is able to further inhibit enzyme function, and subsequently inactivate the GPx enzyme [15, 19]. Although the result obtained in our black women resemble results previously obtained, which indicated an increase in GSH levels and GR activity in hypertensive patients [15], our black women had an added disadvantage of having lower GPx activity when compared to white women. The combination of increased GR activity and decreased GPx activity, which can both result in increased GSH levels, can thus also bear witness to increased oxidative stress in the black women of our study [15].

When we investigated associations of cardiovascular variables with antioxidant enzyme activity, it was found that significantly higher GR activity was independently associated with a thicker carotid intima media in the black men. This finding adds to the result previously obtained by our research team, in which a negative association between CIMT and GSH levels was found in hypertensive black men [9]. This result suggests that attenuated GSH levels may contribute to atherosclerosis development in these participants [9], whereas the positive association we found between CIMT and GR activity may suggest that increased oxidative stress, as indicated by an up-regulation of GR activity, also contributes to the thickening of the arterial wall which is associated with atherosclerosis development [20].

The mechanism to explain the link between CIMT and oxidative stress may involve oxidation of low-density lipoprotein (LDL) cholesterol, which is absorbed by macrophages, and subsequently leads to foam cell formation [5]. Foam cells then accumulate in the sub-endothelial space to form fatty streaks associated with atherosclerosis [5].

In addition to the oxidation of LDL cholesterol and the consequences thereof, hyperglycemia may also enhance the observed oxidative stress in our black men and women. Our results showed significantly higher HbA1c in the black men and women when compared to their white counterparts. Hyperglycaemia has been shown to directly increase ROS through glucose oxidation and non-enzymatic protein glycation [20, 21]. Hyperglycemia can furthermore facilitate  $O_2^{\cdot-}$  leakage from the respiratory chain and activate the NADPH oxidase enzyme [22].

In this study we also indicated an independent association of decreased GPx activity with increased blood pressure in black women. Our results are in line with two previous studies suggesting a link between decreased GPx activity and increased blood pressure as it was shown that the activities of antioxidant enzymes (including GPx) were decreased in hypertensive participants when compared to the normotensive participants [18, 23]. The decreased GPx activity and consequent link with increased blood pressure observed in the black women may be as a result of various contributing factors. These factors include possible inactivation of the GPx enzyme, which may be induced by increased  $O_2^{\cdot-}$  [15, 18]. Decreased GPx activity may also be the result of down-regulated gene expression [23], due to a genetic polymorphism. Such a genetic polymorphism was previously indicated in African Americans [24, 25]. Furthermore, since GPx is a seleno-protein, which require the availability of selenium for its action, the activity of GPx may also be affected by a selenium deficiency in these subjects [26]. The mechanism to explain the link between decreased GPx activity and increased blood pressure may involve the ability of increased ROS, due to decreased antioxidant enzyme activity in this case, to decrease the bio-availability of nitric oxide. Since decreased nitric oxide bio-availability may contribute to endothelial dysfunction through a decrease in

endothelium-dependent vasodilation [4, 27], it may result in increased blood pressure.

This study has to be interpreted within the context of its limitations and strengths. This was a cross-sectional study to investigate the association of cardiovascular variables with antioxidant enzyme activity in a bi-ethnic population, and therefore we cannot infer causality. Although our results were consistent after adjusting for various confounders, we cannot exclude any unknown factors associated with cardiovascular variables and antioxidant enzyme activity. Due to the fact that our study measured total glutathione levels, it is unknown whether GSH or GSSG is increased in our black men and women, which is a limitation that warrants further investigation. Furthermore, our method of ROS measurement is a non-specific method, and improved methods are needed in future studies. Oxidative stress cannot be fully assessed without including a vast number of oxidative stress biomarkers, and further inclusion of such biomarkers could improve the classification of oxidative stress status in our participants. It is also recommended that additional research be performed in order to investigate if the link between oxidative stress and CIMT is mediated through the oxidation of LDL cholesterol in our black population. Further research is also required to confirm whether these results are a common occurrence among the general population, as our study had a very specific cohort. However, our study was well planned and executed under strictly controlled conditions and it sheds light on the poorly researched antioxidant enzyme system in our South African population, which is a great strength in our study.

## **Conclusions**

Black men had significantly higher GR activity, total GSH and ROS, while the black women had significantly higher GR activity, total GSH, but significantly lower GPx activity when compared to their white counterparts. From these results we may deduce that both the black men and black women have higher oxidative stress than their white counterparts. The independent positive association between CIMT and GR in the black men may propose a role for increased oxidative stress, as indicated by increased activity of GR, in arterial remodelling which could enhance the development of atherosclerosis in these participants. Furthermore, the significant negative association between GPx activity and blood pressure in the black women may suggest that oxidative stress as a result of reduced GPx activity may facilitate hypertension development in the black women.

## **Acknowledgements**

The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study would not have been possible without the voluntary collaboration of the participants and the Department of Education, North-West Province, South Africa. We gratefully acknowledge the technical assistance of Mrs Tina Scholtz, Dr Szabolcs Peter and Sister Chrissie Lessing.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged; the National Research Foundation Thuthuka (80643); the North-West University, Potchefstroom; Roche Products (Pty) Ltd, South Africa and the Metabolic Syndrome Institute, France.

## **Declaration of interest**

The authors have no conflicts of interest to disclose. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

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# CHAPTER 4:

# CONCLUDING CHAPTER



## **SUMMARY OF THE MAIN FINDINGS**

Our first hypothesis was that antioxidant enzyme activities would be lower in black participants than in the white participants. Our results indicated that glutathione peroxidase (GPx) activity was lower in black women than in white women, whereas no difference was observed when comparing black and white men. Additionally, glutathione reductase (GR) activity was higher in black men and black women when compared to their white counterparts. This hypothesis is therefore partially accepted based on the significantly lower GPx activity in the black women.

Our second hypothesis was that cardiovascular variables will exhibit an inverse relationship with antioxidant enzyme activity in black participants. We only found significant negative associations between GPx and systolic blood pressure (SBP), and GPx and mean arterial pressure (MAP) in the black women. However we found a positive association between carotid intima media thickness (CIMT) and GR in the black men. This hypothesis is therefore partially accepted based on the inverse relationship between GPx and SBP and GPx and MAP in black women.

## **DISCUSSION OF THE MAIN FINDINGS**

The antioxidant defence mechanism is an intricate cycle of events in which various enzymes work together as links in a chain in order to protect the body from oxidative damage [1, 2]. A malfunction in just one link in this chain could have downstream effects of either increasing the accumulation of ROS, or decreasing the activity of the antioxidant enzymes, with oxidative stress as consequence [1, 3, 4].

Oxidative stress is known to play a role in the development of cardiovascular diseases such as hypertension [5].

Although the literature on this topic is relatively limited in our South African population, previous studies from our research team have shown that black women had higher ROS levels than black men, and hypertensive men had higher reactive oxygen species (ROS) levels than normotensive men. They suggested that the increased ROS was involved in the development of hypertension and arterial stiffness among these participants [6]. Furthermore, it was noted that hypertensive black men exhibited a decreased antioxidant capacity as glutathione (GSH) levels were lower in this group. This decrease in GSH was later associated with an increase in CIMT, thus proposing that atherosclerosis development may be stimulated in these participants [7]. Both of these studies shed light on the relationship between oxidative stress and cardiovascular variables in our population, but we intended to investigate whether the origin of this oxidative stress is from a decreased antioxidant capacity. We suspected that there is a decrease in the antioxidant enzyme activity in the black population, and that this may be associated with cardiovascular variables in these participants.

Our first aim was to compare antioxidant enzyme activities in black and white men, and black and white women. In the present study it was found that there is an increase in the activity of GR in the black men, along with elevated ROS and GSH levels when compared with white men. The observation of increased GR activity and total GSH in the black men of our study can be seen as an indication of increased oxidative stress in this group [8], which is further supported by the significantly higher ROS levels in the black men than in the white men.

In the black women it was found that GPx activity was significantly lower, while GR activity and GSH levels were significantly higher when compared to white women. The increase in GR activity and decrease in GPx activity could both indicate an imbalance in the production of ROS and the antioxidant defence mechanisms, thus also suggesting increased oxidative stress in the black women of our study [8].

When we investigated associations of cardiovascular variables with antioxidant enzyme activity, it was found that significantly higher GR activity was independently associated with a thicker carotid intima media in the black men. This finding adds to the result previously obtained by our research team in which a negative association between CIMT and GSH levels was found in hypertensive black men [9]. Although we did not display attenuated GSH levels, but rather significantly increased GSH levels in our study, the positive association we found between CIMT and GR activity may suggest that increased oxidative stress, as indicated by up-regulated GR activity and increased ROS levels, also contributes to the thickening of the arterial wall. Thus we suggest that although the CIMT is not indicative of atherosclerosis as of yet, there is a possibility for atherosclerosis development among these participants. Results in the women displayed negative associations between GPx and blood pressure in the black women. It is well known that oxidative stress plays an important role in hypertension development [1, 5], possibly due to the development of endothelial dysfunction [1, 9]. We thus suggest that the state of oxidative stress in our black women may facilitate hypertension development in these participants.

## **CONCLUSION**

In conclusion, increased oxidative stress is noted in both black men and black women. The positive association between GR activity and CIMT in black men suggests a possible role of oxidative stress in arterial remodelling, which could eventually lead to atherosclerosis development in the black men. The negative association between GPx and blood pressure in black women suggests that oxidative stress may stimulate hypertension development in these participants.

## **RECOMMENDATIONS FOR FUTURE RESEARCH**

The authors would like to make the following recommendations for further research in the field of cardiovascular variables and antioxidant enzymes:

- As we measured the total GSH levels, it is unknown whether GSH or GSSG is increased in our black men. In future studies these glutathione species should be measured separately in order to evaluate the redox status of our population.
- The method of ROS measurement used in our study is a non-specific method, and more specific methods are needed for advancements in the field of oxidative stress research.
- Oxidative stress cannot be fully assessed without including a vast number of oxidative stress biomarkers, and further inclusion of such biomarkers could improve the classification of oxidative stress status in our participants.

- There are vast opportunities in researching whether oxidized low-density lipoprotein (LDL) cholesterol is involved in the link between CIMT and GR in our population.
- Follow-up studies are needed to confirm cause and effect.
- This study cannot be generalized to a broader population as our study had a very specific cohort. Thus further research is needed to confirm whether these results are a common occurrence among the general population. Our cohort is older, and many participants are already hypertensive. Our association found in the black men could possibly be driven by the hypertensive patients in our cohort; therefore it should be investigated if this association is also present in healthy young black men.

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# APPENDICES



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18 September 2014

Dear Dr Mels

**Ethics Application: NWU-00036-07-A6 "The association of antioxidant enzyme activity with cardiovascular variables in a bi-ethnic population: The SABPA study"**

Thank you for the amendments to your application. Your application to include the sub-study under the SABPA umbrella project has been approved and ethical approval is granted until 31/12/2015.

Yours sincerely

Prof Minrie Greeff  
Health Research Ethics Committee Chairperson

Original details: Prof Minrie Greeff(10187308) C:\Users\13210572\Documents\ETIEK\2012 ETHICS\NWU-00036-12-A6 (C Mels-C van Zyl) - Approval letter.docm  
18 September 2014

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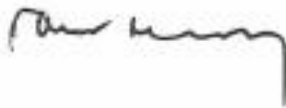
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**Declaration about language editing: Ms Caitlynd van Zyl (student number 22286233)**

I hereby confirm that I have done the language editing of Ms Caitlynd van Zyl's dissertation *The association of antioxidant enzyme activity with cardiovascular variables in a bi-ethnic population: the SABPA study*, submitted for the degree *Magister Scientiae* in Physiology on the Potchefstroom Campus of the North-West University.

I am not an accredited language editor or translator, but over the past few years I have successfully edited a number of theses and dissertations, as well as having done various editing and translation tasks for the NWU itself. In 2009 I was awarded a PHD degree in Pastoral Theology on the Potchefstroom Campus.



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