Comparing glutathione peroxidase and glutathione reductase activity and their associations with cardiovascular measures in Africans and Caucasians: The SABPA study

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Preface

This study serves as the submitted dissertation in fulfilment of the degree Magister Scientiae in Physiology. The article format was used for this dissertation and contains a manuscript which is ready or submission. The peer reviewed journal, Hypertension Research is considered for submission of the manuscript in Chapter 3. The dissertation's structured format is as follows: Chapter 1 consists of the background and motivation of this study. Chapter 2 contains a topic specific literature overview, concluding thoughts on the literature, motivation for the study and lastly the hypotheses. Chapter 3 contains the instructions to authors for the journal Hypertension Research and the actual manuscript of the study, titled: *Comparing glutathione peroxidase and glutathione reductase activity and their associations with cardiovascular measures in Africans and Caucasians: The SABPA study.* The manuscript consists of a cover letter, a title page, abstract, introduction, methods, results, discussion, disclosure and lastly acknowledgements. Chapter 4 contains the concluding remarks. Each chapter contains appropriate references according to the bibliographic style of the journal *Hypertension Research* specified in the authors instructions.

Acknowledgements

"*Wisdom is supreme; therefore get wisdom. Though it cost all you have, get understanding!"*
Proverbs 4, verse 7(NIV)

Thank you heavenly Father for all my blessings and the talents you have entrusted to me, foremost my love of knowledge.

"I will instruct you and teach you in the way you should go; I will counsel you with my loving eye on you." Psalm 32, verse 8 (NIV)

Furthermore I would like to thank:

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Dr Carina Mels, my supervisor, for her motivation, guidance and patience through my journey in learning the basic principles of research. Her knowledge and dedication to research would inspire any student to take on the life of an academic.

"Whoever loves discipline loves knowledge, but whoever hates correction is stupid"
Proverbs 12, verse 1 (NIV)

Prof Johannes van Rooyen, my co-supervisor, I am very privileged to have had the opportunity to learn from him. I stand in awe of his knowledge and his love of God and God's children. He is an inspiration to all and I thank him sincerely for the kindness and understanding he always shows me.

"Wisdom is with aged men, with long life is understanding" Job 12, verse 12 (NIV)

I would also like to thank my parents, Machiel and Zurietta Schoeman. If I could have but a fraction of their faith, compassion, resilience and strength I know I will succeed in every task at hand. I can truly say I am richly blessed with great parents. Thank you for raising me within the fear of God and teaching me to always finish what I have started

"Listen my son, to your father's instructions and do not forsake your mother's teachings

Proverbs 1, verse 8 (NIV)

"Even when I am old and grey, do not forsake me, my God, till I declare your power to the next generation, your mighty acts to all who are to come" Psalm 71, verse 18 (NIV)

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List of Abbreviations

(NAD(P)H)	reduced nicotinamide dinucleotide (phosphate)
$\bullet O_2^-$	superoxide (anion)
$\bullet OH^-$	hydroxyl radical
ACR	albumin to creatinine ratio
AIDS	acquired immunodeficiency syndrome
ABPM	ambulatory blood pressure monitoring
Ang II	angiotensin II
BH_4	tetrahydrobiopterin
BMI	body mass index
BP	blood pressure
BSA	body surface area
BSO	buthionine sulfoximine
BV	blood vessels
CAD	coronary artery disease
CAT	catalase
CHD	coronary heart disease
cIMT	carotid intima-media thickness
CRP	C-reactive protein
CSWA	cross-sectional wall area
Cu/Cu^+	copper/copper -I- ion
CV	cardiovascular
CVD	cardiovascular disease
DBP	diastolic blood pressure
ECG	electrocardiogram
ESC	European Society of Cardiology
ECM	extracellular matrix

eCrCl	estimated creatinine clearance
eNOS/NOS-3	endothelial nitric oxide synthase
ESH	European Society of Hypertension
Fe^{+2}	iron -II- ion
GGT	gamma-glutamyl transferase
GPx	glutathione peroxidase
GPx-1	erythrocyte glutathione peroxidase
GPx-3	plasma glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
H^{+}	hydrogen ion
H_2O	water
H_2O_2	hydrogen peroxide
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HIV	human immunodeficiency virus
HR	heart rate
HT	hypertensive
ICAM	intercellular adhesion molecule
IL-1	interleukin-1
iNOS/NOS-2	inducible nitric oxide synthase
ISH	International Society of Hypertension
LDL-C	low density lipid cholesterol
LVH	left ventricular hypertrophy
MAP	mean arterial pressure
MMP	matrix metalloproteinase

$NAD(P)^+$	oxidized nicotinamide dinucleotide (phosphate)
NCDs	non-communicable diseases
NF_kB	nuclear factor kappa B
nNOS/NOS-1	neuronal nitric oxide synthase
NO/•NO	nitric oxide /radical
NOS	nitric oxide synthase
NT	normotensive
O_2	diatomic/molecular oxygen
O_3	triplet oxygen/ozone
$OONO^-$	peroxynitrite
PKC	protein kinase C
PP	pulse pressure
PRAR-y	peroxisome proliferators activated receptor-gamma
PRAR-α	peroxisome proliferators activated receptor-alpha
PWV	pulse wave velocity
Redox	reduction-oxidation
RNS	reactive nitrogen species
ROS	reactive oxygen species
SABPA	Sympathetic Activity and Ambulatory Blood Pressure in Africans
SAHNES-1	South African National Health and Nutrition Examination Survey
SBP	systolic blood pressure
Se	selenium
SOD	superoxide dismutase
SOD-1	copper/Zinc cofactor dependent superoxide dismutase
SOD-2	manganese cofactor dependent superoxide dismutase
SOD-3	copper/Zinc co-factor dependent (tissue) superoxide dismutase
TC	total cholesterol

TNF	tumour necrosis factor
TPR	total peripheral resistance
VCAM	vascular cell adhesion molecule/protein
WHO	World Health Organization

Contribution of authors

Each researcher contributed to the study as follows:

Ms. ZM Schoeman (BSc Hons) was responsible for the collection of topic-specific literature, execution of statistical analyses, interpretation of results, design and writing of the manuscript.

Dr. CMC Mels (PhD), the study supervisor, gave criticism and professional input with the writing of the literature study, assisted in the initial planning and design of the manuscript, made recommendations and gave guidance in the writing of the manuscript and aided in the interpretation of the results and assisted with technical aspects. Dr. Mels also played a critical part in gaining funding for this project and further served as communicational link among all parties.

Prof JM van Rooyen (DSc), the co-supervisor, gave criticism and professional input with the writing of the literature study, made recommendations in the study methodology and gave guidance in the writing of the manuscript, brought technical faults to the student's attention and aided in the interpretation of the results.

I, ZM Schoeman, hereby declare that the above statement is an accurate representation of my contributions to this study and give my full permission that this manuscript may be published.

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Ms Zurietta M Schoeman (BSc Hons)

The above statement confirms the individual roles of the co-authors, and validates their permission that this manuscript may form part of the dissertation.

Dr Catharina MC Mels	Prof Johannes M van Rooyen

Opsomming

Titel

Vergelyking van glutatioon perokidase en glutatioon reduktase aktiwiteit en die assosiasie met kardiovaskulêre metings in Afrikane en Kaukasiërs: Die SABPA studie

Motivering

Verskeie faktore kan bydra tot die verandering in die voorkoms van kardiovaskulêre (KV) siektes wat in verstedelikte Swart Afrikane waargeneem word. Hierdie faktore sluit ondermeer veranderinge in eetpatrone asook blootstelling aan vrye radikale in. ‘n Beduidende aantal studies dui aan dat oksidatiewe stress op verskeie vlakke betrokke is in die patogenese van hipertensie, insluitende vaskulêre hermodellering en veranderinge in die tonus van weerstandsarteries. Die endogene antiokisidantensieme glutatioon peroksidase en glutatioon reduktase werk gesamentlik om intrasellulêre oksidant opeenhoping asook die gevolge daarvan teen te werk. Beide hierdie ensieme se aktiwiteite verskil op verskillende bloeddrukvlakke. ‘n Verskil in glutatioon peroksidase (GPx) aktiwiteit tussen Kaukasiërs en Afro-Amerikaners is getoon met beduidende laer GPx aktiwiteit in Afrikane. Etniese verskille in GR aktiwiteit is egter nog nie uitgewys nie. Slegs een studie het daarin geslaag om ‘n negatiewe assosiasie tussen ambulatoriese bloeddruk en GPx aktiwiteit aan te toon in hipertensiewe deelnemers. Geen onafhanklike verhouding tussen beide GPx en GR-aktiwiteite en KV-merkers soos sistoliese en diastoliese bloeddruk, intimamedia dikte of die meegewendheid van bloedvate is al aangetoon nie.

Doel

Die doel van die studie is eerstens om GPx en GR-aktiwiteit in Afrikane en Kaukasiërs te bepaal en die twee groepe te vergelyk. Daar is verder ondersoek ingestel of daar ‘n verhouding bestaan tussen kardiovaskulêre metings, insluitende ambulatoriese bloeddrukmetings (SBP, DBP, PP, MAP), karotis intima-mediadikte (cIMT) en polsgolfsnelheid (PWV) met GPx en GR aktiwiteite in beide etniese groepe.

Metode

Hierdie substudie vorm deel van *die Sympathetic Activity and Ambulatory Blood Pressure in Africans* (SABPA) studie, uitgevoer vanaf Februarie 2008 tot Mei 2009. Vierhonderd-en-nege onderwysers (Afrikane, n= 200 en Kaukasiërs, n=209) is ingesluit in die multidissiplinêre vergelykende kohortstudie vanuit die Dr Kenneth Kaunda opvoedingsdistrik, Noordwesprovinsie, Suid-Afrika. Die studieprotokol is deur die etiese komitee van die Noordwes-Universiteit goedgekeur en volg die etiese riglyne soos vervat in die Verklaring van Helsinki. Ambulatoriese metings is vir die periode vanaf 08h00 tot 06h00 geneem en sluit die volgende veranderlikes in: sistoliese bloeddruk (SBP), diastoliese bloeddruk (DBP), gemiddelde arteriële bloeddruk (MAP), polsdruk (PP), en harttempo (HR). Urienmonsters is oornag versamel vir ‘n tydperk van agt ure. Antropometriese metings is vroegoggend geneem en sluit in gewig en lengte; wat gebruik is om die liggaamsmassa-indeks (BMI) te bereken. Rustende karotis dorsalis-pedis polsgolfsnelheid (cdPWV) is daarna geneem. cIMT is bepaal met ultraklank en die gemiddelde karotis deursnit- wandoppervlak is bepaal (CSWA). ‘n Geregistreerde verpleegster het bloedmonsters versamel. Vastende glukose vlakke is in natruimfluoriedplasma bepaal, terwyl hoë sensitiwiteit C-reaktiewe proteïen (CRP), kotinien (metaboliete van nikotien), en gamma-glutamieltransferase (GGT) in serummonsters bepaal is. Urienmonsters is gebruik om kreatinien en albumien vlakke te bepaal en daarvolgens is kreatinien opruiming (eCrCl) bereken. Totale serumperoksides (ROS), GPx aktiwiteit en GR aktiwiteit asook totale glutatioon (GSH) vlakke is bepaal. Die volgende biochemiese metings wat nie normaal versprei is nie, is logaritmies getransformeer: glukose, CRP, hoë-digtheid lipoproteïen (HDL) cholesterol, trigliseriede, GR aktiwiteit, ROS, GGT en eCrCl. Met behulp van basiese statistiese verwerkings (t-toetse en Chi^2- toetse) is die fisiologiese eienskappe tussen die twee etniese groepe vergelyk. Verdere analises is gedoen om korrelasies en onafhanklike verhoudings te ondersoek.

Resultate

Ons doel was om veranderlikes in etniese groepe te vergelyk en daarom is die studiedeelnemers verdeel volgens ras. Kardiovaskulêre metings, insluitende ambulatoriese SBP, DBP, MAP, PP en HR asook vaskulêre funksionele (cdPWV) en strukturele (cIMT en CSWA) merkers is betekenisvol hoër in Afrikane. Oksidatiewe stresmerkers soos ROS, en merkers van anti-oksidant-kapasiteit soos GR en totale GSH is hoër, terwyl GPx aktiwiteit laer is in Afrikane in vergelyking met Kaukasiërs. Verder is gevind dat cIMT betekenisvol korreleer met GR aktiwiteit in Afrikane maar na aanpassing vir ouderdom, geslag, BMI en GGT was die korrelasie nie meer betekenisvol gewees nie. Daar is korrelasies tussen cdPWV en GR aktiwiteit asook eCrCl en GPx aktiwiteit in die Kaukasiërgroep. Nadat aanpassings gemaak is vir ouderdom, geslag, GGT en kotinien het die korrelasie betekenisvol gebly, ‘n korrelasie tussen cdPWV en GPx aktiwiteit ($p<0.01$) was ook nou betekenisvol. Die korrelasie tussen eCrCl en GPx aktiwiteit is nie meer betekenisvol nie. In die voorwaartse stapsgewyse liniêre regressive-analise is aangetoon dat cdPWV onafhanklik negatief geassosieer word met GPx aktiwiteit in Kaukasiërs. Sensitiwiteitsanalise, waar persone wat anti-oksidante of hipertensiemedikasie gebruik sowel as HIV geïnfekteerde persone uitgelaat is, het geen noemenswaardige verandering aan die resultate gemaak nie.

Gevolgtrekking

Biochemiese analise het beduidende verskille getoon tussen die aktiwitiete van GPx en GR tussen Afrikane en Kaukasiërs. Kaukasiërs vertoon hoër GPx aktiwiteit en laer GR aktiwiteit as Afrikane. Die hoër GPx-aktiwiteit is geassosieer met verlaagde cdPWV. Die resultate toon beskermende effekte teen arteriële styfheid in Kaukasiërs. GPx aktiwiteit kan moontlik laer wees in Afrikane as gevolg van ‘n seleniumtekort of ‘n GPx-polimorfisme. In verdere studies moet die seleniumstatus van deelnemers bepaal word voordat die kliniese toepasbaarheid van seleniumaanvullings om GPx aktiwiteit te verhoog ondersoek kan word om sodanig arteriële styfheid te voorkom.

Summary

Title

Comparing glutathione peroxidase and glutathione reductase activity and their associations with cardiovascular measures in Africans and Caucasians: The SABPA study

Motivation

Various factors may contribute to the changing prevalence of cardiovascular disease (CVD) occurring amongst urbanized Black Africans. Changes in eating patterns and exposure to increased levels of oxidants may play a role. Accumulative amounts of data indicate the role that oxidative stress may play in the pathogenesis of hypertension, including changes in vascular tone of resistance arteries and vascular remodelling. The endogenous antioxidant enzymes glutathione peroxidase and glutathione reductase work synergistically to maintain intracellular redox balance. Both these enzymes have been indicated to have varying levels of activities with changes in blood pressure. Furthermore, GPx activity was shown to differ between Caucasians and African-Americans with Africans displaying significantly lower activity of GPx. An ethnic difference in GR activity is yet to be shown. A single study has indicated a negative association between GPx activity and blood pressure. No independent relationships have been established between both enzyme activities and the following cardiovascular measures: systolic and diastolic blood pressure, intima-media thickness and the compliance of arteries.

Aims

The aims of the study were firstly to compare glutathione peroxidase (GPx) and glutathione reductase (GR) activity between a cohort of African and Caucasian participants. A secondary aim was to explore whether relationships exist between cardiovascular measures such as ambulatory systolic and diastolic blood pressure (SBP

and DBP), carotid media thickness (cIMT) and pulse wave velocity (PWV), with these GPx activity and GR activities in both ethnic groups.

Method

This study forms part of the Sympathetic Activity and ambulatory Blood Pressure in Africans (SABPA) study, which was conducted between the period of February 2008 and May 2009. Four hundred and nine teachers (Africans, n= 200 and Caucasians, n=209) formed part of the multi-disciplinary comparative cohort study and hailed from the Kenneth Kaunda educational district, North West Province, South Africa. All procedures were approved by the ethics committee of the North-West University and conformed to the ethical guidelines of the Declaration of Helsinki. Ambulatory blood pressure measurements were taken from 08h00 to 06h00 the next day and included systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP) and heart rate (HR). Urine was collected for a period of eight hours overnight. The participants underwent anthropometric measurements which included: weight and height which were used to calculate body mass index (BMI). Thereafter cardiovascular measurements were performed, that included carotid dorsalis-pedis pulse wave velocity (cdPWV). Carotid intima-media thickness (cIMT) was determined with an ultrasound system and the mean cross-sectional wall area (CSWA) was calculated. Blood samples were taken by a registered nurse to measure fasting glucose levels in sodium fluoride plasma. The following biochemical markers were determined in serum: high sensitivity C-reactive protein (CRP) cotinine (metabolite of nicotine) and gamma glutamyl transferase (GGT). Additionally, albumin and creatinine were measured from urine samples and estimated creatinine clearance (eCrCl) was calculated. Serum peroxides (ROS) and total glutathione (GSH) levels as well as GPx and GR activities were determined. All biochemical measurements with a non-Gaussian spread were logarithmically transformed, including: fasting glucose, CRP, high density lipoprotein (HDL), triglycerides, GR activity, ROS, GGT, and eCrCl. Basic statistical analysis (t-tests and Chi- square tests) were used to depict the characteristics of the study population according to ethnicity. Pearson, partial and multiple regression analyses were used to demonstrate correlations between these two enzymes and cardiovascular variables.

Results

As the aim of this study was to compare variables between ethnic groups, participants were divided according to ethnicity. Cardiovascular measures, including ambulatory SBP, DBP, MAP, PP and HR, were significantly higher in Africans. This was also true for functional (cdPWV) and structural (cIMT and CSWA) vascular markers. ROS and total GSH levels as well as GR activity were higher, while GPx activity was lower in Africans when compared to Caucasians. Furthermore, cIMT significantly correlated with GR activity in Africans; however, after adjustment for age, gender, BMI, cotinine and GGT it was no longer significant. cdPWV and GR activity as well as eCrCl and GPx were shown to correlate in the Caucasian group. The correlation between cdPWV and GR activity remained significant with the addition of a correlation between cdPWV and GPx activity after were made. Forward stepwise linear regression showed an independent negative association between cdPWV and GPx activity in Caucasians. This remained true even after separate exclusion of subjects using anti-hypertensive medication and/or anti-oxidants as well as HIV-infected subjects.

Conclusion

Biochemical analyses indicated significant differences in the activity of GPx and GR between Africans and Caucasians. GPx activity was found to be higher and GR activity lower in Caucasians when compared to Africans. The higher activity of GPx is associated with lower cdPWV, indicating a vascular protective effect against arterial stiffening in Caucasians. GPx activity may be lower in Africans due to selenium deficiency or a polymorphism in the GPx gene. In future studies selenium status should be determined to investigate the clinical application of selenium supplementation as a measure to increase GPx activity and subsequently decrease arterial stiffening.

Chapter 1: Background and motivation

1.1 Background and motivation

Up until recently, statistical evidence indicating the true burden of hypertension among black South Africans (hereby referred to as Africans) has been lacking. Dated as well as recent small cross-sectional data indicate a high prevalence of hypertension among urbanized Africans and this is confirmed by a recently published national report, The South African National Health and Nutrition Examination Survey (SANHANES).[1-3] This study illuminates the current transitional process from optimal to elevated blood pressure levels and the accompanying changes in behavioural risk factors of South African citizens. More than 10 % of the multi-cultural randomly selected group presented with blood pressures exceeding 140/90 mmHg.[3]

Globally, hypertension is said to be the leading cause of morbidity and mortality, however, blood pressure seems to be on the decline since 1980.[4-5] When looking at area-specific data, both male and female African inhabitants have shown an increase in both systolic and diastolic blood pressure.[5] This, however, is no surprise as urbanization and westernization increase in these formally traditional communities.[6-7]

Reactive oxygen species (ROS) are kept at bay by diminutive mechanisms forming part of the antioxidant system.[8] Among various biologically active measures, the endogenous selenium-dependent anti-oxidant enzyme glutathione peroxidase (GPx) and glutathione reductase (GR) together with glutathione (GSH) fulfil an important function in maintaining intracellular reduction-oxidation (redox) balance and protecting against oxidative stress and its consequent damage.[9]

Evidence indicating oxidative stress's role in the pathogenesis of hypertension is substantial.[10-15] GPx activity has been shown to be lower in hypertensive (HT) subjects when compared to normotensive (NT) subjects.[16-18] One study has, however, indicated elevated GPx and GR activities in treated HT subjects when compared to age- and sex-matched controls.[19] Furthermore, ethnicity was shown to independently affect GPx activity[20] and subjects with an African origin had lower GPx activity than their Caucasian counterparts.[20--21] Previously, no association has been shown between GPx and GR activities with blood pressure (BP). However, variants in the GPx-1 gene decreasing

GPx activity have been suggested to be associated with increased carotid intima-media thickness (cIMT).[22]

Accordingly, Africans display higher levels of serum peroxides when compared to Caucasians of the same socioeconomic status.[23] The forementioned correlates with arterial stiffness and is independently positively associated with ambulatory systolic blood pressure (SBP) and pulse pressure (PP) in African men[23]. Total glutathione levels have also been shown to be lower in hypertensive African men and associated with a thicker carotid intima-media thickness (cIMT).[24] The activities of GPx and GR and possible associations with cardiovascular measures have yet to be explored in an African population plagued by hypertension.

We therefore aimed to determine and compare GPx and GR activities between Africans and Caucasians. Additionally, possible independent associations of ambuatory BP and other cardiovascular measures with these antioxidant enzymes were investigated.

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Chapter 2: Literature review

2.1 Oxidative stress and the anti-oxidant system

2.1.1 Reactive oxygen species

The cellular metabolism of the life-sustaining molecule, oxygen (O_2), amongst others, can generate free radicals.[1] Free radicals are molecules that contain one or more unpaired electron(s) in their atomic or molecular orbitals. These molecular species are capable of existing independently and have the ability to either donate or extract an electron from other molecules, thus behaving as oxidants or reductants, respectively. [1-2]

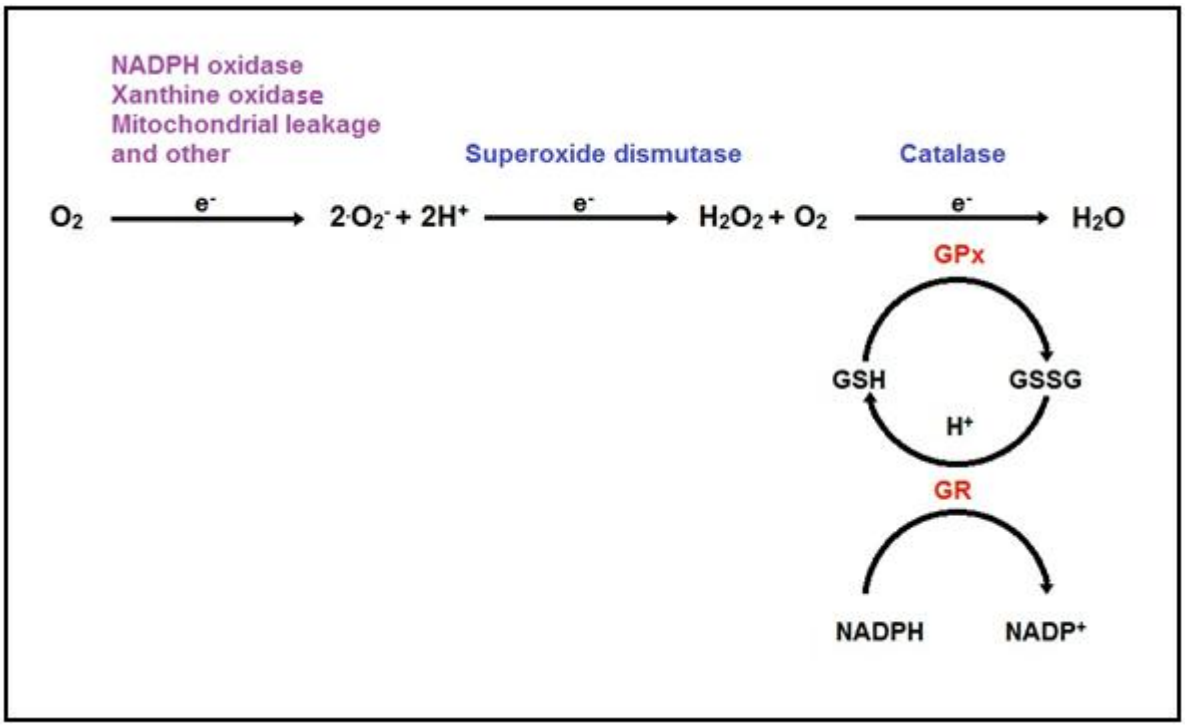

Figure 1: Sequential univalent reduction of molecular oxygen and the key roles that glutathione peroxidase and glutathione reductase play in neutralizing hydrogen peroxide. *Figure adapted from Young & Woodside*[2]

Abbreviations: oxygen (O2); reduced glutathione (GSH); glutathione peroxidase (GPx); glutathione reductase (GR); hydrogen peroxide (H_2O_2); oxidized glutathione (GSSG); superoxide ($\bullet O_2^-$); water (H_2O).

Molecular oxygen (O_2) has the ability to accept four electrons in a tetravalent reduction reaction as it occurs in the inner mitochondrial membrane during aerobic respiration. However, O_2 can also undergo a series of univalent reduction reactions to form free radicals and non-radicals as intermediates in this reduction-oxidation (redox) reaction leading from O_2 to H_2O as indicated in Figure 1.[1] The respective intermediates of the O_2 metabolism form part of a family of highly reactive oxygen products known as reactive oxygen species (ROS).

These include: superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\bullet OH^-$) which correspond to the series of reduction by one, two and three electrons respectively. Singlet oxygen ($^1O_2$) is yet another oxygen-derived radical, formed from ozone (O_3) or ground-state molecular (triplet) oxygen.[1] The forementioned radicals constitute a portion of the family of ROS molecules.[3]

2.1.2 Production of reactive oxygen species and oxidative stress

ROS are constantly being generated and destroyed as a product of both environmental factors and normal physiological processes in biological systems including the human body.[4-6]

2.1.2.1 Endogenous production of reactive oxygen species

Various enzymes are capable of forming ROS which include: xanthine oxidase, (NAD(P)H) oxidase and uncoupled nitric oxide synthase (NOS).

Xanthine oxidase forms part of the myobdoenzyme, xanthine oxidoreductase system.[7] This system consists of two inter-convertible enzymes, xanthine oxidase and xanthine dehydrogenase.[8] Xanthine oxidase reacts with oxygen to form both $\bullet O_2^-$ and H_2O_2 as byproducts of the purine metabolism, and xanthine dehydrogenase reduces oxidized nicotinamide adenine dinucleotide phosphate ($NADP^+$).[9]

The multi-subunit enzyme, (NAD(P)H) oxidase, consists of at least four components which may include: p47phox, p67phox, p40phox, p47phox and the catalytic subunit

gp91phox (now termed Nox2).[10] This enzyme is the key source of ROS in the vasculature and involved in superoxide formation.[7] During this reaction (NAD(P)H) oxidase catalyses a single electron reduction of oxygen, using (NAD(P)H) oxidase (donor) via one of the seven Nox members found in mammalians as the electron transporter.[5,11]

Vascular (NAD(P)H) oxidase activity is regulated by hormonal and hemodynamic forces such as cytokines, growth factors, vaso-active agents stretch, pulsatile strain and shear stress.[5,7,10,12] ROS are also produced by phagocytes including neutrophil and eosinophil granulocytes, monocytes and macrophages, as part of the immune response to foreign organisms, noxious agents and many bacterial strains via phagocytic $NADP^+$ oxidase known as “respiratory burst”.[1,13-15] This enzyme produces large amounts of ROS when activated and serves as part of the immune system’s first line of defence. [7,16]

Uncoupled NOS can also give rise to reactive nitrogen species (RNS), and will be discussed later.

2.1.2.2 Exogenous production of reactive oxygen species

Certain environmental exposures (exogenous factors) can lead to formation of oxidants and ultimately oxidative stress when exposed to in excess. Among other sources, tobacco smoking and the metabolism of pollutants and pesticides and xenobiotics can lead to free radical products.[17-18] Furthermore, ultra-violet light and iodizing radiation result in the formation of free radicals in exposed tissue areas.[19]

2.1.3 Reactive nitrogen species

Reactive nitrogen species (RNS) include a vast range of molecules with opposing and distinct characteristics derived primarily from the reaction of nitric oxide (NO) with physiologically generated free radicals.[20] These compounds include chemically unstable peroxynitrite ($ONOO^-$), from which downstream RNS are formed.[21]

2.1.3.1 Production of reactive nitrogen species

The biologically significant gaseous molecule NO is formed in cells by NOS (Figure 2) in the presence of tetrahydrobiopterin (BH_4) and (NAD(P)H) as co-factors and L-arginine as substrate.[22-23]

Three isoforms of NOS are known. The first two isoforms, endothelial NOS (eNOS or NOS-3) and neuronal NOS (nNOS or NOS-1) are located within the vasculature, brain and myocardium.[24] The first-mentioned releases NO in response to vascular shear stress or eNOS activation induced in response to cytokine activation.[25] While the brain releases NO to facilitate neuro-communication. Inducible NOS (iNOS or NOS-3), the third isoform, has a larger presence and is located throughout the body's immune cells.[24]

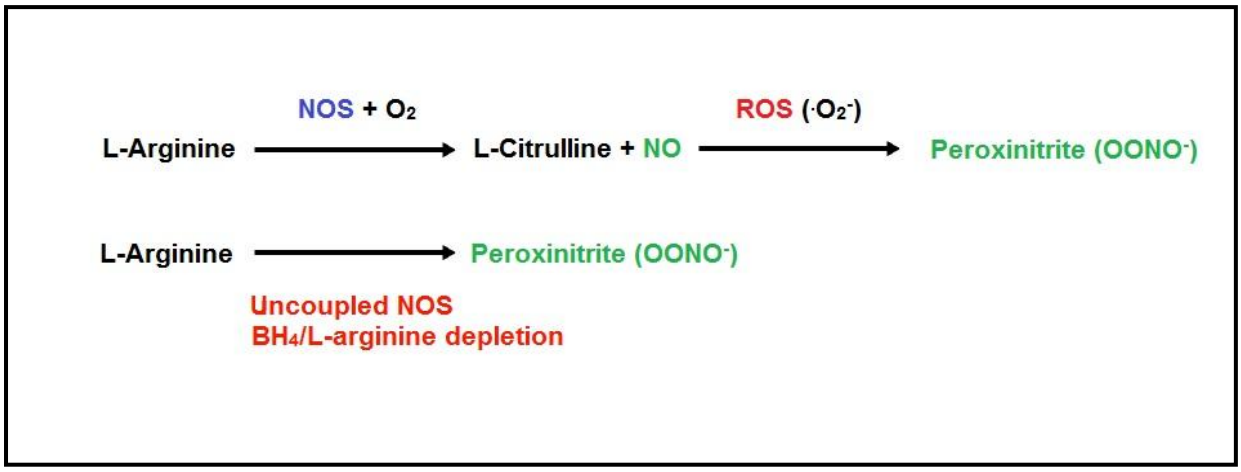

Figure 2: Production of reactive nitrogen species

The first series of reactions indicates the synthesis of the vaso-active, gaseous-free radical, nitric oxide, previously endothelial derived relaxing factor (EDRF) from the conditionally essential amino acid L-Arginine. Peroxynitrite is quickly formed through interaction with present reactive oxygen species such as superoxide. The second equation indicates the product of uncoupled nitric oxide synthase. Here nitric oxide production is ceased and the formation of peroxynitrite is favoured. *Figure adapted from Patel, et al.*[19]

Abbreviations: nitric oxide (NO); nitric oxide synthase (NOS); superoxide ($\bullet O_2^-$); oxygen (O_2) and peroxynitrite ($ONOO^-$).

NOS can contribute to the production of reactive species by formation of RNS through two mechanisms. Firstly, by endothelium derived NO reacting rapidly with ROS molecules thereby diminishing its half-life.[3] NO is bio-inactivated which may lead to the generation of highly-reactive $ONOO^-$.[3] The reaction between the highly reactive and unstable radicals, $\bullet O_2^-$ and NO occurs at an estimated rate of 6.7×10^9 M/sec almost three times the rate of superoxide dismutation.[26]

Secondly, RNS production is mediated directly through NOS-uncoupling where NOS becomes an $ONOO^-$ generator rather than an NO generator, especially in states of sub-optimal levels of L-arginine and BH_4.[5,27-28] This is achieved at the oxygenase domain of NOS-3 which can generate $\bullet O_2^-$ from the dissociation of haeme- ferrous dioxgen complex as well as by flavins in the reductase domain of eNOS.[29-31]

2.1.4 The anti-oxidant system

Under normal physiological conditions the rate and the magnitude of oxidant/radical production are balanced by the rate of oxidant elimination.[32] This constitutes the diminutive function of antioxidants and, as a whole, the antioxidant system. These molecules, in low concentrations compared to high concentrations oxidizable substrate, act by preventing or slowing down the oxidation of other molecules.[32] This is achieved by donating electrons to the oxidant/free radical.[1] This results in the neutralization of the free radicals and prevention of subsequent reduction of other molecules and damage arising as a consequence of these reactions.[2,4].

This system includes anti-oxidant enzymes and non-enzymatic/small molecular weight substances. The latter can be further divided into two groups according to their site of action; either in the lipid bi-layers of cellular membranes (lipid phase) or in the cytosol and extracellular fluids (aqueous phase).[33]

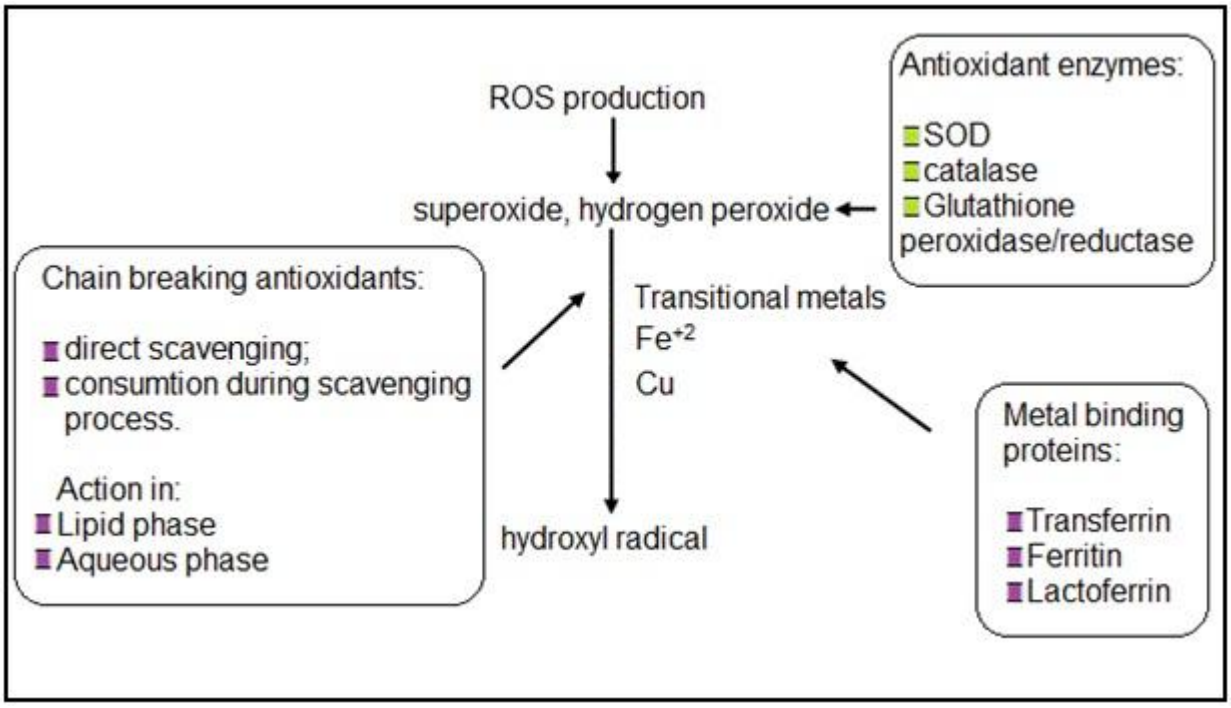

Figure 3: The antioxidant defence system includes various facets that work in on different locations to prevent oxidative stress by diminution of oxidants. *Figure adapted from Young & Woodside* [2]

Abbreviations: copper (Cu); reactive oxygen species (ROS); superoxide dismutase (SOD); Iron-II- ion (Fe^{+2}).

Ascorbate (Vitamin C), tocopherols (Vitamin E), glutathione, billirubin, uric acid, and various other nutritionally derived structures form part of the non-enzymatic defence, which can also be described as chain-breaking or quencher antioxidants.[2,4,34-35] The major antioxidant enzymes found in the vasculature include: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), thioredoxin and peroxiredoxin.[36]

Furthermore, metal-binding proteins also exert anti-oxidative action by binding transitional metal ions such as iron-II-ion (Fe^{+2}) and copper ions (Cu^{+}) and preventing $\bullet OH^{-}$ formation via the Fenton reaction.[2]

Three isoforms of SOD are present in mammalian cells, located in different cells and fluid compartments. All three isoforms are metal ion co-factor dependent. Superoxide dismutase-1 (SOD-1), also called Copper/Zinc co-factor dependent SOD (Cu/ZnSOD), is located in the cytoplasm and organelles of all cells, with two subunits each containing

either a copper or zinc atom.[37] SOD-2 or manganese cofactor dependent SOD (MnSOD) is found in the mitochondria of almost all cells.[38] The last form, SOD-3 or extracellular superoxide dismutase (EC-SOD), also contains copper and zinc co-factors, distinctly different from the previously mentioned SOD-1. SOD-3 is found in the extracellular fluids of only a few cell types including fibroblasts and endothelial cells.[39] SOD, is involved in the dismutation of $\bullet O_2^-$ at a rate of 2 x 10^9 M/sec to form H_2O_2 and O_2 as demonstrated in figure 2. [2,40]

The reaction is then followed by the reduction of H_2O_2 to water and oxygen by either the action of catalase (CAT) or GPx.[41] The tetra-heme protein subunit containing the anti-oxidant enzyme, catalase, catalyses the two-stage conversion of H_2O_2 to H_2O [42]

Selenium (Se) dependent GPx catalyses the oxidation of glutathione (GSH).[4] This nonessential amino-acid composed tripeptide is reduced at the expense of H_2O_2 or other lipid hydroperoxide molecules to its oxidized form (GSSG).[4,43] The flavine nucleotide, GR reduces GSSG to its original state with the help of [NAD(P)H] provided by the pentose phosphate pathway (Figure 1).[44]

The two major forms are GPx-1 and GPx-3.[45] GPx-1 is the ubiquitous intracellular form and a key anti-oxidant enzyme in most cells including the endothelium.[45] GPx-3 is only found in high-density lipoprotein (HDL) particles.[46-48] These enzymes are mostly located within the peroxisomes of cells in the liver and erythrocytes, but are also present in most tissue [2]

2.2 Hypertension and the prevalence of hypertension in South Africans

Hypertension is a term used to refer to a state of chronically elevated blood pressure (BP) exceeding optimal levels. The golden standard for BP measurement is 24 ambulatory blood pressure meassurement. This method differs from clinical office BP measurement as it uses a portable device to measure blood pressure at set time intervals for a continuous period of time. This allows the person to go about theire normal life which removes the whitecoat- effect. Whereas, Office meassurement measures BP only once or multiple times in a short period, with an average calculated.[49]

According to the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC), hypertension can be classified as an ambulatory systolic blood pressure (SBP) and diastolic blood pressure (DBP) in daytime greater than or equal to 130 mmHg and 80 mmHg, and at night-time/sleep of ≥120 mmHg and 70 mmHg, respectively.[49] Other guidelines are also available such as set forth by the World Health Organization (WHO) and the International Society of Hypertension (ISH) in an effort to manage hypertension. [40] It has been suggested that hypertension and cardiovascular disease (CVD) are the current leading causes of mortality and morbidity worldwide.[51] In the 2003 WHO/ISH statement on management of the disease burden, including hypertension, it was claimed that hypertension is as prevalent in many developing countries as in the developed world.[52] This is supported by The African Union (2004) proclaiming hypertension to be the largest health challenge in Africa, second to acquired immunodeficiency syndrome (AIDS). Hypertension affected 10-20 million of the 650 million people in sub-Saharan Africa in 2005.[52]

Up until recently the impact of hypertension on the South African population was elusive. Researchers relied on smaller previously conducted studies to give insight.

In 1996 Seedat already estimated that 6.5 million South Africans had a blood pressure (BP) equal to 130/85 mmHg and a further 3.2 million a BP exceeding 140/95 mmHg.[53] More recent population data regarding the prevalence of hypertension in the South African population and among the different ethnic groups of South Africa are lacking. However, smaller cross-sectional studies conducted in various ethnic groups indigenous to South Africa and various locations demonstrate a higher prevalence among urbanized Africans when compared to their Caucasian counterparts. This was demonstrated by Opie and Seedat (2005) in urbanized Zulu-speaking Africans from Durban, Kwa-Zulu Natal Province and in previously published articles of the Sympathetic Activity and Ambulatory Blood Pressure in South Africans (SABPA) study including Setswana speaking African participants from the Potchefstroom region of the North West Province.[53-55]

More recent evidence indicates a progression of epidemiological transit from infectious to non-communicable diseases (NCDs). Within this report, data from the World Health Organization's Study on Global Ageing and Adult Health (SAGE) it is indicated that

more than 70% of the South African participants (n=2583) had blood pressures of or exceeding 140 mmHg/90 mmHg, indicating hypertension. [56]

2.3 Oxidative and nitrosative stress in the vasculature

Oxidative stress is the term used to refer to a state of reduction-oxidation (redox) dysregulation. This may occur, for example, in the vasculature due to an elevated rate of oxidant production and/or failure of diminutive mechanisms such as the antioxidant system in the vasculature.[1] Oxidative stress as has been described previously a redox state favouring oxidants and leading to subsequent tissue damage, as briefly discussed in the next section.[57]

Similarly to oxidative stress, nitrosative stress refers to excessive levels of RNS due to elevated rates of production and insufficient rates of diminutive mechanism activity.[1]

2.3.1 The vasculature: Oxidative and nitrosative stress's role in hypertension

The blood vessels were first thought of as mere conduits for blood from the heart to tissue. However, it has become apparent that blood vessels are highly specialized organs creating an interface between blood and the vessel wall. It can, furthermore, actively relax and contract in response to the metabolic needs of tissue. This is detected via locally formed compounds or hormonal vaso-active agents, including prostacylin, thromboxane, endothelin, angiotensin, endothelium-derived hyperpolarizing factor, ROS and RNS, bradykinin and NO.[13, 58-59]

ROS is produced in a controlled manner at low concentrations in response to diverse stimuli such as angiotensin II (Ang II). This formation can take place in all three layers (tunica intima, media and adventitia) of the vasculature by virtually all vascular cell types including the endothelium, smooth muscle cells, adventitial fibroblasts and perivascular adipocytes.[13,60-63] ROS (largely H_2O_2) then plays a physiological role by functioning as paracrine or autocrine signalling agents within the vasculature to maintain vascular integrity by regulating endothelial function and vascular tone.[12, 64]

ROS, however, plays a pathophysiological role on factors that may contribute to endothelial injury, vascular contraction and arterial remodelling.[65] Thus, elevated levels of ROS (oxidative stress) have been implicated causatively in the development of hypertension.

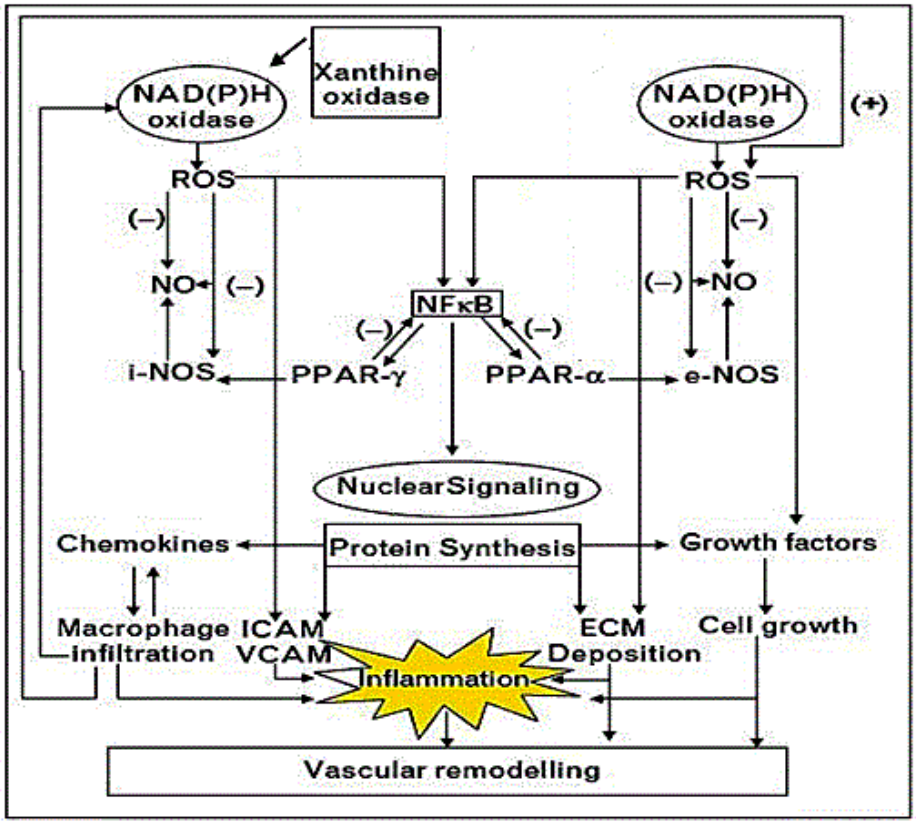

Figure 4: The role of ROS in inflammation and vascular remodelling

Note the effect that ROS has on the vaso-relaxing agent (NO) producer, NOS as well as direct effects on [NO]. ROS promotes vascular inflammation by increasing I-CAM and V-CAM expression and also promoting cell growth. NF_kB effects NO production through its effects on PPAR- α ROS; PPAR- γ. *Adapted from Savoia & Schiffrin.*[70]

Abbreviations: ICAM; VCAM; NF_KB; ECM; NAD(P)H; i-NOS; e-NOS; NO;

This concept is largely based on the disruptive effect ROS has on the micro-environment of the vasculature wall, thereby promoting endothelial dysfunction, vascular contraction and arterial remodelling.

The disruptive effects off ROS on the vasculature wall include lipid oxidation, in particular low-density lipid cholesterol (LDL-C), which is an early event in the formation of vascular lesions.[6] Also, ROS stimulates the expression of adhesion and chemotactic molecules such as vascular chemotactic adhesion molecule (VCAM) and intracellular chemotactic adhesion molecule (ICAM), thereby promoting inflammatory cell (primarily monocytes) invasion/migration into the vessel wall.[6] ROS also promotes vascular smooth muscle growth by proliferation, hypertrophy[66] and increasing the expression of matrix metalloproteinase (MMP) resulting in the induction of fibrosis[10], contributing further to vascular remodelling and also plaque rupturing.[16] Vascular remodelling can be indicated by measurement of the thickness of the two layers (luminal-side) of the vasculature wall known as the tunica intima and tunica media layer. This is a inexpensive, reproducible and noninvasive marker called the intima media thickness (IMT)[67]

Central to this, ROS function as intermediate second messengers of transcriptional factors and pro-inflammatory mediator of nuclear factor kappa B (NF_kB) activation by means of upstream stimuli such as tumour necrosis factor α (TNF-α) and interleukin -1 (IL-1).[68-69] NFkB may be involved in the production of cytokines and the regulation of inflammation (NO attenuates inflammation through modulation of NF_kB) which are both events that are associated with vascular structural changes, such as atherosclerosis.[69]PRAR-α activation decreases cellular inflammation by inhibiting NF_kB signaling pathways [69] (Figure 4).

Furthermore, elevated levels of ROS seem to participate in altered endothelium function. This is evident in the observation that oxidative stress has also been implicated in the regulation of calcium-induced signalling in the vasculature, with consequent effects on calcium-dependent protein kinase (protein kinase-C (PKC)) thus affecting vascular tone and calcineurin.[70-73] In addition to the direct vascular damage and its pro-atherosclerotic effect, ROS has also been shown to affect vascular tone by decreasing the vaso-relaxing agent, NO.[3, 5,74] NO serves a vascular-protector through its role in vascular relaxation (relaxation of vascular smooth muscle cells (VSMC)), and thereby decreasing blood pressure (BP) and increasing blood supply to tissue.[60] NO also mediates various other intracellular reactions that inhibit leukocyte chemotaxis,

platelet adhesion and other coagulant pathways thus serving as a vasodilatory, anti-inflammatory and anti-thrombotic agent.[66, 70-74]

NO bioavailability is reduced through ROS-mediated NOS-uncoupling and by rapid reaction with superoxide, leading to subsequent formation of RNS.[3,5] This may lead to altered vascular tone and increased expression and binding of VCAM, ICAM and inflammatory cells such as monocytes and leukocytes.[75] Altered vascular tone or increased stiffness effects the speed which blood travels from one arterial branch to another. This can be indicated by measurement of the pulse wave velocity (PWV)[76]

An experimental animal study with male Sprague-Dawley rats indicated that inhibition of GSH caused a three-fold increase in GSH levels accompanied by hypertension. Additionally, treatment with vitamin A and C in another group indicated protective effects through its ameliorated effects on blood pressure. The control group showed no difference in GSH- levels or blood pressure. This study thus indicates a causal effect of oxidative stress to hypertension in rats as well as therapeutic treatment of oxidative stress induced hypertension with antioxidants.[77]

Additionally, evaluation of pre-hypertensive rats and patients indicated increased oxidative stress markers.[77-79] These studies may suggest that oxidative stress precedes the elevation of BP to levels categorized as hypertension.[80]

Thus, elevated levels of ROS (oxidative stress) and the subsequent altered redox signalling accompanied by decreased NO bioavailability due to decreased production via NOS-uncoupling and reaction with ROS may lead to endothelial injury and dysfunction, preceding hypertension and other CVD. This supports the notion that ROS may be implicated in having a causative role in the pathogenesis of hypertension.

On the other side, clinical studies have indicated elevated ROS in various types of hypertension, including essential hypertension.[5] These ROS levels returned to normal when blood pressure was reduced to optimum levels, indicating elevated levels of ROS to be a result of hypertension rather than a cause. Furthermore, large clinical studies have failed to provide significant results in the treatment of hypertension with antioxidants.[81] This may be partly due to the irreversible oxidative modification of DNA, proteins and membrane lipids by RNS rendering the treatment useless to lower blood

pressure.[82-84] It has also been hypothesized that anti-oxidant treatment of hypertension may be unsuccessful as the treatment has little or no effect on RNS [83]

From available evidence elevated ROS seems to play a causative role in the pathogenesis of hypertension as well as to be a result of hypertension.

2.3.2 Glutathione peroxidase and glutathione reductase and hypertension

Previous publications from the SABPA study showed higher ROS levels in Africans when compared to Caucasians.[85] This study also found that ROS is positively associated with both 24-hour ambulatory SBP and pulse pressure (PP).[86] A negative association between carotid intima-media thickness (cIMT) and total GSH levels was noted in hypertensive African men of the same population.[87] Thus it is possible that an imbalance between ROS production and anti-oxidative system could contribute to the functional and structural alterations which are present in the hypertensive vasculature.[87]

Of particular importance for this study are the activities of GPx and GR. However, GPx and GR activities and their relation to cardiovascular measures within an African population have yet to be obtained. Furthermore, the activity of GR and its relation to cardiovascular measures has received less attention than that of GPx. Noneteheless; available cross-sectional data on both GPx and GR in Caucasian subjects are presented.

In a small cross-sectional study that included 66 untreated, non-smoking, non-diabetic, hypertensive (HT) and 16 normotensive subjects (NT), both GPx (whole blood and mononuclear cells) activity and the GSSG/GSH ratio were significantly lower in the HT group.[88] Similar results were obtained by Rodrigo et al. (2007), with GPx activity and the GSSG/GSH ratio being significantly lower in hypertensive subjects compared to normotensive subjects.

Additionally, GPx activity and the GSH/GSSG ratio correlated negatively with both 24-hour ambulatory systolic ($r=-0.38$; $p<0.05$) and diastolic blood pressure ($r=-0.42$; $p<0.05$) within the hypertensive group (n=31), whereas no correlations were found in the normotensive group (n=35). However, these relationships were not adjusted for

confounding factors and therefore not independent.[89] Decreased SOD, GPx and CAT activities were observed in another study when comparing pre-hypertensive and HT with NT subjects. Additionally, a negative unadjusted correlation was established between GPx and MAP in HT subjects.[5]

In contradiction to these results, Simic et al. (2006), observed increased GPx activity with increases in severity of hypertension, which would suggest up-regulation of GPx activity in severe oxidative stress states such as essential hypertension.[90] In another study examining the GSH antioxidant defence system in elderly subjects, HT treated elders (n=18) were compared to NT, age and gender matched control subjects (n=15). Results yielded from this study indicated no difference in the activity of GPx when comparing the two groups. They did, however, note a significantly increased activity of GR as well as increased levels of GSH in the treated HT group. This may indicate the ability of anti-hypertensive medication/treatment to lower oxidative stress by up-regulation of some anti-oxidant enzymes, and highlight the importance of the glutathione system in blood pressure regulation.[91] Furthermore up-regulation of antioxidant enzymes have also been noted in HT subjects performing aerobic exercise.[92] Thus, activity of these enzymes may be altered by lifestyle factors such as exercise and anti-hypertensive medication and these factors should be taken into consideration.

In a prospective study it was found that GPx activity is the univariate strongest (comparing GPx activity with SOD activity) predictor of risk for CV events and that the risk for CV events was inversely associated with increasing quartiles of GPx activity. They concluded that sub-optimum GPx activity is independently associated with an elevated risk for CV events in subjects presenting with coronary artery disease (CAD). Within the same study population, decreased GPx activity was found to be associated with increased cardiovascular risk according to the extent of atherosclerosis.[93] Similarly, in another prospective study it was found that GPx activity alongside homocysteine were shown to be the strongest univariate predictor of cardiovascular risk independent of other cardiovascular confounding factors. Their results indicated that subjects with low GPx activity and median homocysteine levels had a threefold increased risk for future CV events.[94] In yet another prospective study it was indicated that low GPx

activity together with low HDL-cholesterol (HDL-C) levels significantly increases the risk of death from CVD.[95]

These studies suggest that disturbances in glutathione-related anti-oxidant enzyme activities occur in hypertensive subjects and may increase the risk of cardiovascular events and death. However, an independent association between GPx and GR with BP or other CV measures is yet to be established.

2.4 Conclusion and motivation

Hypertension contributes largely to the disease burden and mortality rate world-wide.[1] Hypertension in Africans is more common than in Caucasians, and little is known about the factors that contribute to this. Furthermore, both HT and NT African men seem to display increased levels of ROS when compared to Caucasian men, possibly indicating altered anti-oxidant defences. Evidence indicating ROS as a causative factor in the pathogenesis is starting to accumulate and the exact impact of altered GPx and GR activity remains elusive. An unadjusted correlation between GPx activity and BP has been shown, while another study failed to do so. Furthermore, the activities of these enzymes have yet to be explored in an African population burdened by hypertension and strokes. Additionally, it is uncertain whether a relationship exists between BP and other cardiovascular measures with GPx activity and GR activity and whether this could contribute to the burden of hypertension in this ethnic group.

2.5 Purpose of the study

Large amounts of evidence implicate oxidative stress in the pathogenesis of hypertension, which is becoming more prevalent among Africans. Furthermore, Africans display significantly higher levels of ROS when compared to Caucasians. This study explores differences in GPx and GR activity and investigates possible relationships between these enzymes and ambulatory BP variables, and structural and functional vascular markers. This will enable us to investigate whether disturbances in GSH-related endogenous enzymes may contribute to elevated blood pressure and/or altered vascular structure and function as observed in this ethnic group.

2.6 Aims

The general aim of this study was to compare the activities of GPx and GR and to explore possible independent associations between the activities of GPx and GR with ambulatory BP and other cardiovascular measures including PWV and cIMT.

The detailed objectives were:

i To determine and compare the activities of GPx and GR in African and Caucasian participants.

ii To investigate possible associations between GPx and GR with ambulatory BP (systolic, diastolic, mean arterial pressure and pulse pressure).

iii To investigate possible associations between GPx and GR with functional and structural vascular markers including PWV and cIMT.

2.7 Hypotheses

Based on the available literature, the following hypotheses were proposed:

i Africans will display lower GPx activity when compared to Caucasians.

ii Africans will display higher GR activity when compared to Caucasians.

iii Lower GPx activity in Africans will be associated with higher ambulatory BP measures (systolic, diastolic, mean arterial pressure and pulse pressure) as well as PWV and cIMT.

iv Higher GR activity in Africans will be associated with higher ambulatory BP measures (systolic, diastolic, mean arterial pressure and pulse pressure) as well as PWV and cIMT.

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Chapter 3: Manuscript

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Preparation of manuscripts: All papers should be written in concise English but should contain sufficient detail to illustrate how the results were obtained. Manuscripts are considered with the understanding that they have not been published previously in print or electronic format and are not under consideration by another publication or electronic medium. Copies of possibly duplicative materials that have been previously published or are being considered elsewhere must be provided at the time of manuscript submission.

Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964 and Declaration of Tokyo, 1975, as revised in 2008). When reporting the results from experiments on animals indicate whether the experiments were conducted according to the National Research Council's guidelines.

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Title page: The title page should give a concise but informative title, the first and last names and other initials of all authors, as well as their affiliations (but not degrees).Names of grants covering the research described should also be included on this page. The order in which the contributors are listed should be agreed amongst the investigators, and should indicate that the first listed made the greatest contribution to the paper. Full contact details should be provided for the corresponding author. There should be fewer than 10 co-authors. Please provide a running title of no more than 50 characters including spaces.

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

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

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

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

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1. Glodny B, Pauli G. Medullopressin: a new pressor activity from the renal medulla. *Hypertens Res* 2005; 28:827–836.

2. Lender D, Arauz-Pacheco C, Breen L, Mora-Mora P, Ramirez LC, Raskin P. A double-blind comparison of the effects of amlodipine and enalapril on insulin sensitivity in hypertensive patients. *Am J Hypertens* 1999; 12(2):298–303.

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SUBMISSION OF ORIGINAL RESEARCH PAPER

Dear Editor

Please consider the attached manuscript entitled: **"Comparing glutathione peroxidase and glutathione reductase activity and their associations with cardiovascular measures in Africans and Caucasians: The SABPA study"** for publication in ***Hypertension Research.***

Hypertension is the leading cause of mortality and morbidity world-wide. Studies indicate that this lifestyle disease causes a disease burden in South Africa. Various factors may contribute to this; however, this remains to be established. Little research has been done on investigating the possible role of glutathione peroxidase (GPx) and glutathione reductase (GR) activity on cardiovascular measures. This article is the first article, to our knowledge, to compare GPx and GR activity and investigate their relation to cardiovascular measures in a bi-ethnic South African population. Our results indicated a significant difference in GPx and GR activity when comparing Africans and Caucasians. We also indicated a significant relationship between carotid dorsalis-pedis pulse wave velocity and GPx activity in the Caucasians.

We the authors, Zurietta M. Schoeman, Carina M.C. Mels and Johannes M. van Rooyen, hereby confirm that the material submitted is original research. This article has not been published previously and has not been submitted for publication elsewhere while under consideration. We look forward to receiving your comments on the manuscrip in due course.

Yours sincerely

Authors

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Figures: 1

Comparing glutathione peroxidase and glutathione reductase activity and their associations with cardiovascular measures in Africans and Caucasians: The SABPA study

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Abstract

Oxidative stress has been implicated in the pathogenesis of hypertension. In an African population with a high prevalence of hypertension, it was shown that elevated serum peroxides correlated with increased ambulatory systolic blood pressure (BP) and pulse pressure (PP) in African men. In the same population attenuated total glutathione correlated with carotid intima-media thickness in hypertensive African men. It is unknown whether these findings may be the result of attenuated antioxidant enzyme activities. The aim of this study was to compare glutathione peroxidase (GPx) and glutathione reductase (GR) activities and to investigate associations of cardiovascular variables with these antioxidant enzymes in Africans and Caucasians. Our study population included African (n=188) and Caucasian (n=203) teachers. Ambulatory BP and carotid dorsalis-pedis pulse wave velocity (cdPWV) measurements were obtained and GPx and GR activities were determined. Africans presented with a worse cardiovascular profile (higher BP and cdPWV (both $p<0.01$)) and displayed lower GPx ($p<0.05$) and higher GR ($p<0.01$) activities when compared to Caucasians. In Caucasians, an independent negative correlation was indicated between cdPWV and GPx activity ($R^2=0.39$, $\beta=-0.18$, $p<0.01$), whereas Africans indicated no relationship between BP and cardiovascular measures with GPx or GR activities. These results suggest that GPx activity may play an important role in vascular protection against arterial stiffness in the Caucasians. Lower GPx activity in Africans may be the result of a genetic polymorphism, GPx inactivation or selenium deficiency. Since previous findings indicated that deficient dietary selenium intake can predict increased PWV, future investigations on selenium status in Africans may have important clinical implications.

Keywords: antioxidant enzymes, ethnicity, glutathione peroxidase, glutathione reductase, pulse wave velocity.

Introduction

Cardiovascular disease (CVD) and hypertension are the leading causes of mortality and morbidity worldwide.[1] Recently it has been reported that more than 10% of 7030 subjects included in the South African National Health and Nutrition Examination Survey (SANHANES-1) had clinically measured blood pressure exceeding 140/90 mmHg.[2] This is in line with cross-sectional data which indicated a hypertension disease burden among Black Africans.[3-5] This may be due to urbanization leading to changes in dietary habits and possibly elevated exposure to exogenous sources of reactive oxygen species (ROS) or decreased defence against oxidative stress.[6-8]

Evidence highlighting the involvement of oxidative stress in the pathogenesis of hypertension is compelling and various articles on this subject have been published.[9-14] The selenium-dependent endogenous, anti-oxidant enzyme, glutathione peroxidase (GPx) is one of the major reduction-oxidation (redox) buffers in the vasculature, functioning synergistically with glutathione reductase (GR).[15] Contradictory results exist regarding GPx activity in hypertension, with some studies indicating lower GPx activity in hypertensive (HT) subjects,[16-19] while another study indicated increasing GPx activity with increased severity of hypertension [20] GR on the other hand has been investigated less extensively with one study indicating increased GR activity in HT vs. normotensive (NT) subjects[21] while another found similar indirect (GSSG/GSH ratio) results.[17,19] An independent relationship between blood pressure (BP) and other cardiovascular variables with GPx and GR activities is yet to be established.

Previous results from the SABPA study have indicated that higher levels of serum peroxides are independently associated with systolic blood pressure (SBP) and pulse pressure (PP) in the African men [22] whereas GSH levels correlated negatively with carotid intima media thickness (cIMT) in HT African men.[22-23] It is, however, uncertain whether these findings may be a result of attenuated antioxidant enzyme activity.

We therefore aim to firstly compare the activity of GPx and GR enzymes in both Africans and Caucasians and to investigate whether a relationship exists between the activities of these enzymes with BP and other cardiovascular measures such as pulse wave velocity (PWV) and cIMT in both ethnic groups.

Methods

Study population

This study was conducted as part of the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study. The study took place between February 2008 and May 2009; in an effort to minimize seasonal changes. A total of 409 urbanized African and Caucasian educators were recruited from the Dr Kenneth Kaunda Education District in the North West Province of South Africa. This regional selection enabled a homogenous cohort of a similar socio-economic class.

Inclusion and exclusion criteria

All eligible subjects between the age of 25 and 65 years were invited to participate. The exclusion criteria included: elevated tympanic temperature, psychotropic substance abuse or dependence, blood donors, pregnant or lactating women and individuals vaccinated three months prior to the commencement of the study. A total of 200 African and 209 Caucasians were included. Additionally for this sub-study, we also excluded participants with missing data (GPx, GR activities and cardiovascular measurements). In total, eighteen participants were excluded: missing GPx or/and GR activity data (n=5), missing PWV data (n=5), missing cIMT data (n=7) and one participant with an ear temperature exceeding 37.5 °C, leaving a total of (n=391), 188 Africans and 203 Caucasians.

Ethical aspects

Prior to inclusion in the study, each participant was fully informed of the objectives and procedures in English or upon request, in their native tongue. Participants were given an opportunity to ask questions with regard to all aspects of the study. This was then followed by individual signing of an informed consent form. The study complied with all applicable ethical requirements, in particular the Helsinki declaration of 1975 (as revised in 2013) for investigation on human subjects. Furthermore, the study was approved by the ethics review board of the North-West University, Potchefstroom Campus.

Study protocol

During the working week, at approximately 07h00 each morning, ambulatory blood monitoring (ABPM) devices (Meditech CE120® Cardiotens, Budapest, Hungary) were fitted on the participants' non-dominant arm at the workplace. These automated, battery operated apparatuses were programmed to take blood pressure measurements in intervals of 30 minutes during the daytime and one-hour intervals from 22h00 to 06h00. After device fitment, participants were instructed to go about their normal daily routine and to note physical activity, blood pressure-related symptoms and stress levels on a diary card.

On the same day that the ABPM devices were fitted, at approximately 16h30, participants were transported to the Metabolic Unit Research Facility of the North-West University. Participants would overnight in the facility that consists of ten bedrooms, two bathrooms, a living room and a kitchen. Subjects received a standardized dinner and had their last beverage (tea/coffee) at 20h30. Thereafter, they relaxed by reading, watching television or social interaction and refrained from consuming alcohol, caffeine, smoking and doing exercise. They were encouraged to go to bed at around 22h00 as they would undergo various measures the following day. At 05h45 subjects were woken and after the last ABPM measurement had been made at 06h00 the apparatuses were removed and data were downloaded. Blood and urine samples were collected and anthropometric measurements and cardiovascular measurements commenced.

Anthropometric and physical activity measurements

Anthropometric measurements were taken in triplicate with calibrated instruments to the nearest decimal kilogram and centimetre. The following measures were obtained: weight (Invicta precision Health Scale, A & D Company, Tokyo, Japan) and height (Stadiometer, IP 1465, Invicta, London, UK). Thereafter, body mass index (BMI) was calculated as follows: BMI= mass (kg)/ height (m^2).[24] Physical activity as total energy expenditure (TEE) was measured over the period of two days taking metabolic rate into account. Physical activity meters (Actical ® Mini Meter, Bend OR ®, Montréal, Québec) were fitted to participants' hips at 06h00 and remained fastened for eight hours. The physical activity meters were removed the following morning.

Cardiovascular measurements

The Ambulatory BP data (Cardiotens® (Meditech CE120® Cardiotens, Budapest, Hungary) obtained from subjects with a minimum 70% compliance were extracted and downloaded into a database, using the CardioVisions 1.19 Personal Edition (Meditech, Budapest, Hungary). The mean successful inflation rate was 79%. Hypertensive status was classified as SBP and/or DBP ≥130/80 according to the 2013 Guidelines of the European Society of Hypertension/European Society of Cardiology for a 24-hour period**.**

The Complior SP Acquisition System (Artech-Medical, Pantin, France) was used to non-invasively measure cdPWV as an indicator of arterial stiffness, in an arterial segment that includes both elastic and muscular arteries, the carotid dorsalis-pedis, on the left side of each participant after 15-20 minutes in the supine position.

A SonoSite Micromaxx ultrasound system (SonoSite, Bothell, WA, USA) and a 6-13MHz linear array transducer was used to determine cIMT. At least two optimal angles were used to obtain images from the left and right common carotid artery and were analyzed with the Artery Measurement System automated software. The means of measurements (near and far wall) of the carotid arteries were used for in this study. In addition, the cross-sectional wall area (CSWA) as an indicator of structural change of the vasculature was calculated as follows: $CSWA=\pi(d/2+cIMT)^2- \pi(d/2)$, where d denotes luminal diameter.

Biochemical analyses

A registered nurse collected venous blood from the ante-brachial vein branches with the use of a sterile winged infusion set. Plasma and serum samples were obtained by standardized procedures, which were then stored at -80°C until commencement of the analyses.

Serum samples were used for the analysis of total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) triglycerides, C-reactive protein (CRP), γ-glutamyltransferase (GGT) with two sequential multiple analyser computer systems (Unicel DXC 800, Beckman and Coulter, Germany and Konelab^{TM} 20I Sequential Multiple Analyzer Computer, Thermo Scientific, Vantaa, Finland). Low-density

lipoprotein cholesterol (LDL-C) was determined by the Friedawald-formula; $C_{LDL} = C_{plasma} - C_{HDL} - TG/5$.[25] Sodium fluoride plasma samples were used to determine fasting blood glucose with a timed end-point method (Konelab™ 20I Sequential Multiple Analyser Computer, Thermo Scientific, Vantaa, Finland and Unicel DXC 800, Beckman and Coulter, Germany). The major metabolite of nicotine, cotinine, was measured in the serum of each participant using a DRI (Diagnostic Reagents Inc., Sunnyvale, CA, USA) enzyme immunoassay kit.

Serum peroxides (as measures of ROS) were determined with the method as described by Hayashi et al. (2007).[26] Ferric reducing antioxidant power (FRAP) was measured by the method described by Benzie and Strain (1996).[27] Total glutathione levels were determined with the use of BIOXYTECH® GSH/GSSG- 412TM kit (*Oxis*Research™, a division of Health Products, Foster City, CA, USA.). GPx and GR activities were determined with assay kits from Cayman Chemical Company (Ann Arbor, MI, USA). All these assays were performed on a Bio-Tek FL600 Microplate reader (Winooski, VT, USA.).

Creatinine was analysed in serum samples using an enzymatic colorimetric test (Cobas Integra 400 plus, Roche, Basel, Switzerland). Accordingly, we calculated the estimated creatinine clearance by using the Cockcroft–Gault formula as follows: Creatinine clearance ($ml.min^{-1}$) = (140-age) x weight (kg) x constant/serum creatinine ($\mu mol.l^{-1}$), where the constant for females are 1.23 and 1.04 for males.[28]

Statistical analyses

Statistical analyses were performed with Statistica 12 (StatSoft, Talsa, OK, USA). Variables with non-Gaussian distributions (glucose, CRP, HDL-C, triglycerides, eCrCl GR, ROS, GGT and LDL-C) were logarithmically transformed and the central tendency and spread represented by the geometric mean and the 5th and 95th percentile intervals. The mean and standard deviations were used to depict variables with normal distributions. Means and proportions were determined with independent t- and Chi-square tests, respectively, to characterize African and Caucasian groups. In accordance with our aim our groups were divided according to ethnicity, additionally interactions of sex on the relationship between cdPWV and were tested, using multiple

regression analyses. Interaction testing indicated no interaction of sex on the association between cdPWV and GPx (Africans: Adj R^2=0.052; β=-0.048; p=0.858. Caucasians: Adj R^2=0.155; β=0.547; p=0.149).

Associations of ambulatory BP and cardiovascular measures with GPx and GR activity were investigated with Pearson correlations. Partial correlations were performed while adjusting for age, sex, BMI, cotinine and GGT. To determine possible independent associations cdPWV and GPx activity we used a forward stepwise multiple regression analysis. The following covariates were entered in the model: GPx activity, gender, age, BMI, cotinine, GGT, CRP, ambulatory mean arterial pressure (MAP), eCrCl, glucose and TEE. This anylaysis was repeated to explore independent associations between cIMT and GR, ABPM- systolic, diastolic and mean BP with GPx and GR. In separate series of sensitivity analyses the multiple regression analyses were repeated after excluding HIV-infected participants and participants on hypertensive medication and anti-oxidant supplementation.

Results

Characteristics of the study population

In Table 1 the characteristics of the study population are summarized. Africans displayed a significantly higher BMI ($p<0.01$) and an unfavourable cardiovascular profile, with SBP, DBP, MAP, PP and heart rate (all $p<0.01$) being significantly higher in the Africans when compared to the Caucasians. Markers of cardiovascular function and structure such as cdPWV ($p<0.01$), cIMT ($p<0.01$) and CSWA ($p<0.05$), were also higher in the Africans compared to their Caucasian counterparts. Analyses of the oxidative stress markers indicated higher levels of total serum peroxides ($p<0.05$) in Africans accompanied by significantly lower GPx activity ($p<0.01$), while GR activity ($p<0.05$) and total GSH levels ($p<0.01$) were significantly higher in Africans. Lifestyle factors indicated that Africans had higher levels of GGT ($p<0.01$) and a lower level of physical activity measured by total energy expenditure ($p<0.01$).

Unadjusted analyses

In single regression analyses, ambulatory HR ($r=0.15$; $p<0.05$), cIMT ($r=0.22$; $p<0.01$) and CSWA ($r=0.16$; $p<0.01$) correlated positively with GR activity in the African group (Table S1). In the Caucasian group on the other hand, a significant negative association between pulse wave velocity ($r=-0.16$; $p<0.05$) (Figure 1) and estimated creatinine clearance ($r=-0.18$; $p<0.05$) with GPx were shown (Table S1).

Table 1: Characteristics of the study population

	Africans (n= 188)	Caucasian (n=203)	P-values
Age, years	44.5 ± 8.27	44.8 ± 10.9	0.750
Sex, male, n, (%)	93 (49.5)	98 (48.3)	0.814
Anthropometric measurements			
Weight, kg	81.9 ± 18.7	84.1 ± 21.3	0.290
Height, m	1.65 ± 0.09	1.74 ± 0.09	< 0.001
Body mass index, kg/m^2	30.4 ± 7.09	27.7 ± 5.94	< 0.001
Cardiovascular measurements			
Ambulatory systolic blood pressure, mmHg	133 ± 16.4	124 ± 12.0	< 0.001
Ambulatory diastolic blood pressure, mmHg	83.4 ± 10.8	76.7 ± 8.10	< 0.001
Ambulatory mean arterial pressure, mmHg	100 ± 12.2	92.4 ± 8.87	< 0.001
Ambulatory pulse pressure, mmHg	49.9 ± 9.18	47.3 ± 7.00	<0.001
Ambulatory heart rate, beats/min	79.8 ± 10.6	73.8 ± 10.1	< 0.001
Pulse wave velocity, m/s	8.70 ± 1.99	8.01 ± 1.43	< 0.001
Carotid intima-media thickness, mm	0.69 ± 0.12	0.64 ± 0.12	< 0.001
Cross-sectional wall area, mm^2	14.1 ± 4.34	13.1 ± 3.45	0.014
Biochemical analyses			
Glucose, log mmol/l	5.41 (4.10; 10.4)	5.62 (4.70; 6.90)	0.821
C-reactive protein, log mg/l	4.45 (0.57; 31.7)	2.03 (1.00; 9.00)	< 0.001
Total cholesterol, mmol/l	4.60 ± 1.19	5.54 ± 1.28	< 0.001
High density lipoprotein cholesterol, log mmol/l	1.07 (0.66; 1.68)	1.13 (0.64; 1.97)	0.081
Low density lipoprotein cholesterol, log mmol/l	2.79 (1.55; 4.93)	3.81 (2.53; 6.16)	<0.001
Triglycerides, log mmol/l	1.16 (0.50; 3.66)	1.02 (0.46; 2.79)	0.037
Estimated creatinine clearance, log ml/min	115 (73.8; 190)	119 (78.1; 205)	0.241
Oxidative stress markers			
Glutathione reductase, log nmol/min/l	7.06 (2.55; 16.8)	2.54 (0.25; 7.64)	< 0.001
Glutathione peroxidase, nmol/min/l	33.3 ± 14.1	36.2 ± 8.03	0.011
Total serum peroxides, log nmol/min/l	92.5 (57.1; 145)	86.5 (57.4; 137)	0.020
Total glutathione , µM	889 ± 188	821 ± 174	< 0.001
Ferric reducing antioxidant power, µM	389 ± 74.0	435 ±108	< 0.001
Lifestyle and medication			
Cotinine, ng/l	26.7 ± 61.2	20.3 ± 66.2	0.324
γ-glutamyl transferase, log U/l	47.4 (20.3; 184)	19.4 (7.00; 76.0)	< 0.001
HIV-infected, n, (%)	17 (9.04)	0 (0)	< 0.001
Total energy expenditure, kcal	2703 ± 799	3126 ± 1615	< 0.001
Anti-hypertensive medication, n, (%)	42 (22.3)	16 (7.88)	< 0.001
Hypertensive status, n, (%)	126 (67.0)	99 (48.7)	< 0.001
Anti-oxidant supplementation, n, (%)	0 (0)	5 (2.46)	0.030

Data expressed as arithmetic mean ± standard deviation, geometric mean (5^{th} and 95^{th} percentiles) or % of n.

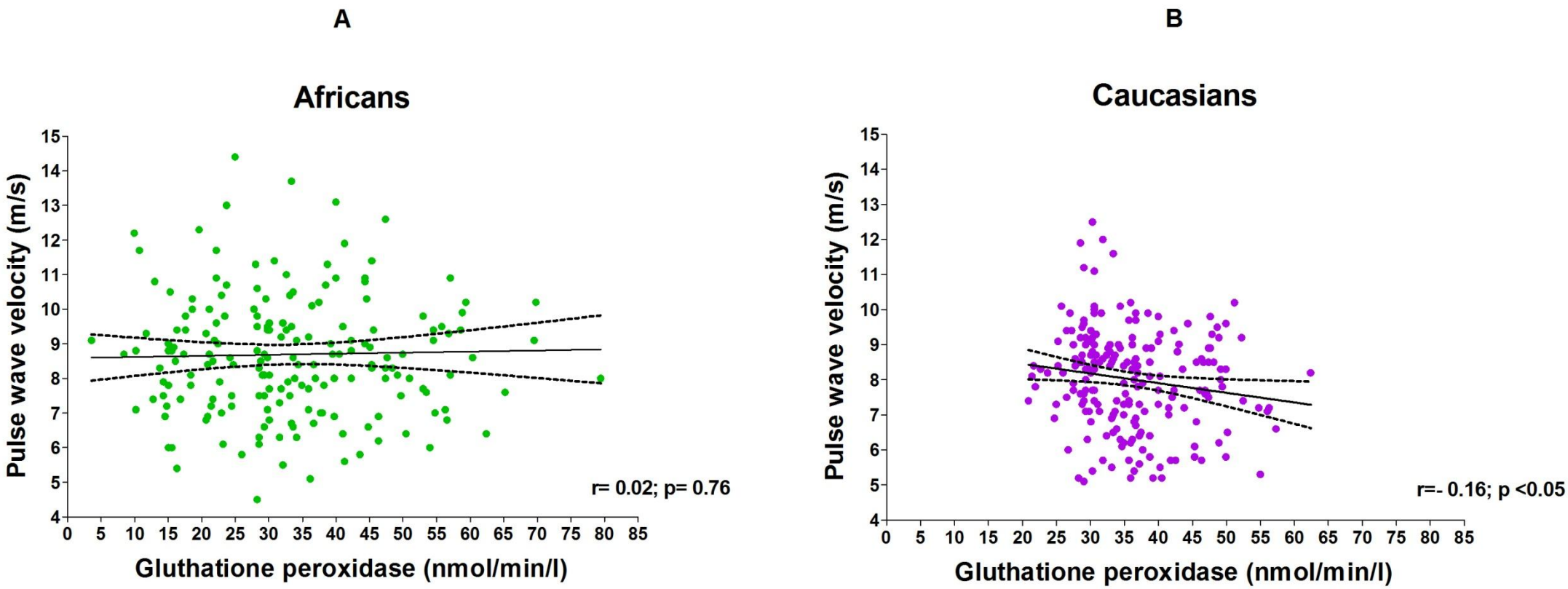

Figure 5: Single regression analyses between (A) glutathione peroxidase vs. pulse wave velocity in Africans; (B) glutathione peroxidase vs. pulse wave velocity in Caucasians.

Table S1: Unadjusted analyses of blood pressure and other cardiovascular measures with glutathione peroxidase and glutathione reductase in Africans and Caucasians.

	Glutathione peroxidase		***Glutathione reductase***	
	Africans	***Caucasians***	***Africans***	***Caucasians***
Ambulatory systolic blood pressure, mmHg	r= -0.12	r= -0.03	r= 0.13	r= -0.11
	p= 0.11	p= 0.67	p= 0.08	p= 0.10
Ambulatory diastolic blood pressure, mmHg	r= -0.07	r= 0.003	r= 0.09	r= -0.10
	p= 0.35	p= 0.97	p= 0.20	p= 0.15
Ambulatory mean arterial pressure, mmHg	r= -0.10	r= 1.00	r= 0.11	r= -0.11
	p= 0.16	p= 0.85	p= 0.16	p= 0.35
Ambulatory pulse pressure, mmHg	r= -0.12	r= -0.07	r= 0.12	r= -0.08
	p= 0.09	p= 0.35	p= 0.12	p= 0.27
Ambulatory heart rate, beats/min	r= 0.04	r= -0.02	r= 0.15	r= -0.05
	p= 0.59	p= 0.81	p= 0.04	p= 0.45
Carotid intima-media thickness, mm	r= -0.11	r= 0.10	r= 0.22	r= 0.01
	p= 0.13	p= 0.14	p= 0.003	p= 0.86
Carotid cross-sectional wall area, mm^2	r= -0.08	r= 0.01	r= 0.16	r= -0.05
	p= 0.26	p= 0.98	p= 0.03	p= 0.46
Pulse wave velocity, m/s	r= 0.02	r= -0.16	r=0.02	r=-0.13
	p= 0.76	p=0.03	p=0.81	p= 0.06
Estimated creatinine clearance, ml/min	r= 0.04	r= -0.18	r= -0.01	r= -0.11
	p= 0.54	p= 0.01	p=0.94	p= 0.10

Adjusted analyses

After adjusting for age, gender, BMI, cotinine and GGT levels, none of the correlations between GR activity and cardiovascular variables remained significant in the African group, although GR activity tended to still correlate positively with cIMT (r=0.14; p=0.051) in this group (Table 2). In Caucasians, the negative correlation between GPx and cdPWV (r=-0.21; p<0.05), and GR activity and ambulatory MAP (r=-0.14; p<0.05) remained significant, whereas a correlation between GR and cdPWV (r=-0.019; p<0.01) became significant (Table 2). In multiple regression analyses the negative association between cdPWV with GPx activity in Caucasians (adjusted R^2= 0.39; β=-0.18; p<0.01) was confirmed to be independent of various covariates, whereas the association between cdPWV and GR activity and between ambulatory MAP and GR activity in this group was no longer significant. In a sensitivity analysis where HIV-infected participants (n=17) (Adj R^2=0.393; β_{GPx}=-0.18; p<0.01) and participants using anti-hypertensive medication (n=158) (Adj R^2=0.396; β_{GPx}=-0.18; p<0.01) or anti-oxidant supplementation (n=5) (R^2=0.396; β_{GPx}=-0.18; p<0.01) were excluded separately, the association between cdPWV and GPx and in the Caucasians did not change.

Table 2: Partial regression analyses between glutathione peroxidase and glutathione reductase with cardiovascular measures Africans and Caucasians.

	Glutathione peroxidase		**Glutathione reductase**	
	Africans	Caucasians	Africans	Caucasians
	n= 188	n=203	n= 188	n=203
Ambulatory systolic blood pressure, mmHg	r= -0.10	r= -0.01	r= 0.004	r= -0.14
	p= 0.19	p= 0.96	p= 0.95	p= 0.05
Ambulatory diastolic blood pressure, mmHg	r= -0.08	r= 0.02	r= -0.05	r= -0.13
	p= 0.30	p= 0.76	p= 0.48	p= 0.07
Ambulatory mean arterial pressure, mmHg	r= -0.09	r= 0.01	r=-0.03	r= -0.14
	p= 0.23	p= 0.91	p= 0.70	p= 0.05
Ambulatory pulse pressure, mmHg	r= -0.08	r= -0.04	r= 0.07	r= -0.07
	p= 0.30	p= 0.57	p= 0.36	p= 0.35
Ambulatory heart rate, beats/min	r= 0.06	r= -0.03	r= 0.11	r= -0.07
	p= 0.46	p= 0.72	p= 0.14	p= 0.32
Carotid intima--media thickness, mm	r= -0.06	r= 0.08	r= 0.14	r= -0.09
	p= 0.46	p= 0.29	p= 0.05	p= 0.23
Carotid cross-sectional wall area, mm^2	r= -0.07	r= -0.02	r= 0.06	r= -0.13
	p= 0.34	p= 0.73	p= 0.40	p= 0.08
Pulse wave velocity, m/s	r= 0.07	r=- 0.21	r=-0.10	r=-0.19
	p= 0.33	p= 0.01	p=0.19	p= 0.01
Estimated creatinine clearance, ml/min	r= 0.06	r= -0.13	r= -0.02	r= -0.02
	p= 0.41	p= 0.07	p= 0.75	p= 0.76

Relationships were adjusted for age, gender BMI, cotinine and GGT.

Table 3: Independent association of pulse wave velocity with glutathione peroxidase activity in the African and Caucasian groups.

Pulse wave velocity **(m/s)**				
	African (n=186)		**Caucasian** (n=202)	
Adjusted **R^2**	0.29		0.39	
	***β* (±95%CI)**	***P-value***	***β* (±95%CI)**	***P-value***
Age, years	0.22 (0.09; 0.35)	0.001	0.37 (0.25; 0.48)	<0.001
Sex	-0.14 (-0.27; 0.00)	0.054	-0.28 (-0.41;-0.15)	<0.001
BMI, kg/m^2			-0.20 (-0.35;-0.06)	0.004
Ambulatory MAP, mmHg	0.26 (0.13; 0.40)	<0.001	0.24 (0.09; 0.38)	<0.001
Glucose, log mmol/l	0.26 (0.13; 0.40)	<0.001	0.15 (0.02; 0.28)	0.031
CRP, log mg/l			0.14 (0.02; 0.27)	0.024
Cotinine, ng/ml	-0.09 (-0.21; 0.03)	0.186		
GPx, nmol/min/l	0.08 (-0.04; 0.21)	0.217	-0.18 (-0.29; -0.07)	0.001

Variables included in the model: pulse wave velocity; age; sex; body mass index (BMI); ambulatory mean arterial pressure (MAP); glucose; cotinine; gamma-glutamyl transferase (GGT); C-reactive protein (CRP); glutathione peroxidase (GPx); estimated creatinine clearance (eCrCl) and physical activity.

Discussion

Our main findings are that GPx activity was significantly lower whereas GR activity was significantly higher in Africans when compared to Caucasians. In the Caucasian group, their higher GPx activity was independently associated with lower cdPWV.

The lower GPx activity is in line with results previously obtained in African-Americans who displayed lower GPx activity when compared to Caucasians.[17] Lower GPx activity can either be explained by a GPx genetic polymorphism, a selenium (cofactor) deficiency or GPx inactivation.

Previous results revealed a genetic GPx polymorphism in African Americans,[29] which was also identified in Black South Africans living in Durban.[30] Furthermore, African origin has been shown to be an independent predictor of reduced GPx activity.[31] On the other hand, previous results indicated that selenium supplementation can increase GPx activity.[32] Since results from studies done in various parts of South Africa have indicated deficient dietary selenium intake,[33-34] or deficient selenium status[35] it may be an important contributor to the lower GPx activity in our African group.

Furthermore, our African group also had an unfavourable CV profile and previous results indicated GPx activity to be lower when comparing HT and NT subjects.[19-20] Although, other contradictory results found GPx activity to progressively increase in accordance to the grade of hypertension,[17] or not to differ between HT and NT subjects.[22]

These contradictory results may be due to the fact that endogenous anti-oxidant enzymes may be differentially expressed in oxidative states such as hypertension[36-38] Additionally, this compensatory mechanism may become overwhelmed and inactivation of GPx may also lead to lower GPx activity.[36-37] Inactivation of GPx activity can take place as a result of elevated levels of superoxide that inhibit peroxide function[36] and/or prolonged exposure to hydrogen peroxide and hydrogen peroxynitrite.[37]

The higher GR activity observed in our African group is contradictory to the results obtained in a study to investigate the effect of racial differences on the activity of anti-oxidant enzymes in children.[38] In this study no differences in the GR activity were found

when comparing African-American and Caucasian children. However, as previously mentioned, our African group also had an unfavourable CV profile and significantly increased GR activity was previously indicated in an elderly treated hypertensive group.[21]

Our second main finding indicates that higher GPx activity is independently associated with lower cdPWV in the Caucasian group suggesting a protective function of GPx against arterial stiffening. Simialarly cdPWV was shown to negatively associate wirh GR activity in Caucasians (partial correlations: $r=-0.19$; $p<0.05$) but failed to indicate an independent relationship between these two variables. This association together with the lower activity of GR in Caucasian further supports studies that have indicated upregulation of enzyme activity [36-38] that may occur in Africans during increased oxidative stress. Caucasians demonstrated more favourable CV profiles thus lower GR activity is to be expected.

To the best of our knowledge we are the first to indicate this protective association between GPx activity and cdPWV. However, it was previously indicated that deficient dietary selenium intake can predict increased PWV independent of various confounding factors.[39] In this case it may also have been due to decreased GPx activity as a result of selenium deficiency. In another study, variation in GPx gene transcription leading to decreased GPx activity was linked with deterioration of vascular structure (increased cIMT) and risk for cardiovascular and peripheral vascular diseases.[40] In the vasculature the enzyme complex NAD(P)H oxidase is the main generator of ROS[41] and in particular superoxide. After the dismutation of superoxide approximately 70% of hydrogen peroxide is detoxified by GPx within endothelial cells.[42] Decreased activity of GPx may therefore result in the accumulation of hydrogen peroxide, which has been shown to play a role in the regulation of vascular tone in various ways.

Firstly, hydrogen peroxide together with other ROS, actively increases intracellular calcium, inducing vascular contraction of vascular smooth muscle cells (VSMC) via its effect on protein kinase-C (PKC).[43-45] Secondly, increased hydrogen peroxide may directly or indirectly, through the formation of other reactive compounds,[46] result in decreased NO bio-availability and subsequent decreased vaso-relaxation.[47]

Additionally, decreased bio-availability of NO (and increased ROS production) does not only impact on vaso-relaxation, but may also lead to inhibition of other vaso-protective effects of NO. These effects include inhibition of platelet adhesion, leucocyte adhesion and vascular smooth muscle cell proliferation.[48-49]

Our study yielded no association between GPx and GR activities with ambulatory SBP or DBP in either Africans or Caucasians. This was also true for other ambulatory measures including MAP, PP or HR. Neither structural nor functional vascular markers, cIMT, CSWA and PWV respectively, indicated a relationship with either GPx activity or GR actitity in Africans even though these variables were significantly higher in Africans. An experimental animal study using GPx-knockout mice (vs. wild type mice) observed endothelial dysfunction to occur in the absence of structural vascular changes, concluding functional changes to precede structural changes.[50] This evidence supports the currently observed relationship between cdPWV and GPx activity in Caucasians and provides insight into possible mechanisms whereby increased anti-oxidant enzyme activity may play a role in protecting the vasculature against endothelial damage and increased arterial stiffness.

The strengths of this study are based on a highly-controlled, ethical study environment and a standardised and reproducible methodology. The study was conducted in two phases to limit seasonal changes. Furthermore, participants were included from the same socio-economic status and no significant difference was found in the age and gender percentages of the two groups. Trained professionals conducted an extensive range of cardiovascular measures on the participants and blood samples were obtained by a registered nurse. Ambulatory blood pressure measurement was used with a minimum of 23 hours which is considered to be the gold standard for clinical blood pressure measurement world-wide.[51]

Limitations of the study include the measurement of PWV in the carotid dorsalis-pedis arterial segment and not the carotid femoral which is the golden standard for stiffness measurement.[52] The unavailability of the selenium status of our participants limited our explanation of the observed lower GPx activity in the Africans. Various studies investigate anti-oxidant enzyme activities by comparing HT and NT participants, in our

study we would have lost statistical power if our groups were further divided by hypertensive status.

Conclusion

In conclusion, the data from our study indicate that Caucasians have a significantly higher activity of the selenium-dependent, endogenous anti-oxidant enzyme, GPx when compared to Africans. This increased GPx activity is protectively associated with arterial stiffness in Caucasians. Furthermore, previous studies indicated decreased GPx activity to be a strong risk predictor for cardiovascular events. The aetiology of the lower GPx activity in our African group may therefore have important clinical implications as selenium supplementation or even food fortification may aid in the prevention of changes in the vascular micro-environment before detrimental structural and functional changes occur.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 4: Concluding chapter

4.1 Summary of main findings

The general aim of this study was to compare the activities of glutathione peroxidase (GPx) and glutathione reductase (GR) and to explore independent associations between GPx and GR activity with ambulatory BP and other cardiovascular measures such as cdPWV and cIMT.

Upon completion of this study, it is now possible to review the hypotheses that were made and to determine whether the hypotheses are supported by the findings of this study.

Hypothesis 1: *Africans display lower GPx activity when compared to Caucasians*

A significant difference in GPx activity was established between African and Caucasian participants, with GPx activity being lower in Africans than in Caucasian participants. Therefore the first hypothesis is accepted. This is in line with data found in African-American subjects who displayed significantly lower GPx activity than their Caucasian counterparts.[1]

Hypothesis 2: Africans *display higher activity of GR when compared to Caucasians*

Our study population indicated a significantly higher GR activity in Africans compared to Caucasians. To the best of our knowledge, our study is the first to indicate ethnic differences in terms of GR activity, with the only other study finding no difference in GR activity.[2] GR activity has been shown to be elevated in states of oxidative stress such as hypertension and Diabetes Mellitus which may be due to increased GR transcription.[3-5] Africans display higher levels of peroxides when compared to Caucasians. Africans also display higher levels of total glutathione. Our study does not distinguish between the oxidised and reduced state, thus not indicating the amount of substrate (GSSG) to be available for GR. It has been suggested that glutathione may accumulate in the cells due to reduced GPx activity which subsequently increased GR activity.[6] Africans displayed significantly higher levels of peroxides as well as higher levels of ambutatory blood pressure together with significantly lower GPx activity. The fore-menthioned may explain the significantly elevated GR activity displayed in Africans. The second hypothesis is therefor accepted.

Hypothesis 3: *Lower GPx activity in Africans will be associated with higher ambulatory BP measures (systolic, diastolic, mean arterial pressure and pulse pressure) as well as PWV and cIMT,*

and

Hypothesis 4: *Higher GR activity in Africans will be associated with higher ambulatory BP measures (systolic, diastolic, mean arterial pressure and pulse pressure) as well as PWV and cIMT.*

Our study is the first to investigate the relationship between both GPx and GR activities with cardiovascular measures in a bi-ethnic population. We can deduce the following from our data:

An independent inverse relationship was established between PWV and GPx in Caucasians which indicates a possible vaso-protective effect of GPx This relationship remained significant even after repetition of the model excluding HIV-infected subjects; subjects using anti-hypertensive medication and subjects using anti-oxidant medication separately. No relationship was established between both GPx activity with neither ambulatory measures nor functional (PWV) and structural vascular measures (cIMT) in Africans. We therefore partially accept the third hypothesis as no independent relationship was established in Africans already indicating less favourable cardiovascular profiles. Thus we could speculate that the protective function of GPx may be to prevent vascular deterioration.

Although Africans had significantly higher GR activity and a less favourable cardiovascular profile, accompanied by elevated serum peroxide levels, we failed to show any relationship between GR activity with BP variables and other cardiovascular measures such as PWV and cIMT. The latter was also true for Caucasians. We therefore reject hypothesis 4.

4.2 Strengths and limitations and recommendations for future studies

4.2.1 Strengths

The strengths of this study are based on a highly-controlled study environment and standardised and reproducible methodology. The study was conducted between February-May 2008 and the second phase in February-May 2009. This was done to limit seasonal changes. Furthermore, participants were included from the same socio-economic status and all participants were between the ages of 25-65 years. These participants stayed overnight in similar rooms of the Metabolic Unit of the North-West University. Potchefstroom Campus, South Africa. Trained professionals conducted an extensive range of cardiovascular measures on the participants and blood samples were obtained by a registered nurse. Ambulatory blood pressure measurements were used with a minimum of 22 hours which is considered to be the gold standard for clinical blood pressure measurement world-wide.[6]

4.2.2 Limitations and confounding

The applicability or extrapolation of the results to the general population is prohibited as this study design is limited (cross-sectional design). Selenium levels were not measured, therefore it is not possible to draw conclusions about cause and effect with respect to changes in the level of GPx activity and/or causes due to genetic variability or even GPx inactivation. Significant provision was made to adjust for several confounding factors, i.e. age, sex, body composition, body mass index (BMI) and exposure to lifestyle factors such as alcohol (gamma- glutamyl transferase) and cigarette smoking (cotinine); however some unidentified factors may have had a confounding effect on the associations found in this study. This study involved both hypertensive and normotensive subjects who were only divided according to ethnicity. Various studies investigating similar topics present their results according to hypertensive status, thus limiting the depth of comparison between these studies and our study. We made use of the carotid dorsalis-pedis segments to measure arterial stiffness. These segments were more accessible and allowed better participation of subjects. This method is, however, not the golden standard that includes the carotid and femoral arteries.[7]

4.3 Recommendations

1. It is firstly recommended that a longitudinal study with a large study population sample be performed to determine cause and effect.

2. Selenium status should be investigated in conjunction with serum peroxide levels and anti-oxidant enzyme activities.

3. The carotid femoral segment should be utilized as this is the golden standard for the measurement of arterial stiffness (PWV).[6]

4. GSSG and GSH levels should be determined to better describe the activities of GPx and GR.

5. Future studies should explore whether a similar relationship exists in young, normotensive subjects, and whether selenium supplementation may be effective in preventing vascular changes that precede hypertension and CVD's.

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1.1 Background and motivation Up until recently, statistical evidence indicating the true burden of hypertension among black South Africans (hereby referred to as Africans) has been lacking. Dated as well as recent small cross-sectional data indicate a high prevalence of hypertension among urbanized Africans and this is confirmed by a recently published national

report, The South African National Health and Nutrition Examination Survey (SANHANES).1 12

3 This study illuminates the current transitional process from optimal to elevated blood pressure levels and the accompanying changes in behavioural risk factors of South African citizens. More than 10 % of the multi-cultural randomly selected group presented with blood pressures exceeding 140/90 mmHg.3 Globally, hypertension is said to be the leading cause of morbidity and mortality, however, blood pressure seems to be on the decline since 1980.4-5 When looking at area-specific data, both male and female African inhabitants have shown an

increase in both systolic and diastolic blood pressure. 8

5 This, however, is no surprise as urbanization and westernization increase in these formally traditional communities.6-7 Reactive oxygen species (ROS) are kept at bay by diminutive mechanisms forming part of the antioxidant system.8 Among various biologically active measures, the endogenous selenium-dependent anti-oxidant enzyme

glutathione peroxidase (GPx) and glutathione reductase (GR) together with glutathione (GSH) 60

fulfil an important function in maintaining intracellular reduction-oxidation (redox) balance and protecting against oxidative stress and its consequent damage.9 Evidence indicating oxidative stress's role in the pathogenesis of hypertension is substantial.10-15 GPx activity has been shown to be lower in hypertensive (HT) subjects when compared to normotensive (NT) subjects.16-18 One study has, however, indicated elevated GPx and GR activity in HT treated subjects when compared to age- and sex- matched control.19 Furthermore, ethnicity was shown to independently affect GPx activity20 and subjects with an African origin had lower GPx activity than their Caucasian counterparts.20--21 Previously, no association has been shown between GPx and GR with blood pressure (BP). However, variants in the GPx-1 gene decreasing GPx activity have been suggested to be associated with increased carotid intima-media thickness (cIMT).22 Accordingly, Africans display higher levels of serum peroxides when compared to Caucasians of the same socioeconomic status.23 The forementioned correlates with arterial stiffness and

is independently positively associated with ambulatory systolic blood pressure (SBP) and pulse pressure (PP) in African 16

men23. Total glutathione levels have also been shown to be lower

in hypertensive African men and associated with a 6

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