The relationship between oxidative stress-related markers and retinal microvascular structure and function in a bi-ethnic population: The SABPA study

VF MAUGANA

23048069

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

Supervisor:	Dr. W Smith
Co-supervisor:	Prof. CMC Mels
Assistant co-supervisor:	Prof. NT Malan

November 2016

It all starts here ™

NORTH-WEST UNIVERSITY ®
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

ACKNOWLEDGEMENTS

As the author of this dissertation, I would like express my gratitude towards:

- My Lord and Savior Jesus Christ for the grace, strength, guidance and courage in making the completion of this dissertation successful. Thank you for the wisdom given unto me liberally without reproach and for the fulfillment of your promises according to *Psalms 32:8 NLT ("The Lord says: I will guide you along the best pathway of your life. I will advise you and watch over you") and Job 22:28 NLT ("You will succeed in whatever you choose to do and light will shine on the road ahead of you").*

- Dr. Wayne Smith, Prof. Carina Mels and Prof. Nico Malan for their inspiration, constant motivation, understanding, guidance, consistency, willingness to assist at all times, patience and encouragement in the process of writing this dissertation.

- The staff and colleagues of the Hypertension in Africa Research Team (HART) for all their input in making the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study a success.

- My parents (Mr Samson Maugana and Mrs Violet Maugana) who believed in me and granted me the opportunity to proceed with my studies. Thanks for your love, prayers and support. I really appreciate it.

- Bishop Desmond Hendricks and Prophetess Gillian Hendricks for all the times they stood in the gap for me in prayer just to bring out the best in me. Thanks for the spiritual intervention in every area of my life. Your love and care for me is highly appreciated.

- My fellow postgraduate students who have made a difference in my life.

CONTRIBUTIONS OF THE AUTHORS

Felicia Maugana: I was responsible for literature searches, performing statistical analyses, processing of data, design, planning and writing of the dissertation. Although I was not involved in obtaining data for the SABPA study, I received training and helped perform some of the cardiovascular measurements in various projects undertaken by the HART including part of the South African leg of the Prospective Urban and Rural Epidemiology (PURE) study that took place in Ganyesa and the ongoing African PRospective study on the Early Detection and Identification of Cardiovascular disease and hyperTension (African-PREDICT) study. These measurements include: pulse wave velocity, blood pressure and electrocardiography. I also had an opportunity to attend a few dynamic vessel analysis (DVA) sessions to improve my understanding on the apparatus and methodology that was used in this dissertation.

Dr. Wayne Smith (Supervisor): He supervised the writing of the literature review and the manuscript. He gave guidance in performing statistical analyses and helped with the interpretation of data. He gave feedback within a reasonable time with recommendations. He also performed DVA measurements and analyzed fundus images and flicker responses of retinal blood vessels.

Prof. Carina Mels (Co-supervisor): She supervised the writing of the literature review and manuscript. She gave recommendations on construction of sentences and helped with interpretation of results. She gave feedback within a reasonable time. Prof. Mels also acquired funding for the analyses of oxidative stress-related markers. (Thuthuka Grant: 80643).

Prof. Nico Malan (Assistant co-supervisor): He gave feedback on the literature review and manuscript. He also provided helpful criticism with regard to scientific language use, sentence construction with clarity and recommendations of critical thinking. He assisted with data collection and analyses as well as performing DVA measurements and analyses of fundus images of retinal blood vessels.

This is a statement from the co-authors confirming their individual role in participation of this study and giving their permission that the manuscript may form part of this dissertation.

_____	_____	_____
Dr. W Smith	Prof. CMC Mels	Prof. NT Malan

TABLE OF CONTENTS

SUMMARY VII

PREFACE XI

LIST OF FIGURES AND TABLES XII

LIST OF ABBREVIATIONS XIII

CHAPTER 1: INTRODUCTION AND MOTIVATION 1

CHAPTER 2: LITERATURE REVIEW 7

2.1 Defining oxidative stress 8

2.2 Different types of reactive species and their metabolism 8

2.3 Oxidative and nitrosative stress 9

2.4 Defense systems against oxidative stress 11

2.5 Oxidative damage to macromolecules 13

2.5.1 Lipid peroxidation 13

2.6 Oxidative stress and the vascular system 16

2.7 Oxidative stress-related markers reported in the SABPA study 17

2.8 The vasculature of the eye 18

2.9 The retinal microvasculature 19

2.10 Retinal vessel calibers 19

2.11 Retinal vessel responses to FLIP 21

2.11.1 Assessment of vascular endothelial function 21

2.12 Confounding factors associated with oxidative stress-related markers, retinal vessel calibers and retinal blood vessel responses to FLIP respectively 23

2.12.1 Age 23

2.12.2 Hypertension 25
2.12.3 Diabetes mellitus 26
2.12.4 Obesity and dyslipidemia 27
2.12.5 Ethnicity 29
2.13 The retinal microvasculature and oxidative stress-related markers 31
2.14 Motivation 32
2.15 Aims and objectives 33
2.16 Hypotheses 34
2.17 References 35
CHAPTER 3: METHODOLOGY 55
CHAPTER 4: MANUSCRIPT OF THE JOURNAL ARTICLE 71
Instructions for authors 72
Title page 74
Abstract 75
Introduction 76
Methods 78
Results 86
Discussion 94
Acknowledgements 98
Conflict of interest 98
References 100
CHAPTER 5: FINAL CONCLUSIONS AND RECOMENDATIONS 106
Introduction 107

Comparison to relevent literature and discussion of main findings 107

Summary 110

Strengths and limitations 111

Recommendations 112

Chance and confounding factors 113

Conclusion 113

References 115

APPENDICES 119

TURN IT IN REPORT 120

LETTER OF APPROVAL FOR THE SUB-STUDY (HART Committee) 121

ETHICS APPROVAL FOR THE SUB-STUDY (Health Research Ethics Committee) 122

ETHICS APPROVAL FOR THE SABPA STUDY (Health Research Ethics Committee) 124

LANGUAGE EDITOR'S CERTIFICATE 125

SUMMARY

The relationship between oxidative stress-related markers and retinal microvascular structure and function in a bi-ethnic population: The SABPA study

Background and aims

Reactive oxygen species (ROS) play a role in regulating endothelial function and vascular tone. Under normal conditions, there is a balance between ROS and antioxidant defense capacity, but any disturbance in this equilibrium may result in oxidative stress which in turn may disrupt redox signaling and lead to molecular damage. This may further disrupt the functioning of the endothelium, contributing to the development of cardiovascular disease (CVD). Since the risk of developing CVD is higher in urban black populations, previous SABPA sub-studies investigated the link between oxidative stress-related markers and components of the macrovasculature. These studies reported associations of higher levels of ROS (serum peroxides) in black men and lower glutathione peroxidase (GPx) activity in black women together with increased blood pressure. Furthermore, reduced glutathione (GSH) levels and up-regulated glutathione reductase (GR) activity were associated with increased carotid intima-media thickness (c-IMT) in black men. These results indicate that blacks are indeed at a higher risk of developing CVD compared to whites. As microvascular functional changes are thought to occur prior to the changes in the macrovasculature, it is valuable to evaluate associations of oxidative stress-related markers and microvascular parameters.

The retina allows easy access and direct visualization of the microvasculature. The Dynamic Vessel Analyzer (DVA) can be used to obtain retinal photographs whereby caliber equivalents of central retinal artery (CRAE), central retinal vein (CRVE) and the subsequent calculation of the ratio between CRAE and CRVE (arterio-venous ratio (AVR)) can be determined. Furthermore, functional responses of retinal blood vessels to a flicker light-induced provocation (FLIP) can be assessed and include maximum artery dilation (Art_max_{dil}) and maximum vein dilation ($Vein_max_{dil}$). Retinal vascular changes are associated with both clinical and subclinical

CVD. Similarly, reduced retinal vessel responses to FLIP have been associated with diabetes, hyperlipidemia, hypertension and coronary artery disease. Both retinal vessel calibers and reduced retinal vessel responses to FLIP are possible early indicators of systemic vulnerability and CVD risk. Detection of these microvascular changes may be useful in predicting future cardiovascular events. Additionally, relevant therapies may be implemented to lower the risk of developing CVD.

Some results on the relationship of retinal vessel calibers and retinal vessel responses to FLIP with oxidative stress-related markers have been reported. Results from these studies suggested that antioxidant defense mechanisms play a cardio-protective role in the retinal microvasculature and regulation of retinal vascular function which may lower the CVD risk. Previous SABPA sub-studies evaluated oxidative stress-related markers in relation to the macrovasculature (as already indicated above). However, there is no available data on the associations of retinal vessel calibers and retinal vessel responses to FLIP with oxidative stress-related markers in Sub-Saharan Africa. The aims of this study were firstly, to compare oxidative stress-related markers in a cohort of black and white men from South Africa and secondly, to explore the relationships of retinal vessel calibers and retinal vessel responses to FLIP with oxidative stress-related markers in this group.

Methods

This cross-sectional study was performed using the data obtained from the follow-up phase of the SABPA study that was conducted between February and May of 2011/2012. Participants were informed about the purpose of the study as well as the procedure of the study and they all signed an informed consent form. A total of 359 black and white teachers from the Kenneth Kaunda Education district in the North-West Province of South Africa took part in the follow-up phase of this study. Thirty three participants were excluded as a result of no available or poor functional retinal vessel analyses data, antioxidant intake and outliers for superoxide dismutase (SOD). Women were also excluded from this study as their status of menopause and/or phase of fluctuating hormone levels during their menstrual cycle were unknown. After these

exclusions, a total of 159 participants were considered eligible for our sub-study of which 82 were black men and 77 were white men. Anthropometric measurements were taken and lifestyle factors were recorded in the form of questionnaires. Standardized procedures were used for biochemical analyses of plasma/serum samples. The following oxidative stress-related markers were measured: ROS (in the form of total peroxides), thiobarbituric acid reactive substances (TBARS), SOD, GR, GPx, total glutathione (tGSH) and the ferric reducing ability of plasma (FRAP). Cardiovascular measurements included: 24h ambulatory blood pressure monitoring. Regarding the retinal vessel analyses, functional responses of retinal vessels to a FLIP were recorded as percentages of baseline and retinal vessel calibers were determined from retinal fundus images using the DVA. Oxidative stress-related markers between black and white men were compared by means of independent t-tests. Chi square tests were used to compare proportions between black and white men. The correlations between retinal vessel calibers and vessel responses to FLIP with oxidative stress-related markers were investigated by means of Pearson and partial analyses. Furthermore, independent associations of oxidative stress-related markers with retinal vessel calibers and responses of retinal vessels to a FLIP were investigated in forward stepwise multiple regression analyses. For the final multiple regressions (which provided the best fit models), variables that entered the stepwise models were used in a separate multiple regression analysis and are presented in tables. Sensitivity analyses was also performed, excluding human immune deficiency virus (HIV) infected participants and also testing the effect of smoking, hypertensive status and using anti-hypertensive medication by adding these factors individually to the models.

Results

Despite higher GPx activity ($p=0.001$) and a tendency of SOD ($p=0.062$) to be higher in black men, they also had higher oxidative damage (TBARS) ($p=0.011$) with lower levels of non-enzymatic antioxidant markers (FRAP and tGSH) (both $p<0.001$) compared to white men. Regarding the functional retinal vessel measurements, Art_max_{dil} ($p=0.036$) was higher in black men compared to white men. Retinal vessel caliber markers showed a smaller AVR and a wider

CRVE (both p<0.001) in black men when compared to white men. An independent association between Art_max_{dil} and SOD (Adjusted R^2=0.19; β=-0.36; p=0.002) was seen in black men only. In the black group, AVR (Adjusted R^2=0.12; β=-0.32; p=0.007) and CRAE (Adjusted R^2=0.27; β=-0.23; p=0.040) inversely associated with ROS.

Conclusion

Art_max_{dil} in response to FLIP was negatively associated SOD in black men. Since SOD is known to be defensive against the effects of $O_2^{\cdot-}$ by increasing the dismutation of $O_2^{\cdot-}$, the up-regulation of SOD may also be needed in the black group to prevent oxidative damage to macromolecules. However, the up-regulated SOD activity may be inadequate to prevent oxidative damage to lipids (reflected by higher TBARS) and also inadequate for the preservation of $NO^{\cdot}$ bioavailability but not to a point where Art_max_{dil} is reduced. Although the retinal vascular function is still maintained, continued exposure to increased oxidative stress may impair Art_max_{dil}. Retinal vessel calibers (AVR and CRAE) were independently associated with ROS in black men only. These results indicate that the narrowing of retinal arteries is mediated by ROS suggesting that oxidative stress may play a role in retinal microvascular changes.

PREFACE

The layout of this dissertation is as follows:

Chapter 1: Provides an introduction, short background and motivation of the study.

Chapter 2: Contains a literature overview of the topic, aims and hypothesis

Chapter 3: Methodology applied in study.

Chapter 4: This chapter contains a manuscript which includes an abstract, introduction, methods, results, discussion, conclusion and acknowledgements of the research study, presented in the format required by the journal: *Free Radical Research*.

Chapter 5: Conclusion based on the main findings of this study as well as recommendations for future research.

Relevant references are given at the end of each chapter, according to the referencing style of the journal: *Free Radical Research.*

LIST OF FIGURES AND TABLES

Chapter 2

Figure 1: The pathways of ROS and RNS formation and clearance 9

Figure 2: Hierarchical model of oxidative stress levels in response to redox cycling chemicals 10

Figure 3: The schematic representation of glutathione redox cycle 12

Figure 4: Schematic illustration of lipid peroxidation process 14

Chapter 3

Figure 1: Retinal fundus image on real-time monitor 62

Figure 2: Retinal vascular reaction in response to FLIP 63

Figure 3: Colour image illustrating retinal blood vessels 64

Figure 4: A monochrome image with a selection of vessel segments between 0.5-1.0 optic disc diameters from the outer margin of the optic disc 65

Chapter 4

Table 1: Characteristics of black and white men 87

Table 2: Partial regression analyses between retinal vessel calibers and functional retinal vessel measurements with oxidative stress-related markers in black and white men 89

Table 3: Independent association of Art_max$_{dil}$ with SOD 92

Table 4: Independent associations of retinal vessel calibers with oxidative stress-related markers 93

Table S1: Single regression analyses between retinal vessel calibers and functional retinal vessel measurements with oxidative stress-related markers in black and white men 99

LIST OF ABBREVIATIONS

ABPM	Ambulatory blood pressure monitoring
Ang II	Angiotensin II
ARE	Antioxidant response element
ARIC	Atherosclerosis Risk In Communities
Art_max_{dil}	Maximum artery dilation
AVR	Arterio-venous ratio
BMI	Body mass index
CAD	Coronary artery disease
CAT	Catalase
cIMT	Carotid intima-media thickness
Cm	Centimeter
CRAE	Central retinal artery equivalent
CRP	C-reactive protein
CRVE	Central retinal vein equivalent
Cu^{2+}	Copper
CVD	Cardiovascular diseases
DBP	Diastolic blood pressure
DNA	Deoxyribonucleic acids
DVA	Dynamic vessel analyzer

DVA	Dynamic vessel analyses
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
Fe^{2+}	Iron
FLIP	Flicker light-induced provocation
FRAP	Ferric reducing ability of plasma
G-6-PDH	Glucose-6-phosphate dehydrogenase
GGT	Gamma glutamyl transferase
GPx	Glutathione peroxidase
GR	Glutathione reductase
tGSH	Total glutathione
GSSG	Glutathione disulphide
HDL	High density lipoprotein
HIV	Human immunodeficiency virus
HNE	4-hydroxynonenal
HOCl	Hypochlorous acid
H_2O_2	Hydrogen peroxide
H_2O	Water
Kg	Kilograms
Kg/m^2	Kilograms per meter squared

LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MESA	Multi-Ethnic Study of Atherosclerosis
m	Meter
mmHg	Millimeter mercury
MPO	Myeloperoxidase
MU	Measuring units
NADPH	Nicotinamide-adenine dinucleotide oxidase
NF-κB	Nuclear factor κB
$NO^{\cdot}$	Nitric oxide
NOS	Nitric oxide synthase
NOx	Nitric oxide metabolite
Nrf-2	Nuclear factor erythroid 2-related factor 2
3-NT	3-Nitrotyrosine
oxLDL	Oxidized low density lipoprotein
O_2	Oxygen
$O_2^{\cdot-}$	Superoxide
$OH^{\cdot}$	Hydroxyl radical
$ONOO^{-}$	Peroxynitrite

8-oxodG	8-Oxo-7,8-dihydro-2'-deoxyguanosine
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SABPA	Sympathetic activity and Ambulatory Blood pressure in Africans
SAfrEIC	South African study regarding the role of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function
SBP	Systolic blood pressure
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
$Vein_max_{dil}$	Maximum vein dilation

CHAPTER 1

INTRODUCTION AND MOTIVATION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide [1,2] but also in sub-Saharan Africa [3]. Many risk factors contribute to the development of CVD with hypertension being one of the most important risk factors [4,5]. The prevalence of hypertension is increasing in South Africa, especially among urban black Africans [6], which may also increase their risk of developing CVD.

Endothelial dysfunction is considered to be an early marker of cardiovascular risk [7] and precedes the manifestation of CVD [8]. Increased oxidative stress is associated with endothelial dysfunction, inflammation, cell proliferation and vascular remodeling [9] and may contribute to the development of cardiovascular pathologies such as hypertension, atherosclerosis, heart failure, stroke and cardiac hypertrophy [10]. Oxidative stress is defined as an imbalance between oxidants and antioxidant defense capacity, favoring the oxidants, which may lead to the damage of macromolecules (such as proteins, deoxyribonucleic acids (DNA) and lipids) and/or disturbance in redox signaling and regulation [11]. The antioxidant defense system is composed of enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) and non-enzymatic antioxidants such as total glutathione (tGSH) and bilirubin, ascorbate, tocopherols and uric acid [9] which can be collectively measured with the ferric reducing ability of plasma (FRAP) assay [12].

Previous sub-studies of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study evaluated the relationships of oxidative stress-related markers with carotid intima-media thickness (c-IMT) (subclinical atherosclerosis) [13,14], blood pressure and arterial stiffness [15], arterial compliance and vascular resistance [16] in blacks and whites. However, the explored relationships were limited to components of the macrovasculature. As changes in microvascular function may occur prior to macrovascular dysfunction [17], it is also important to assess how the antioxidant defense capacity and markers of oxidative damage relate to the retinal microvasculature, as an early marker of CVD risk [18,19], in this sub-Saharan population.

The retinal microvasculature can be directly visualized and studied by using devices such as the Dynamic Vessel Analyzer (DVA) [20]. Retinal fundus images can be obtained to determine the central retinal artery and vein calibers [19] and retinal vessel responses to a flicker light-induced provocation (FLIP) can be assessed [21]. Regarding the retinal vessel calibers, a narrower artery and vein widening has been associated with clinical and subclinical CVD [22-24]. Similarly, studies have indicated that coronary artery disease (CAD) [25], diabetes [26], hyperlipidemia [27] and hypertension [21] may cause a reduction in retinal vessel responses to FLIP. Taken together, retinal vessel calibers and retinal vessel responses to FLIP are possible early indicators of vulnerability to systemic pathologies and the risk of developing CVD [25].

The link between oxidative stress-related markers and the retinal microvasculature has been scarcely studied. Up to date, only a few studies [28,29] have explored these relationships. To our knowledge, there are no studies that have been done on the relationships of retinal vessel calibers and retinal vessel responses to FLIP and oxidative stress-related markers in a cohort of blacks and whites from South Africa.

References

[1] Kengne AP, Amoah AG, Mbanya JC. Cardiovascular complications of diabetes mellitus in sub-Saharan Africa. Circulation 2005;112(23):3592-3601.

[2] Wong J, Wong S. Trends in lifestyle cardiovascular risk factors in women: analysis from the Canadian national population health survey. Int J Nurs Stud 2002;39(2):229-242.

[3] Mbewu A. The burden of cardiovascular disease in sub-Saharan Africa: burden of cardiovascular disease. S Afr Heart 2009;6(1):4-10.

[4] Addo J, Smeeth L, Leon DA. Hypertension in sub-Saharan Africa a systematic review. Hypertension 2007;50(6):1012-1018.

[5] Kannel WB. Blood pressure as a cardiovascular risk factor: prevention and treatment. JAMA 1996;275(20):1571-1576.

[6] Steyn K, Bradshaw D, Norman R, Laubscher R. Determinants and treatment of hypertension in South Africans: the first demographic and health survey. S Afr Med J 2008;98(5):376-380.

[7] Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol 2004;15(8):1983-1992.

[8] Hadi HA, Carr CS, Suwaidi JA. Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. Vasc Health Risk Manag 2005;1(3):183.

[9] Touyz RM, Briones AM. Reactive oxygen species and vascular biology: implications in human hypertension. Hypertens Res 2011;34(1):5-14.

[10] Briones AM, Touyz RM. Oxidative stress and hypertension: current concepts. Curr Hypertens Rep 2010;12(2):135-142.

[11] Jones DP. Redefining oxidative stress. Antioxid Redox Signal 2006;8(9-10):1865-1879.

[12] Benzie IF, Strain J. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. Anal Biochem 1996;239(1):70-76.

[13] van Zyl C, Huisman HW, Mels CM. Antioxidant enzyme activity is associated with blood pressure and carotid intima media thickness in black men and women: The SABPA study. Atherosclerosis 2016;248:91-96.

[14] Schutte R, Schutte AE, Huisman HW, Van Rooyen JM, Malan NT, Péter S, Fourie CM, Van Der Westhuizen FH, Louw R, Botha CA. Blood glutathione and subclinical atherosclerosis in African men: the SABPA Study. Am J Hypertens 2009;22(11):1154-1159.

[15] Kruger R, Schutte R, Huisman H, Van Rooyen J, Malan N, Fourie C, Louw R, Van der Westhuizen F, Van Deventer C, Malan L. Associations between reactive oxygen species, blood pressure and arterial stiffness in black South Africans: the SABPA study. J Hum Hypertens 2012;26(2):91-97.

[16] Mokhaneli MC, Fourie CMT, Botha S, Mels CMC. The association of oxidative stress with arterial compliance and vascular resistance in a bi-ethnic population: the SABPA study. Free Radic Res 2016:920-928.

[17] Gates PE, Strain WD, Shore AC. Human endothelial function and microvascular ageing. Exp Physiol 2009;94(3):311-316.

[18] Wong TY, Klein R, Klein BE, Tielsch JM, Hubbard L, Nieto FJ. Retinal microvascular abnormalities and their relationship with hypertension, cardiovascular disease, and mortality. Surv Ophthalmol 2001;46(1):59-80.

[19] Liew G, Wang JJ, Mitchell P, Wong TY. Retinal vascular imaging a new tool in microvascular disease research. Circ Cardiovasc Imaging 2008;1(2):156-161.

[20] Flammer J, Konieczka K, Bruno RM, Virdis A, Flammer AJ, Taddei S. The eye and the heart. Eur Heart J 2013;34(17):1270-1278.

[21] Nagel E, Vilser W, Lanzl I. Age, blood pressure, and vessel diameter as factors influencing the arterial retinal flicker response. Invest Ophthalmol Vis Sci 2004;45(5):1486-1492.

[22] Ikram MK, Cheung CY, Lorenzi M, Klein R, Jones TL, Wong TY. Retinal vascular caliber as a biomarker for diabetes microvascular complications. Diabetes Care 2013;36(3):750-759.

[23] Tikellis G, Arnett DK, Skelton TN, Taylor HW, Klein R, Couper DJ, Sharrett AR, Wong TY. Retinal arteriolar narrowing and left ventricular hypertrophy in African Americans. The Atherosclerosis Risk in Communities (ARIC) study. Am J Hypertens 2008;21(3):352-359.

[24] Kawasaki R, Cheung N, Wang JJ, Klein R, Klein BE, Cotch MF, Sharrett AR, Shea S, Islam FA, Wong TY. Retinal vessel diameters and risk of hypertension: the Multiethnic Study of Atherosclerosis. J Hypertens 2009;27(12):2386.

[25] Al-Fiadh AH, Farouque O, Kawasaki R, Nguyen T, Uddin N, Freeman M, Patel SK, Burrell LM, Wong TY. Retinal microvascular structure and function in patients with risk factors of atherosclerosis and coronary artery disease. Atherosclerosis 2014;233(2):478-484.

[26] Pemp B, Garhofer G, Weigert Gn, Karl K, Resch H, Wolzt M, Schmetterer L. Reduced retinal vessel response to flicker stimulation but not to exogenous nitric oxide in type 1 diabetes. Invest Ophthalmol Vis Sci 2009;50(9):4029-4032.

[27] Reimann M, Julius U, Bornstein S, Fischer S, Reichmann H, Rüdiger H, Ziemssen T. Regular lipoprotein apheresis maintains residual cardiovascular and microvascular function in patients with advanced atherosclerotic disease. Atheroscler Suppl 2013;14(1):135-141.

[28] Daien V, Carriere I, Kawasaki R, Cristol J-P, Villain M, Fesler P, Ritchie K, Delcourt C. Retinal vascular caliber is associated with cardiovascular biomarkers of oxidative stress and inflammation: the POLA study. PLoS One 2013;8(7):e71089.

[29] Seshadri S, Mroczkowska S, Qin L, Patel S, Ekart A, Gherghel D. Systemic circulatory influences on retinal microvascular function in middle-age individuals with low to moderate cardiovascular risk. Acta Ophthalmol 2015;93(4):e266-e274.

CHAPTER 2

LITERATURE REVIEW

2.1. Defining oxidative stress

Oxidative stress is defined as an imbalance between oxidants and antioxidant defense capacity, in favor of oxidants, which may result in molecular damage and/or altered redox signaling and regulation [1]. Increased oxidative stress is one of the factors associated with pathogenic mechanisms which may lead to the development of cardiovascular disease (CVD) [2-4]. The impact of increased oxidative stress is dependent on several factors such as the type of reactive oxygen species (ROS), the site and intensity of ROS production, antioxidant enzyme activity as well as the capacity of repair systems [5].

2.2. Different types of reactive species and their metabolism

Free radicals are molecules that contain one or more unpaired electron(s) in their outer orbital which leads to high reactivity of these molecules [6]. ROS include free radical molecules such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$) as well as non-radical molecules such as hydrogen peroxide (H_2O_2). Reactive nitrogen species (RNS) include molecules such as nitric oxide ($NO^{\cdot}$) and the non-radical molecule, peroxynitrite ($ONOO^-$) [6-8].

ROS and RNS are formed in all living organisms by enzymatic and non-enzymatic reactions [9,10]. In the reduction-oxidation (redox) process, ROS are formed as intermediates forming water (H_2O) from oxygen (O_2) [2] (Figure 1). One electron reduction of O_2 leads to the production of $O_2^{\cdot-}$ by several enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH), xanthine oxidase and uncoupled endothelial nitric oxide synthase (eNOS) [11]. The enzyme nitric oxide synthase (NOS) metabolize L-arginine to form $NO^{\cdot}$ and citrulline [12]. The rapid reaction of $O_2^{\cdot-}$ with $NO^{\cdot}$ forms $ONOO^-$ [13] which can also be generated directly by NOS when the levels of L-arginine are low [14] or under conditions whereby tetrahydrobiopterin is oxidized [15]. H_2O_2 is formed through the oxidation of two O_2 electrons [11]. Additionally, dismutation of $O_2^{\cdot-}$ by superoxide dismutase (SOD) can also lead to the formation of H_2O_2 [11] which can be eliminated by other antioxidant enzymes such as

glutathione peroxidase (GPx), catalase (CAT) and peroxiredoxin [16]. Hydroxyl radicals are formed when H_2O is exposed to high energy ionizing radiation [17] and when H_2O_2 molecules come in contact with iron (Fe^{2+}) or copper (Cu^{2+}) ions [18]. Furthermore, H_2O_2 can also be metabolized by myeloperoxidase (MPO) to form hypochlorous acid (HOCl) [16].

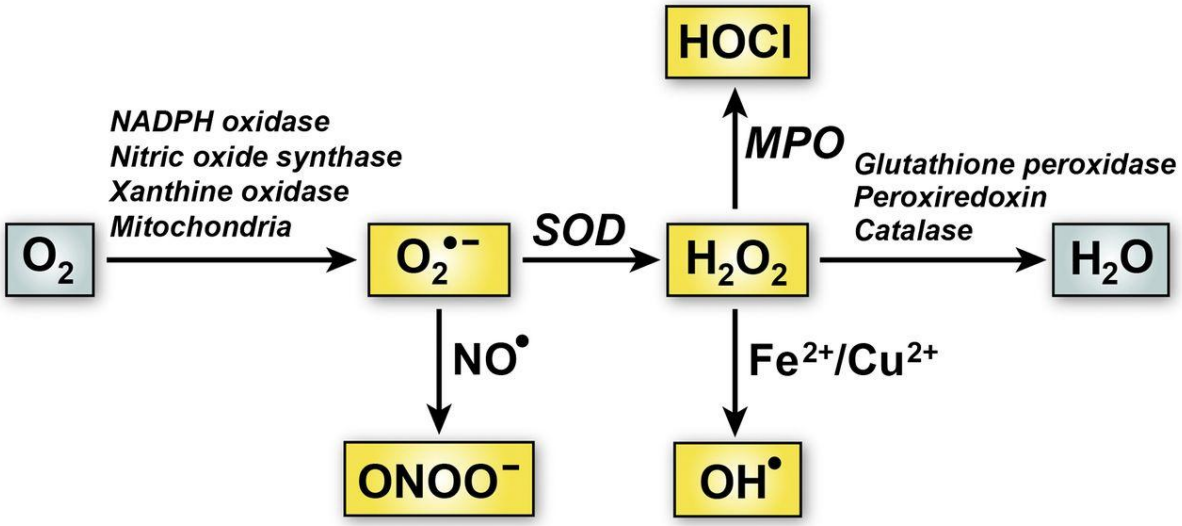


Figure 1: The pathways of ROS and RNS formation and clearance [16]. Superoxide anion ($O_2^{\cdot-}$) is produced via several enzymes such as nicotinamide adenine dinucleotide phosphate NADPH amongst the others. Nitric oxide ($NO^{\cdot}$) formed from the metabolized L-arginine reacts with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$). The enzyme superoxide dismutase (SOD) catalyzes $O_2^{\cdot-}$ to form hydrogen peroxide (H_2O_2) which is eliminated by other enzymes to form water (H_2O). H_2O_2 is further metabolized to hypochlorous acid (HOCl) via myeloperoxidase (MPO). When H_2O_2 come in contact with iron (Fe^{2+}) or copper (Cu^{2+}) ions, hydroxyl radicals ($OH^{\cdot}$) are formed.

2.3. Oxidative and nitrosative stress

ROS generated at lower concentrations can be easily neutralized by antioxidant defense systems [19]. At this lower level of oxidative stress (Figure 2) antioxidant enzymes are induced via the nuclear factor erythroid 2-related factor 2 (Nrf-2)-antioxidant response element (ARE) transcriptional pathway [20]. This transcriptional factor activates the

promoters of genes that encode the antioxidant enzymes [20]. This phase occurs in order to restore cellular redox homeostasis [20]. Any disturbance of this protective response pathway may determine susceptibility of developing CVD during the exposure to oxidative stress [20]. At an intermediate level of oxidative stress, signaling cascades of mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) are activated thereby inducing pro-inflammatory responses [20]. As a consequence, the cell either adapts to oxidative stress by up-regulating antioxidant defense systems or cellular damage takes place [21,22]. At high levels of oxidative stress, ROS production may exceed antioxidant defense capacity which may promote cytotoxicity resulting in cellular apoptosis or necrosis [20]. Cellular apoptosis may result from mitochondrial perturbation [20].

RNS such as $NO^{\cdot}$ are also important in physiological and pathophysiological processes. $NO^{\cdot}$ produced at normal levels play vital roles in the cardiovascular, immune and nervous systems [23,24]. $NO^{\cdot}$ exhibit the ability to participate in physiological processes including neurotransmission, smooth muscle relaxation, immune and blood pressure regulation while other RNS such as $ONOO^{-}$ have the potential to cause nitrosative damage to macromolecules inhibiting their normal function [25-27]. Nitrosative damage to macromolecules results from excessive formation of RNS [28] which exceeds the ability of the antioxidant defense system to combat or eliminate these species [29]. This may occur in pathologies associated with inflammation, ischemia and neurotoxicity [25,30].

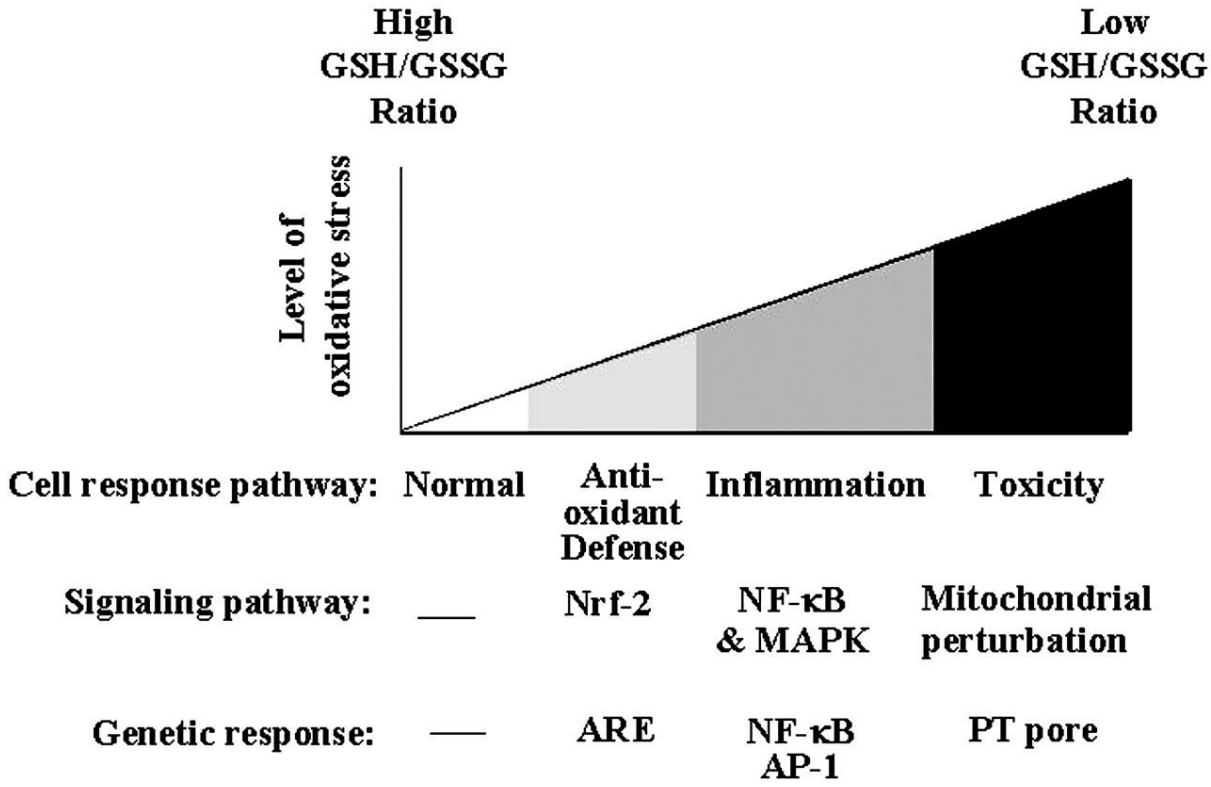


Figure 2: Hierarchical model of oxidative stress levels in response to the redox cycling chemicals [31]. At the lower level of oxidative stress, antioxidant enzymes are activated via the nuclear factor erythroid 2-related factor 2 (Nrf-2)-antioxidant response element (ARE) pathway. The intermediate level of oxidative stress constitutes the activation of mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) which induces pro-inflammatory responses. The higher level of oxidative stress is mediated by mitochondrial perturbation which promotes cytotoxicity.

2.4. Defense systems against oxidative stress

Defense mechanisms such as the antioxidant system are present to counteract increased oxidative stress [6]. An antioxidant is defined as a substance that is capable of delaying or inhibiting the oxidation of a substrate [32]. The antioxidant defense system includes non-enzymatic antioxidants such as total glutathione (tGSH) and the ferric reducing ability of plasma (FRAP) which collectively measures bilirubin, ascorbate, tocopherols and uric acid [2,33]. This defense system also include enzymatic antioxidants such as SOD, GPx found in

the cytosol and mitochondria of the cell, glutathione reductase (GR) and CAT present in the peroxisomes [2].

As a result of increased $O_2^{\cdot-}$ production, SOD catalyzes $O_2^{\cdot-}$ by converting it to H_2O_2 which can in turn be catalyzed by CAT or GPx reactions [34]. CAT converts harmful H_2O_2 to H_2O and O_2 (Figure 3). The glutathione redox system consists of GPx, GSH, glutathione disulphide (GSSG) and GR. GPx catalyzes the conversion of H_2O_2 to form H_2O while GSH is oxidized in the process to form GSSG [6]. To maintain redox homeostasis, GR then reduces GSSG to regenerate GSH, which is a cofactor in the functioning of GPx [35]. Furthermore, glucose-6-phosphate dehydrogenase (G-6-PDH) is responsible for the production of another cofactor for GPx, namely NADPH [35]. In addition, the glutathione redox cycle is a major source of protection against low levels of oxidative stress whereas CAT is more significant in protection against increased oxidative stress [36].

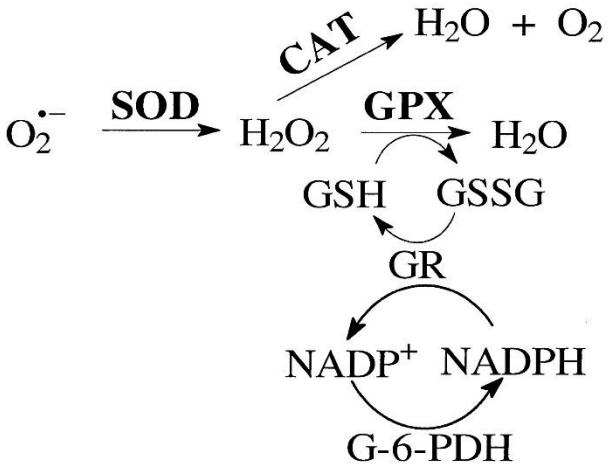


Figure 3: The schematic representation of glutathione redox cycle [35]. Superoxide dismutase (SOD) rapidly reduces superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2). H_2O_2 is converted to water (H_2O) and oxygen (O_2) by the enzyme catalase (CAT). Glutathione peroxidase (GPx) also catalyze the conversion of H_2O_2 to form H_2O, leading to the oxidation of glutathione (GSH). GSH is oxidized to form glutathione disulphide (GSSH). Glutathione reductase (GR) reduces GSSG back to GSH. Glucose-6-phosphate dehydrogenase (G-6-PDH) produces nicotinamide adenine dinucleotide phosphate (NADPH).

2.5. Oxidative damage to macromolecules

Free radicals are produced during normal cellular function but due to their high chemical reactivity, increased production of oxidants and deficiency of antioxidants may lead to oxidative damage of lipids, proteins and deoxyribonucleic acid (DNA) [37]. Oxidative damage of these macromolecules may occur when their structures are altered which may also inhibit their normal function [29]. Most ROS, with the exception of total peroxides cannot be measured directly because of their short half-lives [2], instead, the accumulation of products formed as a result of oxidative damage are frequently measured as markers of oxidative stress [3]. In the current study, total peroxides were measured and are therefore referred to as ROS. Levels of thiobarbituric acid reactive substances (TBARS), 3-nitrotyrosine (3-NT) and 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) can serve as a surrogate markers of oxidative damage to lipids, proteins and DNA respectively [38]. TBARS levels are increased in conditions associated with CVD such as diabetes [39], hypertension [40] and hyperlipidemia [41]. Furthermore, the levels of TBARS also increase as a result of behavioral factors such as cigarette smoking [42], alcohol abuse [43], and obesity [44]. Since the focus in this study was on TBARS as a measure of lipid peroxidation, only the process of lipid peroxidation is discussed in the next section.

2.5.1. Lipid peroxidation

Oxidative damage to lipids occurs via several mechanisms whereby ROS reacts with fatty acids in the membrane lipid bilayer which may lead to membrane leakage and cell death [9]. The breakdown of polyunsaturated fatty acids leads to the formation of malondialdehyde (MDA), which is a product of lipid peroxidation and can be detected by the TBARS assay [45]. During the TBARS assay, the reaction of MDA with thiobarbituric acid (TBA) takes pace to form TBARS which is then measured [45].

Lipid peroxidation is a chain reaction that occurs in three phases, the initiation, propagation and termination phases [32,46,47]. In the initiation phase, a hydrogen atom is abstracted

from the methylene group by a primary reactive radical resulting in the formation of a conjugated diene (step 1) (Figure 4) [47-49]. The propagation phase involves the reaction of carbon-centered fatty acid radicals with molecular oxygen to form highly reactive peroxyl radicals (step 2) [32,47,48]. The peroxyl radicals react with another lipid molecule to form hydroperoxides [50]. In the termination phase, a hydrogen atom is donated to the lipid peroxyl radical species by antioxidants which result in the formation of reactive aldehydes such as 4-hydroxynonenal (HNE) and MDA (step 3) [49,51].

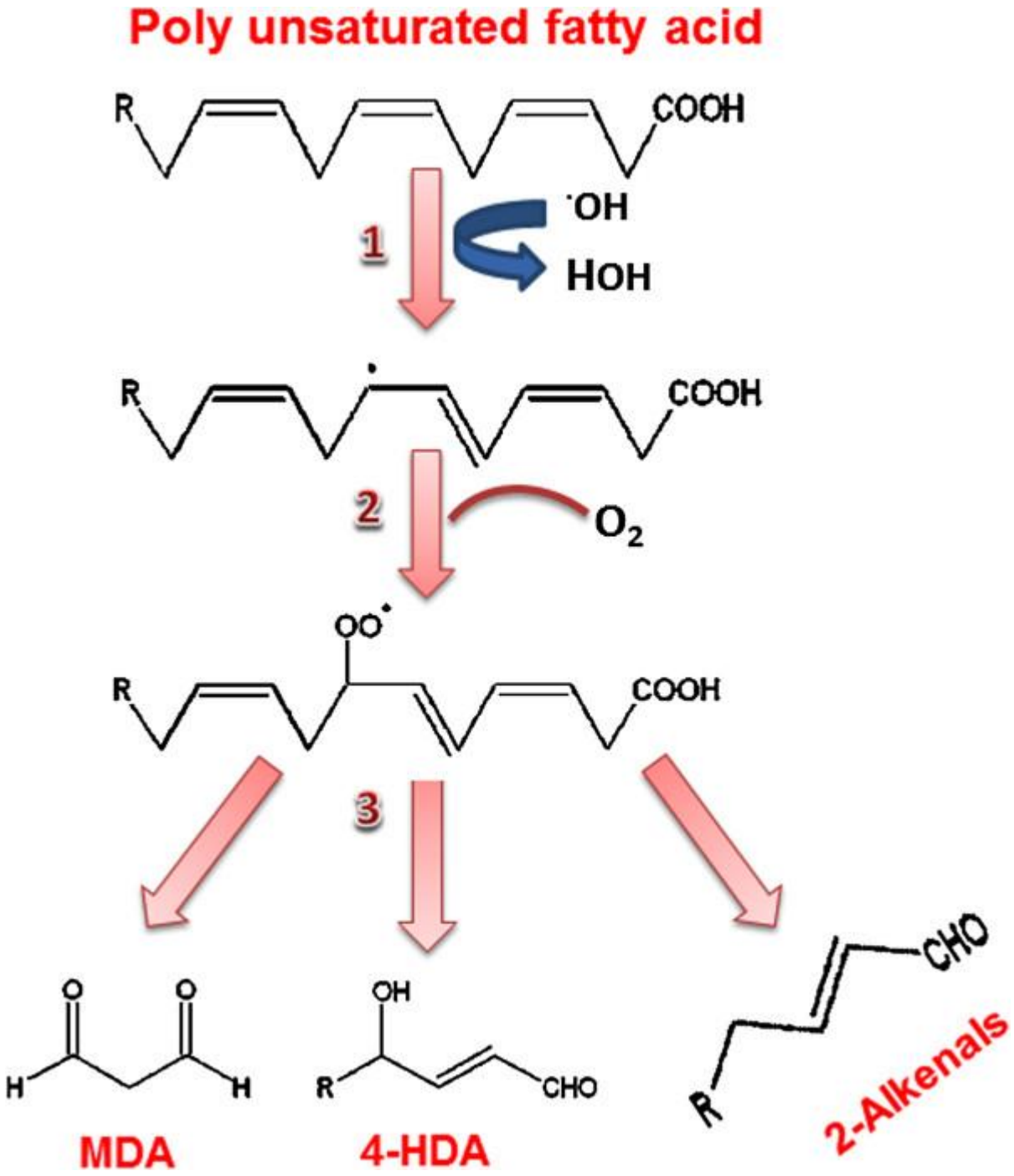


Figure 4: Schematic illustration of lipid peroxidation process [49]. Lipid peroxidation occurs in three phase reactions. ROS, particularly hydroxyl radicals (OH˙), react with lipid membranes and reactive aldehydes including malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are generated.

2.6. Oxidative stress and the vascular system

ROS is produced by all cell types present in each layer of vascular wall including endothelial cells, smooth muscle cells and adventitial fibroblasts [3]. ROS play a physiological role in maintaining vascular integrity [52] by regulating endothelial function and the balance between vascular contraction and relaxation [53]. Processes such as vascular smooth cell growth, apoptosis, migration, inflammation, secretion, and the production of extracellular matrix proteins are modulated by ROS for the regulation of vascular function [52]. In turn, the above mentioned processes may increase the production of ROS and may further lead to endothelial dysfunction and ultimately the development of vascular diseases [54].

When the generation of ROS exceeds the antioxidant defense capacity, the functioning of the endothelium is disrupted [54] which may lead to the impairment of endothelium-dependent vasodilation due to $NO^{\cdot}$ inactivation by $O_2^{\cdot-}$ [55,56]. Furthermore, $O_2^{\cdot-}$ reacts with $NO^{\cdot}$ to yield $ONOO^{-}$ [52,53] which may possibly contribute to cardiovascular pathologies [55]. $NO^{\cdot}$ inactivated by $O_2^{\cdot-}$ may be stabilized to normal levels by an antioxidant enzyme, SOD [57]. However, $NO^{\cdot}$ and $O_2^{\cdot-}$ interact at a rapid rate of 6.7×10^{9} $mol/L^{-1}\cdot s^{-1}$ [58]. This rate is three times faster than the reaction rate for dismutation of $O_2^{\cdot-}$ by SOD [55]. Given this rapid reaction rate, $NO^{\cdot}$ inactivation is more likely to occur with increased $O_2^{\cdot-}$ production which may lead to further vascular damage [55].

Additional to the role of $O_2^{\cdot-}$ in the inactivation of $NO^{\cdot}$, $O_2^{\cdot-}$ can also induce vascular contraction by the activation of arachidonic acid metabolism via the cyclooxygenase pathway [59]. Furthermore, $O_2^{\cdot-}$ may also be involved in the oxidation of low-density lipoprotein (LDL) leading to the formation of oxidized low-density lipoproteins (oxLDL) which may impair the functioning of the endothelium [60] as well as the development of atherosclerosis [61-63]. The exposure of endothelial cells to $O_2^{\cdot-}$ and H_2O_2 induces apoptosis by activating caspase-3 via fatty acid synthase or mitochondria-mediated pathways [64] which may further result in atherogenesis [65]. Apoptosis is also stimulated by oxLDL, angiotensin (Ang) II, high

glucose, and tumor necrosis factor-α which may be inhibited by SOD, CAT and antioxidant vitamins such as vitamin E [65].

The associations of TBARS and the development of CVD are controversial. In a study done on coronary artery disease (CAD) patients (n=634), it was found that higher serum levels of TBARS could predict major cardiovascular events [66]. Other studies also support that the progression of CVD can be predicted by the important role of lipid oxidation [38,67,68]. However, in a small cross-sectional study (n=40), no significant association between elevated TBARS and the presence of CVD was found, after adjusting for blood glucose levels [69], suggesting that glucose may play a role in lipid oxidation in subjects at a risk of developing CVD.

2.7. Oxidative stress-related markers reported in the Sympathetic activity and Ambulatory Blood pressure in Africans (SABPA) study

In the South African population, oxidative stress-related markers have been evaluated in sub-studies which form part of the baseline phase of the SABPA study. Firstly, it was found that ROS levels (in the form of serum peroxides) were higher in black women compared to black men and also in hypertensive black men compared to normotensive black men. Furthermore, higher levels of ROS in hypertensive black men were associated with increased systolic blood pressure (SBP) and pulse pressure. It was hypothesized that increased ROS may be implicated in the development of arterial stiffness and hypertension in the black group [70].

Secondly, in a comparison between hypertensive and normotensive black men, carotid intima-media thickness (c-IMT) was higher in hypertensive black men while GSH levels did not differ between the groups. Furthermore, in hypertensive black men, attenuated GSH levels were associated with increased c-IMT. This results could link reduced antioxidant defense capacity with carotid intima-media thickening which could have future implications in the development of atherosclerosis [71].

Thirdly, it was found that African men had significantly lower FRAP and 8-oxodG levels, a marker of oxidative stress, higher ROS levels (serum peroxides), while their tGSH levels and GR activity were significantly higher. It was proposed that these results may indicate a dose-response relationship in which increased oxidative stress may play a central role in the up-regulation of antioxidant defense and DNA repair mechanisms to prevent oxidative damage [72].

Fourthly, when antioxidant enzyme activities were compared between black and white men and women, GR activity was higher in black men and women whereas GPx activity was lower in black women. Furthermore, it was found that higher GR activity was associated with c-IMT in black men while lower GPx activity in black women was associated with mean arterial pressure, systolic and diastolic blood pressure. These results were thought to indicate a compensatory response of the up-regulated GR activity to prevent arterial remodeling while highlighting the role of reduced antioxidant enzyme activity in the development of hypertension [73].

Lastly, it was found that black participants had higher TBARS and GPx activity when compared to their white counterparts. Increased oxidative damage of lipids (TBARS) was associated with increased vascular resistance (total peripheral resistance) and decreased arterial compliance (windkessel compliance) independent of hypertensive status in black participants. It has been suggested from these results that oxidative stress may play a role in early vascular changes in a black population who are at risk of developing CVD [74].

As this dissertation investigated the relationship between oxidative stress-related markers and the retinal microvasculature, literature regarding the retinal microvasculature is now provided.

2.8. The vasculature of the eye

The eye circulation is divided into four parts: i) the ciliary body of the anterior part of the eye where the aqueous humour is produced, ii) the retina (which is similar to the circulation in the

brain but lacks autonomic innervations), iii) the choroid (which is densely innervated and has fenestrated capillaries), and iv) the optic nerve head [75]. As retinal blood vessels are investigated, the relevant literature is discussed below.

2.9. The retinal microvasculature

The retina is unique in that its microvasculature can be directly visualized and studied [76]. Using devices such as the Dynamic Vessel Analyzer (DVA), vessel calibers can be determined from retinal fundus images [77] and retinal vessel responses to a flicker light-induced provocation (FLIP) can also be assessed [78]. These measurements are possible indicators of early changes occurring in the retinal microvasculature [79]. Importantly, these changes may be related to the development of CVD such as arterial hypertension or coronary heart disease [79]. Furthermore, retinal arteries share similar changes as cerebral and peripheral arteries, particularly in hypertension which may indicate that retinal arterial abnormalities may be a reflection of structural or functional microvascular changes occurring in other organ systems [80-82]. Additionally, retinal microvascular abnormalities, such as generalized arterial narrowing, focal arterial narrowing, arteriovenous (AV) nicking and retinopathy, may be a reflection of damage to the arteries from hypertension (amongst the others) [83-85] and have been hypothesized as markers for CVD [86,87].

2.10. Retinal vessel calibers

Information can be gained regarding the central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE). These variables are summary indexes which reflect the average lumen diameter of central retinal artery and vein respectively. Subsequently, the ratio of these two variables (the arterio-venous ratio (AVR)) is calculated [88].

Traditionally, a reduced AVR was thought to reflect generalized arterial narrowing [89-91] since it was assumed that vein caliber remain unchanged [92]. However, recent studies have suggested a smaller AVR may not only reflect narrower arteries but also wider veins [93,94]

Furthermore, limitations of using the AVR have been noted [93,94]. The use of AVR may capture less information in predicting cardiovascular outcomes than individual retinal vessel calibers [95], and may also lead to incorrect inferences since arterial and vein calibers carry different prognostic information [93,95]. Despite the limitations of using AVR, studies have also found its association with aging [96], cigarette smoking [97], current and past blood pressure [89,98], the development of hypertension [99], progression to severe forms of hypertension [100] incident stroke [101], coronary heart disease [102], and cardiovascular mortality [103]. In addition to the prognostic potential of AVR, diabetes [104], heart failure [105] and metabolic syndrome [106] has been reported to be associated with AVR.

Studies such as the Rotterdam study [93] and the Multi-Ethnic Study of Atherosclerosis (MESA) [107] were conducted to evaluate associations of CVD risk factors with retinal arterial and vein calibers individually. In the Rotterdam study, retinal arterial and vein diameters were found to be decreased with increasing blood and pulse pressures [93]. Furthermore, in both studies, smaller arterial diameters were associated with increased c-IMT [93], higher systolic and diastolic blood pressure, hypertension status, current alcohol consumption, greater body mass index (BMI) while larger vein diameters were associated with higher carotid plaque score, higher levels of high sensitivity C-reactive protein, greater BMI and waist-hip ratio, higher levels of serum glucose, higher total serum cholesterol, higher plasma triglyceride, higher plasma LDL-cholesterol, lower high density lipoprotein (HDL)-cholesterol, and current cigarette smoking [93,107]. These results indicate that retinal artery and vein diameters may play an independent role in predicting cardiovascular disorders.

Changes in retinal vessel caliber have been recognized as an important predictor of systemic damage from elevated blood pressure [108,109]. Generalized arterial narrowing is associated with hypertension characterized by a smaller AVR and CRAE with a wider or unchanged CRVE [110]. These vascular changes [111] may have future implications in the retinal microvasculature which may also aid in predicting CVD [112]. Additionally, studies

have demonstrated that narrower retinal arterial caliber and reduced AVR are associated with both prevalent and incident hypertension [113,114] and wider retinal vein caliber independently predict incident stroke [115].

2.11. Retinal vessel responses to FLIP

The DVA makes use of FLIP to induce retinal vessel dilation [116]. When the retina is stimulated by the flickering light, the dilation of both the arteries and veins occurs [76]. Furthermore, flickering light induces neurovascular coupling, leading to a dilation of capillaries and small arterioles/arteries thereby inducing flow-mediated dilation of larger retinal vessels [76]. Flicker light-induced dilation of the retinal arteries has been shown to be endothelium-and $NO^{\cdot}$-dependent but independent from sympathetic innervations [117]. Indeed it is proposed to provide information regarding microvascular endothelial function [76]. $NO^{\cdot}$ released in the retinal vasculature during FLIP is thought to be the mediator of vessel dilation [118]. *Dorner et al*. [117] conducted a study comprising of 12 health young subjects. These subjects were given an intravenous infusion of either a placebo or N^{G}-monomethyl-L-arginine, a NOS inhibitor. Results have shown that the administration of NOS inhibitor reduced the artery and vein diameter as well as responses of retinal arteries during FLIP [117]. These results indicate the role of $NO^{\cdot}$ in the control of retinal vascular tone and flicker light-induced dilation in humans [117].

2.11.1. Assessment of vascular endothelial function

There are few non-invasive techniques available to measure endothelial function in humans which includes flow-mediated vasodilation of the brachial artery and finger plethysmography [119].

Regarding the microcirculation, retinal vessel dilation in response to FLIP is proposed to be a unique and non-invasive measure of vascular function of the endothelium [76]and although not conclusive, may offer a diagnostic potential in detecting endothelial dysfunction prior to the development of CVD [120]. A few studies have indicated that hypertension [78], diabetes

[121] and hyperlipidemia [122] (all associated with endothelial dysfunction) are related to a reduction in retinal arterial dilation. Furthermore, studies have also reported a reduced vein dilation in conditions associated with CVD risk such as obesity and diabetes [123,124]. *Malan et al*. [125] reported a higher vein dilation but similar arterial dilation in blacks when compared to whites. In the general vasculature, the impairment of endothelium-dependent dilation indicates the loss of the functional integrity of the endothelium [126] and this may then hold true for the retinal microvasculature in response to FLIP. Endothelial dysfunction is a process characterized amongst others by reduced $NO^{\cdot}$ bioavailability whereby the endothelium fails to induce $NO^{\cdot}$-mediated dilation [127,128] and may be a precursor of advanced forms of CVD such as atherosclerosis, hypertension, stroke and CAD [120,127,128]. In this way, endothelial dysfunction enhances the risk of future cardiovascular mortality [129,130].

In addition, vascular function can also be measured using a laser Doppler imagery and iontophoresis. This method measures the skin microvascular response to acetylcholine and sodium nitroprusside [131]. It has been hypothesized that ethnic differences in microvascular function may exist. This was examined in a group of young (18-33 years old), apparently healthy men and women (9 black Africans, 12 mixed ancestry and 12 whites). Microvascular endothelial function was found to be better in whites when compared to black Africans and mixed ancestral descent [131]. These differences were thought to be dependent on skin resistance. However, after adjusting for skin resistance, endothelium-independent response to sodium nitroprusside remained attenuated in the black Africans and mixed ancestry participants. The authors speculated that the smooth muscle may be sensitive to the vasodilatory effect of $NO^{\cdot}$ which may contribute to ethnic differences in the risk of developing CVD [131].

2.12. Confounding factors associated with oxidative stress-related markers, retinal vessel calibers and retinal blood vessel responses to FLIP respectively

2.12.1. Age

Aging is a complex and multifactorial process which leads to a gradual deterioration in physiological functions [132]. In human studies, there are conflicting results with regards to the impact of oxidative stress on aging. The discrepancies between studies may possibly be explained by the influence of lifestyle factors on oxidative stress such as cigarette smoking and alcohol abuse [42,43,133] which may influence biological aging process. However, the precise biological and cellular mechanisms involved in the aging process are unknown. According to *Fontana and Klein* [134], biological and cellular mechanisms are likely to involve oxidative stress-induced protein and DNA damage along with inadequate DNA damage repair. Several studies have found lower antioxidant defense capacity markers such as FRAP [133,135], SOD [136] and GR [137] in older people (41-89 years) whereas some studies found higher antioxidant enzyme activities of SOD [138], CAT and GPx [136] in older people (41-99 years). A negative correlation was shown between SOD activities and age. Conversely, CAT and GPx correlated positively with age [136]. Furthermore, some studies showed no differences in antioxidant defense capacity markers (tGSH, GPx, CAT and SOD) between the older and middle-aged subjects (middle aged 55-70 years; late middle aged 75-85 years; older >85 years) [133,137]. While some studies reported an increase TBARS levels with increasing age [40,139], a decrease in TBARS levels with age increase was also reported [133]. Taken all observations together, higher activities of antioxidant enzymes may play a protective role against exposure to sustained oxidative stress in older subjects but reduced antioxidant defense capacity may be a predictor of oxidative stress risk in this group. These results also suggest that reduced antioxidant defense capacity may enhance oxidative damage to lipids with increasing age.

Consistent findings relating to retinal vascular calibers and their association with age have been observed in older people and older people with increased blood pressure. It has been found that older people (75-84 years) with and without higher blood pressure have narrower arterial diameters [96]. The association of narrowed retinal arteries and higher blood pressure was stronger in younger persons (43-54 years). It has been suggested that the weaker association of retinal arterial diameters and blood pressure in older people may reflect greater retinal arterial sclerosis, preventing arterial narrowing with elevated blood pressure [96]. Retinal vessel calibers have also been examined in subjects aged 49 years and above [91]. Data from this study indicated a decrease in retinal vessel diameters (both CRAE and CRVE) with increasing age. Furthermore, AVR was also reduced for each increasing decade of age, until 79 years. These data suggest that retinal vascular calibers narrow with increasing age [91].

In a study to investigate retinal vessel response to FLIP, 52 clinically healthy volunteers (aged 20-78 years) free of CVD risk factors were recruited [140]. It was found that retinal arterial dilation in older people was reduced. The author speculated that the reduction in arterial dilation could be due to an age-related decline in the regulation of retinal vessel diameter. Furthermore, a reduced arterial dilation in older people can also be attributed to the physiological changes of the vascular endothelial function that occurs with the aging process [140]. These observations were also confirmed by a study conducted in both eyes of 156 healthy volunteers [141]. These volunteers were divided into three age groups: young (n=52, 29.1 ± 5.2 years), middle age (n=54, 44.5 ± 4.3 years) and elderly (n=50, 61.3 ± 5.1 years). Maximum arterial and vein dilations were reduced in the young group compared to the middle age group and also in the elderly group. These results indicate that age plays a role in retinal vessel responses to FLIP [141].

2.12.2. Hypertension

Hypertension is generally associated with increased oxidative stress [142]. In hypertensive subjects, tGSH levels and antioxidant enzyme activities of SOD, GPx and CAT were found to be reduced while GSSG was higher when compared to normotensive subjects [143]. Taken from the above mentioned results, the activity of ROS may increase GSSG leading to a decrease in antioxidant defense capacity [143]. Furthermore, higher GPx activity was also reported in another study performed on subjects with essential hypertension [144]. When the relationship between 24h ambulatory blood pressure and oxidative stress-related markers was further investigated in hypertensive subjects, no significant relationships were observed [143]. It has been suggested that the absence of this relationship indicates that other factors such as hyperinsulinemia or increased Ang II activity rather than blood pressure alone may contribute to the decreased antioxidant defense mechanisms [143].

The relationship between retinal vascular calibers (mainly CRAE) and elevated blood pressure has been reported to be the strongest among other CVD risk factors [89,93,145]. Since retinal arterial narrowing has been proposed to be a reflection of structural damage from chronic hypertension, the relationship of retinal arterial narrowing and AV nicking with past blood pressure (measured three and six years before retinal assessment) has been reported [98]. This relationship was noted to be evident even after the adjustments for current blood pressure were made. This finding suggests that arterial narrowing and AV nicking may be indicators of progressive hypertensive damage [98]. However, data from several longitudinal cohort studies provided evidence that retinal arterial narrowing and widening of veins are not only related to chronic hypertension but may also precede the development of hypertension [99,114,146].

A study of 30 healthy subjects and 15 subjects with untreated essential arterial hypertension was conducted [78]. In this study, retinal arterial dilation in response to FLIP was reduced in subjects with hypertension compared to healthy subjects which the authors suggested could

be due to endothelial cell damage or sclerotic vascular changes in these patients [78]. The authors further speculated that these may lead to the pathogenesis of vascular diseases [78].

2.12.3. Diabetes mellitus

There is evidence indicating increased production of free radicals and reduced antioxidant defense capacity in diabetes. ROS generated as a result of hyperglycaemia may play a role in the pathogenesis of diabetic complications [147,148]. Various studies were conducted with regards to oxidative stress-related markers in diabetic subjects. Higher serum levels of TBARS were found in diabetics, with significantly higher TBARS levels in type II diabetic subjects than type I diabetic subjects [39]. Results from this study suggested that increased formation of free radicals may contribute to enhanced lipid peroxidation in diabetes [39]. Other studies showed lower antioxidant enzyme activities such as CAT and SOD in both types of diabetes [149-151] suggesting that hyperglycaemia may reduce antioxidant enzyme activity presumably by increased glycation [149,152].

The Atherosclerosis Risk In Communities (ARIC) Study showed a higher risk of incident diabetes (independent of other CVD risk factors) in non-diabetic individuals with smaller AVR [153]. However, arterial and vein calibers were not separately examined in this study. The Rotterdam Eye Study proposed that the association found with AVR in the ARIC study may be due to the changes in retinal vein caliber instead of arterial narrowing [154]. This was further confirmed by demonstrating an association of larger retinal vein caliber with impaired fasting glucose levels [154]. Furthermore, various studies have also found an association between wider retinal arterial caliber and diabetes [155-157]. The underlying pathophysiological mechanisms for these observations remain unclear. It has been speculated that endothelial dysfunction and inflammatory processes may play integral roles in the development of wider retinal arterial and vein calibers in diabetes [158].

In a study of 58 healthy volunteers, 68 patients with type I diabetes and 172 patients with type II diabetes, the response of retinal vessels to FLIP was investigated. Results indicated that retinal arterial response to FLIP was reduced regardless of the type of diabetes [159]. Furthermore, a continuous decrease in retinal arterial and vein dilation was shown with increasing stages of diabetic retinopathy [159]. These results indicate that the responses of retinal blood vessels to FLIP may be valuable in the early detection of pathological mechanisms in diabetes before the progression to diabetic retinopathy [159]. Another study was conducted in 224 individuals with diabetes and 103 non-diabetic control subjects [160]. A reduced mean arterial and vein dilation in response to FLIP was indicated in diabetics compared to control subjects. Additionally, diabetic retinopathy was more likely to occur in diabetics with a reduced dilation of retinal vessels to FLIP. The impaired retinal vessel responses to FLIP may also support the notion of endothelial dysfunction as a pathophysiological mechanism underlying diabetes and its microvascular manifestations [160].

2.12.4. Obesity and dyslipidemia

Obesity is related to an increased production of ROS, selectively in the adipose tissue, along with the augmented expression of NADPH oxidase and a decreased expression of antioxidant enzymes [161]. When comparing obese patients with controls, it was indicated that hydroperoxides and the levels of TBARS were higher in obese subjects [162-164]. Additionally, TBARS levels have been shown to positively correlate with BMI and waist circumference [165]. Regarding the antioxidant defense capacity, SOD was shown to positively correlate with weight [166] and BMI (in younger obese subjects aged 20-39 years) [162]. However, in older obese subjects (>60 years), SOD was shown to be negatively correlated with BMI [162]. It has also been noted that antioxidant defense capacity markers such as total antioxidant capacity, tGSH, SOD, CAT and GPx in obese subjects are lower in proportion to the amount of body fat [164,166,167]. In young obese subjects (20-39 years), SOD and CAT activities were higher while activities of these antioxidant enzymes were

reduced in older obese subjects (>60 years), suggesting that oxidative stress may be increased in older obese subjects [162]. When the antioxidant capacity markers were investigated in obese subjects with greater and lower insulin resistance, SOD activity was reduced whereas GPx activity was higher in obese subjects with higher insulin resistance [168]. Furthermore, obese subjects with lower insulin resistance had enhanced CAT activity [168]. The latter results suggest a close association between the degree of insulin resistance and oxidative stress-related markers in obese subjects [168]. Taken together, these observations suggest that the accumulation of excess fat may increase oxidative stress and diminish antioxidant defense capacity which may be a pathogenic mechanism in the development of metabolic syndrome [165]. Furthermore, it was suggested that the up-regulated antioxidant enzyme activities in obese subjects may play a role in counteracting oxidative stress induced by circulating fatty acids but may decrease when the degree of obesity is increased [169].

Variations in retinal vascular calibers may also be associated with the obesity-related factors. Studies have reported associations of larger vein caliber with the components of metabolic syndrome such as obesity (greater BMI and waist-hip ratio) and dyslipidemia (higher levels of plasma triglycerides and LDL cholesterol and lower levels of HDL cholesterol) [93,170]. These associations were also confirmed in population-based studies [171,172]. It was further indicated in the Blue Mountains Eye Study that a larger retinal vein caliber may predict the incidence of obesity over a five-year period, suggesting that attenuated microvascular function may play a role in the pathogenesis of obesity and inflammation [173].

Kotliar et al. [123] assessed the retinal vessel responses to FLIP in 46 obese subjects (49.6 ± 10.0 years) and 46 age and gender-matched healthy controls. A reduced mean retinal arterial and vein dilation was reported in the obese group compared to healthy controls. Furthermore, the time for arteries to dilate maximally was prolonged in obese subjects suggesting a characteristic for changes in retinal vessel reactivity in metabolic syndrome

[123]. A study by *Schiel et al*. [174] reported higher mean arterial and vein dilation in 32 young obese subjects (aged 13.4 ± 2.8 years). The differences in the values of vessel dilatory responses between both studies may be related to the effect of age on retinal vessel responses to FLIP as previously indicated in healthy adult cohorts [140,141].

Cholesterol is one of the most well-established risk factors for early CAD [175,176]. Thus, a strong linear relationship exists between cholesterol levels and CAD risk [177]. In a study of 21 hypercholesterolemic subjects (14 men and 7 women; 57 ± 15 years), acute effects of LDL apheresis (implicated in treatment of severe hypercholesterolemia) on retinal vessel responses to FLIP were investigated. Results of this study have shown that total cholesterol decreased by 58% after LDL apheresis. Initially, more than 75% of all subjects showed impaired retinal vessel dilation in response to FLIP. This impaired flicker light-induced dilation was significantly improved in retinal veins accompanied by a non-significant tendency for improvement in retinal arteries after LDL aphresis. This indicates that retinal vascular function is significantly diminished in subjects with hypercholesterolemia and can be improved by LDL apheresis [122]. These results also suggest that veins may be highly responsive to metabolic changes in the retinal microvasculature [122].

2.12.5. Ethnicity

In an American family-based cohort study of 236 patients, oxidative stress was investigated in black and white participants [178]. Results of this study have shown that the production of H_2O_2 was higher in the white participants than in black participants. These results might be an indication of disturbance in the activities of antioxidant enzymes which may have led to an increased production of H_2O_2 in white participants. However, in a South African study regarding the role of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function (SAfrEIC), it has been reported that serum peroxide levels were significantly higher in blacks than in whites [179]. In addition, another study conducted on 63 European Americans (38 men) and 74 African Americans (35 men), an oxidative stress marker, 8-

hydroxydeoxyguanosine was lower in African Americans while the levels of 8-isoprostane did not differ between the two groups [180]. Taken together, these results imply that race/ethnicity may play a role in the variation of oxidative stress-related markers.

The MESA revealed that AVR was larger in white participants than in black, Hispanic and Chinese participants. Furthermore, both CRAE and CRVE were significantly larger in blacks and Hispanics than in whites and Chinese [172]. In another study, a wider CRAE and CRVE have been noted in African Americans compared to whites [171]. Furthermore, the Sydney Myopia study has observed that retinal vascular caliber (both CRAE and CRVE) was significantly wider in children of East Asian ethnicity compared to white children [181]. This was supportive of other findings from other population-based studies suggesting racial/ethnic differences in retinal vascular calibers [172,182]. However, iris color may influence these results. Dark brown iris color was associated with wider arterial and vein calibers (CRAE and CRVE) in white children only. Despite the observed differences in vascular caliber in Sydney Myopia study, dark brown iris color did not have an influence on retinal arterial caliber (CRAE) when compared between the two racial groups (East Asian and white), although vein caliber (CRVE) was still wider in the East Asian than in the white children [181]. It was further hypothesized that the reported ethnic differences may be due to differences in the retinal pigmentation, represented by the iris color in their study [181]. With regard to the South African studies, *Malan et al*. [183] reported a wider CRVE and a smaller AVR in black men compared to white men whereas CRAE did not differ between the two groups. These observations indicate the ethnic differences in retinal vascular calibers do exist and may be influenced the retinal pigmentation or genetic components [181,184].

Significant differences when CVD risk factors are compared between ethnic groups may explain some of the ethnic differences in retinal vascular calibers [172]. For example, in the MESA study, blacks and Hispanics were more likely to have obesity, diabetes, hyperlipidemia and systemic inflammation than whites [172]. These conditions are

associated with endothelial dysfunction which may also play a role in retinal vascular abnormalities [93,154,158,170].

2.13. The retinal microvasculature and oxidative stress-related markers

The relationship between various retinal microvascular parameters and oxidative stress related markers have been poorly studied in the literature. A study examined the relationship between antioxidant enzyme activity (SOD and GPx-3) and inflammatory markers (C-reactive protein and orosomucoid) with retinal vascular caliber [185]. It was found that larger retinal arterial caliber was associated with higher GPx-3 activity after adjusting for age and gender. There was no significant association between retinal vascular caliber and SOD [185]. It has been noted that a larger retinal artery caliber indicate a lower CVD risk while a wider vein caliber indicate a higher risk of developing CVD [185]. On the other hand, plasma GPx-3 activity play an important role in regulating oxidative stress [186-188] whereas a deficiency in GPx-3 has been associated with an increase in ROS and reduced $NO^{\cdot}$ bioavailability [187]. Findings from this study therefore suggest that the retinal microvasculature is sensitive to systemic oxidative stress, independently of known CVD risk factors [185]. As such, antioxidant enzyme activity may play a role of protecting retinal arteries from oxidative damage related to increased oxidative stress which may also lower the risk of developing CVD [185].

In another study of 102 healthy middle-aged (46-60 years) individuals with low to moderate cardiovascular risk, the relationship between retinal microvasculature in response to FLIP, circulatory markers for CVD risk (triglycerides, plasma total cholesterol, HDL cholesterol, and LDL cholesterol) and antioxidant defense capacity were investigated [189]. Results from this study revealed that the redox index (GSH:GSSG) was positively associated with the arterial dilation slope whereas blood GSH levels were negatively correlated with the overall retinal arterial constriction slope. From the microvascular perspective, endothelial function and redox balance are associated aspects [190,191], of which excessive ROS levels may lead to

the impairment of endothelium-dependent dilation [192,193]. As such, a decrease in GSH:GSSG has been proposed to be indicative of oxidative stress [1,194,195]. Therefore, results from this study suggest that antioxidant defense mechanisms may play a role in the retinal arterial dilatory capacity in response to FLIP possibly through the maintenance of $NO^{\cdot}$ levels in the retinal microvasculature [189]. When normal levels of $NO^{\cdot}$ are maintained, the vascular function is maintained [189]. Furthermore, it was reported that the interaction of thiol antioxidants with ROS in the coronary [196], renal [53] and cerebral [197] levels are associated with endothelial dysfunction and increased vasoconstriction [196]. Similarly, circulating levels of thiol antioxidants seems to have a negative influence on the reactivity of vessels at a microvascular level [189]. Taking these observations together, the relationship between antioxidant defense capacity and retinal vessel responses to FLIP may represent a complex mechanism involved in the regulation of vascular response to oxidative stress at the retinal microvasculature [189].

2.14. Motivation

Although the previously mentioned SABPA sub-studies (section 2.7) evaluated the relationship between oxidative stress-related markers and cardiovascular variables, these explored relationships were limited to the components of the macrovasculature. Controversially, alterations in microvascular function are thought to occur prior to macrovascular dysfunction [198], therefore, it is beneficial to explore the association of oxidative stress markers and antioxidant defense capacity markers with microvascular parameters. To our knowledge, there have been no studies done on the relationships of retinal vessel calibers and vessel responses to FLIP with oxidative stress-related markers in blacks and whites from South Africa.

It was recently found that blacks from South Africa may have attenuated microvascular function compared to whites [131]. Smaller CRAE and wider CRVE in the retinal microvasculature [111,113-115] precede clinical manifestations of CVD [131] and may

predict future cardiovascular complications [102]. Furthermore, reduced retinal arterial and vein dilation are associated with markers of CVD risk [78,121-124]. Therefore it is important to explore the role of oxidative stress-related markers in relation to these microvascular parameters.

The link between oxidative stress-related markers and the retinal microvasculature has been scarcely studied. Up to date, two studies [185,189] have indicated the relationship between retinal vessel calibers, vessel responses to FLIP and antioxidant capacity; but it is of added value to also explore this relationship with markers of oxidative damage. Evaluating both markers of oxidative damage and antioxidant defense capacity may give an indication of which macromolecules are damaged as well as how the antioxidants can defense against increased oxidative stress in the retinal microvasculature.

2.15. Aims and Objectives

1) The aim of this study is to investigate the relationship between retinal vessel calibers and responses of retinal vessels to FLIP with oxidative stress-related markers in a cohort of black and white men from South Africa.

2) The objectives are:

a) To determine whether the levels of oxidative stress markers (TBARS, ROS) and antioxidant defense capacity markers (GPx, GR, tGSH and SOD), differ between black and white men. (Data on the comparisons of levels between oxidative stress-related markers was not yet published at the time of this research being conducted. However, a study by *Mokhaneli et al*. [74] was published with regard to levels of oxidative stress-related markers, although the group stratification was different (black men and women vs white men and women).

b) To explore the relationship between oxidative stress-related markers and retinal vessel calibers and responses of retinal vessels to FLIP.

2.16. Hypotheses

1) The levels of oxidative stress markers will be higher whereas antioxidant defense capacity markers will be lower in the black men than in the white men.

2. a) CRAE and AVR will be negatively associated and CRVE will be positively associated with oxidative stress markers.

b) CRAE and AVR will be positively associated and CRVE will be negatively associated with antioxidant defense capacity markers.

c) Maximum retinal artery and vein dilation will be negatively associated with oxidative stress markers and positively associated with antioxidant defense capacity markers

2.17. References

[1] Jones DP. Redefining oxidative stress. Antioxid Redox Signal 2006;8(9-10):1865-1879.

[2] Touyz RM, Briones AM. Reactive oxygen species and vascular biology: implications in human hypertension. Hypertens Res 2011;34(1):5-14.

[3] Briones AM, Touyz RM. Oxidative stress and hypertension: current concepts. Curr Hypertens Rep 2010;12(2):135-142.

[4] Ballinger SW. Mitochondrial dysfunction in cardiovascular disease. Free Radic Biol Med 2005;38(10):1278-1295.

[5] Ďuračková Z. Oxidants, antioxidants and oxidative stress. Mitochondrial Medicine: Springer; 2008. p 19-54.

[6] Betteridge DJ. What is oxidative stress? Metabolism 2000;49(2):3-8.

[7] Curtin JF, Donovan M, Cotter TG. Regulation and measurement of oxidative stress in apoptosis. J Immunol Methods 2002;265(1):49-72.

[8] Gutteridge J, Halliwell B. Free radicals and antioxidants in the year 2000: a historical look to the future. Ann N Y Acad Sci 2000;899(1):136-147.

[9] Scandalios J. Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Brazilian J Med Biol Res 2005;38(7):995-1014.

[10] Mueller CF, Laude K, McNally JS, Harrison DG. Redox mechanisms in blood vessels. Arterioscler Thromb Vasc Biol 2005;25(2):274-278.

[11] Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. Am J Cardiol 2003;91(3):7-11.

[12] Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. Nat Med 1995;1(8):804-809.

[13] Gu X, El-Remessy AB, Brooks SE, Al-Shabrawey M, Tsai N-T, Caldwell RB. Hyperoxia induces retinal vascular endothelial cell apoptosis through formation of peroxynitrite. Am J Physiol Cell Physiol 2003;285(3):C546-C554.

[14] Arnet UA, McMillan A, Dinerman JL, Ballermann B, Lowenstein CJ. Regulation of endothelial nitric-oxide synthase during hypoxia. J Biol Chem 1996;271(25):15069-15073.

[15] Laursen JB, Somers M, Kurz S, McCann L, Warnholtz A, Freeman BA, Tarpey M, Fukai T, Harrison DG. Endothelial regulation of vasomotion in apoE-deficient mice implications for interactions between peroxynitrite and tetrahydrobiopterin. Circulation 2001;103(9):1282-1288.

[16] Gu SX, Stevens JW, Lentz SR. Regulation of thrombosis and vascular function by protein methionine oxidation. Blood 2015;125(25):3851-3859.

[17] Riley P. Free radicals in biology: oxidative stress and the effects of ionizing radiation. Int J Radiat Biol 1994;65(1):27-33.

[18] Wilson R. Free radicals and tissue damage, mechanistic evidence from radiation studies. Biochem Mechan Liver Injury 1978:123-224.

[19] Halliwell B, Gutteridge J. The chemistry of free radicals and related 'reactive species'. Free Radic Biol Med 1999;3:1-7.

[20] Nel A, Xia T, Mädler L, Li N. Toxic potential of materials at the nanolevel. Science 2006;311(5761):622-627.

[21] Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? British J Pharmacol 2004;142(2):231-255.

[22] Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. Am J Physiol Lung Cell Mol Physiol 2002;283(2):L246-L255.

[23] Mayer B, Hemmens B. Biosynthesis and action of nitric oxide in mammalian cells. Trends Biochem Sci 1997;22(12):477-481.

[24] Bogdan C, Röllinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 2000;12(1):64-76.

[25] Murphy MP. Nitric oxide and cell death. Biochim Biophys Acta 1999;1411(2):401-414.

[26] Patel RP, McAndrew J, Sellak H, White CR, Jo H, Freeman BA, Darley-Usmar VM. Biological aspects of reactive nitrogen species. Biochim Biophys Acta 1999;1411(2):385-400.

[27] Groves JT. Peroxynitrite: reactive, invasive and enigmatic. Curr Opin Chem Biol 1999;3(2):226-235.

[28] Hausladen A, Stamler JS. Nitrosative stress. Methods Enzymol 1999;300:389-395.

[29] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39(1):44-84.

[30] Eiserich JP, Patel RP, O'Donnell VB. Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. Mol Asp Med 1998;19(4):221-357.

[31] Xiao GG, Wang M, Li N, Loo JA, Nel AE. Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem 2003;278(50):50781-50790.

[32] Gutteridge J. Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clin Chem 1995;41(12):1819-1828.

[33] Benzie IF, Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996;239(1):70-76.

[34] Matés J. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 2000;153(1):83-104.

[35] Li S, Yan T, Yang J-Q, Oberley TD, Oberley LW. The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. Cancer Res 2000;60(14):3927-3939.

[36] Kinnula V, Everitt J, Mangum J, Chang L, Crapo J. Antioxidant defense mechanisms in cultured pleural mesothelial cells. Am J Respir, Cell Mol Biol 1992;7(1):95-103.

[37] Matés JM, Pérez-Gómez C, De Castro IN. Antioxidant enzymes and human diseases. Clin Biochem 1999;32(8):595-603.

[38] Ho E, Galougahi KK, Liu C-C, Bhindi R, Figtree GA. Biological markers of oxidative stress: applications to cardiovascular research and practice. Redox Biol 2013;1(1):483-491.

[39] Griesmacher A, Kindhauser M, Andert SE, Schreiner W, Toma C, Knoebl P, Pietschmann P, Prager R, Schnack C, Schemthaner G. Enhanced serum levels of thiobarbituric-acid-reactive substances in diabetes mellitus. Am J Med 1995;98(5):469-475.

[40] Yavuzer H, Yavuzer S, Cengiz M, Erman H, Doventas A, Balci H, Erdincler DS, Uzun H. Biomarkers of lipid peroxidation related to hypertension in aging. Hypertens Res 2016;39(5):342-348.

[41] Yalçln AS, Sabuncu N, Kilinç A, Gülcan G, Emerk K. Increased plasma and erythrocyte lipid peroxidation in hyperlipidemic individuals. Atherosclerosis 1989;80(2):169-170.

[42] Bridges A, Scott N, Parry G, Belch J. Age, sex, cigarette smoking and indices of free radical activity in healthy humans. The Eur J Med 1993;2(4):205-208.

[43] Maneesh M, Dutta S, Chakrabarti A, Vasudevan D. Alcohol abuse-duration dependent decrease in plasma testosterone and antioxidants in males. Indian J Physiol Pharmacol 2006;50(3):291-296.

[44] Vincent HK, Taylor AG. Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. Int J Obes 2006;30(3):400-418.

[45] Buege JA, Aust S. Methods Enzymol 1978;52:302-310.

[46] El-Beltagi HS, Mohamed HI. Reactive oxygen species, lipid peroxidation and antioxidative defense mechanism. Not Bot Horti Agrobo 2013;41(1):44-57.

[47] Repetto M, Boveris A, Semprine J. Lipid peroxidation: chemical mechanism, biological implications and analytical determination. Intech Open Access Publisher; 2012.

[48] Wu D, Cederbaum AI. Alcohol, oxidative stress, and free radical damage. Alcohol Res Health 2003;27:277-284.

[49] Shah D, Mahajan N, Sah S, Nath SK, Paudyal B. Oxidative stress and its biomarkers in systemic lupus erythematosus. J Biomed Sci 2014;21(1):23.

[50] Halliwell B, Gutteridge J. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 1984;219(1):1-14.

[51] McIntyre TM, Hazen SL. Lipid oxidation and cardiovascular disease: introduction to a review series. Circ Res 2010;107(10):1167-1169.

[52] Touyz R, Schiffrin E. Reactive oxygen species in vascular biology: implications in hypertension. Histochem Cell Biol 2004;122(4):339-352.

[53] Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension what is the clinical significance? Hypertension 2004;44(3):248-252.

[54] Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature molecular and cellular mechanisms. Hypertension 2003;42(6):1075-1081.

[55] Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87(10):840-844.

[56] Forgione MA, Cap A, Liao R, Moldovan NI, Eberhardt RT, Lim CC, Jones J, Goldschmidt-Clermont PJ, Loscalzo J. Heterozygous cellular glutathione peroxidase deficiency in the mouse abnormalities in vascular and cardiac function and structure. Circulation 2002;106(9):1154-1158.

[57] Rubanyi G, Vanhoutte P. Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor. Am J Physiol Heart Circ Physiol 1986;250(5):H822-H827.

[58] Thomson L, Trujillo M, Telleri R, Radi R. Kinetics of cytochrome C 2+ oxidation by peroxynitrite: Implications for superoxide measurements in nitric oxide-producing biological-systems. Arch Biochem Biophys 1995;319(2):491-497.

[59] Shepherd J, Katusić Z. Endothelium-derived vasoactive factors: I. Endothelium-dependent relaxation. Hypertension 1991;18(5 Suppl):III76- III85.

[60] Chin JH, Azhar S, Hoffman BB. Inactivation of endothelial derived relaxing factor by oxidized lipoproteins. J Clin Invest 1992;89(1):10-18.

[61] Devaraj S, Jialal I. Oxidized low-density lipoprotein and atherosclerosis. Int J Clin Lab Res 1996;26(3):178-184.

[62] Steinberg D, Witztum JL. Oxidized low-density lipoprotein and atherosclerosis. Arterioscler Thromb Vasc Biol 2010;30(12):2311-2316.

[63] Devasagayam T, Tilak J, Boloor K, Sane KS, Ghaskadbi SS, Lele R. Free radicals and antioxidants in human health: current status and future prospects. J Assoc Physicians India 2004;52:794-804.

[64] Matsura T, Kai M, Fujii Y, Ito H, Yamada K. Hydrogen peroxide-induced apoptosis in HL-60 cells requires caspase-3 activation. Free Radic Res 1999;30(1):73-83.

[65] Dimmeler S, Zeiher AM. Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. Regul Pept 2000;90(1):19-25.

[66] Walter MF, Jacob RF, Jeffers B, Ghadanfar MM, Preston GM, Buch J, Mason RP. Serum levels of thiobarbituric acid reactive substances predict cardiovascular events in patients with stable coronary artery disease: a longitudinal analysis of the PREVENT study. J Am Coll Cardiol 2004;44(10):1996-2002.

[67] Witztum JL, Steinberg D. The oxidative modification hypothesis of atherosclerosis: does it hold for humans? Trends Cardiovasc Med 2001;11(3):93-102.

[68] Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. Free Radic Biol Med 2000;28(12):1815-1826.

[69] Schisterman EF, Faraggi D, Browne R, Freudenheim J, Dorn J, Muti P, Armstrong D, Reiser B, Trevisan M. TBARS and cardiovascular disease in a population-based sample. J Cardiovasc Risk 2001;8(4):219-225.

[70] Kruger R, Schutte R, Huisman H, Van Rooyen J, Malan N, Fourie C, Louw R, Van der Westhuizen F, Van Deventer C, Malan L. Associations between reactive oxygen species, blood pressure and arterial stiffness in black South Africans: the SABPA study. J Hum Hypertens 2012;26(2):91-97.

[71] Schutte R, Schutte AE, Huisman HW, Van Rooyen JM, Malan NT, Péter S, Fourie CM, Van Der Westhuizen FH, Louw R, Botha CA. Blood glutathione and subclinical atherosclerosis in African men: the SABPA Study. Am J Hypertens 2009;22(11):1154-1159.

[72] Mels CM, Schutte AE, Schutte R, Pretorius PJ, Smith W, Huisman HW, van der Westhuizen FH, Fourie CM, van Rooyen JM, Kruger R. 8-Oxo-7, 8-dihydro-2'-deoxyguanosine, reactive oxygen species and ambulatory blood pressure in African and Caucasian men: The SABPA study. Free Radic Res 2014;48(11):1291-1299.

[73] van Zyl C, Huisman HW, Mels CM. Antioxidant enzyme activity is associated with blood pressure and carotid intima media thickness in black men and women: The SABPA study. Atherosclerosis 2016;248:91-96.

[74] Mokhaneli MC, Fourie CMT, Botha S, Mels CMC. The association of oxidative stress with arterial compliance and vascular resistance in a bi-ethnic population: the SABPA study. Free Radic Res 2016:920-928.

[75] Mozaffarieh M, Flammer J. Ocular blood flow and glaucomatous optic neuropathy. Berlin, Springer. 2009: p 45-53.

[76] Flammer J, Konieczka K, Bruno RM, Virdis A, Flammer AJ, Taddei S. The eye and the heart. Eur Heart J 2013;34(17):1270-1278.

[77] Liew G, Wang JJ, Mitchell P, Wong TY. Retinal vascular imaging a new tool in microvascular disease research. Circ Cardiovasc Imaging 2008;1(2):156-161.

[78] Nagel E, Vilser W, Lanzl I. Age, blood pressure, and vessel diameter as factors influencing the arterial retinal flicker response. Invest Ophthalmol Vis Sci 2004;45(5):1486-1492.

[79] Liew G, Wang JJ. Retinal vascular signs: a window to the heart? Rev Esp Cardiol 2011;64(6):515-521.

[80] Rizzoni D, De Ciuceis C, Porteri E, Paiardi S, Boari GE, Mortini P, Cornali C, Cenzato M, Rodella LF, Borsani E. Altered structure of small cerebral arteries in patients with essential hypertension. J Hypertens 2009;27(4):838-845.

[81] Ritt M, Harazny JM, Ott C, Schlaich MP, Schneider MP, Michelson G, Schmieder RE. Analysis of retinal arteriolar structure in never-treated patients with essential hypertension. J Hypertens 2008;26(7):1427-1434.

[82] Heagerty A, Aalkjaer C, Bund S, Korsgaard N, Mulvany M. Small artery structure in hypertension. Dual processes of remodeling and growth. Hypertension 1993;21(4):391-397.

[83] Apple D, Naumann G. Pathology of the eye. New York: Springer; 1986.

[84] Ashton N. The eye in malignant hypertension. Trans Am Acad Ophthalmol Otolaryngol 1972;76(1):17-40.

[85] Tso MO, Jampol LM. Pathophysiology of hypertensive retinopathy. Ophthalmology 1982;89(10):1132-1145.

[86] Goto I, Kimoto K, Katsuki S, Mimatsu T, Hiroshi I. Pathological studies on the intracerebral and retinal arteries in cerebrovascular and noncerebrovascular diseases. Stroke 1975;6(3):263-269.

[87] Wells R, Herman M, Gorlin R. Microvascular changes in coronary artery disease. Lippincott Williams and Wilkins Washington SQ, Philadelphia, PA. 1966: p 237.

[88] Wong TY, Knudtson MD, Klein R, Klein BE, Meuer SM, Hubbard LD. Computer-assisted measurement of retinal vessel diameters in the Beaver Dam Eye Study: methodology, correlation between eyes, and effect of refractive errors. Ophthalmology 2004;111(6):1183-1190.

[89] Sharrett AR, Hubbard LD, Cooper LS, Sorlie PD, Brothers RJ, Nieto FJ, Pinsky JL, Klein R. Retinal arteriolar diameters and elevated blood pressure the Atherosclerosis Risk in Communities Study. Am J Epidemiol 1999;150(3):263-270.

[90] Duncan B, Wong T, Tyroler H, Davis C, Fuchs F. Hypertensive retinopathy and incident coronary heart disease in high risk men. British J Ophthalmol 2002;86(9):1002-1006.

[91] Leung H, Wang JJ, Rochtchina E, Tan AG, Wong TY, Klein R, Hubbard LD, Mitchell P. Relationships between age, blood pressure, and retinal vessel diameters in an older population. Invest Ophthalmol Vis Sci 2003;44(7):2900-2904.

[92] Hubbard LD, Brothers RJ, King WN, Clegg LX, Klein R, Cooper LS, Sharrett AR, Davis MD, Cai J, Group ARiCS. Methods for evaluation of retinal microvascular abnormalities associated with hypertension/sclerosis in the Atherosclerosis Risk in Communities Study. Ophthalmolohy 1999;106(12):2269-2280.

[93] Ikram MK, de Jong FJ, Vingerling JR, Witteman JC, Hofman A, Breteler MM, de Jong PT. Are retinal arteriolar or venular diameters associated with markers for cardiovascular disorders? The Rotterdam Study. Invest Ophthalmol Vis Sci 2004;45(7):2129-2134.

[94] Patton N, Aslam T, MacGillivray T, Dhillon B, Constable I. Asymmetry of retinal arteriolar branch widths at junctions affects ability of formulae to predict trunk arteriolar widths. Invest Ophthalmol Vis Sci 2006;47(4):1329-1333.

[95] Kronmal RA. Spurious correlation and the fallacy of the ratio standard revisited. J of the Royal Statistical Society 1993:379-392.

[96] Wong TY, Klein R, Klein BE, Meuer SM, Hubbard LD. Retinal vessel diameters and their associations with age and blood pressure. Invest Ophthalmol Vis Sci 2003;44(11):4644-4650.

[97] Klein R, Sharrett AR, Klein BE, Chambless LE, Cooper LS, Hubbard LD, Evans G. Are retinal arteriolar abnormalities related to atherosclerosis? The Atherosclerosis Risk in Communities Study. Arterioscler Thromb Vasc Biol 2000;20(6):1644-1650.

[98] Leung H, Wang JJ, Rochtchina E, Wong TY, Klein R, Mitchell P. Impact of current and past blood pressure on retinal arteriolar diameter in an older population. J Hypertens 2004;22(8):1543-1549.

[99] Wong TY, Shankar A, Klein R, Klein BE, Hubbard LD. Prospective cohort study of retinal vessel diameters and risk of hypertension. BMJ 2004;329(7457):79.

[100] Smith W, Wang JJ, Wong TY, Rochtchina E, Klein R, Leeder SR, Mitchell P. Retinal arteriolar narrowing is associated with 5-year incident severe hypertension the Blue Mountains Eye Study. Hypertension 2004;44(4):442-447.

[101] Wong TY, Klein R, Couper DJ, Cooper LS, Shahar E, Hubbard LD, Wofford MR, Sharrett AR. Retinal microvascular abnormalities and incident stroke: the Atherosclerosis Risk in Communities Study. Lancet 2001;358(9288):1134-1140.

[102] Wong TY, Klein R, Sharrett AR, Duncan BB, Couper DJ, Tielsch JM, Klein BE, Hubbard LD. Retinal arteriolar narrowing and risk of coronary heart disease in men and women: the Atherosclerosis Risk in Communities Study. JAMA 2002;287(9):1153-1159.

[103] Wang JJ, Liew G, Wong TY, Smith W, Klein R, Leeder S, Mitchell P. Retinal vascular calibre and the risk of coronary heart disease-related death. Heart 2006;92(11):1583-1587.

[104] Nguyen TT, Wang JJ, Islam FA, Mitchell P, Tapp RJ, Zimmet PZ, Simpson R, Shaw J, Wong TY. Retinal arteriolar narrowing predicts incidence of diabetes the Australian diabetes, obesity and lifestyle (AusDiab) study. Diabetes 2008;57(3):536-539.

[105] Wong TY, Rosamond W, Chang PP, Couper DJ, Sharrett AR, Hubbard LD, Folsom AR, Klein R. Retinopathy and risk of congestive heart failure. JAMA 2005;293(1):63-69.

[106] Wong TY, Duncan BB, Golden SH, Klein R, Couper DJ, Klein BE, Hubbard LD, Sharrett AR, Schmidt MI. Associations between the metabolic syndrome and retinal microvascular signs: the Atherosclerosis Risk In Communities Study. Invest Ophthalmol Vis Sci 2004;45(9):2949-2954.

[107] Wong TY, Islam FA, Klein R, Klein BE, Cotch MF, Castro C, Sharrett AR, Shahar E. Retinal vascular caliber, cardiovascular risk factors, and inflammation: the Multi-Ethnic Study of Atherosclerosis (MESA). Invest Ophthalmol Vis Sci 2006;47(6):2341-2350.

[108] Walsh JB. Hypertensive retinopathy: description, classification, and prognosis. Ophthalmology 1982;89(10):1127-1131.

[109] Dodson P, Lip G, Eames S, Gibson J, Beevers D. Hypertensive retinopathy: a review of existing classification systems and a suggestion for a simplified grading system. J of Hum Hypertens 1996;10(2):93-98.

[110] Wong TY, McIntosh R. Systemic associations of retinal microvascular signs: a review of recent population-based studies. Ophthalmic Physiol Opt 2005;25(3):195-204.

[111] Ikram MK, Ong YT, Cheung CY, Wong TY. Retinal vascular caliber measurements: clinical significance, current knowledge and future perspectives. Ophthalmology 2013;229(3):125-136.

[112] Witt N, Wong TY, Hughes AD, Chaturvedi N, Klein BE, Evans R, McNamara M, Thom SAM, Klein R. Abnormalities of retinal microvascular structure and risk of mortality from ischemic heart disease and stroke. Hypertension 2006;47(5):975-981.

[113] Cheung CY-l, Ikram MK, Sabanayagam C, Wong TY. Retinal microvasculature as a model to study the manifestations of hypertension. Hypertension 2012;60(5):1094-1103.

[114] Ding J, Wai KL, McGeechan K. Retinal vascular caliber and the development of hypertension: a meta-analysis of individual participant data. J Hypertens 2014;32(2):207-215.

[115] Baker ML, Hand PJ, Wang JJ, Wong TY. Retinal signs and stroke revisiting the link between the eye and brain. Stroke 2008;39(4):1371-1379.

[116] Dorner GT, Garhöfer G, Huemer KH, Riva CE, Wolzt M, Schmetterer L. Hyperglycemia affects flicker-induced vasodilation in the retina of healthy subjects. Vis Res 2003;43(13):1495-1500.

[117] Dorner GT, Garhofer G, Kiss B, Polska E, Polak K, Riva CE, Schmetterer L. Nitric oxide regulates retinal vascular tone in humans. Am J Physiol Heart Circ Physiol 2003;285(2):H631-H636.

[118] Delles C, Michelson G, Harazny J, Oehmer S, Hilgers KF, Schmieder RE. Impaired endothelial function of the retinal vasculature in hypertensive patients. Stroke 2004;35(6):1289-1293.

[119] Flammer AJ, Anderson T, Celermajer DS, Creager MA, Deanfield J, Ganz P, Hamburg NM, Lüscher TF, Shechter M, Taddei S. The assessment of endothelial function from research into clinical practice. Circulation 2012;126(6):753-767.

[120] Lim M, Sasongko MB, Ikram MK, Lamoureux E, Wang JJ, Wong TY, Cheung CY. Systemic associations of dynamic retinal vessel analysis: a review of current literature. Microcirculation 2013;20(3):257-268.

[121] Pemp B, Garhofer G, Weigert Gn, Karl K, Resch H, Wolzt M, Schmetterer L. Reduced retinal vessel response to flicker stimulation but not to exogenous nitric oxide in type 1 diabetes. Invest Ophthalmol Vis Sci 2009;50(9):4029-4032.

[122] Reimann M, Julius U, Bornstein S, Fischer S, Reichmann H, Rüdiger H, Ziemssen T. Regular lipoprotein apheresis maintains residual cardiovascular and microvascular function in patients with advanced atherosclerotic disease. Atheroscler Suppl 2013;14(1):135-141.

[123] Kotliar KE, Lanzl IM, Schmidt-Trucksäss A, Sitnikova D, Ali M, Blume K, Halle M, Hanssen H. Dynamic retinal vessel response to flicker in obesity: a methodological approach. Microvasc Res 2011;81(1):123-128.

[124] Garhöfer G, Zawinka C, Resch H, Kothy P, Schmetterer L, Dorner G. Reduced response of retinal vessel diameters to flicker stimulation in patients with diabetes. British J Ophthalmol 2004;88(7):887-891.

[125] Malan L, Hamer M, von Känel R, Schlaich MP, Reimann M, Frasure-Smith N, Lambert GW, Vilser W, Harvey BH, Steyn F. Chronic depression symptoms and salivary NOx are associated with retinal vascular dysregulation: The SABPA study. Nitric Oxide 2016;55:10-17.

[126] Quyyumi AA. Prognostic value of endothelial function. Am J Cardiol 2003;91(12):19-24.

[127] Mudau M, Genis A, Lochner A, Strijdom H. Endothelial dysfunction: the early predictor of atherosclerosis: review article. Cardiovasc J Afr 2012;23(4):222-231.

[128] Yang G, Lucas R, Caldwell R, Yao L, Romero MJ, Caldwell RW. Novel mechanisms of endothelial dysfunction in diabetes. J Cardiovasc Dis Res 2010;1(2):59-63.

[129] Al Suwaidi J, Hamasaki S, Higano ST, Nishimura RA, Holmes DR, Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 2000;101(9):948-954.

[130] Schächinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation 2000;101(16):1899-1906.

[131] Pienaar P, Micklesfield L, Gill J, Shore A, Gooding K, Levitt N, Lambert E. Ethnic differences in microvascular function in apparently healthy South African men and women. Exp Physiol 2014;99(7):985-994.

[132] Poljak K, Milisav I. Aging, oxidative stress and antioxidants. Intech 2013;14:331-353.

[133] Andriollo-Sanchez M, Hininger-Favier I, Meunier N, Venneria E, O'Connor J, Maiani G, Coudray C, Roussel A. Age-related oxidative stress and antioxidant parameters in middle-aged and older European subjects: the ZENITH study. Eur J Clin Nutr 2005;59:S58-S62.

[134] Fontana L, Klein S. Aging, adiposity, and calorie restriction. JAMA 2007;297(9):986-994.

[135] Mutlu-Türkoğlu Ü, İlhan E, Öztezcan S, Kuru A, Aykaç-Toker G, Uysal M. Age-related increases in plasma malondialdehyde and protein carbonyl levels and lymphocyte DNA damage in elderly subjects. Clin Biochem 2003;36(5):397-400.

[136] İnal ME, Kanbak G, Sunal E. Antioxidant enzyme activities and malondialdehyde levels related to aging. Clin Chim Acta 2001;305(1):75-80.

[137] Andersen HR, Nielsen JB, Nielsen F, Grandjean P. Antioxidative enzyme activities in human erythrocytes. Clin Chem 1997;43(4):562-568.

[138] Mecocci P, Polidori MC, Troiano L, Cherubini A, Cecchetti R, Pini G, Straatman M, Monti D, Stahl W, Sies H. Plasma antioxidants and longevity: a study on healthy centenarians. Free Radic Biol Med 2000;28(8):1243-1248.

[139] Junqueira VB, Barros SB, Chan SS, Rodrigues L, Giavarotti L, Abud RL, Deucher GP. Aging and oxidative stress. Mol Asp Med 2004;25(1):5-16.

[140] Kneser M, Kohlmann T, Pokorny J, Tost F. Age related decline of microvascular regulation measured in healthy individuals by retinal dynamic vessel analysis. Med Sci Monit 2009;15(8):CR436-CR441.

[141] Lanzl I, Kotliar K, Bock A, Halle M, Vilser W, Schmidt-Trucksäss A. Age dependence of retinal vessel reaction to flicker. Invest Ophthalmol Vis Sci 2007;48(13):2263-2263.

[142] Kizhakekuttu TJ, Widlansky ME. Natural antioxidants and hypertension: promise and challenges. Cardiovasc Ther 2010;28(4):e20-e32.

[143] Redón J, Oliva MR, Tormos C, Giner V, Chaves J, Iradi A, Sáez GT. Antioxidant activities and oxidative stress byproducts in human hypertension. Hypertension 2003;41(5):1096-1101.

[144] Russo C, Olivieri O, Girelli D, Faccini G, Zenari ML, Lombardi S, Corrocher R. Anti-oxidant status and lipid peroxidation in patients with essential hypertension. J Hypertens 1998;16(9):1267-1271.

[145] Gepstein R, Rosman Y, Rechtman E, Koren-Morag N, Segev S, Assia E, Grossman E. Association of retinal microvascular caliber with blood pressure levels. Blood Pressure 2012;21(3):191-196.

[146] Ikram MK, Witteman JC, Vingerling JR, Breteler MM, Hofman A, de Jong PT. Retinal vessel diameters and risk of hypertension the Rotterdam study. Hypertension 2006;47(2):189-194.

[147] Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes 1991;40(4):405-412.

[148] Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. Diabetes Care 1996;19(3):257-267.

[149] Škrha J, Hodinar A, Kvasnička J, Hilgertova J. Relationship of oxidative stress and fibrinolysis in diabetes mellitus. Diabetes Med 1996;13(9):800-805.

[150] Atalay M, Laaksonen D, Niskanen L, Uusitupa M, Hänninen O, Sen C. Altered antioxidant enzyme defences in insulin-dependent diabetic men with increased resting and exercise-induced oxidative stress. Acta Physiol Scand 1997;161(2):195-201.

[151] Santini SA, Marra G, Giardina B, Cotroneo P, Mordente A, Martorana GE, Manto A, Ghirlanda G. Defective plasma antioxidant defenses and enhanced susceptibility to lipid peroxidation in uncomplicated IDDM. Diabetes 1997;46(11):1853-1858.

[152] Wells-Knecht MC, Lyons TJ, McCance DR, Thorpe SR, Baynes JW. Age-dependent increase in ortho-tyrosine and methionine sulfoxide in human skin collagen is not accelerated in diabetes. Evidence against a generalized increase in oxidative stress in diabetes. J Clin Invest 1997;100(4):839.

[153] Wong TY, Shankar A, Klein R, Klein BE, Hubbard LD. Retinal arteriolar narrowing, hypertension, and subsequent risk of diabetes mellitus. Arch Intern Med 2005;165(9):1060-1065.

[154] Ikram MK, Janssen JA, Roos AM, Rietveld I, Witteman JC, Breteler MM, Hofman A, Van Duijn CM, de Jong PT. Retinal vessel diameters and risk of impaired fasting glucose or diabetes The Rotterdam Study. Diabetes 2006;55(2):506-510.

[155] Nguyen TT, Wang JJ, Sharrett AR, Islam FA, Klein R, Klein BE, Cotch MF, Wong TY. Relationship of retinal vascular caliber with diabetes and retinopathy The multi-ethnic study of atherosclerosis (MESA). Diabetes Care 2008;31(3):544-549.

[156] Tikellis G, Wang J, Tapp R, Simpson R, Mitchell P, Zimmet P, Shaw J, Wong T. The relationship of retinal vascular calibre to diabetes and retinopathy: the Australian diabetes, obesity and lifestyle (AusDiab) study. Diabetes 2007;50(11):2263-2271.

[157] Jeganathan VSE, Sabanayagam C, Tai ES, Lee J, Lamoureux E, Sun C, Kawasaki R, Wong TY. Retinal vascular caliber and diabetes in a multiethnic Asian population. Microcirculation 2009;16(6):534-543.

[158] Klein R, Klein BE, Knudtson MD, Wong TY, Tsai MY. Are inflammatory factors related to retinal vessel caliber?: The Beaver Dam Eye Study. Arch Ophthalmol 2006;124(1):87-94.

[159] Mandecka A, Dawczynski J, Blum M, Müller N, Kloos C, Wolf G, Vilser W, Hoyer H, Müller UA. Influence of flickering light on the retinal vessels in diabetic patients. Diabetes Care 2007;30(12):3048-3052.

[160] Nguyen TT, Kawasaki R, Wang JJ, Kreis AJ, Shaw J, Vilser W, Wong TY. Flicker light–induced retinal vasodilation in diabetes and diabetic retinopathy. Diabetes Care 2009;32(11):2075-2080.

[161] Fernández-Sánchez A, Madrigal-Santillán E, Bautista M, Esquivel-Soto J, Morales-González Á, Esquivel-Chirino C, Durante-Montiel I, Sánchez-Rivera G, Valadez-Vega C, Morales-González JA. Inflammation, oxidative stress, and obesity. Int J Mol Sci 2011;12(5):3117-3132.

[162] Karaouzene N, Merzouk H, Aribi M, Merzouk S, Berrouiguet AY, Tessier C, Narce M. Effects of the association of aging and obesity on lipids, lipoproteins and oxidative stress biomarkers: a comparison of older with young men. Nutr Metabol Cardiovasc Dis 2011;21(10):792-799.

[163] Olivares-Corichi IM, Viquez MR, Gutierrez-Lopez L, Ceballos-Reyes GM, Garcia-Sanchez JR. Oxidative stress present in the blood from obese patients modifies the structure and function of insulin. Horm Metabol Res 2011;43(11):748-753.

[164] Ozata M, Mergen M, Oktenli C, Aydin A, Sanisoglu SY, Bolu E, Yilmaz MI, Sayal A, Isimer A, Ozdemir IC. Increased oxidative stress and hypozincemia in male obesity. Clin Biochem 2002;35(8):627-631.

[165] Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 2004;114(12):1752-1761.

[166] Viroonudomphol D, Pongpaew P, Tungtrongchitr R, Phonrat B, Supawan V, Vudhivai N, Schelp FP. Erythrocyte antioxidant enzymes and blood pressure in relation to overweight and obese Thai in Bangkok. Southeast Asian J Trop Med Public Health 2000;31(2):325-334.

[167] Chrysohoou C, Panagiotakos DB, Pitsavos C, Skoumas I, Papademetriou L, Economou M, Stefanadis C. The implication of obesity on total antioxidant capacity in apparently healthy men and women: the ATTICA study. Nutr Metabol Cardiovasc Dis 2007;17(8):590-597.

[168] Tinahones FJ, Murri-Pierri M, Garrido-Sánchez L, García-Almeida JM, García-Serrano S, García-Arnés J, García-Fuentes E. Oxidative stress in severely obese persons is greater in those with insulin resistance. Obesity 2009;17(2):240-246.

[169] Savini I, Catani MV, Evangelista D, Gasperi V, Avigliano L. Obesity-associated oxidative stress: strategies finalized to improve redox state. Int J Mol Sci 2013;14(5):10497-10538.

[170] Sun C, Wang JJ, Mackey DA, Wong TY. Retinal vascular caliber: systemic, environmental, and genetic associations. Survey of Ophthalmol 2009;54(1):74-95.

[171] Liew G, Sharrett AR, Wang JJ, Klein R, Klein BE, Mitchell P, Wong TY. Relative importance of systemic determinants of retinal arteriolar and venular caliber: the Atherosclerosis Risk in Communities study. Arch Ophthalmol 2008;126(10):1404-1410.

[172] Wong TY, Islam FA, Klein R, Klein BE, Cotch MF, Castro C, Sharrett AR, Shahar E. Retinal vascular caliber, cardiovascular risk factors, and inflammation: the multi-ethnic study of atherosclerosis (MESA). Invest Ophthalmol Vis Sci 2006;47(6):2341.

[173] Wang JJ, Taylor B, Wong TY, Chua B, Rochtchina E, Klein R, Mitchell P. Retinal vessel diameters and obesity: a population-based study in older persons. Obesity 2006;14(2):206-214.

[174] Schiel R, Vilser W, Kovar F, Kramer G, Braun A, Stein G. Retinal vessel response to flicker light in children and adolescents with type 1 diabetes mellitus and overweight or obesity. Diabetes Res Clin Pract 2009;83(3):358-364.

[175] Cui Y, Blumenthal RS, Flaws JA, Whiteman MK, Langenberg P, Bachorik PS, Bush TL. Non–high-density lipoprotein cholesterol level as a predictor of cardiovascular disease mortality. Arch Intern Med 2001;161(11):1413-1419.

[176] Kannel WB, Castelli WP, Gordon T. Cholesterol in the prediction of atherosclerotic disease: new perspectives based on the Framingham study. Ann Intern Med 1979;90(1):85-91.

[177] Group MRFITR. Multiple risk factor intervention trial. JAMA 1982;248(12):1465-1477.

[178] Lacy F, Kailasam MT, O'connor DT, Schmid-Schönbein GW, Parmer RJ. Plasma hydrogen peroxide production in human essential hypertension role of heredity, gender, and ethnicity. Hypertension 2000;36(5):878-884.

[179] Butler CJ, Schutte R, Glyn MC, van der Westhuizen FH, Gona P, Schutte AE. Exploring the link between serum peroxides and angiogenesis in a bi-ethnic population from South Africa: The SAfrEIC study. J Am Soc Hypertens 2013;7(4):267-275.

[180] Kapuku G, Treiber F, Raouane F, Halbert J, Davis H, Young-Mayes S, Robinson V, Harshfield G. Race/ethnicity determines the relationships between oxidative stress markers and blood pressure in individuals with high cardiovascular disease risk. J Hum Hypertens 2016;31(1):70-75.

[181] Rochtchina E, Wang JJ, Taylor B, Wong TY, Mitchell P. Ethnic variability in retinal vessel caliber: a potential source of measurement error from ocular pigmentation?—the Sydney Childhood Eye Study. Vasc Dis 2008;49(4):1362-1366.

[182] Cheung N, Islam FA, Saw SM, Shankar A, de Haseth K, Mitchell P, Wong TY. Distribution and associations of retinal vascular caliber with ethnicity, gender, and birth parameters in young children. Invest Ophthalmol Vis Sci 2007;48(3):1018-1024.

[183] Malan NT, Smith W, von Känel R, Hamer M, Schutte AE, Malan L. Low serum testosterone and increased diastolic ocular perfusion pressure: a risk for retinal microvasculature. VASA 2015;44(6):435-443.

[184] Larsson M, Pedersen NL, Stattin H. Importance of genetic effects for characteristics of the human iris. Twin Res 2003;6(3):192-200.

[185] Daien V, Carriere I, Kawasaki R, Cristol J-P, Villain M, Fesler P, Ritchie K, Delcourt C. Retinal vascular caliber is associated with cardiovascular biomarkers of oxidative stress and inflammation: the POLA study. PLoS One 2013;8(7):e71089.

[186] Jin RC, Mahoney CE, Anderson LC, Ottaviano F, Croce K, Leopold JA, Zhang Y-Y, Tang S-S, Handy DE, Loscalzo J. Glutathione peroxidase-3 deficiency promotes platelet-dependent thrombosis in vivo. Circulation 2011;123(18):1963-1973.

[187] Wolin MS. Plasma glutathione peroxidase activity is potentially a key regulator of vascular disease-associated thrombosis. Circulation 2011;123(18):1923-1924.

[188] Buijsse B, Lee D-H, Steffen L, Erickson RR, Luepker RV, Jacobs Jr DR, Holtzman JL. Low serum glutathione peroxidase activity is associated with increased cardiovascular mortality in individuals with low HDLc's. PLoS One 2012;7(6):e38901.

[189] Seshadri S, Mroczkowska S, Qin L, Patel S, Ekart A, Gherghel D. Systemic circulatory influences on retinal microvascular function in middle-age individuals with low to moderate cardiovascular risk. Acta Ophthalmol 2015;93(4):e266-e274.

[190] Frisard MI, Broussard A, Davies SS, Roberts LJ, Rood J, De Jonge L, Fang X, Jazwinski SM, Deutsch WA, Ravussin E. Aging, resting metabolic rate, and oxidative damage: results from the Louisiana Healthy Aging Study. J Gerontol A Biol Sci Med Sci 2007;62(7):752-759.

[191] Salmon AB, Richardson A, Pérez VI. Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? Free Radic Biol Med 2010;48(5):642-655.

[192] Prasad A, Andrews NP, Padder FA, Husain M, Quyyumi AA. Glutathione reverses endothelial dysfunction and improves nitric oxide bioavailability. J Am Coll Cardiol 1999;34(2):507-514.

[193] Griscavage JM, Fukuto JM, Komori Y, Ignarro LJ. Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. J Biol Chem 1994;269(34):21644-21649.

[194] Jones DP. Extracellular redox state: refining the definition of oxidative stress in aging. Rejuvenation Res 2006;9(2):169-181.

[195] James SJ, Rose S, Melnyk S, Jernigan S, Blossom S, Pavliv O, Gaylor DW. Cellular and mitochondrial glutathione redox imbalance in lymphoblastoid cells derived from children with autism. FASEB J 2009;23(8):2374-2383.

[196] Yamada S, Saitoh S-i, Machii H, Mizukami H, Hoshino Y, Misaka T, Ishigami A, Takeishi Y. Coronary artery spasm related to thiol oxidation and senescence marker protein-30 in aging. Antioxid Redox Signal 2013;19(10):1063-1073.

[197] Ong PK, Melchior B, Martins YC, Hofer A, Orjuela-Sánchez P, Cabrales P, Zanini GM, Frangos JA, Carvalho LJ. Nitric oxide synthase dysfunction contributes to impaired cerebroarteriolar reactivity in experimental cerebral malaria. PLoS Pathog 2013;9(6):e1003444.

[198] Gates PE, Strain WD, Shore AC. Human endothelial function and microvascular ageing. Exp Physiol 2009;94(3):311-316.

CHAPTER 3

METHODOLOGY

Study design and population

This cross-sectional study forms part of the three year follow-up phase of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) prospective cohort study that was conducted between February and May of 2011/2012. The objectives of the SABPA study were to conduct the first psychophysiological study in sub-Saharan Africa addressing the brain-heart link and neural response pathways to describe mechanisms for cardiometabolic morbidity and mortality in a bi-ethnic cohort which includes both sexes. Further details regarding the SABPA study protocol have been published [1]. Shortly, at baseline phase, participants from the Kenneth Kaunda Education district in the North-West Province of South Africa were fully informed about the objectives and procedures of the study prior to their recruitment. These procedures were also explained verbally in their native language. The exclusion criteria were an ear temperature above 37℃, blood donors, psychotropic substance dependence or abuse, pregnant and lactating women and individuals vaccinated within three months prior to commencement of the study. At baseline phase, all participants were teachers with the attempt to obtain participants from a similar socio-economic status. All participants who took part in the baseline phase of the SABPA study were re-invited to take part in the follow-up study. The volunteering teachers were contacted again at their schools three months prior to the data collection of the follow-up phase. A total of 359 black and white teachers (aged 20-65 years) of both sexes returned for follow-up measurements. The volunteers meeting inclusion and exclusion criteria signed an informed consent form explaining the procedures and objectives before commencement of the follow-up phase of the study. Since sex hormones are known to have antioxidant properties [2], these hormones may play a protective role in the regulation of vascular oxidative stress [3]. For example, oestrogens (which are present in highest levels in premenopausal women) have been reported to lower vascular oxidative stress by modulating the expression and function of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, and also antioxidant enzymes [3]. Therefore, evaluating the role of oxidative stress in women may influence the results possibly because they may be less

sensitive to oxidative stress [4] due to the antioxidant effect of oestrogens (even through hormone replacement therapy), which protects the tissues from oxidative damage [5-7]. Furthermore, the role of oxidative stress in women may be masked by the menstrual cycle variation of oxidative stress-related markers since sex hormones are known to fluctuate in women during their menstrual cycle [2,8]. For this particular sub-study, women were excluded as their menopausal status and/or the phase of hormone fluctuations during their menstrual cycle were unknown. Participants with no functional retinal vessel analyses or poor retinal vessel analyses (n=15), participants using antioxidants (n=12) and participants with outliers (mean +/- 3 x the standard deviation) for superoxide dismutase (SOD) (n=6) were also excluded. Although some participants had one retinal vessel parameter (e.g. calibers from retinal fundus images but no data of retinal vessel responses to FLIP), they were still included.

Permission to participate in the follow-up study was granted by the North-West Education Department and support ensured from the South African Democratic Teachers Union. The study complied with the requirements of Helsinki declaration of 1975 (revised in 2008). Ethical clearance for the SABPA study was obtained from the North-West University (Potchefstroom campus) Ethics committee (NWU-00036-07-S6). Following approval of this sub-study by the scientific research committee of the Hypertension in Africa Research Team, a separate ethical application for this sub-study was approved by the Health Research Ethics Committee of the North-West University (NWU-00036-07-A6).

Organizational procedure

All measurements were taken over two consecutive days. During the working week, 24h ambulatory blood pressure monitoring (ABPM) was conducted. An ABPM device (Cardiotens CE120®, Meditech, Budapest, Hungary) was attached to the participant's non-dominant arm every morning of day one (from Monday to Thursday) between 07h00-8h00. At 14h00 of day one, participants were transported to the North-West University and retinal vessel analyses

commenced at 15h00. Thereafter they were transported to the Metabolic Unit Research Facility of the North-West University which consists of ten bedrooms, two bathrooms, a living room and a kitchen. A standardized dinner was provided to the participants and they had their last beverages at 20h30. Thereafter, they relaxed and were asked not to consume alcohol or coffee, not to smoke or perform exercise till the following day. Participants were advised to go to bed at 22h00. At 06h00 of day two, participants were woken and the ABPM device was removed at 07h30. A battery of cardiovascular measurements were performed (07h30-08h30). This was followed by blood sampling (08h30-09h00) taken by registered nurses. For the assessment of physical activity, participants were fitted with Actical accelerometers (Actiheart®, CamNTech Ltd, UK) around their hips, 24hrs/day for seven consecutive days and the average total energy expenditure was calculated.

Questionnaires

A General Health Questionnaire was completed by each participant. This questionnaire was used to determine self-reported lifestyle habits (such as smoking and alcohol use), medication usage and antioxidant intake.

Anthropometrical measurements

Body height in meter (m), body weight in kilogram (kg) and waist circumference in centimeter (cm) were measured by calibrated instruments such as a Stadiometer (Invicta Stadiometer, IP 1465, UK), weight scale (Precision Health Scale, A & D Company, Japan) and Holtain unstretchable, flexible 7 mm wide metal tape (A & D Company, Japan) respectively. Body mass index (BMI) was calculated in kilogram per meter squared (kg/m^2). According to standard methods by registered level two anthropometrists [9], all measurements were done in triplicate.

Blood pressure measurements

Blood pressure measurements were obtained at 30 minute intervals during the day (between 07h00-08h00 to 22h00) and every hour during the night (22h00 to 07h00-08h00) [10]. This was done by a programmed ABPM device (Cardiotens CE120®, Meditech, Budapest, Hungary) with the cuff being attached to the participant's non-dominant arm. The participants were requested to continue with their day-to-day activities as well as noting down any abnormalities and discomfort such as headache, nausea and stress. The Cardio Visions 1.15 Personal Edition software was then used to analyze data. Participants were regarded as hypertensive if their 24h ABPM values of systolic blood pressure (SBP) were ≥ 130 and/or diastolic blood pressure (DBP) ≥ 80 millimeter mercury (mmHg) (European society of hypertension guidelines, 2013) [11]. On day one, hypertensive participants took their hypertension medication as usual but on day two, they were requested to take their medication after clinical measurements.

Retinal vessel analyses

Participants were instructed not to eat, drink and smoke or to perform exercise one hour prior to the measurement. They were also asked to consume their lunch an hour prior to the measurement to avoid being in a fasting state.

The retinal vessel measurements were conducted in a half-lit temperature-controlled room. A drop of Mydriacyl (Tropicamide 1% from Alcon) was administered to the right eye of each participant but the left eye was used for participants who had conditions such as cataract, previous eye injury and lens replacement. When the measurement was taking place, a cotton wool eye patch was placed on the eye from which the measurement was not taken in order to avoid any possible distraction. After mydriatic conditions were achieved, the researchers proceeded with functional retinal vessel measurements followed by retinal photography for the determination of retinal vessel calibers.

Flicker light-induced retinal vessel response measurements

The Dynamic Vessel Analyzer (DVA) (Zeiss Fundus Camera FF-450 Plus (Imedos, Jena, Germany) and RVA 4.10 software (Imedos, Jena, Germany) were used to record functional retinal vessel measurements in response to a flicker light-induced provocation (FLIP). The fundus camera was fixed at a 30° angle and participants were asked to focus on the tip of fixation bar within the camera. A selection of artery and vein segment was made, in the superior or inferior temporal region of the retina. These segments are found along an unbranched vessel segment located between 0.5–2.0 optic disc diameters from the margin of the optic disc. Mean vessel diameters of these selected segments were automatically recorded when the FLIP protocol was initiated (Figure 1). Baseline vessel diameter was measured first for 50 seconds followed by the stimulation of blood vessels which continued for 20 seconds. The FLIP cycle was repeated three times separated by a non-flicker period for 80 seconds. The quality of the measurement was assessed by means of a subjective scoring method (Personal Communication between Dr W Smith and Prof KE Kotliar). The criteria were as follows: 1) The possibility of raw data to be analyzed, 2) the availability of at least one good flicker recording (baseline/flicker pattern), 3) the availability of consistent measuring points during the flicker, 4) low noise and 5) almost no gaps. For each category, a score was given (0, 0.5 or 1) which carried the same weight for a total of 5 points (0 = poor quality and 5 = excellent quality; in extreme cases a negative score could be allocated). For this study, the only participants included were those with a vessel segment score of 2.5 or above.

The raw numerical data generated from the DVA was exported to a Microsoft Excel template with macros allowing further filtering, processing and data analysis. Data was extracted from all three FLIP curves and median values were generated for each time point. This enabled the researchers to construct an average summary curve (Figure 2). To get good quality data curves, a running median curve with a window of 5 seconds from the previously mentioned

average summary curve was calculated. For the purposes of this study, the following parameters were determined:

1. Absolute vessel diameters (measured in measuring units (MU) where 1 MU = 1 micrometer (µm) of the normal Gullstrand eye) of the vessel segment selected in the measurement. This was calculated individually as the median value over the last 30 seconds of the first baseline phase prior to FLIP.

Retinal microvascular structure is essentially the measurement of lumen diameter. The retina reflects the illumination light from the fundus camera entering the eye's pupil. The vessels present on the surface of the retina are filled with blood which absorbs a certain amount of reflected light. There is a column of red blood cells inside the vessel walls. Under imaging conditions, this column of red blood cells is invisible for the fundus camera. The retinal vessel analyzer therefore measures the diameter of the column of red blood cells, termed as the vessel diameter [12].

2. Maximum artery dilation (Art_max_{dil}) and maximum vein dilation ($Vein_max_{dil}$) in response to FLIP. This maximum point was taken between the start of the FLIP and 40 seconds after FLIP was initiated. The values were expressed as percentage of baseline.

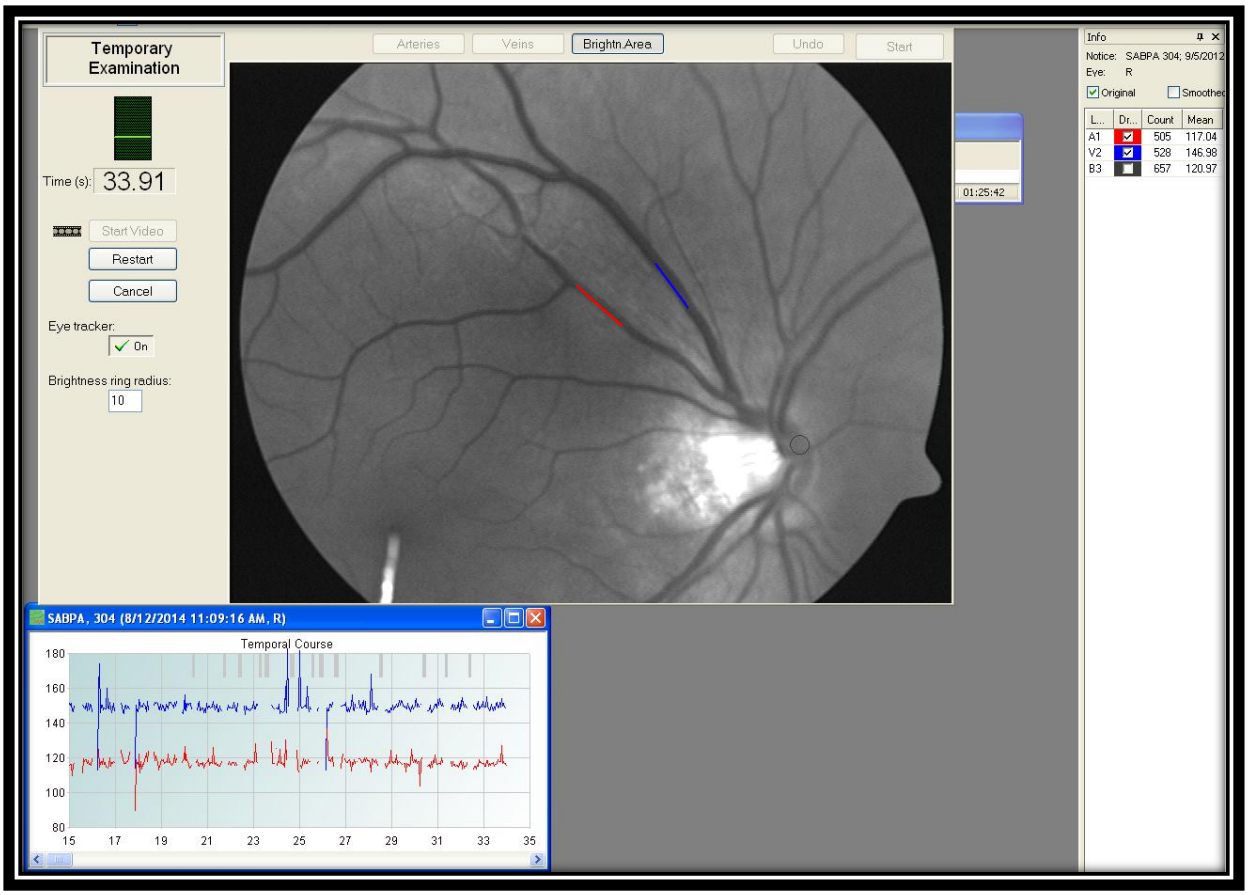


Figure 1: Retinal fundus image on real-time monitor. Participants were asked to focus on the tip of fixation bar (monochrome image; bottom-left). An artery (red) and vein (blue) segment was selected by the operator. A FLIP protocol was initiated where vessel diameters of these selected segments were recorded (graph; bottom-left).

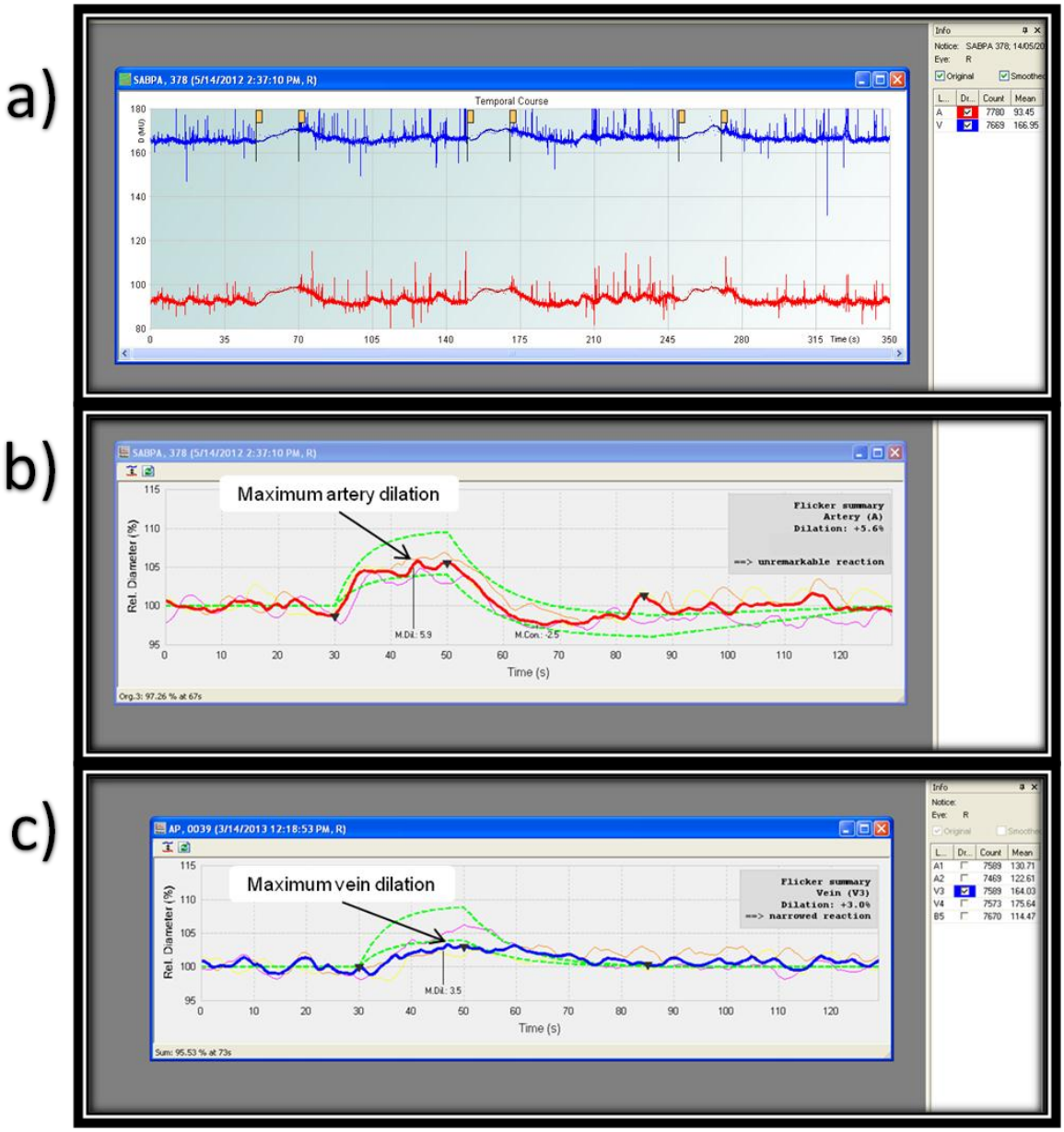


Figure 2: Retinal vascular reaction to FLIP. The images display a) temporal course of the artery (red) and vein (blue) diameter changes over three FLIP cycles b) summary curve of arteries (in red) over the three FLIP cycles c) summary curve of veins (in blue) over the three FLIP cycles (thin lines in orange, pink and yellow depicting the three individual flicker cycles).

Retinal vessel caliber measurements

Visualis 2.81 software (Imedos Systems, Jena, Germany) was used to capture retinal images. Color and monochrome images (Figure 3 and 4) were taken with the camera set at

an angle of 50°. The VesselMap2 software version 3.02 (Imedos Systems, Jena, Germany) was used to analyze the monochrome image. Vessel segments located within 0.5-1.0 optic disc diameters from the outer margin of the optic disc were selected as artery or vein. The trunks of the vessels were used. The six largest arteries and veins in the measuring area were used for the calculation of central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) using the Knudtson formula [13]. The arterio-venous ratio (AVR) was then calculated. When the measurement was completed, a cotton wool eye patch was placed on the eye from which the measurement was taken to prevent any possible damage to the retina from bright light.

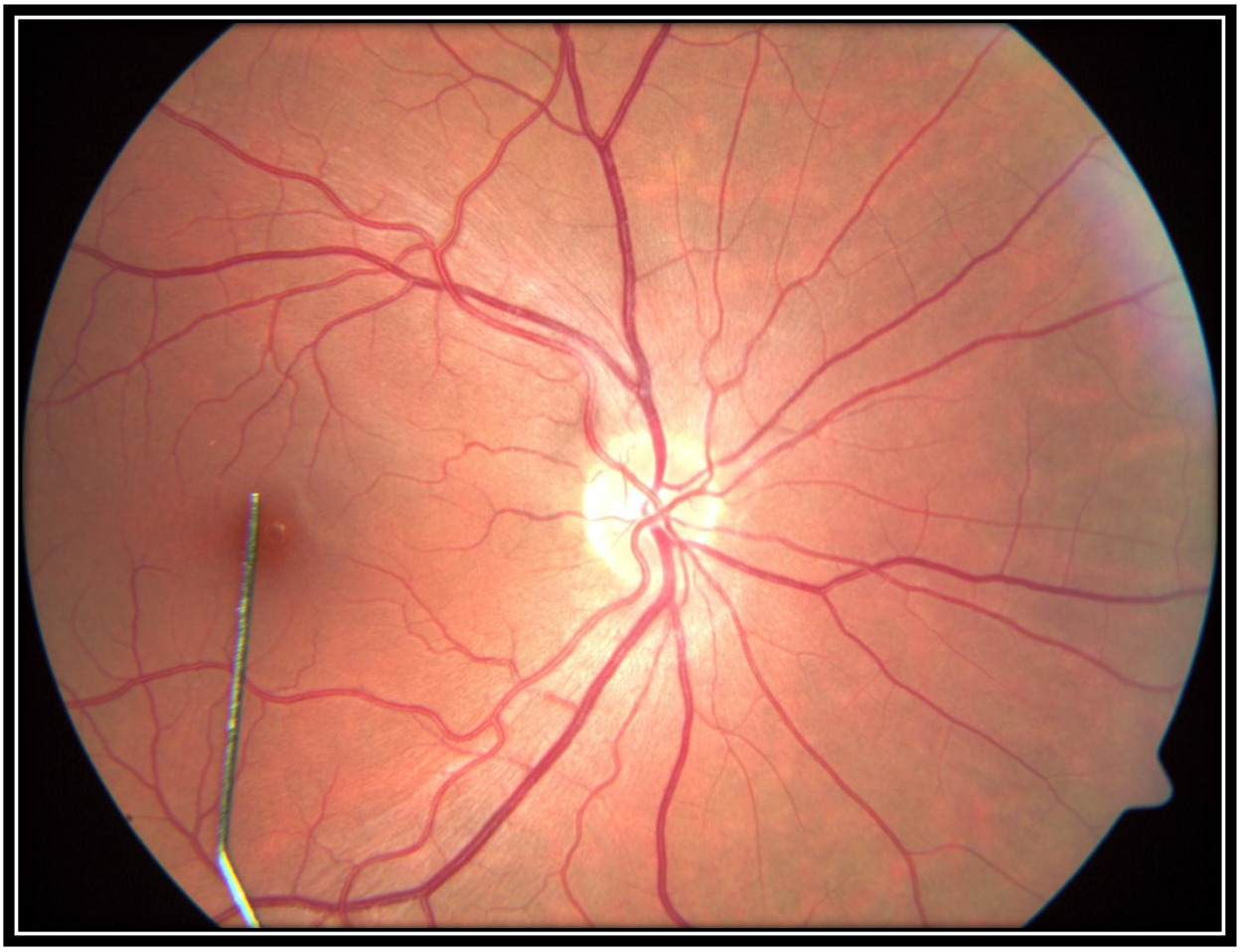


Figure 3: Color image illustrating retinal blood vessels

Figure 4: A monochrome image with a selection of vessel segments between 0.5-1.0 optic disc diameters from the outer margin of the optic disc

There are controversies on the use of terminology regarding the retinal microvasculature. Some researchers refer to the terms ‘arterioles’ and ‘venules’, while others refer to ‘arteries’ and ‘veins’. For this study, the latter two terms were used.

Biochemical analyses

On day one, participants were in a fasting state from 22h00 until 08h30-09h00 the next morning. Blood samples were obtained from the antebrachial vein branches by registered nurses using a sterile winged infusion set. Serum, sodium fluoride plasma and ethylenediaminetetraacetic acid (EDTA) plasma were prepared according to standard procedures and aliquots were stored at -80°C until biochemical analyses were performed.

Reactive oxygen species (ROS) species (in the form of serum peroxides) was determined by means of a high-throughput spectrophotometric assay [14]. The derivatives of reactive oxygen metabolites (D-ROM) test was used to determine the amount of oxygen-centered free radicals in blood samples. Five µl of either serum or hydrogen peroxide (H_2O_2) was added to 0.1 M sodium acetate buffer in 1 well of a 96-well microtiter plate to give a mixed solution which contains alkylamine and metals. Subsequently, the mixed solution containing alkylamine and metals was incubated at 37˚C for a minute using a spectrophotometric plate reader and absorbance was measured at 505 nm for a fixed time with 15 seconds interval. The calibrated curve was constructed from the slopes which were calculated from the varying absorbance at 505 nm with each time (in minutes) which corresponded to the concentration of H_2O_2. ROS levels were calculated from the calibration curve by a spectrophotometric plate reader and expressed as equivalent to levels of H_2O_2 (1 unit = 1.0 mg H_2O_2/l). ROS levels determined by this assay system correlate with the amount of free radicals and peroxides which react with various molecules in the body forming stable metabolites. In this study, serum peroxides are referred to as ROS. Thiobarbituric acid reactive substances (TBARS), a marker of lipid peroxidation, was determined spectrophotometrically [15]. Ferric reducing ability of plasma (FRAP), a measure of antioxidant power, was measured with FRAP assay [16]. The reduction of ferric to ferrous ion at low pH forms a colored ferrous-tripyridyltriazine complex. FRAP values were obtained by comparing change in absorbance at 593 nm in test reaction mixtures with those containing ferrous iron in known concentrations. Whole blood samples were used to determine total GSH (tGSH) levels by means of BIOXYTECH® GSH/GSSG-412™ kit supplied by OxisResearch™, (Foster City, CA). Assay kits from Cayman chemical company (Ann Arbor, MI, USA) were used to determine the activities of glutathione peroxidise (GPx) (EDTA plasma), glutathione reductase (GR) (EDTA plasma) and SOD (serum). An assay based on the enzymatic conversion of nitrate to nitrite by nitrate reductase was used to determine serum nitric oxide metabolite (NOx) levels. Nitrite, an azo dye product of the Griess reaction was detected colorimetrically. The Griess reaction is based on the two-step

diazotization reaction in which acidified nitrite (NO_2^-) produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540-570 nm (R & D Systems, Minneapolis, MN, USA). Before analysis, proteins were removed by ultrafiltration using 10 000 molecular weight cut-off filters. All of these assays were performed on a Synergy HT microplate reader (Bio-Tek, Winooski, VT, USA).

Sodium fluoride plasma samples were used to measure glucose levels whereas serum samples were used to determine total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, high sensitivity C-reactive protein (CRP) and gamma-glutamyl transferase (GGT) (Cobas Integra 400 plus, Basel, Switzerland). The first response HIV card test 1-2.0 (PMC Medical, India Pvt Ltd) was used to determine the infection with Human immunodeficiency virus (HIV). The results were also confirmed by the Pareekshak HIV triline test (UCB Pharma, India).

Statistical analyses

Statistical analyses were performed using Statistica version 12 (Statsoft inc., Tulsa, OK, USA). Visual inspection of data was used to test the normality of data distribution and non-Gaussian variables (BMI, TBARS, GR, SOD, GPx:SOD, GR:GPx, total NOx, glucose, CRP, GGT, triglycerides, HDL cholesterol, total cholesterol:HDL and total energy expenditure) were logarithmically transformed. Descriptive statistics were done and arithmetic means with standard deviations were calculated for normally distributed data while geometric mean with 5th and 95th percentile intervals were calculated for logarithmically transformed variables. Comparisons of mean values and proportions between black and white men were done using independent *t*-tests and Chi-square tests respectively. Pearson and partial correlations were used to explore relationships between retinal vessel calibers and retinal vessel responses to FLIP and oxidative stress-related markers. In addition to partial correlations, adjustments were made for age and BMI in the case of retinal vessel caliber markers

whereas adjustments were made for age and waist circumference in the case of retinal vessel responses to FLIP. These adjustments were based on BMI and waist circumference being strongly related to retinal vessel calibers and functional retinal vessel measurements respectively. Independent associations of retinal vessel calibers and retinal vessel responses to FLIP with oxidative stress-related markers in both groups were evaluated by performing forward stepwise multiple regression analyses. Participants with missing data for all the oxidative stress-related markers and retinal vessel variables were not excluded in order to keep our statistical power in the regression models. As such multivariate regression analyses were performed using casewise deletion of the data. The selection of covariates for entry in multiple regression models were based on their bivariate relationship with the main variables. The following covariates were included in the models: age, BMI (retinal vessel calibers), waist circumference (retinal vessel responses to FLIP), total cholesterol, CRP, GGT, glucose, 24h SBP. As arterial and vein calibers are highly correlated [17], it may also be challenging to provide valid conclusions regarding their associations with factors of CVD risk. Furthermore, when analyzing vessel calibers separately, possible confounding effects of the correlated component variable may exist. *Liew et al*. [17] suggested that when CRAE was the main independent variable, adjustment for vein caliber (CRVE) must be made and vice versa. This was done to avoid any confounding effect that may exist between CRAE and CRVE. To provide the best fit models for the final multiple regressions, only the variables that entered the stepwise models and were used in a separate regression analysis are presented in the tables. In sensitivity analyses, an exclusion was done for HIV infected participants (n=14) and multiple regression analyses were repeated. The effect of anti-hypertensive medication usage, smoking and hypertensive status was further tested on our results by adding these factors individually as covariates to the models.

References

[1] Malan L, Hamer M, Frasure-Smith N, Steyn HS, Malan NT. Cohort profile: Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) prospective cohort study. Int J Epidemiol 2015;44(6):1814-1822.

[2] Wactawski-Wende J, Schisterman EF, Hovey KM, Howards PP, Browne RW, Hediger M, Liu A, Trevisan M. BioCycle study: design of the longitudinal study of the oxidative stress and hormone variation during the menstrual cycle. Paediatr Perinat Epidemiol 2009;23(2):171-184.

[3] Miller AA, De Silva TM, Jackman KA, Sobey CG. Effect of gender and sex hormones on vascular oxidative stress. Clin Exp Pharmacol Physiol 2007;34(10):1037-1043.

[4] Gerschman R, Gilbert DL, Nye SW, Dwyer P, Fenn WO. Oxygen poisoning and x-irradiation: a mechanism in common. Science 1954;119(3097):623-626.

[5] Yagi K. Female hormones act as natural antioxidants-a survey of our research. Acta Biochim Pol 1997;44(4):701-709.

[6] Yagi K, Komura S. Inhibitory effect of female hormones on lipid peroxidation. Biochem Int 1986;13(6):1051-1055.

[7] Leal M, Díaz J, Serrano E, Abellán J, Carbonell LF. Hormone replacement therapy for oxidative stress in postmenopausal women with hot flushes. Obstet Gynecol 2000;95(6 Pt 1):804-809.

[8] Browne RW, Bloom MS, Schisterman EF, Hovey K, Trevisan M, Wu C, Liu A, Wactawski-Wende J. Analytical and biological variation of biomarkers of oxidative stress during the menstrual cycle. Biomarkers 2008;13(2):160-183.

[9] Norton K, Olds T. Anthropometrica: a textbook of body measurement for sports and health courses. UNSW Press; 1996.

[10] Kohara K, Nishida W, Maguchi M, Hiwada K. Autonomic nervous function in non-dipper essential hypertensive subjects evaluation by power spectral analysis of heart rate variability. Hypertension 1995;26(5):808-814.

[11] Mancia G, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Böhm M, Christiaens T, Cifkova R, De Backer G, Dominiczak A. 2013 ESH/ESC guidelines for the management of arterial hypertension: the Task force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Blood Pressure 2013;22(4):193-278.

[12] Seifert BU, Vilser W. Retinal vessel analyzer (RVA)-design and function. Biomed Tech 2002;47 (Suppl 1 Pt 2):678-681.

[13] Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. Curr Eye Res 2003;27(3):143-149.

[14] Hayashi I, Morishita Y, Imai K, Nakamura M, Nakachi K, Hayashi T. High-throughput spectrophotometric assay of reactive oxygen species in serum. Mutat Res 2007;631(1):55-61.

[15] Buege JA, Aust S. Methods Enzymol 1978;52:302-310.

[16] Benzie IF, Strain J. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. Anal Biochem 1996;239(1):70-76.

[17] Liew G, Sharrett AR, Kronmal R, Klein R, Wong TY, Mitchell P, Kifley A, Wang JJ. Measurement of retinal vascular caliber: issues and alternatives to using the arteriole to venule ratio. Invest Ophthalmol Vis Sci 2007;48(1):52-57.

CHAPTER 4

MANUSCRIPT OF THE JOURNAL ARTICLE

Instructions for authors; *Free Radical Research*

Manuscripts should be compiled in the following order: title page; abstract; main text; acknowledgments; declaration of interest statement; appendices (as appropriate); references; tables with captions (on separate pages); figure captions (as a list); figures.

Title Page

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

Key Words

Five key terms that are not in the title should also be included on the title page.

Abstract

All articles should start with an abstract of less than 250 words, summarizing the central core of knowledge that is the focus of the paper. It should be written in an informative style permitting its use, without revision, by abstracting services, give essential details of research findings without further reference to the text, and avoid generalisations and nonessential information.

Main Text

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

References

References should follow the Council of Biology Editors (CBE) style. References should then be listed in numerical order at the end of the article as follows:

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

Book chapter: [2] Kuret JA, Murad F. Adenohypophyseal hormones and related substances. In: Gilman AG, Rall TW, Nies AS, Taylor P, editors. The pharmacological basis of therapeutics. 8th ed. New York: Pergamon; 1990. p 1334–1360.

Conference proceedings: [3] Irvin AD, Cunningham MP, Young AS, editors. Advances in the control of Theileriosis. International Conference held at the International Laboratory for Research on Animal Diseases; 1981 Feb 9-13; Nairobi. Boston: Martinus Nijhoff Publishers; 1981. 427 p.

Dissertations or Thesis: [4] Mangie ED. A comparative study of the perceptions of illness in New Kingdom Egypt and Mesopotamia of the early first millennium [dissertation]. Akron (OH): University of Akron; 1991. 160 p. Available from: University Microfilms, Ann Arbor MI; AAG9203425.

Journal article on internet: [5] Loker WM. "Campesinos" and the crisis of modernization in Latin America. Jour of Pol Ecol [serial online] 1996; 3(1). Available: http://www.library.arizona.edu/ej/jpe/volume_3/ascii-lokeriso.txt via the INTERNET. Accessed 1996 Aug 11.

Webpage: [6] British Medical Journal [Internet]. Stanford, CA: Stanford Univ; 2004 July 10 - [cited 2004 Aug 12]; Available from: http://bmj.bmjjournals.com/

Internet databases: [7] Prevention News Update Database [Internet]. Rockville (MD): Centers for Disease Control and Prevention (US), National Prevention Information Network. 1988 Jun - [cited 2001 Apr 12]. Available from: http://www.cdcnpin.org/db/public/dnmain.htm Periodical abbreviations should follow the style given by Index Medicus.

The relationship between oxidative stress-related markers and retinal microvascular structure and function in a bi-ethnic population: The SABPA study

Felicia Maugana, Carina Mels, Nico Malan and Wayne Smith

Hypertension in Africa Research Team (HART)

North-West University; Potchefstroom; South Africa.

Brief title: Oxidative stress and the retinal microvasculature

Source Funding: The financial assistance of the National Research Foundation (NRF), NRF Thuthuka (80643), Medical Research Council (MRC) and Roche Diagnostics towards this research is hereby acknowledged. Expressed opinions and conclusions arrived at, are those of the authors and are not necessarily to be attributed to the NRF.

Conflict of interest: None to declare

Abstract word count: 232

Number of tables: 4

Number of figures: 0

Supplementary tables: 1

Correspondence:

Dr. Wayne Smith

Hypertension in Africa Research Team (HART),

Private bag x6001,

North-West University

Potchefstroom,

2520

South Africa

Telephone: +27-18-299-2053

Fax: +27-18-285-2432

E-mail: Wayne.Smith@nwu.ac.za

ABSTRACT

Oxidative stress is implicated in the pathogenesis of macrovascular diseases, while little is known about its role in the retinal microvasculature. Retinal microvascular abnormalities may indicate early cardiovascular disease risk. We compared oxidative stress-related markers in a cohort of black and white South African men and investigated whether oxidative stress-related markers are associated with retinal vessel calibers and vessel responses to flicker light-induced provocation (FLIP) in these groups. We included 82 black and 77 white men and 24h ambulatory blood pressure was measured. Retinal maximum artery (Art_max_{dil}) and vein ($Vein_max_{dil}$) dilation in response to FLIP were assessed. Central retinal artery equivalent (CRAE), central retinal vein equivalent (CRVE) and arterio-venous ratio (AVR) were determined. Oxidative stress-related markers included: thiobarbituric acid reactive substances (TBARS), reactive oxygen species (ROS) (serum peroxides), total glutathione (tGSH), ferric reducing ability of plasma (FRAP), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR). Although black men had higher TBARS ($p=0.011$), they also had higher GPx ($p=0.001$) and SOD activities (tendency; $p=0.062$) with lower FRAP and tGSH levels (both $p<0.001$) compared to white men. Multiple regression analyses revealed an independent association between SOD activity and Art_max_{dil} (Adjusted $R^2=0.19$; $\beta=-0.36$; $p=0.002$) whereas AVR (Adjusted $R^2=0.12$; $\beta=-0.32$; $p=0.007$) and CRAE (Adjusted $R^2=0.27$; $\beta=-0.23$; $p=0.040$) were independently associated with ROS. The up-regulated SOD activity in black men may contribute in maintaining retinal vascular function while retinal arterial narrowing is mediated via ROS.

Key words: flicker light-induced retinal vessel dilation, retinal vessel calibers, vascular function, reactive oxygen species, superoxide dismutase.

INTRODUCTION

Reactive oxygen species (ROS) play a physiological role in the regulation of endothelial function and vascular tone [1,2]. Normally, the balance between ROS and antioxidant capacity is maintained, but when this equilibrium is disturbed, it may result in oxidative stress [3]. This may lead to endothelial dysfunction, inflammation, vascular remodeling [4] and ultimately the development of cardiovascular disease (CVD) [5].

The antioxidant defense system is composed of non-enzymatic and enzymatic antioxidants. Some of the non-enzymatic antioxidants include glutathione (GSH), and bilirubin, ascorbate, tocopherols and uric acid which can be accumulatively measured with the ferric reducing ability of plasma (FRAP) assay [6]. The enzymatic antioxidant system includes superoxide dismutase (SOD) which is involved in the dismutation of superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2). In turn H_2O_2 can be further metabolized by the enzymes catalase (CAT) and glutathione peroxidase (GPx) [7]. During the reaction catalysed by GPx, GSH is oxidized to form glutathione disulphide (GSSG) which can be recycled by the action of the enzyme glutathione reductase (GR) [8]. When the antioxidant capacity is overwhelmed by the production of ROS it may lead to oxidative damage to macromolecules, such as proteins or deoxyribonucleic acids (DNA) and lipids (with thiobarbituric acid reactive substances (TBARS) being a marker of lipid peroxidation) [3].

Since the prevalence of hypertension and CVD is rapidly increasing in urban black populations [9], previous studies investigated the link between oxidative stress and the macrovasculature. These studies indicated that higher levels of ROS in hypertensive black men and lower GPx activity in black women were associated with increased blood pressure [10,11]. Furthermore, attenuated total glutathione levels and up-regulated GR activity were associated with increased carotid intima-media thickness (c-IMT) [10,12]. Since alterations in microvascular function may occur prior to macrovascular dysfunction [14], it is important to evaluate associations of oxidative stress-related markers with microvascular parameters.

The retina provides a unique site where the microvasculature can be visualized directly [13]. Retinal vessel function can be assessed by evaluating reactivity to a flicker light-induced provocation (FLIP) [14]. Alternatively from retinal fundus images, vessel calibers can be determined [15]. With regard to retinal vessel calibers, large population-based studies have shown that retinal arterial narrowing and vein widening are associated with a wide range of subclinical and clinical CVD [16-18]. Similarly, reduced retinal vessel responses to FLIP, as a possible measure of vascular endothelial function [13] have been associated with diabetes [19], hypertension [14], hyperlipidemia [20] and coronary artery disease (CAD) [21].

Limited information on the relationship between the retinal microvasculature parameters as listed above and oxidative stress-related markers are available. The available studies indicated the importance of antioxidant defense mechanisms in retinal microvascular functional responses during FLIP [22] and retinal microvascular calibers [23].

We, therefore, aimed to compare oxidative stress-related markers in a cohort of black and white men from South Africa and to investigate the relationships between retinal vessel calibers and retinal vessel responses to FLIP with oxidative stress-related markers in this group.

METHODS

Study population

This cross-sectional study uses data generated during the follow-up phase of the Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) study. This phase of the SABPA study was conducted between February and May of 2011/2012 and included teachers from the Kenneth Kaunda Education district in the North West Province of South Africa. A detailed description of the SABPA study protocol has been published [24]. In short, the procedures were explained to the participants in the language of their choice prior to their recruitment. Participants with an ear temperature above 37℃, blood donors, psychotropic substance dependence or abuse, pregnant and lactating women and individuals vaccinated within three months prior to commencement of the study were excluded. All participants who took part in the baseline phase of the SABPA study (aged 20-65 years) were re-invited to participate and a total of 359 black and white teachers of both sexes participated in the follow-up phase. Since oxidative stress-related markers are known to vary in women during their menstrual cycle [25,26] and because oestrogens have a protective effect against oxidative damage [27-29], women were excluded from the sub-study as their menopausal status and/or fluctuating hormone levels during their menstrual phase were unknown. In addition, participants with no available retinal vessel analyses or poor retinal vessel analyses (n=15), participants using antioxidants (n=12) and those with outliers (mean +/- 3 x the standard deviation) for SOD (n=6) were excluded.

An informed consent form explaining the procedures and objectives was signed by all participants before commencement of the follow-up phase of the study. The study complied with the requirements of the Helsinki declaration of 1975 revised in 2008. The Health Research Ethics Committee of the North-West University (Potchefstroom campus) approved this sub-study (NWU-00036-07-A6).

Organizational procedure

The measurements were taken over the course of two consecutive days. 24h Ambulatory blood pressure monitoring (ABPM) was conducted during the working week. On day one, every morning (from Monday to Thursday) between 07h00-08h00, an ABPM device was attached to the participant's non-dominant arm. Participants were transported to the North-West University at 14h00 and retinal vessel analyses commenced at 15h00. Thereafter they were transported to the Metabolic Unit Research Facility of the North-West University. Participants were advised to go to bed at 22h00 and they were also requested not to consume any food from that time till the following day. On day two, participants were woken at 06h00 and the ABPM device was removed at 07h30. Thereafter a battery of cardiovascular measurements were performed (07h30-08h30) followed by blood sampling (08h30-09h00). Participants were also fitted with Actical accelerometers (Actiheart®, CamNTech Ltd, UK) around their hips to assess their physical activity during a normal working day for seven consecutive days and the average total energy expenditure was calculated.

Questionnaires

Each participant completed a General Health Questionnaire which was used to determine self-reported lifestyle habits (such as smoking and alcohol use), medication usage and antioxidant intake.

Anthropometrical measurements

Calibrated instruments such as a stadiometer (Invicta Stadiometer, IP 1465, UK), weight scale (Precision Health Scale, A & D Company, Japan) and Holtain unstretchable, flexible 7 mm wide metal tape (A & D Company, Japan) were used to measure body height in meter (m), body weight in kilogram (kg) and waist circumference in centimeter (cm) respectively. Body mass index (BMI) was calculated in kilograms per meter squared (kg/m^2). All

measurements were done in triplicate according to standard methods by registered level two anthropometrists [30].

Blood pressure measurements

An ABPM device (Cardiotens®, CE0120, Meditech, Budapest, Hungary) was programmed to obtain blood pressure measurements at 30 minute intervals during the day (between 07h00-08h00 to 22h00) and every hour during the night (22h00 to 07h00-08h00) [31]. The cuff was attached to the participant's non-dominant arm. The participants were requested to proceed with their daily activities and to note of any abnormalities and discomfort. Data was then analyzed using Cardio Visions version 1.15 Personal Edition software. Participants with 24h ABPM values of systolic blood pressure (SBP) ≥ 130 and/or diastolic blood pressure (DBP) ≥ 80 millimeter mercury (mmHg) were regarded as hypertensive (European society of hypertension guidelines, 2013) [32]. On day one, participants with hypertension took their medication as usual but on day two, their medication for hypertension was taken after clinical measurements.

Retinal vessel analyses

Participants were instructed to abstain from food and liquid intake and not to smoke or perform physical exercise for one hour prior to the measurement. They were also asked to consume their lunch an hour before the measurement commenced so as not to be in a fasting state.

The retinal vessel measurements were conducted in a half-lit temperature-controlled room. A drop of Mydriacyl (Tropicamide 1% from Alcon) was administered to the right eye of each participant. The left eye was used for participants who had problems with their right eye such as cataract, previous eye injury and lens replacement. During the measurement, a cotton wool eye patch was placed on the eye from which the measurement was not taken. This was done to help the participant stay focused on the tip of the fixation bar within the camera and

to avoid any possible distraction. After achieving mydriatic conditions, the researchers proceeded with functional retinal vessel measurements followed by retinal vessel caliber measurements.

Flicker light-induced retinal vessel responses

Retinal vessel measurements in response to a FLIP were recorded using the Dynamic Vessel Analyzer (DVA) (Zeiss Fundus Camera FF450 Plus, Imedos, Jena, Germany) and RVA 4.10 software (Imedos, Jena, Germany). With the fundus camera fixed at a 30° angle, participants were asked to focus on the tip of a fixation bar within the camera. Artery and vein segments were selected in the superior or inferior temporal region of the retina. These segments occurred along an unbranched vessel segment located between 0.5–2.0 optic disc diameters from the margin of the optic disc. A FLIP protocol was initiated where mean vessel diameters of these selected segments were automatically recorded. Baseline vessel diameter was measured first for 50 seconds. Stimulation of blood vessels began by FLIP cycles for 20 seconds. The FLIP was repeated three times separated by a recovery period of 80 seconds. To aid with the quality control of the measurement, a subjective scoring method (Personal Communication between Dr W Smith and Prof KE Kotliar) was used. The raw data from each participants recording was evaluated using the following criteria: 1) the raw data is possible to analyze, 2) at least one good flicker recording is present (baseline/flicker pattern), 3) consistent measuring points during the flicker is available, 4) there is low noise and 5) there is almost no gaps. A score (0, 0.5 or 1) was given for each category carrying a similar weight for a total of 5 points (0 = poor quality and 5 = excellent quality; in extreme cases a negative score could be allocated). For this study, only participants with a vessel segment score of 2.5 or above were included.

The raw numerical data generated from the DVA was exported into Microsoft Excel template with macros which further filters, process and analyze the data to achieve the study's endpoints. Data was extracted from all three FLIP curves and median values were

generated for each time point which enabled the researchers to construct an average summary curve. A running median curve was calculated with a window of 5 seconds from the previously mentioned average summary curve to ensure good quality data curves. For the purposes of this study, the following parameters were determined: 1) absolute vessel diameters (measured in measuring units (MU) where 1 MU = 1 micrometer (µm) of the normal Gullstrand eye) of the vessel segment selected in the measurement, 2) maximum artery (Art_max_{dil}) and vein dilation (Ven_max_{dil}) in response to FLIP.

Retinal vessel caliber measurements

Retinal images were captured using Visualis 2.81 software (Imedos Systems, Jena, Germany). A monochrome and color image was taken with the camera set at an angle of 50°. Vessels were located within 0.5-1.0 optic disc diameters from the outer margin of the optic disc. These vessels were identified by two experienced scientists who had to agree on the type of vessel before the final identification. The monochrome image was analyzed using VesselMap2 software version 3.02 (Imedos Systems, Jena, Germany). Each retinal image gives information regarding, central retinal artery equivalent (CRAE), central retinal vein equivalent (CRVE) and the arterio-venous ratio (AVR) is then calculated. The Knudtson formula was used to calculate CRAE and CRVE, where the six largest arteries and veins in the measuring area were used [33]. After the measurement, a cotton wool eye patch was placed on the eye from which the measurement was taken. This was done to prevent any possible damage to the retina.

Biochemical analyses

Participants remained in a fasting state from 22h00 of day one until 08h30-09h00 the next morning when a blood sample was obtained from the antebrachial vein by registered nurses. Blood was collected in the appropriate tubes and centrifuged according to standard procedures to obtain serum, sodium fluoride plasma and ethylenediaminetetraacetic acid (EDTA) plasma.

One of the measurable ROS species, namely total peroxides, were determined in serum samples by means of a high-throughput spectrophotometric assay [34]. For the purposes of this paper, serum peroxides are referred to as ROS. TBARS as a measure of lipid peroxidation [35] and FRAP as measure of antioxidant power was measured [6]. Total GSH (tGSH) levels were determined in whole blood samples with the BIOXYTECH® GSH/GSSG-412^{TM} kit supplied by OxisResearch^{TM}, (Foster City, CA). GPx and GR (EDTA plasma) and SOD activity (serum) were determined with assay kits from Cayman chemical company (Ann Arbor, MI, USA). Serum nitric oxide metabolite (NOx) levels were determined with an assay based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess Reaction is based on the two-step diazotization reaction in which acidified nitrite (NO_2^-) produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540-570 nm (R&D Systems, Minneapolis, MN, USA). Proteins were removed before analysis by ultrafiltration using 10 000 molecular weight cut-off filters. All of these assays were performed on a Synergy HT microplate reader (Bio-Tek, Winooski, VT, USA).

Glucose levels were measured in sodium fluoride plasma whereas high sensitivity C-reactive protein (CRP), gamma-glutamyl transferase (GGT), triglycerides, total cholesterol and high density lipoprotein (HDL) cholesterol were determined in serum samples (Cobas Integra 400 plus, Basel, Switzerland). Human immunodeficiency virus (HIV) status was determined by the first response HIV card test 1-2.0 (PMC Medical, India Pvt Ltd) which was also confirmed by the use of Pareekshak HIV triline test (UCB Pharma, India).

Statistical analyses

Statistica version 12 (Statsoft inc., Tulsa, OK, USA) was used to perform the statistical analyses. Normality of data distribution was tested with visual inspection of data and non-

Gaussian distributed variables were logarithmically transformed (BMI, TBARS, GR, SOD, GPx:SOD, GR: GPx, NOx, glucose, CRP, GGT, triglycerides, HDL cholesterol, total cholesterol:HDL and total energy expenditure). Descriptive statistics were done to calculate the arithmetic mean with standard deviation for normally distributed variables and the geometric mean with the 5th and 95th percentile intervals for logarithmically transformed variables. Mean values and proportions of the two ethnic groups (black and white men) were compared using independent *t*-tests and Chi-square tests respectively. Relationships between retinal vessel calibers and retinal vessel responses to FLIP and oxidative stress-related markers were explored using Pearson and partial correlations. In partial correlations with retinal vessel calibers (AVR, CRAE and CRVE), adjustments were made for age and BMI, whereas adjustments made for retinal vessel responses to FLIP (Art_max_{dil} and $Vein_max_{dil}$) were age and waist circumference. Retinal vessel calibers were more strongly related to BMI and retinal vessel responses to FLIP were more strongly related to waist circumference. Forward stepwise multiple regression analyses were performed to evaluate independent associations of retinal vessel calibers and vessel responses to FLIP with oxidative stress-related markers in both groups (black and white men). To keep our statistical power in the regression models, participants with missing data for all the oxidative stress-related markers and retinal vessel variables were not excluded. As such forward stepwise multivariate regression analyses were performed using a casewise deletion of the data. Covariates considered for entry in multiple regression models were based on their bivariate relationship with the main variables. Covariates considered in the models were age, BMI (retinal vessel calibers), waist circumference (retinal vessel responses to FLIP), total cholesterol, CRP, GGT, glucose, 24h SBP. When CRAE was the main independent variable, CRVE was included in the model and vice versa [36]. For the final multiple regression models, only the variables that entered the stepwise models were used in a separate regression analysis and presented in the tables in order to provide the best fit models. In sensitivity analyses, HIV infected participants (n=14) were excluded and multiple regression analyses were repeated. Additionally, the effect of anti-hypertensive medication usage,

smoking and hypertensive status was also tested on our results by adding these factors individually as covariates to the models.

RESULTS

Characteristics of black and white men

Black men had a lower BMI and waist circumference compared to white men (p≤0.007) (Table 1). Overall, black men presented with higher blood pressure, higher prevalence of hypertension and higher glucose, CRP, GGT, triglycerides and total cholesterol levels when compared to white men (all p<0.026). Although the activity of the antioxidant enzyme GPx (p=0.001) was significantly higher while SOD activity tended to be higher (p=0.062), other non-enzymatic capacity markers such as FRAP and tGSH (both p<0.001) were significantly lower in black men than in white men. Furthermore, the lipid peroxidation marker, TBARS (p=0.011) were higher in black men when compared to white men. With regards to functional retinal vessel measurements, Art_max_{dil} (p=0.036) was significantly higher in black men than in white men. When retinal vessel caliber measurements were compared, AVR was lower and CRVE was higher in black men than in white men (both p<0.001).

Table 1: Characteristics of black and white men

	Black men (n=82)	White men (n=77)	*p*
Age (years)	46.5 ± 7.4	49.1 ± 10.0	0.062
ANTHROPOMETRICAL MEASUREMENTS			
Body mass index (kg/m^2)	27.5 (20.9; 38.4)	30.2 (24.2; 42.9)	0.007
Waist circumference (cm)	98.5 ± 15.1	106.9 ± 13.4	<0.001
RETINAL MEASUREMENTS			
Flicker light-induced vessel response measurements			
Artery diameter (MU)	113 ± 15	114 ± 14	0.45
Maximum artery dilation (%)	4.1 ± 2.3	3.8 ± 1.8	0.036
Vein diameter (MU)	155 ± 22	149 ± 22	0.11
Maximum vein dilation (%)	4.4 ± 1.9	3.8 ± 1.7	0.052
Retinal vessel caliber measurements			
Arterio-venous ratio	0.59 ± 0.06	0.64 ± 0.04	<0.001
Central retinal artery equivalent (MU)	148 ± 13	151 ± 12	0.10
Central retinal vein equivalent (MU)	251 ± 19	238 ± 19	<0.001
CARDIOVASCULAR MEASUREMENTS			
24h systolic blood pressure (mmHg)	138 ± 17	128 ± 11	<0.001
24h diastolic blood pressure (mmHg)	87 ± 10	79 ± 7	<0.001
Hypertension status, n (%)	65 (79.3)	37 (48.1)	<0.001
OXIDATIVE STRESS-RELATED MARKERS			
ROS (1 unit = 1.0 mg/L H_2O_2)	75.20 ± 21.75	75.70 ± 17.11	0.88
TBARS (mg/g creatinine)	0.11 (0.03; 0.45)	0.08 (0.03; 0.18)	0.011
Ferric reducing ability of plasma (µM)	467.36 ± 84.83	523.57 ± 80.60	<0.001
Total glutathione (µM)	855.76 ± 211.21	1092.30 ± 357.70	<0.001
Glutathione peroxidase (nmol/min/ml)	36.99 ± 16.31	30.46 ± 6.58	0.001
Glutathione reductase (nmol/min/ml)	3.98 (0.76; 13.75)	3.31 (0.76; 57.56)	0.27
Superoxide dismutase (U/ml)	2.75 (0.77; 8.17)	2.19 (0.63; 5.76)	0.062
GPx:SOD	12.30 (2.56; 57.20)	13.49 (4.54; 52.97)	0.46
GR:GPx	0.12 (0.02; 0.42)	0.11 (0.03; 1.56)	0.74
Nitric oxide metabolites (µmol/L)	8.32 (0.00; 34.24)	10.96 (1.59; 128.97)	0.22
BIOCHEMICAL ANALYSES			
Glucose (mmol/l)	5.75 (4.54; 11.41)	4.57 (2.96; 8.49)	<0.001
C-reactive protein (mg/l)	2.82 (0.30; 27.15)	1.23 (0.18; 7.05)	<0.001
Gamma glutamyl transferase (U/l)	60.26 (22.95; 252.30)	26.92 (9.50; 97.30)	<0.001
Triglycerides (mmol/l)	1.48 (0.67; 3.42)	1.23 (0.59; 3.02)	0.026
Total cholesterol (mmol/l)	4.68 ± 1.02	4.21 ± 1.04	0.005
HDL cholesterol (mmol/l)	0.89 (0.54; 1.49)	0.81 (0.53; 1.28)	0.051
TC:HDL	5.13 (2.89; 8.65)	5.01 (3.04; 8.00)	0.68
LIFESTYLE MEASUREMENTS			
Total energy expenditure (kCal/day)	3090 (1955; 6059)	3890 (2399; 7790)	<0.001
Anti-hypertensive medication use, n (%)	26 (31.7)	19 (24.7)	0.33
HIV infected, n (%)	14 (17.1)	0 (0.00)	<0.001
Alcohol use, n (%)	57 (69.5)	47 (61.0)	0.26
Smoking, n (%)	20 (24.4)	13 (16.9)	0.24

*1 MU, measuring units = 1 µm in the normal Gullstrand eye. Values are arithmetic mean ± standard deviation or geometric mean (5th to 95th percentile interval); n (%); n, number of participants; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; GPx:SOD, glutathione peroxidase:superoxide dismutase; GR:GPx, glutathione reductase:glutathione peroxidase; HDL, high density lipoprotein; TC: HDL, total cholesterol:high density lipoprotein; HIV, human immunodeficiency virus.

Correlations

Single regression analyses are presented in supplementary table 1. In partial regression analyses (Table 2), Art_max_{dil} correlated negatively with SOD in black men (r=-0.37; p=0.006). Additionally, a tendency to have a negative correlation was seen between $Vein_max_{dil}$ and ROS in black men (r=-0.24; p=0.06). AVR tended to correlate negatively with ROS in black men (r=-0.26; p= 0.06). Also in black men, CRVE showed a tendency to have a positive correlation with GPx (r=0.23; p=0.09). In white men, CRAE correlated positively with NOx (r=0.24; p= 0.049).

Table 2: Partial regression analyses between retinal vessel calibers and functional retinal vessel measurements with oxidative stress-related markers in black and white men

			Black men		
	AVR	**CRAE**	**CRVE**	**Art_max$_{dil}$**	**Vein_max$_{dil}$**
ROS	r = -0.26*	r = -0.15	r = 0.15	r = 0.12	r = -0.24*
TBARS	r = 0.06	r = 0.15	r = 0.10	r = -0.05	r = 0.06
FRAP	r = -0.03	r = -0.10	r = -0.07	r = -0.06	r = 0.17
tGSH	r = -0.07	r = 0.04	r = 0.15	r = -0.04	r = -0.07
GPx	r = -0.16	r = 0.02	r = 0.23*	r = -0.18	r = 0.06
GR	r = 0.07	r = 0.10	r = 0.01	r = 0.15	r = 0.11
SOD	r = -0.0003	r = -0.08	r = -0.10	r = -0.37†	r = -0.14
GPx:SOD	r = -0.03	r = 0.14	r = 0.19	r = 0.20	r = 0.16
GR:GPx	r = 0.11	r = 0.07	r = -0.07	r = 0.22	r = 0.06
NOx	r = -0.06	r = -0.10	r = -0.01	r = 0.05	r = -0.07
			White men		
	AVR	**CRAE**	**CRVE**	**Art_max$_{dil}$**	**Vein_max$_{dil}$**
ROS	r = -0.10	r = 0.03	r = 0.10	r = -0.08	r = -0.002
TBARS	r = -0.07	r = 0.05	r = 0.02	r = -0.02	r = 0.08
FRAP	r = -0.17	r = -0.13	r = 0.03	r = -0.11	r = -0.13
tGSH	r = 0.04	r = 0.19	r = 0.14	r = -0.07	r = 0.06
GPx	r = 0.002	r = -0.05	r = -0.04	r = -0.05	r = -0.02
GR	r = 0.01	r = 0.02	r = -0.02	r = 0.09	r = 0.11
SOD	r = 0.03	r = 0.09	r = 0.12	r = -0.19	r = -0.06
GPx:SOD	r = -0.04	r = -0.10	r = -0.14	r = 0.16	r = 0.05
GR:GPx	r = 0.01	r = 0.04	r = 0.03	r = 0.11	r = 0.12
NOx	r = 0.17	r = 0.24‡	r = 0.10	r = -0.07	r = -0.11

p≤0.01 but p≥0.001 † p≤0.05 but p>0.01 ‡ p≤0.10 but p>0.05 *

Retinal vessel calibers were adjusted for age and BMI, while functional retinal vessel responses to FLIP were adjusted for age and waist circumference.

AVR, arterio-venous ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; Art_max$_{dil}$, maximum artery dilation; Vein_max$_{dil}$, maximum vein dilation; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; FRAP, ferric reducing ability of plasma; tGSH, total glutathione, GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; GPx:SOD, glutathione peroxidase:superoxide dismutase; GR:GPx, glutathione reductase: glutathione peroxidase; NOx, nitric oxide metabolites

Multiple regression analyses

Forward stepwise multiple regression analyses were performed to test for independent associations of retinal vessel measurements with oxidative stress-related markers. In black men, Art_max$_{dil}$ was independently associated with SOD (Adjusted R^2=0.19; β=-0.36; p=0.002) (Table 3). Furthermore, a negative association tended to exist between Vein_max$_{dil}$ and ROS (Adjusted R^2=0.18; β= -0.21; p=0.07) while Vein_max$_{dil}$ tended to associate positively with FRAP (Adjusted R^2=0.18; β=0.20; p=0.07) in black men (data not shown).

Regarding the associations of retinal vessel calibers with oxidative stress-related markers in black men, independent negative associations of AVR with ROS (Adjusted R^2=0.12; β=-0.32; p=0.007) and CRAE with ROS (Adjusted R^2=0.27; β=-0.23; p=0.040) were found (Table 4). While a negative association between CRAE and FRAP (Adjusted R^2=0.57; β=-0.16; p=0.049) was noted in white men (data not shown). The association between CRAE and NOx was no longer evident in white men.

Sensitivity analyses revealed that the relationship observed between the Art_max$_{dil}$ and SOD in black men remained the same when HIV infected participants were excluded and when hypertension status, anti-hypertensive medication use and smoking were separately added to the models. Exceptionally, the relationship between Vein_max$_{dil}$ and ROS (Adjusted R^2=0.19; β=-0.31; p=0.014) in black men became significant whereas the relationship between Vein_max$_{dil}$ and FRAP (Adjusted R^2=0.14; β=-0.20; p=0.10) lost significance after the exclusion of HIV infected participants (n=14). When hypertension status, anti-hypertensive medication use and smoking were separately added to the models, both results (Vein_max$_{dil}$ and ROS; Vein_max$_{dil}$ and FRAP) remained borderline significant.

Regarding similar sensitivity analyses with the retinal vessel caliber; the results in black men remained significant with the exception of the relationship between CRAE and ROS which became borderline significant when hypertension status (Adjusted R^2=0.32; β=-0.21; p=0.055) and anti-hypertensive medication usage (Adjusted R^2=0.27; β=-0.20; p=0.09) were

added to the model. The negative association of CRAE with FRAP obtained in white men remained robust in all sensitivity analyses.

Table 3: Independent association of Art_max$_{dil}$ with SOD

	Art_max$_{dil}$			
	Black men (n=69)		**White men (n=69)**	
	Adjusted R^2= 0.19		**Adjusted R^2= 0.09**	
	β (95% CI)	*p*	*β (95% CI)*	*p*
Superoxide dismutase	-0.36 (-0.57; -0.14)	0.002	-0.18 (-0.42; 0.06)	0.15
Age	-0.33 (-0.54; -0.12)	0.004	-0.21 (-0.44; 0.22)	0.08
Glucose	-	-	0.18 (-0.06; 0.41)	0.15
Total cholesterol	-	-	0.13 (-0.10; 0.37)	0.26

Art_max$_{dil}$, maximum artery dilation; SOD, superoxide dismutase. Covariates included in the best fit model were age, glucose and total cholesterol.

Table 4: Independent associations of retinal vessel calibers with oxidative stress-related markers

	AVR			
	Black men (n=70)		**White men (n=72)**	
	Adjusted R^2= 0.12		**Adjusted R^2= 0.14**	
Model 1	*β (95% CI)*	*p*	*β (95% CI)*	*p*
Reactive oxygen species	-0.32 (-0.54; -0.10)	0.007	-	-
Total cholesterol	-0.24 (-0.47; -0.02)	0.037	-0.32 (-0.53; -0.10)	0.006
Body mass index	-	-	-0.18 (-0.40; 0.05)	0.13
Age	-	-	0.11 (-0.10; 0.33)	0.30
C-reactive protein	-	-	-0.12 (-0.34; 0.11)	0.31
	CRAE			
	Black men (n=70)		**White men (n=72)**	
	Adjusted R^2= 0.27		**Adjusted R^2= 0.55**	
Model 2	*β (95% CI)*	*p*	*β (95% CI)*	*p*
Reactive oxygen species	-0.23 (-0.44; -0.01)	0.040	-	-
Central retinal vein equivalent	0.34 (0.13; 0.55)	0.003	0.68 (0.52; 0.84)	<0.001
Glucose	0.18 (-0.05; 0.40)	0.13	-	-
Total cholesterol	-0.14 (-0.36; 0.07)	0.20	-0.27 (-0.41; -0.10)	0.002
24h systolic blood pressure	-0.44 (-0.67; -0.20)	0.001	-0.16 (-0.33; 0.02)	0.09
Body mass index	0.13 (-0.09; 0.36)	0.26	-0.09 (-0.27; 0.09)	0.32

AVR, arterio-venous ratio; CRAE, central retinal artery equivalent. Covariates included in the best fit model were total cholesterol, body mass index, age and C-reactive protein in model 1; glucose, total cholesterol, 24h systolic blood pressure and body mass index in model 2.

DISCUSSION

The main aim of this study was to investigate the associations of retinal vessel calibers and vessel responses to FLIP with oxidative stress-related markers in a cohort of black and white men from South Africa. Our main results indicate that despite the tendency of SOD activity to be higher in black men, an inverse association exists between Art_max$_{dil}$ and SOD activity whereas AVR and CRAE associated inversely with ROS in this group. These results suggest that SOD activity may be up-regulated to maintain retinal vessel dilation. As retinal vessel dilation parameters were higher in black men and because of the negative relationship noted between SOD and Art_max$_{dil}$, our data suggest that with sustained exposure to oxidative stress, adverse retinal microvascular changes may ensue in this black group. The data therefore highlight the importance of adequate antioxidant defense capacity in maintaining retinal vessel function (retinal blood vessel calibers and reactivity to FLIP).

Black men with a higher cardiometabolic profile unexpectedly displayed higher (albeit by less than a percentage) vessel dilatory responses to FLIP in the retinal artery with a similar tendency in veins. In line with this, *Malan et al*. [37] reported higher vein dilation but similar artery dilation in the same cohort although different exclusion criteria and different mode of analyses were used. However, available information on the retinal vessel responses to FLIP has indicated reduced dilations of retinal arteries in conditions associated with endothelial dysfunction [14,19,20], with this being limited to white populations. The reason for higher Art_max$_{dil}$ values noted in the black group of our study is unknown but may reflect a compensatory mechanism to maintain the functioning of the endothelium. Alternatively, functional arterial narrowing may occur in the black cohort which may result in a greater dilation due to a greater dilatory reserve as observed in this study. In this regard, a wider CRAE and CRVE has been noted in African Americans compared to whites [38]. Although the CRAE and the artery segment diameter were comparable between black and white participants in the present study, the normal age-related values for CRAE and CRVE of this group are unknown. Given the findings in African Americans, it could be that black

participants have a reduced CRAE relative to their ethnic specific value. Indeed, it had been previously reported that urbanized black males from South Africa have elevated total peripheral resistance [39]. Retinal arterial narrowing may be a reflection of generalized arterial remodeling [40]. Additionally, retinal microvascular remodeling may exhibit similarities as those in small resistance arteries of other organs [41]. As peripheral vascular resistance increases primarily in small arteries and arterioles, arterial narrowing might contribute to blood pressure elevation which may eventually lead to hypertension [40]. Therefore, a dynamic vicious cycle may occur in the microvasculature whereby the initial increase in blood pressure may be maintained or enhanced [42]. As a final speculation, since HDL has an anti-inflammatory effect in the vasculature [43], the tendency for it to be higher in the black group could possibly contribute to the higher Art_max$_{dil}$.

With regards to retinal vessel calibers, a smaller AVR and wider CRVE noted in black men are in agreement with previous research indicating these adverse changes as characteristic of hypertensive status [44]. The observations relating to CRVE and CRAE had previously been reported in the SABPA study [45].

We found higher GPx and SOD (tendency; p=0.062) activities with lower non-enzymatic antioxidant markers (tGSH and FRAP) in black men compared to white men. Our results confirm previous findings suggesting that there may be an up-regulation of antioxidant enzyme activity in blacks [46,47]. The up-regulation of antioxidant enzymes is an adaptive mechanism when oxidative stress increases to prevent oxidative damage to macromolecules [48,49] and disrupted redox signaling [50]. However, our results further suggest that the up-regulated antioxidant enzyme system along with the lower levels of non-enzymatic antioxidants may be inadequate to minimize the oxidative damage as indicated by the higher levels of TBARS in the black group.

A beneficial relationship between Art_max$_{dil}$ and SOD was initially expected but surprisingly an inverse relationship in black men was found. SOD is known to be the first line of defense

against the effects of $O_2^{\cdot-}$ [47] and the up-regulation of SOD may be needed to increase the dismutation of $O_2^{\cdot-}$ to aid in the prevention of oxidative damage to macromolecules [47]. However, in addition to the up-regulation of antioxidant enzymes (such as SOD) to counteract the effects of $O_2^{\cdot-}$ being insufficient to prevent lipid peroxidation, it may also be insufficient to preserve $NO^{\cdot}$ bioavailability but not to the point that Art_max_{dil} is reduced significantly in the black group. Overtime, this may lead to a greater susceptibility of this group to oxidative stress which may disturb the maintenance of Art_max_{dil}. The reaction of $O_2^{\cdot-}$ with $NO^{\cdot}$ proceeds at a higher rate [51] than the dismutation of $O_2^{\cdot-}$ by SOD [52] therefore favoring $NO^{\cdot}$ inactivation. When $NO^{\cdot}$ is inactivated, oxidative cellular damage may increase further, eventually leading to cell death [53]. This may also attenuate the regulation of endothelial function in retinal blood vessels thus reducing the vessel dilatory responses to FLIP which may contribute to the pathogenesis of vascular disease in the future [54]. Although direct comparisons with the following result cannot be made, *Seshadri et al*. [22] found a positive relationship between retinal arterial dilation slope and the redox index (GSH:GSSG). This author suggested that in addition to other processes, antioxidant defense mechanisms may play a co-operative role in the retinal artery dilation during a FLIP, possibly by maintaining normal $NO^{\cdot}$ levels in the retinal microvasculature [22]. Both results from our study and the above mentioned study suggest that the balance between oxidative stress and antioxidant defense capacity plays a role in the regulation of microvascular function of retinal blood vessels in response to FLIP.

$Vein_max_{dil}$ tended to associate negatively with ROS in black men and after the exclusion of HIV infected participants, this relationship became significant. Since HIV-infection is associated with increased inflammation, one would expect that inflammation may have masked this relationship. However, as this relationship was independent of inflammation, other factors such as stages of HIV infection [55] and duration of antiretroviral treatment [56] might have played a role in masking the above mentioned relationship. Therefore, elucidation of this relationship is needed in future studies.

Inverse associations of AVR and CRAE with ROS were indicated only in black men. Furthermore, these associations seem to be largely independent of hypertensive status in sensitivity analyses. Our results therefore suggest that ROS may play a vital role in retinal arterial narrowing independent of hypertension status. This may contribute to endothelial dysfunction which may provide insight to early microvascular changes prior to the onset of CVD [57]. Since hypertension is generally characterized by a smaller AVR, CRAE and/or wider or unchanged CRVE [44], it had been previously reported that these changes might be indicative of structural damage as a result of elevated blood pressure [58]. With previous research investigating the link between retinal vessel caliber and antioxidant capacity, *Daien et al*. [23] considered higher GPx-3 activity as being cardioprotective since it was related to wider retinal artery caliber which was further associated with a lower CVD risk [23]. Taking both results together, these observations suggest that ROS and blood pressure may be mediators of early microvascular changes whereas antioxidant enzyme activity may play a role in protecting the retinal blood vessels from vascular damage.

Despite retinal artery caliber being comparable between the two groups with higher FRAP levels in white men, we however found a weak inverse association between CRAE and FRAP in white men. This result is difficult to explain and requires further investigation.

This study has to be interpreted within the context of its limitations and strengths. This is a cross-sectional study and therefore, the cause and effect cannot be inferred. Results obtained in this population cannot be generalized as participants were only recruited in the North-West province of South Africa. These participants were of the teaching profession with the attempt to obtain a similar socio-economic status. The strengths of this study include a young bi-ethnic population without advanced and established CVD and the use of more oxidative stress-related markers (both markers of oxidative damage and antioxidant defense capacity).

In conclusion, the negative relationship between Art_max$_{dil}$ and SOD in black men implies that the up-regulation of SOD is required for compensation of increased levels of oxidative stress. The up-regulated SOD activity may contribute to the maintenance of vascular function by preserving $NO^{\cdot}$ bioavailability. Although Art_max$_{dil}$ is still maintained, sustained exposure to oxidative stress may lead to attenuated Art_max$_{dil}$ overtime. Both AVR and CRAE associated with ROS in the black men indicating that ROS mediates retinal arterial narrowing. Since these associations were only evident in black men, it may suggest that the higher cardiometabolic profile observed in the black group might have played a role in the link between oxidative stress and the retinal microvasculature.

ACKNOWLEDGEMENTS

We would like to thank the Department of Education, North-West Province, South Africa and all the participants of this study together with the Hypertension in Africa Research Team (HART) staff and colleagues for their contributions in making the SABPA study a success. This study was supported by the National Research Foundation, South Africa; NRF Thukhutha (80643); North-West University, Potchefstroom, South Africa; ROCHE Diagnostics, and the Metabolic Syndrome Institute, France.

CONFLICT OF INTEREST

None declared.

Supplementary table 1: Single regression analyses between retinal vessel calibers and functional retinal vessel measurements with oxidative stress-related markers in black and white men

			Black men		
	AVR	**CRAE**	**CRVE**	**Art_max$_{dil}$**	**Vein_max$_{dil}$**
ROS	r = -0.29‡	r = -0.20	r = 0.16	r = -0.007	r = -0.24‡
TBARS	r = 0.07	r = 0.13	r = 0.08	r = -0.02	r = 0.07
FRAP	r = -0.04	r = -0.10	r = -0.06	r = -0.03	r = 0.16
tGSH	r = -0.08	r = 0.03	r = 0.16	r = -0.06	r = -0.08
GPx	r = -0.17	r = -0.01	r = 0.23*	r = -0.20*	r = 0.06
GR	r = 0.09	r = 0.12	r = 0.0004	r = 0.16	r = 0.11
SOD	r = 0.009	r = -0.07	r = -0.10	r = -0.33‡	r = -0.13
GPx:SOD	r = -0.05	r = 0.11	r = 0.20	r = 0.14	r = 0.15
GR:GPx	r = 0.13	r = 0.09	r = -0.08	r = 0.24‡	r = 0.07
NOx	r = -0.04	r = -0.09	r = -0.02	r = 0.03	r = -0.06
			White men		
	AVR	**CRAE**	**CRVE**	**Art_max$_{dil}$**	**Vein_max$_{dil}$**
ROS	r = -0.16	r = 0.06	r = 0.18	r = -0.007	r = -0.04
TBARS	r = -0.05	r = 0.05	r = 0.008	r = -0.04	r = 0.10
FRAP	r = -0.21*	r = -0.13	r = 0.06	r = -0.07	r = -0.15
tGSH	r = 0.01	r = 0.21*	r = 0.18	r = -0.03	r = 0.04
GPx	r = -0.004	r = -0.05	r = -0.04	r = -0.04	r = -0.02
GR	r = 0.02	r = -0.009	r = -0.01	r = 0.06	r = 0.11
SOD	r = 0.01	r = 0.05	r = 0.05	r = -0.23*	r = -0.03
GPx:SOD	r = -0.01	r = -0.07	r = -0.06	r = 0.20	r = 0.02
GR:GPx	r = 0.02	r = 0.003	r = -0.006	r = 0.07	r = 0.12
NOx	r = 0.17	r = 0.16	r = 0.02	r = -0.12	r = -0.10
p≤0.01 but p≥0.001 †		p≤0.05 but p>0.01 ‡		p≤0.10 but p>0.05 *	

AVR, arterio-venous ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; Art_max$_{dil}$, maximum artery dilation; Vein_max$_{dil}$, maximum vein dilation; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; FRAP, ferric reducing ability of plasma; tGSH, total glutathione, GPx, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; GPx:SOD, glutathione peroxidase:superoxide dismutase; GR:GPx, glutathione reductase:glutathione peroxidase; NOx, nitric oxide metabolites

References

[1] Förstermann U. Oxidative stress in vascular disease: causes, defense mechanisms and potential therapies. Nat Clin Pract Cardiovasc Med 2008;5(6):338-349.

[2] Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension what is the clinical significance? Hypertension 2004;44(3):248-252.

[3] Betteridge DJ. What is oxidative stress? Metabolism 2000;49(2):3-8.

[4] Touyz RM, Briones AM. Reactive oxygen species and vascular biology: implications in human hypertension. Hypertens Res 2011;34(1):5-14.

[5] Briones AM, Touyz RM. Oxidative stress and hypertension: current concepts. Curr Hypertens Rep 2010;12(2):135-142.

[6] Benzie IF, Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996;239(1):70-76.

[7] Matés J. Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. Toxicology 2000;153(1):83-104.

[8] Li S, Yan T, Yang J-Q, Oberley TD, Oberley LW. The role of cellular glutathione peroxidase redox regulation in the suppression of tumor cell growth by manganese superoxide dismutase. Cancer Res 2000;60(14):3927-3939.

[9] Opie LH, Seedat YK. Hypertension in sub-Saharan African populations. Circulation 2005;112(23):3562-3568.

[10] van Zyl C, Huisman HW, Mels CM. Antioxidant enzyme activity is associated with blood pressure and carotid intima media thickness in black men and women: The SABPA study. Atherosclerosis 2016;248:91-96.

[11] Kruger R, Schutte R, Huisman H, Van Rooyen J, Malan N, Fourie C, Louw R, Van der Westhuizen F, Van Deventer C, Malan L. Associations between reactive oxygen species, blood pressure and arterial stiffness in black South Africans: the SABPA study. J Hum Hypertens 2012;26(2):91-97.

[12] Schutte R, Schutte AE, Huisman HW, Van Rooyen JM, Malan NT, Péter S, Fourie CM, Van Der Westhuizen FH, Louw R, Botha CA. Blood glutathione and subclinical atherosclerosis in African men: the SABPA Study. Am J Hypertens 2009;22(11):1154-1159.

[13] Flammer J, Konieczka K, Bruno RM, Virdis A, Flammer AJ, Taddei S. The eye and the heart. Eur Heart J 2013;34(17):1270-1278.

[14] Nagel E, Vilser W, Lanzl I. Age, blood pressure, and vessel diameter as factors influencing the arterial retinal flicker response. Invest Ophthalmol Vis Sci 2004;45(5):1486-1492.

[15] Liew G, Wang JJ, Mitchell P, Wong TY. Retinal vascular imaging a new tool in microvascular disease research. Circ Cardiovasc Imaging 2008;1(2):156-161.

[16] Ikram MK, Cheung CY, Lorenzi M, Klein R, Jones TL, Wong TY. Retinal vascular caliber as a biomarker for diabetes microvascular complications. Diabetes Care 2013;36(3):750-759.

[17] Tikellis G, Arnett DK, Skelton TN, Taylor HW, Klein R, Couper DJ, Sharrett AR, Wong TY. Retinal arteriolar narrowing and left ventricular hypertrophy in African Americans. The Atherosclerosis Risk in Communities (ARIC) study. Am J Hypertens 2008;21(3):352-359.

[18] Kawasaki R, Cheung N, Wang JJ, Klein R, Klein BE, Cotch MF, Sharrett AR, Shea S, Islam FA, Wong TY. Retinal vessel diameters and risk of hypertension: the Multiethnic Study of Atherosclerosis. J Hypertens 2009;27(12):2386.

[19] Pemp B, Garhofer G, Weigert Gn, Karl K, Resch H, Wolzt M, Schmetterer L. Reduced retinal vessel response to flicker stimulation but not to exogenous nitric oxide in type 1 diabetes. Invest Ophthalmol Vis Sci 2009;50(9):4029-4032.

[20] Reimann M, Julius U, Bornstein S, Fischer S, Reichmann H, Rüdiger H, Ziemssen T. Regular lipoprotein apheresis maintains residual cardiovascular and microvascular function in patients with advanced atherosclerotic disease. Atheroscler Suppl 2013;14(1):135-141.

[21] Al-Fiadh AH, Farouque O, Kawasaki R, Nguyen T, Uddin N, Freeman M, Patel SK, Burrell LM, Wong TY. Retinal microvascular structure and function in patients with risk factors of atherosclerosis and coronary artery disease. Atherosclerosis 2014;233(2):478-484.

[22] Seshadri S, Mroczkowska S, Qin L, Patel S, Ekart A, Gherghel D. Systemic circulatory influences on retinal microvascular function in middle-age individuals with low to moderate cardiovascular risk. Acta Ophthalmol 2015;93(4):e266-e274.

[23] Daien V, Carriere I, Kawasaki R, Cristol J-P, Villain M, Fesler P, Ritchie K, Delcourt C. Retinal vascular caliber is associated with cardiovascular biomarkers of oxidative stress and inflammation: the POLA study. PLoS One 2013;8(7):e71089.

[24] Malan L, Hamer M, Frasure-Smith N, Steyn HS, Malan NT. Cohort profile: Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) prospective cohort study. Int J Epidemiol 2015;44(6):1814-1822.

[25] Browne RW, Bloom MS, Schisterman EF, Hovey K, Trevisan M, Wu C, Liu A, Wactawski-Wende J. Analytical and biological variation of biomarkers of oxidative stress during the menstrual cycle. Biomarkers 2008;13(2):160-183.

[26] Wactawski-Wende J, Schisterman EF, Hovey KM, Howards PP, Browne RW, Hediger M, Liu A, Trevisan M. BioCycle study: design of the longitudinal study of the oxidative stress and hormone variation during the menstrual cycle. Paediatr Perinat Epidemiol 2009;23(2):171-184.

[27] Yagi K. Female hormones act as natural antioxidants-a survey of our research. Acta Biochim Pol 1997;44:701-709.

[28] Yagi K, Komura S. Inhibitory effect of female hormones on lipid peroxidation. Biochem Int 1986;13(6):1051-1055.

[29] Leal M, Díaz J, Serrano E, Abellán J, Carbonell LF. Hormone replacement therapy for oxidative stress in postmenopausal women with hot flushes. Obstet and Gynecol 2000;95(6 Pt 1):804-809.

[30] Norton K, Olds T. Anthropometrica: a textbook of body measurement for sports and health courses. UNSW Press; 1996.

[31] Kohara K, Nishida W, Maguchi M, Hiwada K. Autonomic nervous function in non-dipper essential hypertensive subjects evaluation by power spectral analysis of heart rate variability. Hypertension 1995;26(5):808-814.

[32] Mancia G, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Böhm M, Christiaens T, Cifkova R, De Backer G, Dominiczak A. 2013 ESH/ESC guidelines for the management of arterial hypertension: the Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Blood Pressure 2013;22(4):193-278.

[33] Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. Curr Eye Res 2003;27(3):143-149.

[34] Hayashi I, Morishita Y, Imai K, Nakamura M, Nakachi K, Hayashi T. High-throughput spectrophotometric assay of reactive oxygen species in serum. Mutat Res 2007;631(1):55-61.

[35] Buege JA, Aust S. Methods Enzymol 1978;52:302-310.

[36] Liew G, Sharrett AR, Kronmal R, Klein R, Wong TY, Mitchell P, Kifley A, Wang JJ. Measurement of retinal vascular caliber: issues and alternatives to using the arteriole to venule ratio. Invest Ophthalmol Vis Sci 2007;48(1):52-57.

[37] Malan L, Hamer M, von Känel R, Schlaich MP, Reimann M, Frasure-Smith N, Lambert GW, Vilser W, Harvey BH, Steyn F. Chronic depression symptoms and salivary NOx are associated with retinal vascular dysregulation: The SABPA study. Nitric Oxide 2016;55:10-17.

[38] Liew G, Sharrett AR, Wang JJ, Klein R, Klein BE, Mitchell P, Wong TY. Relative importance of systemic determinants of retinal arteriolar and venular caliber: the atherosclerosis risk in communities study. Arch Ophthalmol 2008;126(10):1404-1410.

[39] van Rooyen J, Huisman H, Eloff F, Laubscher P, Malan L, Steyn H, Malan N. Cardiovascular reactivity in Black South-African males of different age groups: the influence of urbanization. Ethn Dis 2001;12(1):69-75.

[40] Ding J, Wai KL, McGeechan K. Retinal vascular caliber and the development of hypertension: a meta-analysis of individual participant data. J Hypertens 2014;32(2):207-215.

[41] Sabanayagam C, Shankar A, Klein BE, Lee KE, Muntner P, Nieto FJ, Tsai MY, Cruickshanks KJ, Schubert CR, Brazy PC. Bidirectional association of retinal vessel diameters and estimated GFR decline: the Beaver Dam CKD Study. Am J Kidney Dis 2011;57(5):682-691.

[42] Pries AR, Secomb TW. Structural adaptation of microvascular networks and development of hypertension. Microcirculation 2002;9(4):305-314.

[43] Hafiane A, Genest J. HDL, atherosclerosis, and emerging therapies. Cholesterol 2013;2013:891403.

[44] Wong TY, McIntosh R. Systemic associations of retinal microvascular signs: a review of recent population-based studies. Ophthalmic Physiol Opt 2005;25(3):195-204.

[45] Malan NT, Smith W, von Känel R, Hamer M, Schutte AE, Malan L. Low serum testosterone and increased diastolic ocular perfusion pressure: a risk for retinal microvasculature. VASA 2015;44(6):435-443.

[46] Mels CM, Schutte AE, Schutte R, Pretorius PJ, Smith W, Huisman HW, van der Westhuizen FH, Fourie CM, van Rooyen JM, Kruger R. 8-Oxo-7, 8-dihydro-2'-deoxyguanosine, reactive oxygen species and ambulatory blood pressure in African and Caucasian men: The SABPA study. Free Radic Res 2014;48(11):1291-1299.

[47] Surapaneni KM, Venkataramana G. Status of lipid peroxidation, glutathione, ascorbic acid, vitamin E and antioxidant enzymes in patients with osteoarthritis. Indian J Med Sci 2007;61(1):9-14.

[48] Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? British J Pharmacol 2004;142(2):231-255.

[49] Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. Am J Physiol Lung Cell Mol Physiol 2002;283(2):L246-L255.

[50] Jones DP. Redefining oxidative stress. Antioxid Redox Signal 2006;8(9-10):1865-1879.

[51] Huie RE, Padmaja S. The reaction of NO with superoxide. Free Radic Res 1993;18(4):195-199.

[52] Cudd A, Fridovich I. Electrostatic interactions in the reaction mechanism of bovine erythrocyte superoxide dismutase. J Biol Chem 1982;257(19):11443-11447.

[53] Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. Apoptosis 2007;12(5):913-922.

[54] Lim M, Sasongko MB, Ikram MK, Lamoureux E, Wang JJ, Wong TY, Cheung CY. Systemic associations of dynamic retinal vessel analysis: a review of current literature. Microcirculation 2013;20(3):257-268.

[55] Amarnath S, Dong L, Li J, Wu Y, Chen W. Endogenous TGF-β activation by reactive oxygen species is key to Foxp3 induction in TCR-stimulated and HIV-1-infected human CD4+ CD25-T cells. Retrovirology 2007;4(1):57.

[56] Pathai S, Weiss HA, Lawn SD, Peto T, D'Costa LM, Cook C, Wong TY, Gilbert CE. Retinal arterioles narrow with increasing duration of anti-retroviral therapy in HIV infection: a novel estimator of vascular risk in HIV? PLoS One 2012;7(12):e51405.

[57] Nguyen TT, Wang JJ, Wong TY. Retinal Vascular Changes in Pre-Diabetes and Prehypertension New findings and their research and clinical implications. Diabetes Care 2007;30(10):2708-2715.

[58] Wong TY, Klein R, Klein BE, Meuer SM, Hubbard LD. Retinal vessel diameters and their associations with age and blood pressure. Invest Ophthalmol Vis Sci 2003;44(11):4644-4650.

CHAPTER 5

FINAL CONCLUSIONS AND RECOMMENDATIONS

Introduction

This chapter entails the summary of the main findings of our study with the discussion compared to the relevant literature. Strengths and limitations of this study are stated as well as recommendations for future research.

This study aimed to compare oxidative stress-related markers and investigated the relationships of retinal vessel calibers and retinal vessel responses to a flicker light-induced provocation (FLIP) with oxidative stress-related markers in a cohort of black and white men from South Africa.

Hypothesis 1: The levels of oxidative stress markers (reactive oxygen species (ROS) and thiobarbituric acid reactive substances (TBARS)) will be higher whereas antioxidant defense capacity (the ferric reducing ability of plasma (FRAP), total glutathione (tGSH), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR)) will be lower in the blacks than in the whites.

Higher levels of oxidative damage (TBARS) and lower non-enzymatic antioxidant markers (FRAP and tGSH) were demonstrated in the black men when compared to white men. However, with regards to the enzymatic antioxidant defense system, GPx was higher while SOD tended to be higher in the black men than white men. ROS levels and GR were similar in both groups. Therefore, we partially accept our first hypothesis.

When our results were compared with data from other studies, it is evident that certain findings confirm while others differ from previous observations. A finding of higher antioxidant enzyme activity (GPx) in black men compared to white men is in agreement with previous research suggesting that there is an up-regulation of antioxidant defense capacity in blacks [1,2]. Previous Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) sub-studies have indicated decreased GPx activity [3,4] and higher ROS levels (in the form of serum peroxides) in black men with and without hypertension [2,5] which could also indicate an increase in other ROS such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radicals

(OH˙) in this groups. Since oxidative stress is known to occur when the production of ROS exceeds the antioxidant defense mechanisms [6], the up-regulation of antioxidant enzyme activity in the black group may be an adaptive mechanism for the defense against increased oxidative stress [7,8]. In addition to the up-regulation of antioxidant enzyme activity, this may also prevent further oxidative damage to macromolecules [7,8]. However, the up-regulated enzyme activity in black men may be insufficient for the prevention of oxidative damage since TBARS levels were higher in this group. Along with the up-regulated enzyme activity, lower non-enzymatic antioxidant capacity markers were found in black men compared to white men, which may also contribute to the insufficiency of antioxidant defense capacity to reduce the oxidative damage in the black group. As a result, redox signaling and control may be disturbed [9]. Consequently, the disturbance in redox signaling and control may lead to pathogenic mechanisms contributing to the development of cardiovascular disease (CVD) [10-12].

Hypothesis 2: a) Arterio-venous ratio (AVR) and central retinal artery equivalent (CRAE) will be negatively associated and central retinal vein equivalent (CRVE) will be positively associated with oxidative stress markers.

AVR and CRAE were inversely associated with ROS in black men only. No associations were found with CRVE. Therefore, we partially accept our hypothesis.

Since this study is the first to investigate relationships of retinal vessel calibers and vessel responses to FLIP with oxidative stress-related markers in black and white South Africans, literature on these relationships remain limited. Studies have reported that a smaller AVR and CRAE are associated with CVD risk [13,14] while higher ROS levels are associated with higher blood pressure [5], but this study is the first to show an inverse relationship of AVR and CRAE with ROS. Sensitivity analysis revealed that these associations were independent of hypertension status. Our results suggest that ROS may play a role in mediating retinal arterial narrowing independently of hypertension status. In the literature, generalized arterial

narrowing has been associated with hypertension which is characterized by smaller AVR and CRAE with a wider or unchanged CRVE [15]. Similar results of smaller AVR and wider CRVE found in this study and in a study by *Malan et al*. [16] may also be a reflection of hypertension. These vascular changes resulting from increased blood pressure [17] may contribute to endothelial dysfunction providing an indication of early microvascular changes before the development of CVD [18].

b) AVR and CRAE will be positively associated and CRVE will be negatively associated with antioxidant defense capacity markers.

CRAE was negatively associated with FRAP in white men only. There were no further independent associations observed. Therefore, we reject this hypothesis.

In contrast to our findings, *Daien et al*. [19] reported an association between larger retinal vessel caliber and higher GPx-3 activity. This results suggest that retinal arteries may be sensitive to oxidative stress [19] as decreased antioxidant defense activity may lead to an increase in ROS levels and decreased $NO^{\cdot}$ bioavailability [20]. GPx-3 activity, an important regulator of oxidative stress [20-22] may play a role in protecting retinal arteries against increased oxidative stress which may also reduce the risk of developing CVD [19].

c) Art_max$_{dil}$ and Ven_max$_{dil}$ will be negatively associated with oxidative stress markers and positively associated with antioxidant defense capacity markers.

Art_max$_{dil}$ was inversely associated with SOD in black men only. We therefore, reject this hypothesis.

We initially expected a beneficial relationship between antioxidant defense capacity markers and retinal vessel responses to FLIP, however; a negative relationship between Art_max$_{dil}$ and SOD was found. It is known that SOD plays a role in the defense against the effects $O_2^{\cdot-}$ [1]. When SOD activity is up-regulated, the dismutation of $O_2^{\cdot-}$ by SOD may be increased which may aid in the prevention of oxidative damage [1]. However, our result suggests that

the insufficiency of up-regulated SOD activity in preventing lipid peroxidation. Additionally, the up-regulation of SOD may also be insufficient to preserve $NO^{\cdot}$ bioavailability (explaining the negative association between SOD and Art_max_{dil}), but not to a point where vascular function is impaired (as Art_max_{dil} was higher in the black group). Overtime, the black group may be exposed to increased oxidative stress which might lead to an attenuation of Art_max_{dil}. Although direct comparisons cannot be made, a study by *Seshadri et al*. [23] reported a positive association between retinal artery dilation slope in response to FLIP with redox index. This result suggests that antioxidant defense mechanisms may play a role in the capacity of retinal arteries to dilate during FLIP via maintenance of $NO^{\cdot}$ levels in the retinal microvasculature [23]. Antioxidant defense mechanisms are involved in maintaining the redox homeostasis by controlling the redox status [24]. In the same way, the balance in redox index may be needed for the maintenance of vascular function [25,26] and for the prevention of attenuated endothelium-dependent dilation resulting from excessive ROS levels [27,28]. Taken together, these results suggest that the balance between oxidative stress and antioxidant defense mechanisms may play a role in regulating retinal microvascular function in response to FLIP.

Summary

The knowledge about the relationships of retinal microvascular parameters with oxidative stress-related markers still remains limited. This study however added to the literature by showing a negative relationship between Art_max_{dil} and SOD and negative associations of CRAE and AVR with ROS in black men only. Since this is a cross-sectional design, the cause and effect cannot be inferred. However, the possible reason that associations were only prominent in the black group may be that this group had higher oxidative damage in the retinal microvasculature. This can also be explained by the figure below (Figure 1) which was previously discussed in the literature (Chapter 2, section 2.3). Shortly, antioxidant enzymes are up-regulated when oxidative stress increases [7,8,29]. These antioxidant enzymes may not be up-regulated simultaneously in both racial groups. White men may

have been in the normal level of oxidative stress (Figure 1) whereas black men may been in the intermediate level of oxidative stress (Figure 1; tier 2) since they had higher cardiometabolic profile (as indicated by higher blood pressure, higher prevalence of hypertension and higher glucose, CRP, GGT, triglycerides and total cholesterol levels) which might have exposed them to oxidative lipid damage. Furthermore, increased oxidative stress may lead to pathogenic mechanisms which may contribute to the risk of developing CVD [10-12]. Given the link between oxidative stress and the retinal microvasculature, our results suggest the role of oxidative stress and antioxidant defense capacity in early vascular changes and functional responses of blood vessels to FLIP.

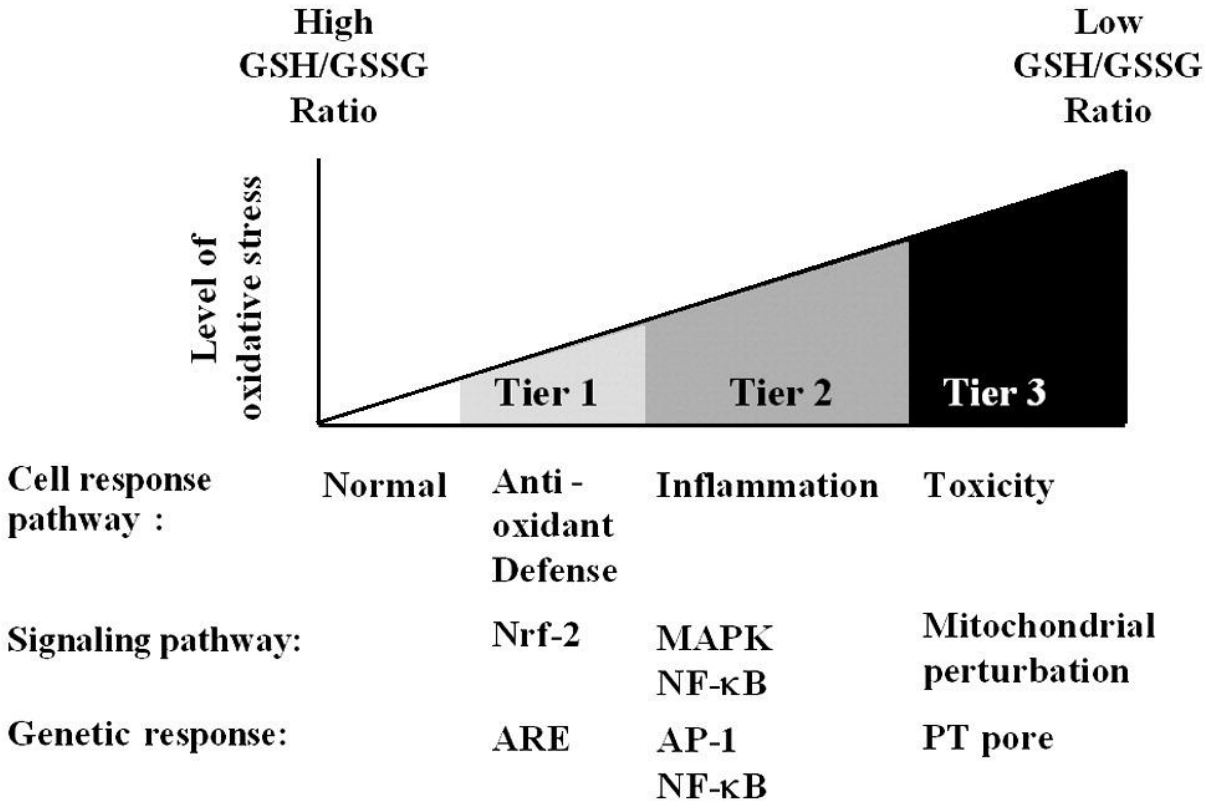


Figure 1: Hierarchical model of oxidative stress levels [30].

Strengths and limitations

To the best of our knowledge, this is the first study investigating the relationship between retinal vessel parameters and oxidative stress-related markers in black and white South Africans. This was a highly controlled study comprising of a relatively young a bi-ethnic

population group without established CVD. This study made use of both oxidative stress markers and antioxidant defense capacity markers which may represent a better profile for investigation. Both retinal vessel calibers and retinal vessel responses to FLIP were evaluated as these parameters may indicate early risk of developing CVD.

The possible limitations of this study might be the small subgroups. A specific population group (teachers in the North-West area) was used and the results could not be generalized in the whole population. Therefore, a more representative sample of participants should be included in future studies. This is a cross-sectional study design which could be a limitation because the cause and effect relationship cannot be determined.

Recommendations

- FRAP and ROS are generalized markers, therefore future studies should explore the relationships of retinal vessel calibers and retinal vessel responses to FLIP with additional markers of oxidative stress (markers of protein and DNA damage; 3-nitrotyrosine, 8-Oxo-7,8-dihydro-2'-deoxyguanosine respectively) and individual non-enzymatic markers collectively measured by FRAP (bilirubin, ascorbate, tocopherols and uric acid) for a more complete profile. The evaluation of a profile including these additional markers may be valuable in assessing the balance between oxidative stress and antioxidant defense capacity. This may also provide insight on the extent or the rate of oxidative damage as well as the potential of defense mechanisms in minimizing the oxidative damage in the retinal microvasculature.
- Since the explored relationships of retinal vessel calibers and retinal vessel responses to FLIP still remain limited, longitudinal studies may provide clarity on these relationships.
- Relatively larger population groups are needed to investigate the relationships of microvascular parameters with oxidative stress-related markers in black and white South Africans for more representative results.

- Responses of retinal arteries to FLIP have been proposed to be a measure of vascular function (Flicker light-induced vasodilation in the retinal artery has been shown to be endothelium and $NO^{\cdot}$-dependent, however, independent from sympathetic innervations) [31]. Therefore, validity of this proposed measurement to be a measure of endothelial function is needed.
- Retinal vessel calibers and reduction in vessel responses to FLIP may indicate an early vulnerability of systemic pathologies and CVD risk [32]. However, information on the normal and abnormal values with regard to retinal vessel calibers and retinal vessel responses to FLIP is lacking [33]. Knowledge on the normal and abnormal values might be useful, aiding in the ability to assess an individual risk of developing CVD.
- CVD in adulthood has been suggested to be predisposed to early life [34], therefore, evaluating retinal vessel calibers and retinal vessel responses to FLIP in healthy children may provide insight on early vascular changes and attenuated vascular function.

Chance and confounding factors

Several factors might have affected the results in this study. The study was performed on participants from the North-West Province which cannot be representative of the entire South African population. The study population was stratified by ethnicity. Statistical significance of our results is only an indication of probabilities. Not all statistical significant results in this study may indicate physiological importance. Therefore, an understanding of physiological mechanisms is crucial when interpreting the observations in this study.

Conclusion

Although SOD activity may have been up-regulated to maintain vascular function possibly via the preservation of $NO^{\cdot}$ bioavailability, the up-regulation was inadequate to defend against increased oxidative damage. This may lead to further damage to macromolecules

which may with time impair the dilation of retinal blood vessels contributing to the pathogenesis of vascular disease. Inverse associations of AVR and CRAE with ROS, suggest that retinal arterial narrowing is mediated by the presence of ROS. These results also suggest that oxidative stress may play a role in retinal microvascular changes.

References

[1] Surapaneni KM, Venkataramana G. Status of lipid peroxidation, glutathione, ascorbic acid, vitamin E and antioxidant enzymes in patients with osteoarthritis. Indian J Med Sci 2007;61(1):9-14.

[2] Mels CM, Schutte AE, Schutte R, Pretorius PJ, Smith W, Huisman HW, van der Westhuizen FH, Fourie CM, van Rooyen JM, Kruger R. 8-Oxo-7, 8-dihydro-2'-deoxyguanosine, reactive oxygen species and ambulatory blood pressure in African and Caucasian men: The SABPA study. Free Radic Res 2014;48(11):1291-1299.

[3] Lammertyn L, Mels CM, Pieters M, Schutte AE, Schutte R. Ethnic-specific relationships between haemostatic and oxidative stress markers in black and white South Africans: The SABPA study. Clin Exp Hypertens 2015;37(6):511-517.

[4] van Zyl C, Huisman HW, Mels CM. Antioxidant enzyme activity is associated with blood pressure and carotid intima media thickness in black men and women: The SABPA study. Atherosclerosis 2016;248:91-96.

[5] Kruger R, Schutte R, Huisman H, Van Rooyen J, Malan N, Fourie C, Louw R, Van der Westhuizen F, Van Deventer C, Malan L. Associations between reactive oxygen species, blood pressure and arterial stiffness in black South Africans: the SABPA study. J Hum Hypertens 2012;26(2):91-97.

[6] Betteridge DJ. What is oxidative stress? Metabolism 2000;49(2):3-8.

[7] Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? British J Pharmacol 2004;142(2):231-255.

[8] Comhair SA, Erzurum SC. Antioxidant responses to oxidant-mediated lung diseases. Am J Physiol Lung Cell Mol Physiol 2002;283(2):L246-L255.

[9] Jones DP. Redefining oxidative stress. Antioxid Redox Signal 2006;8(9-10):1865-1879.

[10] Touyz RM, Briones AM. Reactive oxygen species and vascular biology: implications in human hypertension. Hypertens Res 2011;34(1):5-14.

[11] Briones AM, Touyz RM. Oxidative stress and hypertension: current concepts. Curr Hypertens Rep 2010;12(2):135-142.

[12] Ballinger SW. Mitochondrial dysfunction in cardiovascular disease. Free Radic Biol Med 2005;38(10):1278-1295.

[13] Cheung CY-l, Ikram MK, Sabanayagam C, Wong TY. Retinal microvasculature as a model to study the manifestations of hypertension. Hypertension 2012;60(5):1094-1103.

[14] Ding J, Wai KL, McGeechan K. Retinal vascular caliber and the development of hypertension: a meta-analysis of individual participant data. J Hypertens 2014;32(2):207-215.

[15] Wong TY, McIntosh R. Systemic associations of retinal microvascular signs: a review of recent population-based studies. Ophthalmic Physiol Opt 2005;25(3):195-204.

[16] Malan NT, Smith W, von Känel R, Hamer M, Schutte AE, Malan L. Low serum testosterone and increased diastolic ocular perfusion pressure: a risk for retinal microvasculature. VASA 2015;44(6):435-443.

[17] Wong TY, Klein R, Klein BE, Meuer SM, Hubbard LD. Retinal vessel diameters and their associations with age and blood pressure. Invest Ophthalmol Vis Sci 2003;44(11):4644-4650.

[18] Nguyen TT, Wang JJ, Wong TY. Retinal vascular changes in pre-diabetes and pre-hypertension new findings and their research and clinical implications. Diabetes Care 2007;30(10):2708-2715.

[19] Daien V, Carriere I, Kawasaki R, Cristol J-P, Villain M, Fesler P, Ritchie K, Delcourt C. Retinal vascular caliber is associated with cardiovascular biomarkers of oxidative stress and inflammation: the POLA study. PLoS One 2013;8(7):e71089.

[20] Wolin MS. Plasma glutathione peroxidase activity is potentially a key regulator of vascular disease-associated thrombosis. Circulation 2011;123(18):1923-1924.

[21] Jin RC, Mahoney CE, Anderson LC, Ottaviano F, Croce K, Leopold JA, Zhang Y-Y, Tang S-S, Handy DE, Loscalzo J. Glutathione peroxidase-3 deficiency promotes platelet-dependent thrombosis in vivo. Circulation 2011;123(18):1963-1973.

[22] Buijsse B, Lee D-H, Steffen L, Erickson RR, Luepker RV, Jacobs Jr DR, Holtzman JL. Low serum glutathione peroxidase activity is associated with increased cardiovascular mortality in individuals with low HDLc's. PLoS One 2012;7(6):e38901.

[23] Seshadri S, Mroczkowska S, Qin L, Patel S, Ekart A, Gherghel D. Systemic circulatory influences on retinal microvascular function in middle-age individuals with low to moderate cardiovascular risk. Acta Ophthalmol 2015;93(4):e266-e274.

[24] Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82(1):47-95.

[25] Frisard MI, Broussard A, Davies SS, Roberts LJ, Rood J, De Jonge L, Fang X, Jazwinski SM, Deutsch WA, Ravussin E. Aging, resting metabolic rate, and oxidative damage: results from the Louisiana healthy aging study. J Gerontol Biol Sci Med Sci 2007;62(7):752-759.

[26] Salmon AB, Richardson A, Pérez VI. Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? Free Radic Biol Med 2010;48(5):642-655.

[27] Prasad A, Andrews NP, Padder FA, Husain M, Quyyumi AA. Glutathione reverses endothelial dysfunction and improves nitric oxide bioavailability. J Am Coll Cardiol 1999;34(2):507-514.

[28] Griscavage JM, Fukuto JM, Komori Y, Ignarro LJ. Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. J Biol Chem 1994;269(34):21644-21649.

[29] Xiao GG, Wang M, Li N, Loo JA, Nel AE. Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem 2003;278(50):50781-50790.

[30] Li N, Xia T, Nel AE. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic Biol Med 2008;44(9):1689-1699.

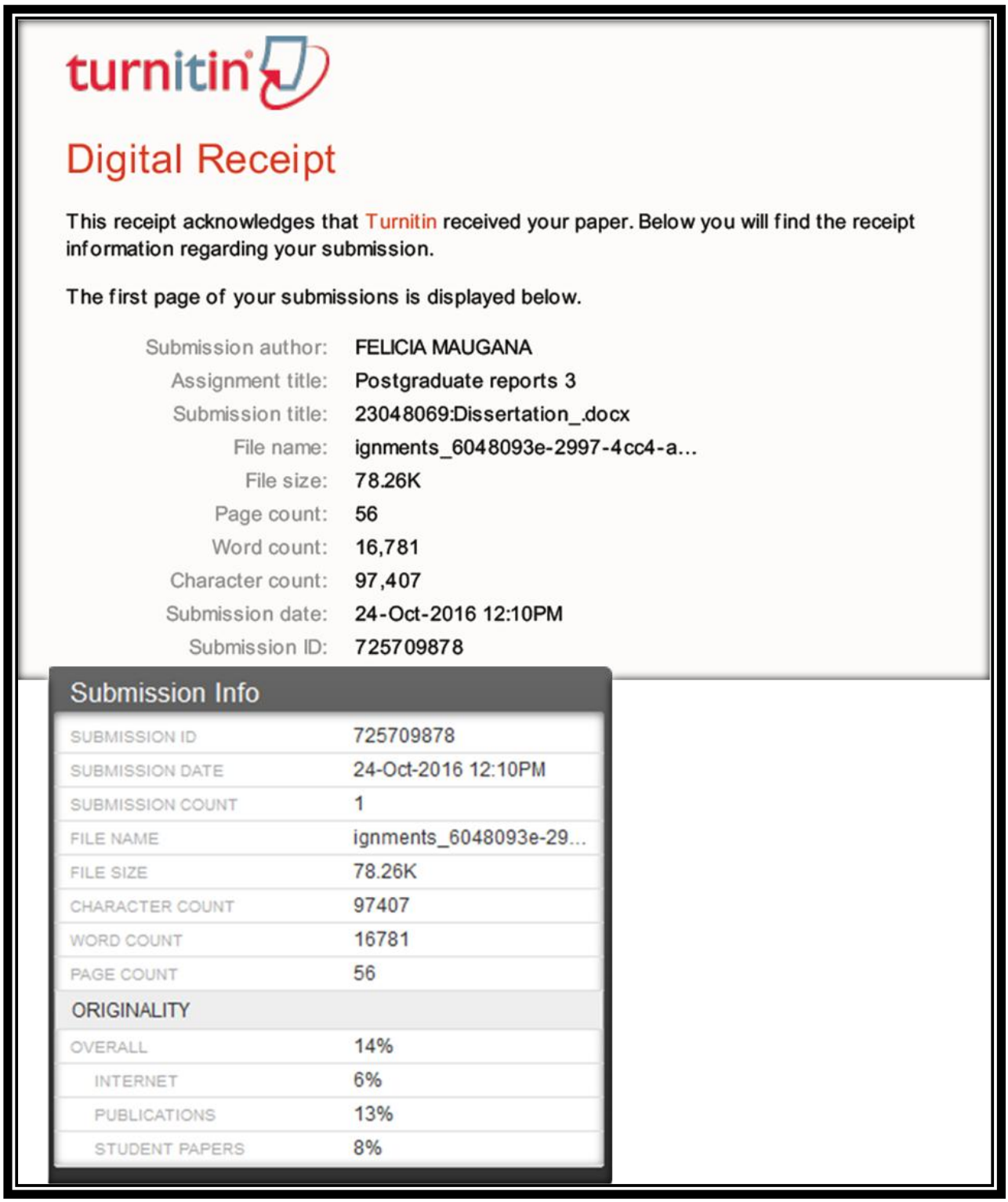
[31] Flammer J, Konieczka K, Bruno RM, Virdis A, Flammer AJ, Taddei S. The eye and the heart. Eur Heart J 2013;34(17):1270-1278.

[32] Al-Fiadh AH, Farouque O, Kawasaki R, Nguyen T, Uddin N, Freeman M, Patel SK, Burrell LM, Wong TY. Retinal microvascular structure and function in patients with risk factors of atherosclerosis and coronary artery disease. Atherosclerosis 2014;233(2):478-484.

[33] Ikram MK, Ong YT, Cheung CY, Wong TY. Retinal vascular caliber measurements: clinical significance, current knowledge and future perspectives. Ophthalmologica 2013;229(3):125-136.

[34] Li LJ, Lee YS, Wong TY, Cheung CYL. Can the retinal microvasculature offer clues to cardiovascular risk factors in early life? Acta Paediatr 2013;102(10):941-946.

APPENDICES



turnitin

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	FELICIA MAUGANA
Assignment title:	Postgraduate reports 3
Submission title:	23048069:Dissertation_.docx
File name:	ignments_6048093e-2997-4cc4-a...
File size:	78.26K
Page count:	56
Word count:	16,781
Character count:	97,407
Submission date:	24-Oct-2016 12:10PM
Submission ID:	725709878

Submission Info

SUBMISSION ID	725709878
SUBMISSION DATE	24-Oct-2016 12:10PM
SUBMISSION COUNT	1
FILE NAME	ignments_6048093e-29...
FILE SIZE	78.26K
CHARACTER COUNT	97407
WORD COUNT	16781
PAGE COUNT	56
ORIGINALITY	
OVERALL	14%
INTERNET	6%
PUBLICATIONS	13%
STUDENT PAPERS	8%

HART
Hypertension in Africa Research Team

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOMKAMPUS

Private bag X6001, Potchefstroom
South Africa, 2520

Hypertension in Africa Research Team (HART)
Tel: 018 299-2435
Web: http://www.nwu.ac.za/hart
Email: hugo.huisman@nwu.ac.za

Date 21-04-2015

Dear Prof Minrie Greeff

HART ETHICS PANEL REVIEW:

Candidate: Ms Vuledzani Felicia Maugana

Project title: The relationship between oxidative stress-related markers and retinal microvascular structure and function in a bi-ethnic population.

The scientific committee of the Hypertension in Africa Research Team (HART) has evaluated the abovementioned protocol of the postgraduate candidate that will make use of secondary data analyses under the umbrella project:

Sympathetic activity and Ambulatory Blood Pressure in Africans (**SABPA**)	☒
African PRospective study on the Early Detection and Identification of Cardiovascular disease and HyperTension (**African-PREDICT**)	☐
Prospective Urban and Rural Epidemiology study (**PURE**)	☐
South African study regarding the influence of Sex, Age and Ethnicity on Insulin sensitivity and Cardiovascular function (**SAfrEIC**)	☐

The HART scientific committee has reviewed all contents including the literature review, motivation, methodology, statistical analyses and time line of this project and all members involved have accepted that this study continues after approval from the HREC of the NWU (Potchefstroom) has been granted.

Scientific committee members:

Proff HW Huisman, AE Schutte, JM van Rooyen, L Malan, NT Malan, Drs CMT Fourie, CMC Mels, R Kruger, W Smith; Me S Botha, L Lammertyn, LF Gafane

Date of meeting: 21 April 2015

Kind regards,

Prof HW Huisman
Research Director
Hypertension in Africa Research Team (HART)

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: 018 299-1111/2222
Web: http://www.nwu.ac.za

Ethics Office
Tel: 018-299 2092
Fax: 018-299 2088
Email: Minnie.Greeff@nwu.ac.za

25 October 2015

Dr W Smith
HART

Dear Dr Smith

HREC APPROVAL OF YOUR APPLICATION

Ethics number: NWU-00036-07-A6 Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the Health Research Ethics Committee (HREC) secretariat.

Project title: The relationship between oxidative stress-related markers and retinal microvascular structure and function in a bi-ethnic population: The SABPA study

Project leader/supervisor: Dr W Smith

Student: VF Maugana

Application type: Sub-study

Risk level descriptor: Minimal

You are kindly informed that at the meeting held on 14/05/2015 of the HREC, Faculty of Health Sciences, the aforementioned was approved.

The period of approval for this project is from 25/10/2015 to 31/12/2016.

After ethical review:

Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC (if applicable).

The HREC requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the protocol or other associated documentation must be submitted to the HREC prior to implementing these changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form.

A progress report should be submitted within one year of approval of this study and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the HREC must be notified if the study is temporarily suspended

or terminated. The progress report template is obtainable from Carolien van Zyl at Carolien.VanZyl@nwu.ac.za. Annually a number of projects may be randomly selected for an external audit.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process.

Please note that for any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

The HREC complies with the South African National Health Act 61 (2003), the regulations on Research with Human Participants of 2014 of the Department of Health and Principles, the Declaration of Helsinki, 2013, the Belmont Report and the Ethics in Health Research: Principles, Structures and Processes (SANS document).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Ethics Office at Carolien.VanZyl@nwu.ac.za or 018 299 2089.

Yours sincerely

Prof Minrie Greeff
HREC Chairperson

Current details: (13210572) C:\Users\13210572\Documents\HREC\HREC - Applications\2015 Applications\Applications 04 - 14 May 2015\NWU-00035-07-A6 (W Smith-VF Maugana)\NWU-00035-07-A6 (W Smith-VF Maugana) - ALNWU-00035-07-A6 (W Smith-VF Maugana) - AL.docm
25 October 2015

File reference: 9.1.5.3

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

Private Bag X6001, Potchefstroom
South Africa 2520

Tel: (018) 299-4900
Faks: (018) 299-4910
Web: http://www.nwu.ac.za

Ethics Committee
Tel +27 18 299 2542
Fax +27 18 297 5308
Email Ethics@nwu.ac.za

Dr L Malan

Dear Dr Malan

6 February 2008

ETHICS APPROVAL OF PROJECT

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

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

Ethics number: N W U - 0 0 0 3 6 - 0 7 - S 6

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

Special conditions of the approval (if any): None

General conditions:

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

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

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

Yours sincerely

Prof M M J Lowes
(chair NWU Ethics Committee)

DECLARATION

I, C Vorster (ID: 710924 0034 084), Language editor and Translator, and member of the South African Translators' Institute (SATI member number 1003172), herewith declare that I did the language editing of the dissertation of ms VF MAUGANA from the North-West University (student number 23048069).

Title of the dissertation: The relationship between oxidative stress-related markers and retinal microvascular structure and function in a bi-ethnic population: The SABPA study

C Vorster _____ 18 Oct 2016 _____

C Vorster *Date*