

Does smoking impact on the association between oxidative stress and vascular function in young normotensives? The African-Predict Study

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PREFACE

This dissertation contains five chapters of which the research results are presented in article-format. The first chapter provides an introduction consisting of a brief background and problem statement motivating the purpose of this study. Chapter 2 comprises a comprehensive literature overview relevant to the topic of this dissertation, aims objectives and the hypotheses. Chapter 3 is an overview of the study protocol combined with all applicable information on the materials and methods used to acquire the data. Chapter 4 consists of the research article (abstract, introduction, methods, results, discussion and conclusion). The manuscript will be submitted for publication to the *Journal of Hypertension Research*. Chapter 5 is the concluding chapter, which contains the summary of key findings and recommendations for future studies. The supervisor and co-supervisor are included as co-authors of the article. The first author was accountable all the parts of this dissertation, as well as literature searches, statistical analyses, the interpretation of results in addition to writing the research paper. All co-authors gave their permission for the research articles to form part of this dissertation. After every chapter, relevant references are provided in the format set forth by the *Journal of Hypertension Research*.

CONTRIBUTION OF AUTHORS

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

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Responsible for the literature review, statistical analysis, design and arrangement of the manuscript, interpretation of results and the writing up of the manuscript.

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The following is a statement from the co-authors confirming their individual roles in this study and granting permission that the article may form part of this dissertation.

Hereby, I declare that I approved aforementioned manuscript and that my role in this study, as stated above, is representative of my actual contribution. I also give my consent that this manuscript may be published as part of the Master's dissertation of Moliehi Mothae.

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Summary

Motivation

Cigarette smoking is one of the most important modifiable risk factors for the development of cardiovascular disease (CVD). Smoking not only plays a role in the onset of CVD, but it has a significant contribution to disease progression and fatal cardiovascular outcomes such as stroke. Globally, smoking kills 6 million people annually. In 2002, it was estimated that 15% of South Africans are smokers.

Tobacco products contain a variety of toxic chemicals as well as reactive oxygen species (ROS) with the possibility of sustaining cardiovascular injury. Tobacco use increases the amount of oxidative stress by producing ROS and weakening the antioxidant defence system. Elevated levels of ROS react with membrane lipids, proteins, and nucleic acids, causing cellular dysfunction and death. Oxidative stress is one of the major contributors in the link between smoking and CVD. Oxidative stress has also been shown to increase pulse wave velocity (PWV). Higher levels of antioxidant enzymes such as glutathione peroxidase-3 (GPx-3) is associated with wider central retinal artery equivalent (CRAE).

Cigarette smoke is associated with increased PWV and decreased compliance, suggesting an increase in arterial stiffness. Cigarette smoking also adversely impacts on the microvasculature, it has been linked to a wider central retinal vein equivalent (CRVE), and to a lesser extent larger or unchanged CRAE.

The role of smoking on the association of oxidative stress with micro- and macrovasculature in smokers in a young South African population that does not yet present cardiovascular dysfunction is not well investigated.

Methodology

We included 237 non-smokers (108 black and 129 white) and 145 smokers (78 black and 67 white) from Potchefstroom, South Africa aged between 20 and 30 years. All participants gave written informed consent prior to any measurements being performed. Anthropometric measurements (weight, height, waist circumference and body mass index) were measured. Carotid femoral PWV (cfPWV) was determined using a SphyMoCor Xcel device, whereas CRAE and CRVE were determined by using retinal imaging with a Dynamic Vessel Analyser. Serum ROS was analyzed in order to describe oxidative stress and the Synergy H4 hybrid microplate reader was used. Serum cotinine levels were determined to

differentiate smokers from non-smokers. Cotinine was analyzed using Chemiluminescence method of the Immulite.

Two-way ANOVA and Chi-square tests were performed to compare means and proportions between groups. Person and partial correlation analyses were done and in partial correlations, adjustments were made for age, gender and body mass index (BMI). PWV was additionally adjusted for mean arterial pressure (MAP). Forward stepwise multiple regression analysis was performed to determine independent associations. Variables that were included in the multiple regression models were age, gender, BMI, systolic blood pressure (SBP), MAP, C-reactive protein (CRP), total cholesterol and ROS. PWV, CRAE, CRVE and AVR

Results

Regardless of smoking status, PWV, CRVE and ROS were similar between black and white groups, whereas CRAE and arterio-venous ratio (AVR) were higher in white than in black non-smokers. In single regression analysis, ROS correlated positively with the CRVE ($r=0.29$; $p=0.014$) and inversely with AVR ($r=-0.37$; $p=0.002$) in black smokers only. After partially correcting for age, gender and BMI only the association with AVR remained, and a positive correlation emerged between ROS and PWV in black smokers ($r=0.24$; $p=0.042$). In black smokers, we confirmed the independent associations of ROS with AVR (Adj. $R^2=0.19$; $\beta=-0.33$; $p=0.004$), PWV (Adj. $R^2=0.35$; $\beta=-0.24$; $p=0.042$), and an independent relationship of ROS and CRAE (Adj. $R^2=0.18$; $\beta=-0.25$; $p=0.034$) emerged.

Conclusions

Our results suggest that cigarette smoking modifies the relationship between ROS and vascular function and may contribute to a potential acceleration of increasing cardiovascular morbidities, especially among the black population.

Key words: race, reactive oxygen species, arterial stiffness, retinal vessel calibres

TABLE OF ABBREVIATIONS

AASI	Ambulatory arterial stiffness index
ABPM	Ambulatory blood pressure monitoring
African-PREDICT	African P rospective study on the E arly D etection and I dentification of C ardiovascular disease and hyper T ension
AIx	Augmentation index
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ARIC	The Atherosclerosis Risk in Communities Study
AVR	Arterio-Venous ratio
BMI	Body mass index
CHD	Coronary heart disease
CHS	Cardiovascular Health Study
CO	Carbon monoxide
COHb	Carboxyhaemoglobin
CRAE	Central retinal artery equivalent
CRP	C-reactive protein
CRVE	Central retinal vein equivalent
CVD	Cardiovascular disease
CYP 2A6	Cytochrome P450 2A6
DBP	Diastolic blood pressure

EC	Endothelial cell
ECG	Electrocardiogram
eNOS	Endothelial NOS
GPx-3	Glutathione peroxidase-3
H ₂ O ₂	Hydrogen peroxide
HART	Hypertension in Africa research team
HbA1c	Glycated haemoglobin A1c
HDL	High density lipoprotein
hsCRP	high sensitivity CRP
IL-6	Interleukin-6
IMT	Intima media thickness
kg/m ²	kilograms per meter squared
LDL	Low-density lipoprotein
m/s	Meters per second
m ²	Meter squared
MAP	Mean arterial pressure
mg/g	milligrams per gram
mg/H ₂ O ₂	milligrams per hydrogen peroxide
mg/L	milligram per liter
ml/min	millilitre per minute
mmHg	millimetre of Mercury
mmol/L	millimol per liter

MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MU	Measuring unit
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
O ₂	Oxygen
O ⁻²	Superoxide
PWV	Pulse wave velocity
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SOD	Superoxide dismutase
SST	Serum separation tubes
VEGF	Vascular endothelial growth factor
VO _{2max}	Maximum O ₂ uptake
VSMC	Vascular smooth muscle cell
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION AND MOTIVATION

1.1 Motivation and problem statement

Cigarette smoking is the foremost cause of avoidable morbidity and premature death¹ whether due to respiratory diseases, lung cancer or cardiovascular disease (CVD)²⁻⁴. Smoking was shown to play a role not only in the onset of CVD, but also significantly adds to disease progression and fatal cardiovascular outcomes such as coronary heart disease (CHD) and stroke^{3, 5}. Globally, smoking kills 6 million people every year with almost 10% of those deaths related to second-hand smoking³. In South Africa cigarette smoking ranks fourth out of 17 mortality risk factors^{6, 7}. Other mortality risk factors included in this list are excessive alcohol abuse, high blood pressure, diabetes and hypercholesterolemia⁸. In 2002, it was estimated that 7 million (15%) people in South African are active smokers⁹.

The key aspects of the cardiovascular pathophysiology linked to smoking include endothelial dysfunction, a prothrombotic state, inflammation, altered lipid metabolism and hypoxia¹⁰. Numerous mechanisms have been recommended to be involved in the etiological connection between smoking and CVD¹¹, with oxidative stress regarded as one of the major contributors¹². Tobacco smoke contains several toxic, carcinogenic and mutagenic chemicals, as well as stable and unstable radicals and reactive oxygen species (ROS) with the likelihood of sustaining cardiovascular injury¹³. Tobacco use has further been shown to augment the amount of oxidative stress, not only through the production of ROS present in smoke but also through weakening of the antioxidant defence system¹⁴. The NOX family of ROS-generating NADPH oxidases has been shown to contribute the most to cigarette-induced ROS¹¹. Elevated levels of ROS react directly with membrane lipids, proteins and nucleic acid, causing cellular dysfunction and death (both through apoptosis and necrosis)¹⁵.

Pulse wave velocity (PWV), a surrogate measure of arterial stiffness, increases with age and in specific disease states that are themselves related to elevated cardiovascular risk, such as hypercholesterolemia, hypertension and diabetes mellitus¹⁶. As changes can be identified prior to the presence of clinically apparent vascular disease, arterial stiffness might also act as a marker for the onset and development of future atherosclerotic diseases or could be more directly involved in the process of atherosclerosis¹⁷. Traditional risk factors such as elevated body mass index (BMI) and smoking have been implicated in augmented arterial stiffening. Though, findings with regard to risk factors for arterial stiffness except age and blood pressure are inconsistent¹⁸.

Acute and chronic smoking induces oxidative stress, modifying vascular tone and increasing arterial stiffness¹⁹. Oxidative stress may be the primary causal pathway leading directly or indirectly to loss of elasticity in the arterial wall²⁰. Excess oxidant burden modifies

DNA transcription resulting in cellular proliferation and interruption of numerous redox-sensitive signalling pathways that influence arterial remodelling ²¹.

It has been suggested that microcirculatory modifications are closely related to cardiovascular outcomes ^{22, 23}. Retinal photography, by permitting a direct observation of retinal vessels, might establish a practical and non-invasive method for the investigation of early alterations in human microcirculation ²⁴. Changes in retinal vessel calibre have been shown to reflect cardiovascular risk ²⁵. Cigarette smoking has also been shown to have an adverse impact on the microvasculature ²⁶⁻²⁸ and retinal microvasculature might serve as an early indicator of cardiovascular dysfunction. It was indicated that cigarette smoking associated with larger central retinal vein equivalent (CRVE) and to a lesser extent larger central retinal arteriolar equivalent (CRAE) ²⁶. Evidence exist suggesting that CRVE might be influenced by systemic markers of inflammation, obesity and dyslipidaemia ²⁹ and retinal arteriolar narrowing is assumed to reflect structural damage from prolonged hypertension ³⁰. In the Atherosclerosis Risk in Communities Study (ARIC) and the Cardiovascular Health Study (CHS) black individuals were reported to have lower arterio-venous ratio (AVR) than white individuals, which was suggested to reflect a more severe degree of arteriolar narrowing associated with chronic hypertension in blacks ^{31, 32}. Larger CRAE has been shown to associate with higher glutathione peroxidase-3 (GPx-3) (marker of oxidative stress) activity, suggesting lower risk of CVD ²⁴.

To our knowledge only one study has investigated the link between ROS and arterial stiffness in a South African population ³³. This study found that levels of ROS were higher in black women when compared with men, but higher in hypertensive men when compared with normotensive men. Increased levels of ROS were associated with increased arterial stiffness (as measured by pulse pressure, PWV and ambulatory arterial stiffness index (AASI)) in black hypertensive men only. This study, however, focused on older (between the ages of 25 and 65 years) individuals and smoking status was not considered. The role of smoking on the association of oxidative stress with the micro- and macrovasculature is not well investigated in especially a young South African population that does not yet present any cardiovascular dysfunction.

1.2 References

1. Ward K. Smoking cessation: evidence based pharmacy practice. *SAPJ* 2008; **60**: 8–13.
2. Powell JT. Vascular damage from smoking: disease mechanisms at the arterial wall. *Vasc Med* 1998; **3**: 21–28.
3. Messner B, Bernhard D. Smoking and cardiovascular disease mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol* 2014; **34**: 509–515.
4. Phillips DH, Hewer A, Martin CN, Garner RC, King MM. Correlation of DNA adduct levels in human lung with cigarette smoking. *Nature* 1988; **336**:790–792.
5. Ockene IS, Miller NH. Cigarette smoking, cardiovascular disease, and stroke a statement for healthcare professionals from the American Heart Association. *Circ* 1997; **96**: 3243–3247.
6. Sitas F, Urban M, Bradshaw D, Kielkowski D, Bah S, Peto R. Tobacco attributable deaths in South Africa. *Tob Control* 2004; **13**: 396–399.
7. Pacella-Norman R, Urban M, Sitas F, Carrara H, Sur R, Hale M, et al. Risk factors for oesophageal, lung, oral and laryngeal cancers in black South Africans. *Br J Cancer* 2002; **86**: 1751–1756.
8. MRC Burden of Disease Research Unit. 17 risk factors: South African comparative risk assessment: Summary Report. 2008.
9. van Walbeek C. Recent trends in smoking prevalence in South Africa-some evidence from AMPS data. *S Afr Med J* 2002; **92**: 468–472.
10. U.S Department of Health and Human Services. Preventing Tobacco Use among Young People: A Report of the Surgeon General. 2012.
11. Kim M, Han CH, Lee MY. NADPH oxidase and the cardiovascular toxicity associated with smoking. *Toxicol Res* 2014; **30**:149–157.
12. De Rosa S, Pacileo M, Sasso L, Di Palma V, Maietta P, Paglia A, et al. Insights into pathophysiology of smoke-related cardiovascular disease. *Monaldi Arch Chest Dis* 2016; **70**: 59–67.
13. Valavanidis A, Vlachogianni T, Fiotakis K. Tobacco Smoke: Involvement of Reactive Oxygen Species and Stable Free Radicals in Mechanisms of Oxidative Damage, Carcinogenesis and Synergistic Effects with Other Respirable Particles. *Int J Environ Res Public Health* 2009; **6**: 445–462.

14. Isik B, Ceylan A, Isik R. Oxidative stress in smokers and non-smokers. *Inhal Toxicol* 2007; **19**: 767–769.
15. Zhang M, Shah AM. Acute Heart Failure. In Mebazaa A, Gheorghiade M, Zannad FM, Parrillo JE (eds), *Reactive Oxygen Species in Heart Failure*, Springer: London, LN, UK, 2008, 118–123.
16. Glasser SP, Arnett DK, McVeigh GE, Finkelstein SM, Bank AJ, Morgan DJ, et al. Vascular Compliance and Cardiovascular Disease: A Risk Factor or a Marker? *Am J Hypertens* 1997; **10**: 1175–1189.
17. Mackenzie I, Wilkinson I, Cockcroft J. Assessment of arterial stiffness in clinical practice. *QJM* 2002; **95**: 67–74.
18. Cecelja M, Chowienzyk P. Role of arterial stiffness in cardiovascular disease. *JRSM Cardiovasc Dis* 2012; **1**: e11. doi: 10.1258/cvd.2012.012016.
19. Cacciola RR, Guarino F, Polosa R. Relevance of endothelial-haemostatic dysfunction in cigarette smoking. *Curr Med Chem* 2007; **14**: 1887–1892.
20. Patel RS, Al Mheid I, Morris AA, Ahmed Y, Kavtaradze N, Ali S, et al. Oxidative stress is associated with impaired arterial elasticity. *Atheroscler* 2011; **218**: 90–95.
21. Thomas SR, Witting PK, Drummond GR. Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signaling* 2008; **10**: 1713–1766.
22. Cheung N, Bluemke DA, Klein R, Sharrett AR, Islam FA, Cotch MF, et al. Retinal arteriolar narrowing and left ventricular remodeling: the multi-ethnic study of atherosclerosis. *J Am Coll Cardiol* 2007; **50**: 48–55.
23. Cheung N, Sharrett AR, Klein R, Criqui MH, Islam FA, Macura KJ, et al. Aortic distensibility and retinal arteriolar narrowing the multi-ethnic study of atherosclerosis. *Hypertens* 2007; **50**: 617–622.
24. Daien V, Carriere I, Kawasaki R, Cristol JP, Villain M, Fesler P, et al. Retinal Vascular Caliber Is Associated with Cardiovascular Biomarkers of Oxidative Stress and Inflammation: The POLA Study. *PLoS One* 2013; **8**: e71089. doi: 10.1371/journal.pone.0071089.
25. Wong TY, Klein R, Sharrett AR, Manolio TA, Hubbard LD, Marino EK, et al. The prevalence and risk factors of retinal microvascular abnormalities in older persons: The Cardiovascular Health Study. *Ophthalmol* 2003; **110**: 658–666.
26. Kifley A, Liew G, Wang JJ, Kaushik S, Smith W, Wong TY, et al. Long-term effects of smoking on retinal microvascular caliber. *Am J Epidemiol* 2007; **166**: 1288–1297.
27. Liew G, Sharrett AR, Wang JJ, Klein R, Klein BE, Mitchell P, et al. Relative importance of systemic determinants of retinal arteriolar and venular caliber: the atherosclerosis risk in communities study. *Arch Ophthalmol* 2008; **126**: 1404–1410.

28. von Hanno T, Bertelsen G, Sjølie AK, Mathiesen EB. Retinal vascular calibres are significantly associated with cardiovascular risk factors: the Tromsø Eye Study. *Acta Ophthalmol* 2014; **92**: 40–46.
29. 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**: 87–94.
30. Wong TY, Shankar A, Klein R, Klein BE, Hubbard LD. Prospective cohort study of retinal vessel diameters and risk of hypertension. *BMJ* 2004; **329**: e79. doi: 10.1136/bmj.38124.682523.55.
31. Klein R, Sharrett AR, Klein BE, Chambless LE, Cooper LS, Hubbard LD, et al. Are retinal arteriolar abnormalities related to atherosclerosis? The Atherosclerosis Risk in Communities Study. *ArteriosclerThromb Vasc Biol* 2000; **20**: 1644–1650.
32. Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. *Curr Eye Res* 2003; **27**:143–149.
33. Kruger R, Schutte R, Huisman H, Van Rooyen J, Malan N, Fourie C, et al. Associations between reactive oxygen species, blood pressure and arterial stiffness in black South Africans: the SABPA study. *J Hum Hypertens* 2012; **26**: 91–97.

CHAPTER 2

LITERATURE STUDY

2.1 General Introduction

It has been recognized that tobacco use has harmful health consequences ¹ and it is a common cause of untimely death whether from respiratory disease, lung cancer or cardiovascular disease ². Cigarette smoke has been shown to act interdependently with other cardiovascular risk factors to augment the prevalence of cardiovascular disease (CVD) ³. Even in the absence of other risk factors, the risk attributable to smoking continues ⁴. This makes cigarette smoking one of the most important modifiable risk factors for CVD ².

Globally, active and second-hand smoking contributes to mortality ⁵. However, a number of people persist or even start smoking ⁶. The number of smokers worldwide increased from 721 million in 1980 to 967 million in 2012 and the number of cigarettes smoked increased from 4.96 trillion to 6.25 trillion as a result of population growth ⁷. A World Health Organisation (WHO) report from 2002 reported that worldwide, approximately 20% of young teenagers (13-15 years) smoke. About 80 000 to 100 000 children start smoking every day. About half of those who start smoking in their youth years are anticipated to continue smoking for 15 to 20 years ⁸.

According to the WHO, CVD was shown as the number one cause of death worldwide ⁹. In South Africa, the prevalence of CVD is very common, especially in urban areas where two thirds of urban Africans may present with numerous risk factors for CVD ¹⁰. Black South Africans seem to run a higher risk of developing CVD ^{11, 12} and in the presence of modifiable risk factors such as smoking; this risk increases ^{13, 14}. Smoking not only play a role in the initiation of CVD, but it also contributes to the development of disease and fatal cardiovascular outcomes such as coronary heart diseases, abdominal aortic aneurysms and stroke ^{5, 15}.

For the design of this dissertation, we will be using data from the African prospective study on the early detection and identification of cardiovascular disease and hypertension (African-PREDICT). This study included clinically normotensive black and white men and women from the North-West province between the ages of 20 and 30 years. A comparison between these groups will be drawn to determine whether possible differences and links between ROS and measures of micro- and macrovascular function exists in apparently healthy young smokers versus non-smokers. This chapter contains the relevant literature to supply the necessary background and is an addition to the introduction of the manuscript. The main focus of the literature review will be on the links of ROS and measures of micro- and macrovascular function pertaining to the role of smoking in those relationships. A brief motivation for the research article will also be included in this chapter. To conclude this chapter the aims and the hypotheses of the manuscript will be presented.

2.2 Cigarette Smoke

2.2.1 Properties of cigarette smoke

There are two phases contained in cigarette smoke – the tar and gas phases ¹⁶. The tar phase is characterized as the material that is entrapped when the smoke stream is passed through the cambridge glass-fibre filter that contains 99.9% of all tar material with a size $> 0.1\mu\text{m}$ ^{16, 17} and the gas phase is defined as the material that passes through the filter ¹⁷. The tar phase contains $> 10^{17}$ free radicals/g and the gas phase consists of $> 10^{15}$ free radicals/puff ¹⁶. The free radicals connected with the tar phase have a long half-life (hours to months), whereas the radicals linked to the gas phase have a shorter lifespan (seconds) ¹⁸. Mainstream smoke is the term used to describe the cigarette smoke that is drawn through the tobacco into an active smoker's airways ¹⁷. Smoke emitted from the burning ends of a cigarette is known as side stream ¹⁶. Mainstream cigarette smoke does not contain high concentrations of toxic gaseous components when compared with side stream cigarette smoke ¹⁷.

Table 2.1 Chemical components of side stream and mainstream tobacco smoke

Type of compound			
Known human carcinogen	Probable human carcinogen		Toxic compounds
Benzene	Formaldehyde	1,3-Butadiene	Carbon monoxide
2-Naphthylamine	Hydrazine	Aniline	Acrolein
4-Aminobiphenyl	<i>N</i> -nitrosodimethylamine	Benzopyrene	Ammonia
Nickel	<i>N</i> -nitrosodiethylamine	<i>N</i> -nitrosodiethanolamine	Nitrogen oxide
Polonium-210	<i>N</i> -nitrosopyrrolidine	Cadmium	-

Components shaded in grey are side-stream components ¹⁹.

2.2.2 Nicotine Metabolism

Nicotine is the principal tobacco alkaloid ²⁰ and is widely metabolized to numerous metabolites (Figure 2.1) by the liver. Only about six of these metabolites have been identified. Quantitatively speaking, the most significant metabolite of nicotine in most mammalian species is cotinine. In humans, about 70–80% of nicotine is converted to cotinine ²¹.

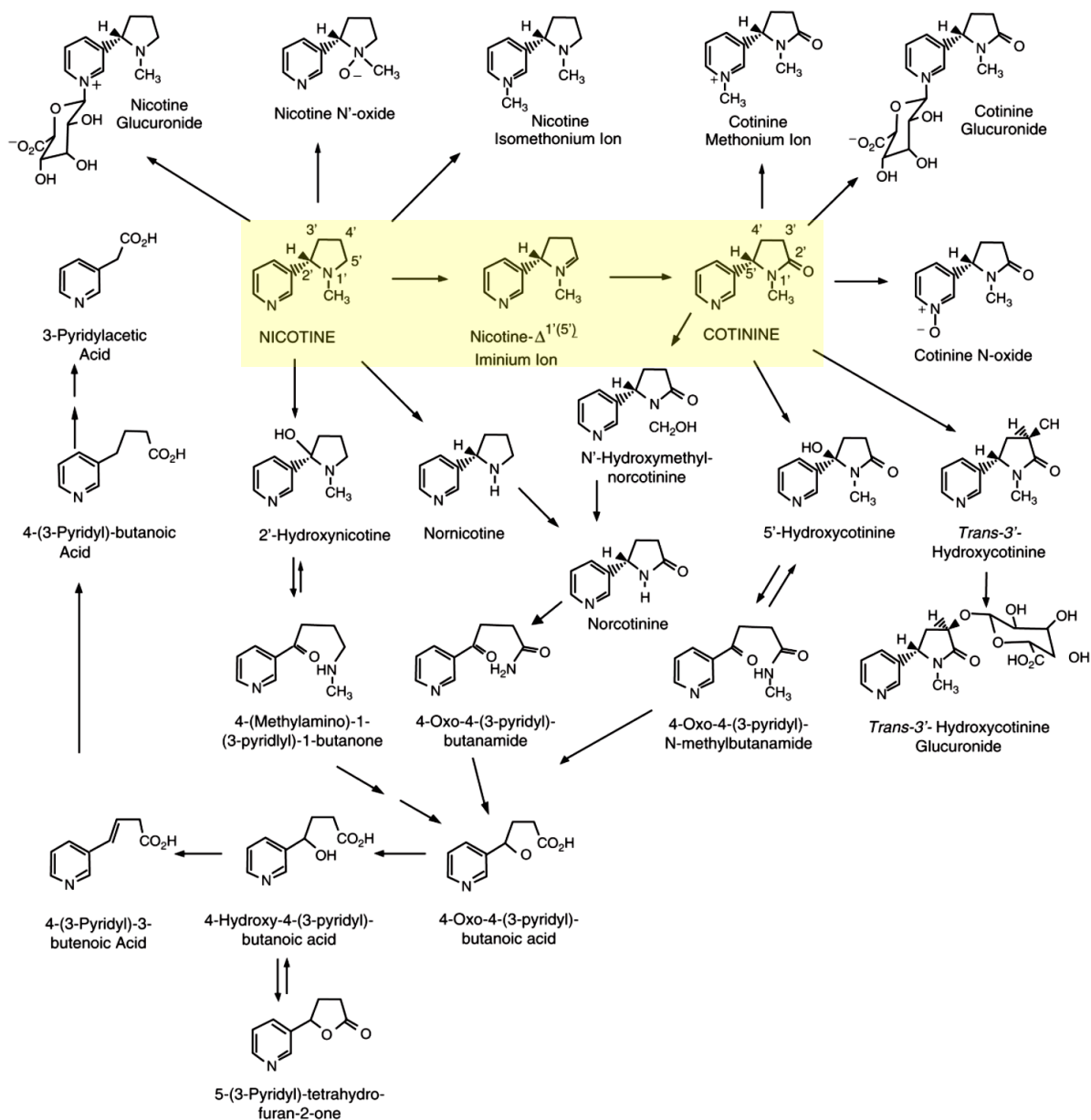


Figure 2.1 | Pathways of nicotine and cotinine metabolism

The rate at which nicotine is metabolized can be determined by measuring nicotine blood levels after administration of a known dose thereof ²². Total clearance of nicotine averages about 1200 ml.min⁻¹. Non-renal clearance is approximately 70% of liver blood flow. The metabolism of nicotine is much faster than that of cotinine. Clearance of cotinine averages about 45 ml.min⁻¹ ²⁰.

There are racial differences in the metabolism of nicotine and cotinine²³⁻²⁵. In a study by Benowitz et al., (2009) and Perez-Stable et al., (1998), the metabolism of nicotine and cotinine in blacks and whites were compared. ^{23, 25}, Black individuals seemed to have a considerably lower total and non-renal clearance rate of cotinine compared to that of whites

(total clearance 0.57 vs. 0.76 ml.min⁻¹.kg⁻¹). Blacks also seemed to have a lower fractional and metabolic clearance of nicotine when compared with cotinine, compared to their white counterparts. Slower metabolism of cotinine in part offers an explanation for the higher cotinine levels per cigarette noticed in blacks than in whites²⁶⁻²⁸. One other explanation for the slower metabolism of cotinine in blacks is the considerably higher quantity of menthol cigarettes smoked among blacks than among whites^{23, 29}. This is true for blacks from America, as studies in South Africa regarding the use of menthol cigarettes is lacking. Menthol cigarette smoking has been shown to hinder nicotine oxidation and glucuronidation³⁰. Another explanation on higher cotinine levels observed in blacks is that there could be a racial genetic difference in cotinine metabolism²⁵. (*See description in the next section*)

2.2.3 Biomarkers of tobacco exposure

Nicotine measurement is exceedingly precise for tobacco use or exposure (in the absence of nicotine medication use), but because of nicotine's short lifespan (2 h) the measurement of nicotine is not recommended for general use. Cotinine is an extremely specific and sensitive marker for tobacco use (in the absence of nicotine medication use) and has the benefits of a moderately long half-life (16 h)²⁰. Because of its long half-life, cotinine has been utilised as a biomarker for daily cigarette smoke intake, both in active cigarette smokers and those who have been exposed to second-hand tobacco smoke³¹. There is a high link among cotinine concentrations measured in plasma, saliva, and urine and measurements in any of these biological fluids can be used as a marker of nicotine exposure³¹. There is, on the other hand, individual inconsistency in the quantitative association linking steady state cotinine levels and nicotine intake³². This is due to the fact that different people convert different percentages of nicotine to cotinine (usual range 50–90%), and because different people metabolize cotinine differently at different rates (usual clearance range 20–75 ml min⁻¹)³¹.

Despite the fact that cotinine functions quite well as a biomarker of nicotine intake, due to racial genetic differences²⁵ and individual variation in its metabolism, it is not without fault. The metabolism of cotinine has been shown to be affected by factors such as race, gender, genetic variation in the liver enzyme CYP2A6 (cytochrome P450 2A6), and/or by the presence of pregnancy, liver or kidney disease. There is another limitation to the use of cotinine, given an average half-life of 16 hours, cotinine levels indicate rather a short-term exposure to tobacco (that is, over the past 3–4 days)²⁰. The cut-off values to differentiate between smokers and non-smokers are: non-smokers are those individuals with cotinine values of less than

10ng/ml. Secondary smokers and active smokers are individuals with cotinine values of 101-300 ng/ml and >300 ng/ml, respectively ³³.

2.3 The role of various components of tobacco smoke in cardiovascular disease

Nicotine, carbon monoxide (CO) and oxidant gases are the three components of cigarette smoke that have been given the most attention, as possible contributors to CVD ¹⁷.

2.3.1 Nicotine

Nicotine exerts its cardiovascular effects via sympathetic neural stimulation ³. Nicotine has been shown to increase blood pressure, heart rate and cardiac output resulting in an increased myocardial oxygen demand. Nicotine augments heart rate both acutely (up to 10-15 beats/min) ³⁴ and as well as throughout the day with regular dosing (average increase 7 beats/min) as determined on ambulatory blood pressure monitoring ³. Nicotine has been reported to have unpredictable effects on nitric oxide (NO) ³⁵ which may result in endothelial dysfunction among tobacco users ³⁶.

Nicotine might decrease NO production directly through nicotinic-receptor activation of nitroxiidergic nerves ³⁷ or bypass receptor activation by directly connecting with biochemical pathways in endothelial cells. On the other hand, nicotine might decrease activity of the enzyme responsible for the formation of NO (NOS) indirectly through ROS production ³⁵. Therefore, because nicotine can cross cell membranes, it is possible that nicotine could directly affect NO production through interacting with NOS and altering the redox state of endothelial cells ³⁸.

2.3.2 Carbon Monoxide

Exposure to CO has been involved in the process of atherosclerosis, contributing to the accumulation of cholesterol in the aorta and coronary arteries ³⁹, by considerably increasing endothelial membrane permeability ⁴⁰. Furthermore, exposure to CO increases endothelial damage, resulting in detrimental effects in the presence of ischaemic heart or peripheral vascular disease ⁴¹. The harmful effects of CO are more reflective in the myocardium than peripheral tissues due to the extremely high oxygen extraction by the myocardium ³⁹. Hypoxia has been shown to be the main mechanism by means of which CO causes heart disease ⁴². Inhaling cigarette smoke, either actively or passively amplifies the levels of carboxyhaemoglobin (COHb) in the blood, resulting in decreased supply of oxygen

(O₂) uptake to the tissues ⁴³. Additionally, myoglobin binds CO so that the heart muscle does not take up the necessary O₂ preventing it from performing optimally ³⁹. The reduction of O₂ uptake as a consequence of smoking, leads to the decrease in peak aerobic capacity and to a significant decrease in maximum O₂ uptake (VO_{2max}) ³⁹.

2.3.3 Oxidant Gases

In a setting of cigarette smoking, free radicals could arise from:

- ❖ The tar or gas phases of cigarette smoke,
- ❖ circulating or in situ-activated macrophages and neutrophils; and/or
- ❖ endogenous sources of ROS such as uncoupled endothelial nitric oxide synthase (eNOS), xanthine, oxidase and the mitochondrial electron transport chain ¹⁶.

The major mediators of endothelial dysfunction in smokers are oxidizing chemicals, as well as oxides of nitrogen and numerous free radicals present at high levels in cigarette smoke ¹⁷.

2.4 Cigarette Smoking and Reactive Oxygen Species

Extreme production of ROS such as superoxide and hydrogen peroxide and their products have been involved as the last common pathway for the development of endothelial dysfunction by a variety of cardiovascular risk factors. ROS may be liable for the observed reduction in NO biosynthesis, as well as the upregulation of eNOS ⁴³. ROS has been shown to uncouple eNOS and uncoupled eNOS produces superoxide instead of NO ⁴⁴. It has been shown that ROS is not present in either unburned tobacco leaves or in cigarette ash. The ROS in major cigarette brands is produced through burning of the cigarette. ROS exists in the gas phase of cigarette smoke or are connected to the suspended tar phase ⁴⁵. Short-lived ROS, such as superoxide radical and nitrogen oxide (both of which immediately react to form highly reactive peroxynitrite) are mostly contained within the gas phase of cigarette smoke ⁴⁶. In contrast, the tar phase contains the long-lived hydroquinones that undergo redox-cycling to form superoxide radicals and hydrogen peroxide via semiquinones; thus resulting in constant oxidative stress ⁴⁷.

2.5 Oxidative Stress and Antioxidant Defence System

Extracellular and intracellular antioxidant capacity has been shown to decrease as a result of cigarette smoke. For instance, exposure to cigarette reduces blood levels of antioxidants⁴⁹. In *in vitro* studies it has been shown that free radicals found in cigarette smoke diminish some plasma antioxidants^{50, 51} and a number of studies found decreased antioxidant concentrations in smokers⁵²⁻⁵⁴. Some cigarette smoke components become involved in oxidative stress only after they are chemically modified by metabolic processes *in vivo*. For example, benzo [a] pyrene can be metabolized to its corresponding quinone, which can produce ROS via a redox cycling mechanism^{55, 56}.

The shift in balance between oxidant/antioxidant in support of oxidants is known as oxidative stress⁵⁷. This happens when the production of ROS increases and antioxidants decrease⁵⁸ (Figure 2.2). When oxidative stress arises, cells attempt to stabilise the oxidant effects and restore the redox balance by activating or silencing genes encoding defensive enzymes, transcription factors and structural proteins⁵⁹. The human body is armed with a number of antioxidants that aid to counterbalance the effect of oxidants⁵⁷. An antioxidant is a molecule able to slow down or prevent the oxidation of other molecules⁶⁰. Scavenging of ROS entails antioxidative enzymes, regularly occurring with metal ions in their active sites, directly contributing to redox reactions⁶¹.

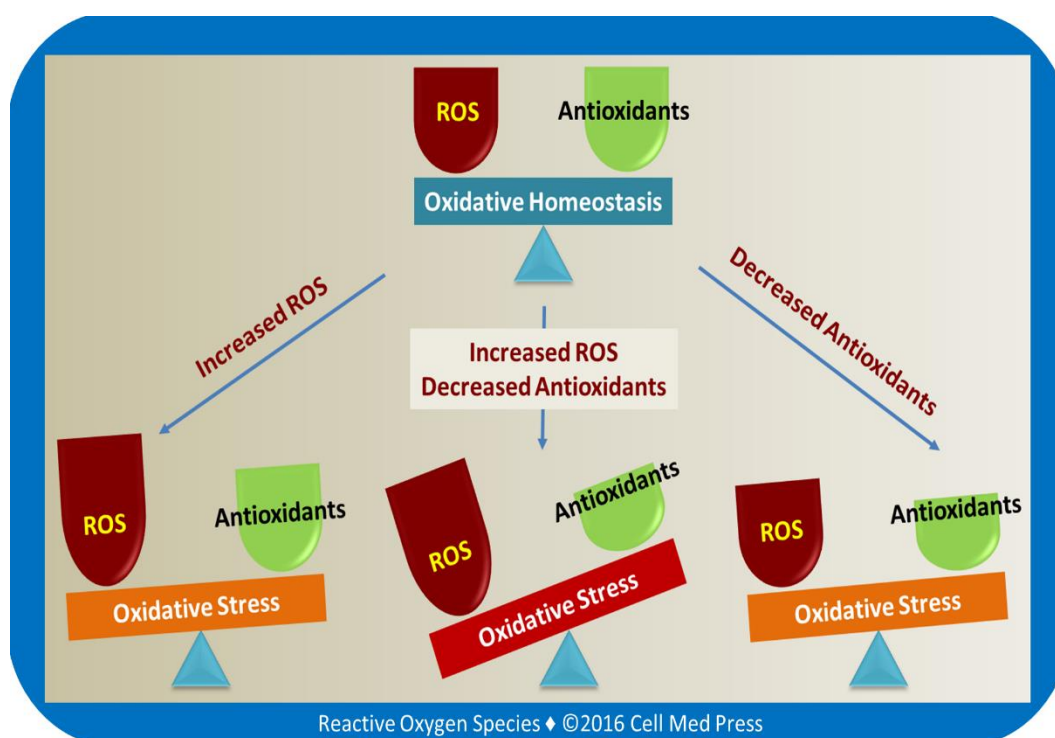


Figure 2.2 | Illustration of how oxidative stress occurs⁵⁸

In low quantities ROS alter and polish up intracellular signalling, and their potentially adverse effects are prohibited by the various cellular antioxidant systems. When ROS are prevalent in higher quantities, if their production is extreme or if the antioxidative systems are inadequate, oxidative stress results ⁶². Lifestyle risk factors such as smoking augment the production of ROS, leading to a state of oxidative stress ⁵⁷. In the defence of cellular elements against ROS, there are cellular antioxidative systems that play a vital role ⁶² (table 2.2):

Table 2.2 The major ROS molecules and their metabolism

ROS molecule	Main sources	Enzymatic defence system	Product(s)
Superoxide ($O_2^{\cdot-}$)	'Leakage' of electrons from the electron transport chain Activated phagocytes Xanthine oxidase Flavoenzymes	Superoxide dismutase (SOD)	$H_2O_2 + O_2$
Hydrogen peroxide (H_2O_2)	From $O_2^{\cdot-}$ via SOD NADPH-oxidase (neutrophils) Glucose oxidase Xanthine oxidase	Glutathione peroxidase Catalases Peroxiredoxins (Prx)	$H_2O + GSSG$ $H_2O + O_2$ H_2O
Hydroxyl radical ($\cdot OH$)	From $O_2^{\cdot-}$ and H_2O_2 via transition metals (Fe^{2+} or Cu^{2+})		
Nitric oxide (NO)	Nitric oxide synthase	Glutathione/TrxR	GSNO

2.6 The Effects of Cigarette Smoking and Elevated Reactive Oxygen Species (oxidative stress) Levels on Vascular Function

CVD is the leading cause of death with 17 million deaths worldwide from a total of 57 million deaths annually ⁶³. Behavioural risk factors are accountable for 80% of all diagnoses of CHD and cerebrovascular disease ⁶⁴. Even though unhealthy diet, physical inactivity and damaging use of alcohol play a role; the leading behavioural risk factor for CVD is smoking ⁶⁵. Smokers run a 2 to 4 times increased risk of heart disease and stroke when compared with non-smokers ¹⁶. In a disease free state the production of ROS is low and it acts as a signalling molecule that regulates the contraction and relaxation of vascular smooth muscle cell (VSMC) and it also contributes to VSMC growth⁶⁶⁻⁶⁸. In a pathophysiological state, ROS plays a vital role in a number of disease states such as atherosclerosis, ischemic heart disease, arrhythmias, ischemia reperfusion injury, cardiomyopathy and congestive heart failure ⁶⁹.

Smoking stimulates an immunological response to vascular damage, resulting in lipid peroxidation, and endothelial cell dysfunction (decrease in NO generation and bioavailability) and foam cell proliferation in the tunica media ^{70, 71}. Furthermore, smoking enhances platelet aggregation, impairing lipoprotein metabolism, resulting in a reduction of high-density lipoprotein (HDL) cholesterol and indices of distensibility of the vessel walls ⁷². Smoking enhances the production of ROS, resulting in a state of oxidative stress ⁷³. Cigarette smoking is associated with enhanced levels of inflammatory markers ⁷⁴. During the acute phase of the inflammatory state, there are quantifiable rises in C-reactive protein (CRP), white blood-cell count and fibrinogen and a reduction in serum albumin ⁷⁴. (Figure 2.3). ROS are important signalling molecules that play a vital role in the development of inflammatory disorders. A heightened ROS production at the site of inflammation results in endothelial dysfunction and tissue injury ⁷⁵.

2.6.1 Smoking and the Vasculature

The innermost layer of a blood vessel that is in direct contact with the blood is known as the endothelium ⁷⁶. The function of endothelium is to sustain vessel integrity, manage vascular tone and the vascular inflammatory process ⁷⁷. In response to enhanced blood flow and acetylcholine, the NOS in the cells of the endothelium utilizes L-arginine to produce NO in the endothelium, leading to vasodilation ⁷⁷. Vasoconstriction by the endothelium is mediated by endothelin in response to epinephrine ⁷⁶. Interruption of these regular endothelial physiological processes results in endothelial dysfunction, which eventually results in cardiovascular diseases through numerous mechanisms ^{3, 76}.

Cigarette smoking has been shown to cause endothelial dysfunction ⁷⁸. The consequence of endothelial dysfunction includes a decreased production and release of NO ⁷⁹. Endothelium-derived vasodilators, especially NO, and ROS have to be in balance in order to modulate endothelial function, therefore an imbalance of NO and ROS is involved in endothelial dysfunction through reduced NO production ⁷³. Vascular function is reliant on the balance of oxidant and antioxidant mechanisms ⁸⁰.

In the microvasculature, endothelial dysfunction could have an impact on retinal venular calibre size. The associations of endothelial dysfunction and retinal vascular calibre are inconsistent ⁸¹. One study found an association between larger venular calibre with markers of endothelial dysfunction (e.g. soluble intercellular adhesion molecule-1) ⁸². In other studies, this association was not evident⁸³⁻⁸⁵.

Blood pressure is described as the force applied by the blood against the vessel wall ^{77, 86} millimeter of mercury (mmHg) is the unit used to express blood pressure ⁸⁶. Increases in myocardial contractility, SBP, diastolic blood pressure (DBP) and heart rate are acute haemodynamic responses to cigarette smoking. One mechanism entails the binding of nicotine to nicotinic receptors in the adrenal medulla. Nicotine has been shown to enhance the flow of catecholamines into the bloodstream, which leads to an increase in blood pressure, myocardial contractility and heart rate ^{3, 4}. This results in an increase in myocardial work, which then leads to an increase in myocardial blood flow. Additionally, the sensitivity of the baroreflex function is decreased by acute cigarette smoking. This damage could possibly be linked directly to smoking-related reduced arterial distensibility and the resulting loss of stretch receptor responsiveness, all which adds in part to increases in blood pressure variability and hinders muscle nerve activity ⁸⁷.

Hypertension has been shown to be associated with oxidative stress ⁸⁸. It has been suggested that oxidative stress plays a major role in the pathogenesis of hypertension ^{89, 90}.

Oxidative stress promotes vascular smooth muscle cell proliferation and hypertrophy and collagen deposition, leading to thickening of the vascular media and narrowing of the vascular lumen. Moreover, enhanced oxidative stress may adversely affect the endothelium and endothelium-dependent vascular relaxation and augment vascular contractile activity. All these effects on the vasculature may explain how augmented oxidative stress can cause hypertension ⁸⁸.

It has long been acknowledged that increased blood pressure exerts intense effects on the retinal microcirculation. An important issue is whether retinal vascular calibre alterations are markers of increasing, long-term blood pressure damage or only reveal a temporary result of acutely raised blood pressure ⁸¹. Alterations in retinal vascular calibre, specifically retinal arteriolar narrowing and venular widening, are independently and significantly associated with an augmented risk of incident hypertension ⁹¹ and smaller CRAE and AVR precede the clinical stage of hypertension and predict the development of hypertension in originally normotensive persons ⁹²⁻⁹⁴. Both lower AVR and narrowed CRAE were independently correlated with past blood pressure levels, proposing that retinal arteriolar calibre alterations reveal persistent damage from long-term hypertension⁹⁵⁻⁹⁷. It has also been shown that retinal arteriolar narrowing might be associated with increased aortic stiffness, left ventricular hypertrophy and left ventricular remodelling ^{98, 99}.

Arterial stiffness is the phrase used to explain the decreased capability of an artery to expand and contract in response to pressure changes. Compliance, distensibility and PWV are the parameters that describe vessel stiffness ^{100, 101}. It has been shown that compliance of both large and medium arteries reduces immediately after just smoking one cigarette ¹⁰² and causes short-term increases in arterial wall stiffness that might be damaging to the artery and increase the risk for plaque rupture ¹⁰³. Chronic cigarette smoking has been shown to be associated with increased arterial stiffness ¹⁰⁴. Arterial stiffness as measured by PWV is acknowledged as a significant precursor of CVD and is an independent predictor of cardiovascular events ^{104, 105}. The result of cigarette smoking on the heart rate, blood pressure and PWV in chronic smokers suggests that cigarette smoking can have damaging effects on the cardiovascular system by stiffening the arteries ^{76, 77}. Whether these relationships are known in a young normotensive and apparently healthy group of individuals are not known, yet and smoking status has only been used to quantify lifestyle risk in statistical models ^{106, 107}. Evidence of a link between arterial stiffness, self-reported smoking and cotinine never has existed.

The retinal vasculature permits direct non-invasive imaging of the body's microcirculation ¹⁰⁸. Since the retina and other end organs, for instance the kidney and brain

share comparable anatomical features and physiological properties, the retinal vessels offer an exceptional and easily reachable window to study the health and disease of the human microcirculation ¹⁰⁹. It has been demonstrated that adverse changes in retinal microvasculature calibre are related to an increased risk for cardiovascular disease ¹¹⁰. Variations in retinal vascular calibre could predict a variety of cardiovascular diseases, regardless of traditional risk factors ^{109, 111}.

Wider CRVE is independently associated with an increased risk of stroke events ¹¹² and it has also been linked to systemic inflammation as measured by highly sensitive CRP (hsCRP), plasma fibrinogen and interleukin 6 (IL-6) ⁸². Cigarette smoking has been connected to a larger retinal venular calibre and to a lesser degree to wider retinal arteriolar calibre ^{83, 113}. It has been hypothesised that the relationship of smoking with venular dilation could involve elevated CO levels and endothelium-dependent relaxation, which may possibly lead to a reduction in oxygen supply to the retinal tissue, consequently resulting in retinal venular dilatation ⁸¹. Lower AVR has been linked to aging ¹¹⁴, cigarette smoking ⁸⁵ past and current blood pressure ^{115, 116} and cardiovascular outcome such as stroke ¹⁰⁹. Smoking is the utmost significant contributor to endothelial dysfunction and microvascular disease throughout the body, caused partly by impairment of vascular endothelial growth factor (VEGF), with consequent production of ROS and decreased NO release ¹¹⁷. However, cigarette smoke induced oxidative stress has not yet been explored as a potential contributor to changes in CRAE and CRVE.

2.6.2 Smoking, Inflammation and Reactive Oxygen Species

Inflammation is described as part of the complex biological response that takes place as a result of any kind of bodily injury ¹¹⁸. By removing the injurious stimuli as well as initiating the healing process, is the organism's way of attempting to protect itself ⁷⁷. Inflammation is important to the body's protection against infection ¹¹⁹. Environmental factors, for instance smoking, have been reported to change the host response to injury and hence change progression, severity and outcome ¹¹⁹. Inflammation results in increased lipids, platelets and CRP, which in turn cause CHD, atherosclerosis and thrombosis ⁷⁷. Cigarette smoking has been shown to be linked to CRP, interleukin-6 and tumour necrosis factor alpha ¹⁶. ROS in cigarette smoke results in inflammation by upregulating ROS-sensitive transcription factors, and by recruiting inflammatory macrophages and neutrophils. These macrophages and neutrophils in turn generate extra ROS via the actions of enzymes, such as xanthine oxidase, NADPH oxidase and myeloperoxidase (MPO), which contribute to additional oxidative stress and additional recruitment of inflammatory cells (Figure 2.4) ¹²⁰.

Oxidative stress and inflammation are associated with numerous chronic diseases as well as cardiovascular diseases (including hypertension), neurodegenerative diseases and cancer ¹²¹. There is no hesitation that chronic low-grade inflammation plays a fundamental role in the pathogenesis of various chronic diseases ¹²¹. Inflammatory reactions induce the generation of ROS and the reverse sequence of these events holds true ¹²². The inflammatory response does not only indicate atherosclerotic potential, but may accelerate the development of atherosclerosis ⁷⁴.

Inflammation has been rated as the main causal mechanism of both large and small vessel disease ¹²³. It has been indicated that systemic inflammatory markers (e.g. CRP, white cell count, IL-6) are associated with larger retinal venules^{83-85, 124, 125}. An association of larger venular calibre with CRP, plasma fibrinogen and IL-6 was observed in the MESA study, regardless of age, smoking status, lipid profile and other factors ¹²⁵.

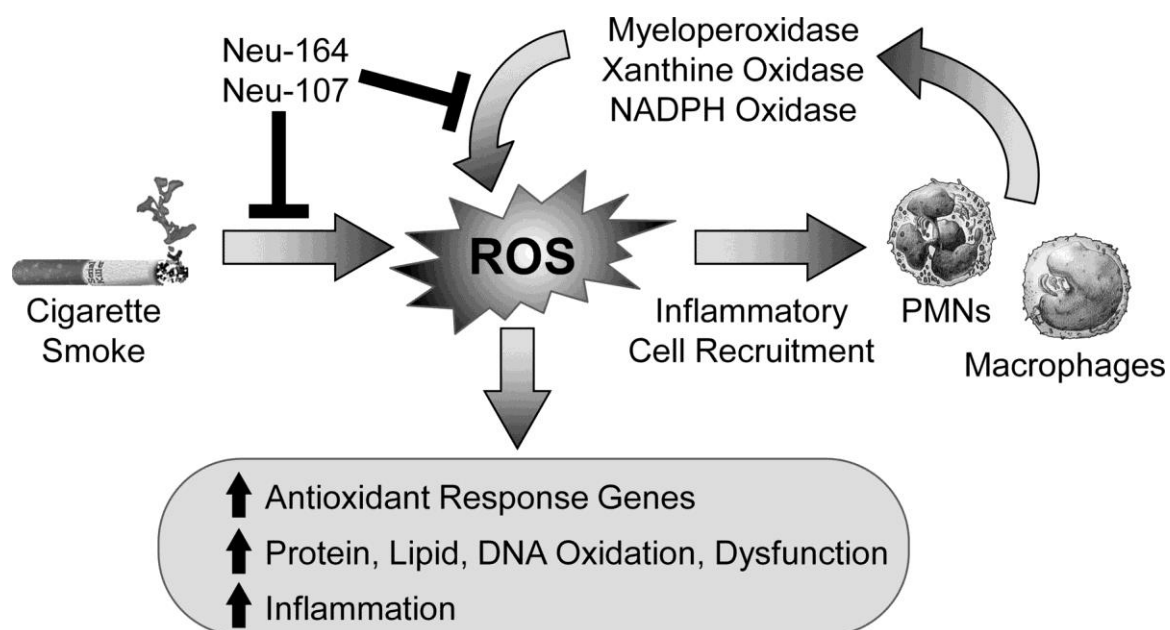


Figure 2.4 | Mechanisms of how cigarette smoking leads to inflammation ¹²⁰

2.6.3 Smoking, Atherosclerosis and Reactive Oxygen Species

Atherosclerosis is characterized as a chronic immune inflammatory disease of the medium and large arteries stimulated by lipids ¹²⁶. Cells of the endothelium, leucocytes and intimal smooth muscle cells are the key players in the development of atherosclerosis ¹²⁷. Atherosclerosis starts in childhood and develops from fatty streaks to raised lesions in

adolescence and young adulthood ¹²⁸. As an individual progresses into middle age, raised lesions increase in size by constant accumulation of lipids and become vulnerable to rupture, an event resulting in occlusive thrombosis and ischemic injury to the brain, heart or extremity ¹²⁹.

Cigarette smoking has been shown to influence all phases of atherosclerosis from endothelial dysfunction to clinical events ¹⁶. Exposure to cigarette smoke triggers numerous mechanisms predisposing to atherosclerosis, together with insulin resistance and dyslipidaemia, thrombosis, vascular inflammation, angiogenesis and abnormal vascular growth, in addition to loss of endothelial integrity and regenerative functions ¹³⁰.

Exposure to cigarette smoke results in endothelial dysfunction, and damage and death of ECs (endothelial cells), generating sites for deposition of lipids and inflammatory cells. Cigarette smoking furthermore leads to elevated platelet number and increased sensitivity of platelets and leukocytes for activation. Elevated inflammation and stimulation of matrix metalloproteinases (MMPs) results in the formation of rupture-prone 'vulnerable plaques'. As the plaque ruptures, acute atherothrombosis ensues through adhesion and aggregation of platelets, and activation of the coagulation cascade ¹³¹. (Figure 4.5). Atherosclerosis represents a state of heightened oxidative stress characterized by lipid and protein deposits in the vascular wall ¹³². Atherosclerosis is the end product of oxidative modification of low density-lipoproteins (LDL) in the arterial wall by ROS ¹³³.

Retinal vessel diameters have been associated with atherosclerotic risk markers ⁸³. Reduced AVR correlated with carotid artery plaque and carotid arterial stiffness, but not with carotid intima-media thickness (IMT)^{85, 134, 135}. In contrast, Ikram et al., (2004) found that a lower AVR was associated with higher carotid IMT and greater carotid plaque score defined an independent association of wider venular calibre with greater carotid plaque score and elevated levels of aortic calcification. In the same study, there was an association of reduced arteriolar diameters with increased carotid IMT ⁸³, Liao et al., (2004) described that the association between larger venular calibre and carotid IMT became non-significant after adjusting for cardiovascular risk factors ¹³⁶. Smoking and oxidative stress were not considered in these studies.

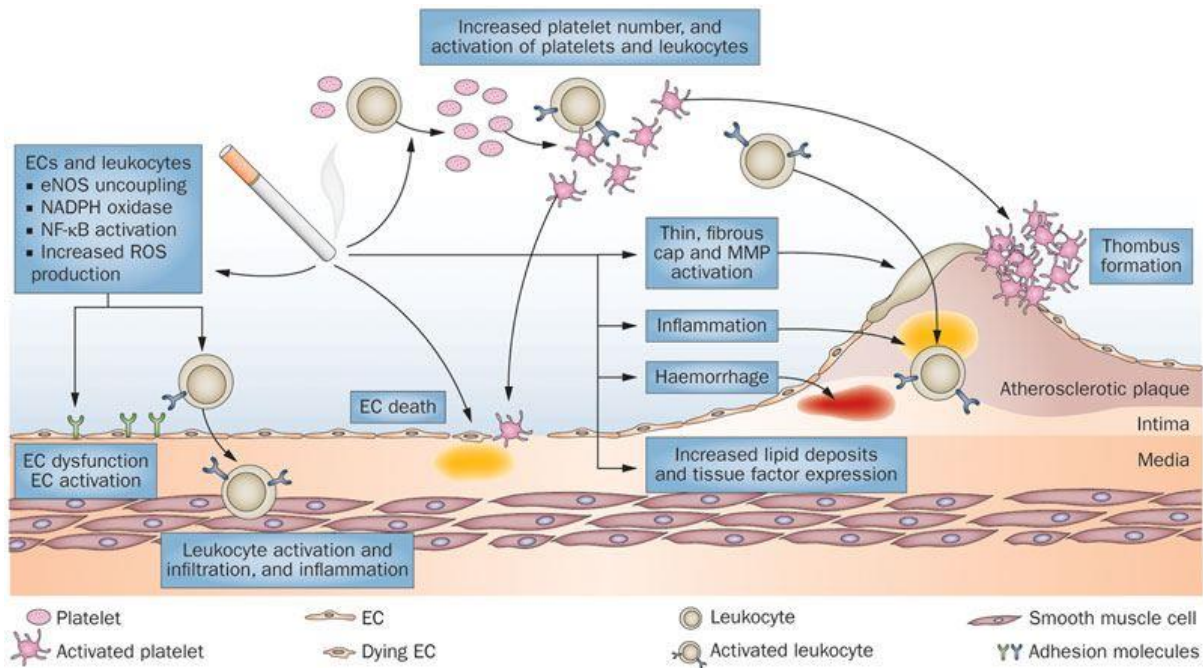


Figure 2.5 | Setting the stage for atherosclerotic plaque formation ¹³¹

The common risk factors (such as cigarette smoking) for atherosclerosis enhance the generation of ROS (oxidative stress) by endothelial, vascular smooth muscle and adventitial cells ¹³⁷. ROS are key mediators of signalling pathways that underlie vascular inflammation in atherogenesis, beginning from the initiation of fatty streak development, through lesion progression, to final plaque rupture ¹³².

2.7 Smoking Cessation

It has been well established that cigarette smoking enhances the production of ROS ¹³⁷ and when ROS overwhelmed the regular cellular or tissue defences, oxidative stress ensues, resulting in pathophysiological processes. Certainly, oxidative stress is a vital pathophysiological mechanism underlying a number of human diseases. In this respect, it is feasible that reducing the levels of ROS or preventing the oxidative damage via using antioxidant-based strategies could offer significant beneficial effects ⁵⁷. However, other strategies (such as smoking cessation) could reverse the health effects of ROS found in cigarette smoke. With smoking, the reversibility of health effects is affected by a number of factors, such as smoking exposure (the number of cigarettes per day and the period of smoking) and physiologic susceptibility ¹³⁸. The existence of other diseases, genetic variables and even nutritional factors also enter into susceptibility assessment ¹³⁹. Smoking cessation

brings benefits at any age, but there are threshold quantities of smoking that permanently increase the risk for some diseases.

In a study by Johnson (2010), they found that individuals who have stopped smoking for one year experienced a significant improvement in endothelial function (as measured by flow-mediated vasodilation). Improvements in endothelial function could facilitate some of the reduced CVD risks observed after smoking cessation.¹⁴⁰ It has also been shown that ambulatory blood pressure decreases only one week after smoking cessation¹⁴¹. Damaging effects of smoking on arterial stiffness are reversible, but it could take more than ten years to achieve levels of stiffness comparable to that of never smokers¹⁴². In a study on Japanese women, they found that wider CRVE was associated with smoking, but this association became non-significant after ten or more years of smoking cessation, suggesting that the impact of smoking on retinal venular dilation is reversible following long-term smoking cessation¹⁴³.

Since smoking impacts adversely on the cardiovascular system via augmenting the production of ROS, it results in a state of oxidative stress. It is worth looking into smoking cessation programmes that could inhibit the adverse effects of smoking in young, apparently healthy, individuals who do not yet present with major cardiovascular dysfunction.

2.8 Aims and Hypotheses

2.8.1 Aims Objectives and Hypotheses

The overall aim of this study was to explore the association of reactive oxygen species with markers of microvascular status and arterial stiffness in young smoking and non-smoking black and white South Africans.

In normotensive black and white South African smokers and non-smokers, the objectives are:

- ❖ To compare ROS levels between these groups.
- ❖ To compare measures of micro- and macrovascular markers and
- ❖ to investigate the independent associations of ROS with measures of microvascular status (central retinal artery equivalent [CRAE]); central retinal vein equivalent (CRVE) and aortic pulse wave velocity in smokers versus non-smokers.

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

- ❖ Smokers will have higher levels of ROS when compared with non-smokers.
- ❖ Smokers will present with adverse micro- and macrovascular variables when compared with non-smokers.
- ❖ ROS will adversely relate to micro and macrovascular markers in smokers only.

2.9 References

1. Saha SP, Bhalla DK, Whayne TF, Gairola C. Cigarette smoke and adverse health effects: An overview of research trends and future needs. *Int J Angiol* 2007; **16**: 77-83.
2. Powell JT. Vascular damage from smoking: disease mechanisms at the arterial wall. *Vasc Med* 1998; **3**: 21–28.
3. Benowitz NL. Cigarette smoking and cardiovascular disease: pathophysiology and implications for treatment. *Prog Cardiovasc Dis* 2003; **46**: 91-111.
4. Burns DM. Epidemiology of smoking-induced cardiovascular disease. *Prog Cardiovasc Dis* 2003; **46**: 11–29.
5. Messner B, Bernhard D. Smoking and cardiovascular disease mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol* 2014; **34**: 509–515.
6. Chen Z, Boreham J. Smoking and cardiovascular disease. *Semin Vasc Med* 2002; **2**: 243–252.
7. Ng M, Freeman MK, Fleming TD, Robinson M, Dwyer-Lindgren L, Thomson B, et al. Smoking prevalence and cigarette consumption in 187 countries, 1980-2012. *JAMA* 2014; **311**: 183–192.
8. World Health Organization. Smoking Statistics. World Health organisation regional office for the Western Pacific. 2002.
9. World Health Organisation. Global status report on noncommunicable diseases 2014.
10. Stewart S, Libhaber E, Carrington M, Damasceno A, Abbasi H, Hansen C, et al. The clinical consequences and challenges of hypertension in urban-dwelling black Africans: insights from the Heart of Soweto Study. *Int J Cardiol* 2011; **146**: 22–27.
11. Opie LH, Seedat YK. Hypertension in sub-Saharan African populations. *Circ* 2005; **112**: 3562–3568.
12. Seedat Y, Seedat M, Hackland D. Prevalence of hypertension in the urban and rural Zulu. *J Epidemiology Community Health* 1982; **36**: 256–261.
13. Buttar HS, Li T, Ravi N. Prevention of cardiovascular diseases: Role of exercise, dietary interventions, obesity and smoking cessation. *Exp Clin Cardiol* 2005; **10**: 229–249.
14. Erhardt L. Cigarette smoking: an undertreated risk factor for cardiovascular disease. *Atheroscler* 2009; **205**: 23–32.
15. Ockene IS, Miller NH. Cigarette smoking, cardiovascular disease, and stroke a statement for healthcare professionals from the American Heart Association. *Circ* 1997; **96**: 3243–3247.
16. Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: An update. *J Am Coll Cardiol* 2004; **43**: 1731–1737.

17. Salahuddin S, Prabhakaran D, Roy A. Pathophysiological Mechanisms of Tobacco-Related CVD. *Glob Heart* 2012; **7**: 113–120.
18. Pryor WA, Stone K. Oxidants in Cigarette Smoke Radicals, Hydrogen Peroxide, Peroxynitrate, and Peroxynitrite. *Ann N Y Acad Sci* 1993; **686**(1): 12–27.
19. Florescu A, Ferrence R, Einarson T, Selby P, Soldin O, Koren G. Methods for quantification of exposure to cigarette smoking and environmental tobacco smoke: focus on developmental toxicology. *Ther Drug Monit* 2009; **31**: 14–30
20. Benowitz NL, Hukkanen J, Jacob III P. Nicotine chemistry, metabolism, kinetics and biomarkers. *Handb Exp Pharmacol* 2009; **192**: 29–60.
21. Shigenaga M, Trevor A, Castagnoli N. Metabolism-dependent covalent binding of (S)-[5-3H] nicotine to liver and lung microsomal macromolecules. *Drug Metabo Dispos* 1988; **16**: 397–402.
22. Hukkanen J, Jacob P, Benowitz NL. Metabolism and disposition kinetics of nicotine. *Pharmacol Rev* 2005; **57**: 79–115.
23. Benowitz NL, Bernert JT, Caraballo RS, Holiday DB, Wang J. Optimal serum cotinine levels for distinguishing cigarette smokers and nonsmokers within different racial/ethnic groups in the United States between 1999 and 2004. *Am J Epidemiol* 2009; **169**: 236–48.
24. Benowitz NL, Perez-Stable EJ, Fong I, Modin G, Herrera B, Jacob P. Ethnic differences in N-glucuronidation of nicotine and cotinine. *J Pharmacol Exp Ther* 1999; **291**: 1196–1203.
25. Perez-Stable EJ, Herrera B, Jacob III P, Benowitz NL. Nicotine metabolism and intake in black and white smokers. *JAMA* 1998; **280**: 152–156.
26. Caraballo RS, Giovino GA, Pechacek TF, Mowery PD, Richter PA, Strauss WJ, et al. Racial and ethnic differences in serum cotinine levels of cigarette smokers: Third National Health and Nutrition Examination Survey, 1988-1991. *JAMA* 1998; **280**: 135–139.
27. English PB, Eskenazi B, Christianson RE. Black-white differences in serum cotinine levels among pregnant women and subsequent effects on infant birthweight. *Am J Public Health* 1994; **84**: 1439–1443.
28. Wagenknecht LE, Cutter GR, Haley NJ, Sidney S, Manolio TA, Hughes GH, et al. Racial differences in serum cotinine levels among smokers in the Coronary Artery Risk Development in (Young) Adults study. *Am J Public Health* 1990; **80**: 1053–1036.
29. Giovino GA, Sidney S, Gfroerer JC, O'Malley PM, Allen JA, Richter PA, et al. Epidemiology of menthol cigarette use. *Nic Tob Res* 2004; **6** (Suppl 1): 67–81.
30. Benowitz NL, Herrera B, Jacob P. Mentholated cigarette smoking inhibits nicotine metabolism. *J Pharmacol Exp Ther* 2004; **310**: 1208–1215.
31. Benowitz NL. Cotinine as a biomarker of environmental tobacco smoke exposure. *Epidemiol Rev* 1996; **18**: 188–204.

32. World Health Organisation. The Scientific Basis of Tobacco Product Regulation. 2007.
33. Human Sciences Research Council MRC. South African National Health and Nutrition Examination Survey: SANHANES-1 2013.
34. Kool MJF, Hoeks APG, Struijker Boudier HAJ, Reneman RS, Van Bortel LMAB. Short and long-term effects of smoking on arterial wall properties in habitual smokers. *J Am Coll Cardiol* 1993; **22**: 1881–1886.
35. Mayhan WG, Sharpe GM. Chronic exposure to nicotine alters endothelium-dependent arteriolar dilatation: effect of superoxide dismutase. *J Appl Physiol* 1999; **86**: 1126–1134.
36. Neunteufl T, Heher S, Kostner K, Mitulovic G, Lehr S, Khoschsorur G, et al. Contribution of nicotine to acute endothelial dysfunction in long-term smokers. *J Am Coll Cardiol* 2002; **39**: 251–256.
37. Toda N, Okamura T. Regulation by nitroxidergic nerve of arterial tone. *Physiol* 1992; **7**: 148–152.
38. Tonnessen BH, Severson SR, Hurt RD, Miller VM. Modulation of Nitric-Oxide Synthase by Nicotine. *J Pharmacol Exp Ther* 2000; **295**: 601–606.
39. Papathanasiou G, Mamali A, Papafloratos S, Zerva E. Effects of smoking on cardiovascular function: the role of nicotine and carbon monoxide. *Health Sci J* 2014; **8**: 274–290.
40. Sarma J, Tillmanns H, Ikeda S, Bing R. The effect of carbon monoxide on lipid metabolism of human coronary arteries. *Atheroscler* 1975; **22**: 193–198.
41. Zevin S, Saunders S, Gourlay SG, Jacob Iii P, Benowitz NL. Cardiovascular effects of carbon monoxide and cigarette smoking. *J Am Coll Cardiol* 2001; **38**: 1633–8.
42. Townsend C, Maynard R. Effects on health of prolonged exposure to low concentrations of carbon monoxide. *Occup Environ Med* 2002; **59**: 708–711.
43. Castleden C, Cole P. Carboxyhaemoglobin levels of smokers and non-smokers working in the City of London. *Br J Ind Med* 1975; **32**: 115–118.
44. Barua RS, Ambrose JA, Srivastava S, DeVoe MC, Eales-Reynolds L-J. Reactive oxygen species are involved in smoking-induced dysfunction of nitric oxide biosynthesis and upregulation of endothelial nitric oxide synthase an in vitro demonstration in human coronary artery endothelial cells. *Circ* 2003; **107**: 2342–2347.
45. Taverne YJ, Bogers AJ, Duncker DJ, Merkus D. Reactive oxygen species and the cardiovascular system. *Oxid Med Cell Longev* 2013; **2013**. doi: 10.1155/2013/862423
46. Huang MF, Lin WL, Ma YC. A study of reactive oxygen species in mainstream of cigarette. *Indoor Air* 2005; **15**:135–140.
47. Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. *Environ Health Perspect* 1985; **64**: 111–126.

48. Nakayama T, Church DF, Pryor WA. Quantitative analysis of the hydrogen peroxide formed in aqueous cigarette tar extracts. *Free Radic Biol Med* 1989; **7**: 9–15.
49. Aoshiba K, Nagai A. Oxidative stress, cell death, and other damage to alveolar epithelial cells induced by cigarette smoke. *Tob Induc Dis* 2003; **1**: 1–8.
50. Frei B, Forte TM, Ames BN, Cross CE. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. *Biochem J* 1991; **277**: 133–138.
51. Eiserich JP, van der Vliet A, Handelman GJ, Halliwell B, Cross CE. Dietary antioxidants and cigarette smoke-induced biomolecular damage: a complex interaction. *Am J Clin Nutr* 1995; **62**: 1490–1500.
52. Marangon K, Herbeth B, Lecomte E, Paul-Dauphin A, Grolier P, Chancerelle Y, et al. Diet, antioxidant status, and smoking habits in French men. *Am J Clin Nutr* 1998; **67**: 231–239.
53. Lykkesfeldt J, Christen S, Wallock LM, Chang HH, Jacob RA, Ames BN. Ascorbate is depleted by smoking and repleted by moderate supplementation: a study in male smokers and nonsmokers with matched dietary antioxidant intakes. *Am J Clin Nutr* 2000; **71**: 530–536.
54. Ross M, Crosley L, Brown K, Duthie S, Collins A, Arthur J, et al. Plasma concentrations of carotenoids and antioxidant vitamins in Scottish males: influences of smoking. *Eur J Clin Nutr* 1995; **49**: 861–865.
55. Briede JJ, Godschalk RW, Emans MT, De Kok T, Van Agen E, Van Maanen J, et al. In vitro and in vivo studies on oxygen free radical and DNA adduct formation in rat lung and liver during benzo [a] pyrene metabolism. *Free Radic Res* 2004; **38**: 995–1002.
56. Winston G, Church D, Cueto R, Pryor W. Oxygen consumption and oxyradical production from microsomal reduction of aqueous extracts of cigarette tar. *Arch Biochem Biophys* 1993; **304**: 371–378.
57. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative Stress and Antioxidant Defense. *World Allergy Organ J* 2012; **5**: 9–19.
58. Li YR, Jia Z, Trush MA. Defining ROS in biology and medicine. *ROS* 2016; **1**: 9–21.
59. Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. *Ann Rev Pharmacol Toxicol* 1999; **39**: 67–101.
60. Kabel AM. Free Radicals and Antioxidants: Role of Enzymes and Nutrition. *World J Nutr Health* 2014; **2**: 35–38.
61. Stańczyk M, Gromadzińska J, Wasowicz W. Roles of reactive oxygen species and selected antioxidants in regulation of cellular metabolism. *Int J Occup Med Environ Health* 2005; **18**: 15–26.
62. Nordberg J, Arner ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 2001; **31**: 1287–312.

63. Bovet P, Paccaud F. Cardiovascular disease and the changing face of global public health: a focus on low and middle income countries. *Public Health Rev* 2012; **33**:397–415.
64. World Health Organization. Global health risks: mortality and burden of disease attributable to selected major risks: World Health Organization; 2009.
65. Health and Social Care Information Centre (HSCIC) LS. Statistics on Smoking: England, 2012. 2012.
66. Touyz RM, Schiffrin EL. Ang II–stimulated superoxide production is mediated via phospholipase D in human vascular smooth muscle cells. *Hypertens* 1999; **34**: 976–982.
67. Rao GN, Berk BC. Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ Res* 1992; **70**:593–599.
68. Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG, et al. Role of NADH/NADPH oxidase–derived H₂O₂ in angiotensin II–induced vascular hypertrophy. *Hypertens* 1998; **32**: 488–495.
69. Lakshmi SV, Padmaja G, Kuppusamy P, Kutala VK. Oxidative stress in cardiovascular disease. *Ind J Biochem Biophys* 2009; **46**: 421–440.
70. Libby P. Inflammation in atherosclerosis. *Nature* 2002; **420**: 868–874.
71. Mayhan WG, Sharpe GM. Effect of cigarette smoke extract on arteriolar dilatation in vivo. *J Appl Physiol* 1996; **81**: 1996–2003.
72. Bøttcher M, Falk E. Pathology of the coronary arteries in smokers and non-smokers. *Eur J Cardiovasc Risk* 1999; **6**: 299–302.
73. Higashi Y, Noma K, Yoshizumi M, Kihara Y. Endothelial function and oxidative stress in cardiovascular diseases. *Circ J* 2009; **73**: 411–418.
74. Bakhru A, Erlinger TP. Smoking Cessation and Cardiovascular Disease Risk Factors: Results from the Third National Health and Nutrition Examination Survey. *PLoS Med* 2005; **2**: e160.doi: 10.1371/journal.pubmed.0020160.
75. Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxid Redox Signaling* 2014; **20**: 1126–1167.
76. Barnoya J, Glantz SA. Cardiovascular effects of secondhand smoke nearly as large as smoking. *Circ* 2005; **111**: 2684–2698.
77. Opie LH. Heart physiology: from cell to circulation. Lippincott Williams & Wilkins, Philadelphia, USA, 2004, 431-459.
78. Pittilo M. Cigarette smoking, endothelial injury and cardiovascular disease. *Int J Exp Pathol* 2000; **81**: 219–230.
79. Drexler H, Hornig B. Endothelial dysfunction in human disease. *J Molec Cell Cardiol* 1999; **31**: 51–60.
80. Ogita H, Liao JK. Endothelial function and oxidative stress. *Endothelium* 2004; **11**: 123–32.

81. Sun C, Wang JJ, Mackey DA, Wong TY. Retinal Vascular Caliber: Systemic, Environmental, and Genetic Associations. *Surv Ophthalmol* 2009; **54**: 74–95.
82. Wong TY, Islam FA, Klein R, Klein BE, Cotch MF, Castro C, et al. Retinal vascular caliber, cardiovascular risk factors, and inflammation: the multi-ethnic study of atherosclerosis (MESA). *Invest Ophthalmology Vis Sci* 2006; **47**: 2341–2350.
83. Ikram MK, de Jong FJ, Vingerling JR, Witteman JC, Hofman A, Breteler MM, et al. Are retinal arteriolar or venular diameters associated with markers for cardiovascular disorders? The Rotterdam Study. *Invest Ophthalmol Vis Sci* 2004; **45**: 2129–2134.
84. Klein R, Klein BK, Knudtson MD, Wong TY, Tsai MY. Are inflammatory factors related to retinal vessel caliber?: The beaver dam eye study. *Arc Ophthalmol* 2006; **124**: 87–94.
85. Klein R, Sharrett AR, Klein BE, Chambless LE, Cooper LS, Hubbard LD, et al. Are retinal arteriolar abnormalities related to atherosclerosis? The Atherosclerosis Risk in Communities Study. *Arterioscler Thromb Vasc Biol* 2000; **20**: 1644–1650.
86. Guyton A. Textbook of medical physiology, 11th edn Elsevier Saunders: Philadelphia, USA, 2005.
87. Burns DM. Epidemiology of smoking-induced cardiovascular disease. *Prog Cardiovasc Dis* 2003; **46**: 11–29.
88. Gropelli A, Giorgi DM, Omboni S, Parati G, Mancia G. Persistent blood pressure increase induced by heavy smoking. *J Hypertens* 1992; **10**: 495–499.
89. Grossman E. Does increased oxidative stress cause hypertension? *Diabetes Care* 2008; **31** (Suppl 2): 185–189.
90. Montezano AC, Touyz RM. Oxidative stress, Noxs, and hypertension: experimental evidence and clinical controversies. *Ann Med* 2012; **44** (Suppl 1): 2–16.
91. Rodrigo R, González J, Paoletto F. The role of oxidative stress in the pathophysiology of hypertension. *Hypertens Res* 2011; **34**: 431–440.
92. 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**: 207–215.
93. Ikram MK, Witteman JCM, Vingerling JR, Breteler MMB, Hofman A, de Jong PTVM. Retinal Vessel Diameters and Risk of Hypertension: The Rotterdam Study. *Hypertens* 2006; **47**: 189–194.
94. Smith W, Wang JJ, Wong TY, Rochtchina E, Klein R, Leeder SR, et al. Retinal Arteriolar Narrowing Is Associated With 5-Year Incident Severe Hypertension. The Blue Mountains Eye Study. *Hypertens* 2004; **44**: 442–447.
95. Smith W, Wang JJ, Wong TY, Rochtchina E, Klein R, Leeder SR, et al. Retinal arteriolar narrowing is associated with 5-year incident severe hypertension the Blue Mountains Eye Study. *Hypertens* 2004; **44**: 442–447.

96. 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**: 1543–1549.
97. Sharrett AR, Hubbard LD, Cooper LS, Sorlie PD, Brothers RJ, Nieto FJ, et al. Retinal arteriolar diameters and elevated blood pressure the atherosclerosis risk in communities study. *Am J Epidemiol* 1999; **150**: 263–270.
98. Wong T, Hubbard L, Klein R, Marino E, Kronmal R, Sharrett A, et al. Retinal microvascular abnormalities and blood pressure in older people: the Cardiovascular Health Study. *Br J Ophthalmol* 2002; **86**: 1007–1013.
99. Cheung N, Bluemke DA, Klein R, Sharrett AR, Islam FA, Cotch MF, et al. Retinal arteriolar narrowing and left ventricular remodeling: the multi-ethnic study of atherosclerosis. *J Am Coll Cardiol* 2007; **50**: 48–55.
100. Cheung N, Sharrett AR, Klein R, Criqui MH, Islam FMA, Macura KJ, et al. Aortic Distensibility and Retinal Arteriolar Narrowing. The Multi-Ethnic Study of Atherosclerosis. *Hypertens* 2007; **50**: 617–622.
101. Cecelja M, Chowienzyk P. Role of arterial stiffness in cardiovascular disease. *JRSM Cardiovasc Dis* 2012; **1**: e11. doi: 10.1258/cvd.2012.012016.
102. Miyatani M, Masani K, Oh PI, Miyachi M, Popovic MR, Craven BC. Pulse Wave Velocity for Assessment of Arterial Stiffness Among People With Spinal Cord Injury: A Pilot Study. *J Spinal Cord Med* 2009; **32**: 72–78.
103. Mahmud A, Feely J. Effect of smoking on arterial stiffness and pulse pressure amplification. *Hypertens* 2003; **41**:183–187.
104. Kool MJ, Hoeks AP, Boudier HAS, Reneman RS, Van Bortel LM. Short and long-term effects of smoking on arterial wall properties in habitual smokers. *J Am Coll Cardiol* 1993; **22**: 1881–1886.
105. Prabha V, Buhutkar P, Bhutkar M, Sivagami G. A comparative study of arterial stiffness indices between smokers & non smokers *Al Ameen J Med Sci* 2014; **7**: 207–212.
106. Yun M, Li S, Sun D, Ge S, Lai CC, Fernandez C, et al. Tobacco smoking strengthens the association of elevated blood pressure with arterial stiffness: the Bogalusa Heart Study. *J Hypertens* 2015; **33**: 266–274.
107. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circ* 1998; **97**: 1837–1847.
108. Onat A. Risk factors and cardiovascular disease in Turkey. *Atheroscler* 2001; **156**: 1–10.
109. Cheung CY-I, Ikram MK, Sabanayagam C, Wong TY. Retinal microvasculature as a model to study the manifestations of hypertension. *Hypertens* 2012; **60**: 1094–1103.

110. 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**: 59–80.
111. Gishiti O, Jaddoe VW, Felix JF, Klaver CC, Hofman A, Wong TY, et al. Retinal microvasculature and cardiovascular health in childhood. *Pediatrics* 2015; **135**: 678–85.
112. Wong TY, Shankar A, Klein R, Klein BE, Hubbard LD. Prospective cohort study of retinal vessel diameters and risk of hypertension. *BMJ*. 2004; **329**: e79. doi: 10.1136/bmj.38124.682523.55.
113. Kawasaki R, Xie J, Cheung N, Lamoureux E, Klein R, Klein BE, et al. Retinal Microvascular Signs and Risk of Stroke The Multi-Ethnic Study of Atherosclerosis (MESA). *Stroke* 2012; **43**: 3245–3251.
114. Kifley A, Liew G, Wang JJ, Kaushik S, Smith W, Wong TY, et al. Long-term effects of smoking on retinal microvascular caliber. *Am J Epidemiol* 2007; **166**: 1288–1297.
115. 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**: 4644–4650.
116. Hubbard LD, Brothers RJ, King WN, Clegg LX, Klein R, Cooper LS, et al. Methods for evaluation of retinal microvascular abnormalities associated with hypertension/sclerosis in the Atherosclerosis Risk in Communities Study. *Ophthalmol* 1999; **106**: 2269–2280.
117. Sharrett AR, Hubbard LD, Cooper LS, Sorlie PD, Brothers RJ, Nieto FJ, et al. Retinal Arteriolar Diameters and Elevated Blood Pressure: The Atherosclerosis Risk in Communities Study. *Am J Epidemiol* 1999; **150**: 263–270.
118. Harris B, Klein R, Jerosch-Herold M, Hoffman EA, Ahmed FS, Jacobs Jr DR, et al. The association of systemic microvascular changes with lung function and lung density: a cross-sectional study. *PloS one* 2012; **7**: e50224. doi: 10.1371/journal.pone.0050224.
119. Ferrero-Miliani L, Nielsen O, Andersen P, Girardin S. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1 β generation. *Clin Exp Immunol* 2007; **147**: 227–235.
120. Gonçalves R, Coletta R, Silvério K, Benevides L, Casati M, Da Silva J, et al. Impact of smoking on inflammation: overview of molecular mechanisms. *Inflamm Res* 2011; **60**: 409–424.
121. Thatcher TH, Hsiao H-M, Pinner E, Laudon M, Pollock SJ, Sime PJ, et al. Neu-164 and Neu-107, two novel antioxidant and anti-myeloperoxidase compounds, inhibit acute cigarette smoke-induced lung inflammation. *Am J Physiol Lung Cell Molec Physiol*. 2013; **305**: 165–174.

122. Biswas SK. Does the Interdependence between Oxidative Stress and Inflammation Explain the Antioxidant Paradox? *Oxid Med Cell Longev* 2016; **2016**. doi: 10.1155/2013/862423.
123. Hakim J. [Reactive oxygen species and inflammation.] *CR Seances Soc Biol Fil* 1993 **187**: 286–295.
124. Ross R. Atherosclerosis — An Inflammatory Disease. *N Eng J Med* 1999; **340**: 115–126.
125. De Jong FJ, Ikram MK, Witteman J, Hofman A, De Jong PT, Breteler M. Retinal vessel diameters and the role of inflammation in cerebrovascular disease. *Ann Neurol* 2007; **61**: 491–495.
126. Wong TY, Amirul Islam FM, Klein R, Klein BEK, Cotch MF, Castro C, et al. Retinal Vascular Caliber, Cardiovascular Risk Factors, and Inflammation: The Multi-Ethnic Study of Atherosclerosis (MESA). *Invest Ophthalmol Vis Sci* 2006; **47**: 2341–2350.
127. Glass CK, Witztum JL. Atherosclerosis: the road ahead. *Cell*. 2001;104(4):503-16.
128. Falk E. Pathogenesis of atherosclerosis. *J Am Coll Cardiol* 2006; **47**: C7-C12.
129. McGill HC, McMahan CA, Malcom GT, Oalmann MC, Strong JP, Group PR. Effects of serum lipoproteins and smoking on atherosclerosis in young men and women. *Arterioscler Thromb Vasc Biol* 1997;**17**: 95–106.
130. Constantinides P. Plaque fissures in human coronary thrombosis. *J Atheroscler Res* 1966; **6**: 1–17.
131. Lee J-E, Cooke JP. The role of nicotine in the pathogenesis of atherosclerosis. *Atheroscler* 2011; **215**: 281–283.
132. Csordas A, Bernhard D. The biology behind the atherothrombotic effects of cigarette smoke. *Nature Revi Cardiol* 2013; **10**: 219–30.
133. Bonomini F, Tengattini S, Fabiano A, Bianchi R, Rezzani R. Atherosclerosis and oxidative stress. *Histol Histopathol* 2008; **23**: 381–390.
134. Vogiatzi G, Tousoulis D, Stefanadis C. The role of oxidative stress in atherosclerosis. *Hellenic J Cardiol* 2009; **50**: 402–409.
135. Klein R, Sharrett AR, Klein BEK, Chambless LE, Cooper LS, Hubbard LD, et al. Are Retinal Arteriolar Abnormalities Related to Atherosclerosis? The Atherosclerosis Risk in Communities Study. *Atheroscler Thromb Vis Biol* 2000; **20**: 1644–1650.
136. Liao D, Wong TY, Klein R, Jones D, Hubbard L, Sharrett AR. Relationship Between Carotid Artery Stiffness and Retinal Arteriolar Narrowing in Healthy Middle-Aged Persons. *Stroke* 2004; **35**: 837–842.
137. van Hecke MV, Dekker JM, Nijpels G, Stolk RP, Henry RM, Heine RJ, et al. Are retinal microvascular abnormalities associated with large artery endothelial dysfunction and intima-media thickness? The Hoorn Study. *Clin Sci* 2006;**110**: 597–604.

138. Harrison D, Griendling KK, Landmesser U, Hornig B, Drexler H. Role of oxidative stress in atherosclerosis. *Am J Cardiol* 2003; **91**: 7–11.
139. Brodish PH. The Irreversible Health Effects of Cigarette Smoking: American Council on Science and Health; 1998.
140. Morabia A, Wynder EL. Dietary habits of smokers, people who never smoked, and exsmokers. *Am J Clin Nutr* 1990; **52**: 933–937.
141. Johnson HM, Gossett LK, Piper ME, Aeschlimann SE, Korcarz CE, Baker TB, et al. Effects of smoking and smoking cessation on endothelial function: 1-year outcomes from a randomized clinical trial. *J Am Coll Cardiol* 2010; **55**: 1988–1995.
142. Minami J, Ishimitsu T, Matsuoka H. Effects of smoking cessation on blood pressure and heart rate variability in habitual smokers. *Hypertens* 1999; **33**:586–590.
143. Jatoi NA, Jerrard-Dunne P, Feely J, Mahmud A. Impact of smoking and smoking cessation on arterial stiffness and aortic wave reflection in hypertension. *Hypertens* 2007; **49**: 981–985.
144. Yanagi M, Misumi M, Kawasaki R, Takahashi I, Itakura K, Fujiwara S, et al. Is the Association Between Smoking and the Retinal Venular Diameter Reversible Following Smoking Cessation? Smoking Effect on Venular Caliber. *Investigat Ophthalmol Vis Sci* 2014; **55**: 405–411.

CHAPTER 3

STUDY PROTOCOL AND PROCEDURES

3.1 Student's Contributions

Research projects performed within the Hypertension in Africa Research Team (HART) are large multidisciplinary projects and involve a number of people. As part of my Masters training, I was involved in the data collection for the Prospective Urban and Rural Epidemiological Study (PURE). I took measurements for PWV. I was also involved in the African **PR**ospective study on the **E**arly **D**etection and **I**dentification of **C**ardiovascular disease and **H**ypertension study (African-PREDICT), where I performed blood pressure measurements, augmentation index, conducted urine analysis, rapid tests (glucose and cholesterol) and determination of blood group type. I also conducted health and physical activity questionnaires. I also performed electrocardiogram (ECG) measurements and did data capturing for ECG measurements. I also helped in the lab with the centrifuging of samples and also with the storage of those samples in freezers.

3.2 Protocol and Procedures for the Current Study

3.2.1 Study Design

The African **PR**ospective study on the **E**arly **D**etection and **I**dentification of **C**ardiovascular disease and hyper**T**ension (African-PREDICT) is a longitudinal study that started in 2013, and is expected to continue for 12+ years. A total of 1200 young and normotensive black and white participants between ages 20-30 years are included to track early cardiovascular deterioration and hypertension development.

African-PREDICT consists of two phases. The first phase is the screening of all the individuals recruited by field workers, whereas the second phase was the research study including the individuals fulfilling the eligibility criteria in the screening phase. Information leaflets were distributed to participants a few days prior to the measurements. The procedures were explained to the participants in their own language (performed by skilled and experienced fieldworkers). Participants were given the opportunity to ask questions before signing informed consent. On the day of the screening, participants underwent blood pressure measurements, rapid tests for detecting cholesterol and glucose levels. They gave a spot urine sample, blood for the determination of blood type. Anthropometric measurements lasted approximately 15 minutes and HIV pre-counselling, testing and post-counselling approximately 30 minutes.

The exclusion criteria included individuals that were hypertensive, of Indian, Asian or mixed origin ethnicity, if they were not a permanent resident of Potchefstroom or surrounding area (i.e. intend to move to another area), had type 1 or 2 Diabetes Mellitus, elevated glucose

> 5.6 mmol/L and confirmed glycated haemoglobin (HbA1c) $\geq$ 6.5%, HIV infected, fever (internal ear temperature $\geq$ 37.5°C on the research day), known liver disease, cancer, tuberculosis or renal disease, microalbuminuria > 30mg/ml in spot morning urine or proteinuria, medication use for chronic disease i.e. antihypertensive, anti-diabetic, antiretroviral or anti-inflammatory medication, pregnant or lactating women, recent surgery or trauma (within the past three months) and had previous history of stroke, angina pectoris or myocardial infarction. The reason for this was to exclude any potential high risk-individuals or those already using chronic medication, as the healthy individuals will be the baseline of the African-PREDICT study in order to investigate the onset and development of cardiovascular disease (CVD) in this cohort.

Participants that passed the screening were invited back for advanced measurements. Participants were required to fast overnight. All the procedures were explained to the participants in their home language. Participants were again required to sign informed consent forms. A registered nurse took blood samples from the brachial vein while participants were lying down. Participants were requested to complete General Health Questionnaires (of which the duration was approximately 30 minutes). A maximum of 4 participants were measured per day.

In a *priori* power analysis, using the G*power v3.1.9.2 software, a sample size of N was computed as a function of the required power level. The preselected power was 80%. The pre-specified significance level was estimated at $\alpha=0.05$. The population effect size was also detected at the probability of $1 - \beta$ (in this case 0.5) for arterial stiffness as the main outcome measure. The a priori analysis calculated that an N value per group of 64 or population size of 128 is sufficient to test the hypothesis of this sub-study.

The study fulfilled all relevant requirements of International Regulations, specifically the Helsinki Declaration of 1975 (as revised in 2008), for the investigation of human participants. The Ethics Committee of North-West University (NW-00001-12-A1) (Potchefstroom campus) approved this sub-study.

3.3 Material and Methods

A brief breakdown of this sub-study is displayed below:

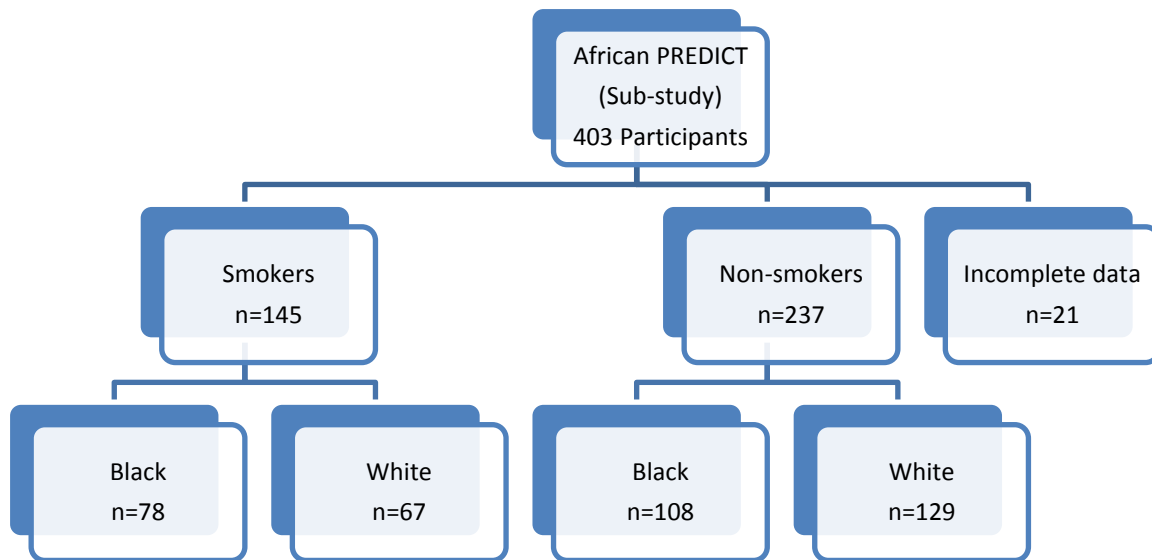


Figure 3.1 | Number of participants included in this African-PREDICT sub-study

3.3.1 Anthropometric Measurements

Qualified personnel measured the body height, body mass as well as waist and hip circumference of each participant in accordance with standard procedures ¹. The circumferences were measured in triplicate using a non-flexible measuring tape (Holtain tape) and recorded to the nearest 0.1 cm. The median of the three waist circumference recordings were used in subsequent analyses. Maximum height was measured to the nearest 1.0 cm using SECA stadiometer (SECA stadiometer, Birmingham U.K.). Weight was measured to the nearest 0.1 kg using a digital scale (SECA electronic scales; Birmingham U.K.). Body mass index (BMI) was calculated using the standard $weight\ (kg)/height\ (m^2)$ formula, and waist to height ratio was calculated using $waist\ circumference\ (cm)/height\ (cm)$.

3.3.2 Cardiovascular Measurements

Validated CardioXplore devices (CardioXplore, MediTech, Hungary) were used for the collection of 24-hour blood pressure measurements programmed to take recordings every 30 minutes during the day (6 am to 10pm) and every hour during the night (10 pm to 6am). Ambulatory blood pressure measurements were undertaken to confirm that participants are normotensive at the baseline of this study and also to detect masked hypertension. The 24-hours ambulatory blood pressure monitoring is required to see how participants' blood pressure fluctuated during the course of the day. The ambulatory blood pressure monitor (ABPM) is recognised as the best method determining blood pressure and it gives a better prediction of risk than office measurements ². The ABPM was fitted to each participant at approximately the same time every day (late morning), using an appropriate sized cuff, which was fitted to the non-dominant upper arm. Only participants with >70% of valid blood pressure measurements, >20 day measurements and >7 night measurements were included in the final statistical analysis. This blood pressure method was chosen because it accurately and noninvasively measures blood pressure over 24 hours.

Carotid femoral PWV (cfPWV) is the golden standard for determining arterial stiffness ³ and it has been linked to cardiovascular morbidity and mortality. Therefore this was a good measure of outcome. PWV was captured using the non-invasive SphygmoCor XCEL device (SphygmoCor EXCEL, AtCor Medical, Australia). PWV was captured at the femoral and carotid arterial pulse points. The femoral artery wave form was captured via an appropriate size cuff placed around the thigh, and the carotid arterial waveform was captured simultaneously via applanation tonometry. The PWV was measured on the left side of each participant, while the participant was in a supine position.

Retinal vessel calibres were determined by capturing retinal images with a Dynamic Vessel Analyser (Plus 12100003 with Zeiss Fundus Camera FF 450 Plus Mydriatic, Imendos, Jena, Germany). As far as probable the right eye was selected. Participants were not in fasting state at the time of the measurement, but did not eat, drink, smoke or perform exercise an hour prior to the measurement. Mydriasis was induced 30 minutes before the measurements took place. Optic disc-centred colour images were used to determine retinal vessel calibres using specific software. In the event that the colour image was of insufficient quality, the grey-scale image was used. Analysis included manually registering all first-order artery or vein vessel-segments located within the inner zone of a measuring ring (0.5 – 1.0 optic disc diameters from the margin of the optic disc) using VesselMap 2 software. The software then determined the central retinal artery or vein equivalent (CRAE and CRVE) using the Knudtson variation of the Parr-Hubbard formula ⁴. The arterio-to-venous ratio (AVR) was also

automatically calculated (CRAE/CRVE). Vessel calibres were expressed in measuring units (MU) where 1 MU is equivalent to 1 μ M in the normal Gullstrand eye.

3.3.3 Blood Collection and Biochemical Analyses

Participants were required to fast for at least 8 hours prior to any measurements being taken. Fasted venous blood samples were collected from the brachial antecubital vein into serum separation tubes (SST) (cotinine, lipids, CRP and ROS) and sodium fluoride plasma tubes (glucose). Serum and plasma samples were prepared and stored at -80°C until further biochemical analyses could be performed. Serum blood glucose, C-reactive protein, serum creatinine, serum albumin, urinary albumin and urinary creatinine were determined later in the laboratory with a Cobas Integra 400plus (Roche, Basel, Switzerland).

Serum cotinine was determined with the Chemiluminescence method with the Immulite (Siemens, Erlangen, Germany), this metabolite was measured in order to support self-reported smoking data obtained from basic health questionnaires. If cotinine values were indicated as <10 ng/ml, they were changed to 1 ng/ml and if values were indicated as >500 ng/ml, they were changed to 500 ng/ml. Participants were categorised according to their smoking status using the serum cotinine levels. Smokers were classified as individuals who currently smoked (active smokers), past smokers and secondary smokers. Non-smokers were those individuals who had never smoked and those with cotinine values of less than 10ng/ml. Secondary smokers and active smokers were identified by cotinine levels between 101-300 ng/ml and >300 ng/ml, respectively ⁵. Serum peroxides were determined by an advanced assay system created on the principle of the derivatives of reactive oxygen metabolites test, which is accepted as an effective technique for assessing oxidative stress in the body. This spectrophotometric method was performed on a Synergy H4 hybrid microplate reader (BioTek, Winooski, VT, USA). ROS levels are represented in Units, where 1.0 mgL⁻¹ H₂O₂ represents one unit of ROS ⁶.

3.3.4 Statistical Analyses

All statistical analyses were performed using Statistica version 12. Variables with a non-Gaussian distribution were logarithmically transformed and the central tendency and spread were represented by the geometric mean and the 5th and 95th percentile intervals. The associations between macro- and micro-variables with ROS were tested for interaction with ethnicity by introducing appropriate interaction terms by means of ANCOVA analyses. Two-way ANOVA was performed to compare means. Chi-square tests were performed to compare

proportions between groups. Unadjusted and adjusted correlations were performed between ROS and macro- and micro-variables. Multivariable linear regression models with forward stepwise selection were performed with PWV, CRAE, CRVE and AVR as the main dependent variables. Variables included in the model were age, gender BMI, SBP, CRP, glucose and ROS. MAP was used in the PWV model instead of SBP. If the CRVE was the main independent, then CRAE was adjusted for and vice versa, as there is a likelihood of confounding by the other fellow component variable ⁷.

3.4 References

1. Norton K, Olds T. Anthropometrica: a textbook of body measurement for sports and health courses, UNSW press, Sydney, Australia, 1996.
2. Pickering TG, Hall JE, Appel LJ, Falkner BE, Graves J, Hill MN, et al. Recommendations for blood pressure measurement in humans and experimental animals part 1: blood pressure measurement in humans: a statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research. *Hypertens* 2005; **45**: 142–161.
3. Huybrechts SA, Devos DG, Vermeersch SJ, Mahieu D, Achten E, de Backer TL, et al. Carotid to femoral pulse wave velocity: a comparison of real travelled aortic path lengths determined by MRI and superficial measurements. *J Hypertens* 2011; **29**: 1577–1582.
4. Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. *Curr Eye Res* 2003; **27**: 143–149.
5. Human Sciences Research Council MRC. South African National Health and Nutrition Examination Survey: SANHANES-1 2013.
6. Hayashi I, Morishita Y, Imai K, Nakamura M, Nakachi K, Hayashi T. High-throughput spectrophotometric assay of reactive oxygen species in serum. *Muta Res/Genetic Toxicol Environ Muta* 2007; **631**: 55–61.
7. Liew G, Sharrett AR, Kronmal R, Klein R, Wong TY, Mitchell P, et al. Measurement of retinal vascular caliber: issues and alternatives to using the arteriole to venule ratio. *Invest Ophthalmol Vis Sci* 2007; **48**: 52–57.

CHAPTER 4

RESEARCH ARTICLE

Does smoking impact on the association between oxidative stress and vascular function in young normotensives?

The African-PREDICT study

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4.1 Abstract

Objective: The role of oxidative stress in vascular remodelling is known, but less is known about the contribution of cigarette smoking, a modifiable lifestyle risk factor in this relationship. We explored the associations of oxidative stress with markers of vascular function in young, normotensive black and white non-smokers and smokers.

Design and Methods: We included 237 non-smokers and 145 smokers from South Africa between ages 20 and 30 years. We measured femoral pulse wave velocity and retinal vessel calibres. Serum cotinine levels were measured to define smoking status and reactive oxygen species to describe oxidative stress.

Results: Regardless of smoking status, PWV and ROS were similar between black and white groups, whereas CRAE and AVR was higher in white non-smokers vs black non-smokers. In single regression analysis, ROS correlated positively with CRVE and inversely with AVR ($r=-0.37$; $p=0.002$) in black smokers only. After partially correcting for age, sex and body mass index only the association with AVR remained, and a positive correlation further emerged with ROS and PWV in black smokers ($r=0.24$; $p=0.042$). In black smokers, we confirmed independent associations of ROS with AVR (Adj. $R^2=0.15$; $\beta=-0.37$; $p=0.002$) and, PWV (Adj. $R^2=0.35$; $\beta=-0.24$; $p=0.042$). An independent association of ROS with CRAE (Adj. $R^2=0.18$; $\beta=-0.25$; $p=0.034$) also emerged in black smokers.

Conclusion: Our results suggest that cigarette smoking adversely modifies the relationship of oxidative stress with retinal microvascular calibre and arterial stiffness in young normotensive black individuals.

4.2 Introduction

Cigarette smoking and other forms of tobacco use are major modifiable risk factors for cardiovascular disease (CVD) ¹. Along with smoking, other modifiable risk factors such as unhealthy diet, sedentary behaviour and excessive alcohol intake are responsible for the increase in CVD and related morbidity and mortality ². The adverse impact of smoking can be observed at both micro- and macrovascular levels ^{3, 4} and a number of factors such as oxidative stress may mediate this relationship ⁴.

Studies indicated that excessive cigarette smoking impacts adversely on the cardiovascular system ⁵⁻⁷, contributing to the initiation and progression of CVD, and possibly even an earlier onset of CVD ⁸. Smoking was further shown to increase the amount of oxidative stress, not only through the production of reactive oxygen species (ROS) present in smoke, but also through weakening of the antioxidant defence system ⁹. Many pathophysiological changes caused by smoking can be reversed or improved by smoking cessation ¹.

Changes to retinal microvasculature (retinal vessel calibre) occur with aging ¹⁰ and may even precede hypertension ¹¹. Both smoking and oxidative stress may contribute to retinal vessel calibre changes^{3,12}, but the role of oxidative stress in smokers on the microvasculature is not well studied. Likewise, at a macrovascular level, smoking contributes to an acute increased arterial stiffness ¹³. These vascular actions related to smoking are normally observed in middle-aged population groups^{3, 14-16}, but the mediating role of oxidative stress in smokers in the initiation of vascular changes in young adults has not been fully explored.

Studies have used smoking status as a means to quantify lifestyle risk in statistical models ^{17, 18}, but have failed to show a direct link between arterial stiffness, self-reported smoking and/or a metabolite of nicotine, namely cotinine. Furthermore, it is known that the metabolic rate of nicotine (catabolism) differs among ethnic groups ¹⁹, where black individuals have increased levels of cotinine due to the decreased production of cytochrome P450 enzyme ¹⁹. In a South African context, reactive oxygen species have previously been linked to arterial stiffness in an apparently healthy South African population between the ages of 25 and 65 years, but smoking status was not considered ²⁰. A study by Zatu et al. (2011) reported increased arterial stiffness (as measured by means of carotid-radialis pulse wave velocity and carotid-dorsalis pedis pulse wave velocity) in black South African smokers when compared with non-smokers, but found no correlation between cotinine and arterial stiffness nor included oxidative stress as potential mediator in this relationship ²¹.

Since tobacco use among young South Africans is a major concern ²², our study is motivated by the lack of potential underlying mechanisms in the direct link between smoking,

oxidative stress and cardiovascular function in young healthy individuals. We aimed at exploring the association of reactive oxygen species with measures of micro- and macrovascular function in a young black and white South African cohort stratified by smoking status as determined by cotinine levels.

4.3 Methods

4.3.1 Study Design and Population Sample

Cross-sectional data from the African prospective study on the early detection and identification of cardiovascular disease and hypertension (African-PREDICT) was used and included the first 403 available participants. In the current analysis, 21 participants were excluded due to missing data. Participants were required to undergo screening to determine eligibility for participation in the African-PREDICT study. This study was approved by the Health Research Ethics Committee of North-West University (NW-00001-12-A1) (Potchefstroom campus) and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki (revised 2008) for the investigation of human participants.

Individuals between ages 20 and 30 years, apparently healthy, black and white men and women, with normal clinic blood pressure (i.e. < 140/90 mmHg), no known cardiovascular disease, not on any anti-hypertensive medication, no chronic disease (or treatment thereof), HIV-negative, tuberculosis-free and not pregnant or currently breast feeding were included from the Potchefstroom district in the North West Province of South Africa.

4.3.2 Procedure

All participants gave written informed consent prior to any measurements performed. On the scheduled morning the participants arrived at the North West University's (Potchefstroom campus) Hypertension Clinic at approximately 07:45 am where all procedures were further explained and performed. Fasting blood samples and a midstream spot urine sample were first collected. Body composition of each participant was collected, after which they were given time to complete a battery of validated questionnaires including the general health survey, which involved demographics, self-reported smoking and alcohol use.

4.3.2.1 Body composition

In a private room, weight (kg) was measured to the nearest 0.01 kg (SECA electronic scales; Birmingham, UK); height (cm) was measured to the nearest 1.0 cm (SECA stadiometer; Birmingham, UK); and waist circumference (cm) was measured in triplicate using a non-flexible tape measure (Holtain, UK) and recorded to the nearest 0.1 cm. The median of the three waist circumference recordings were used in subsequent analyses. These measurements were conducted by trained anthropometrists according to standard procedures.

4.3.2.2 Cardiovascular Measurements

Validated CardioXplore devices (CardioXplore, MediTech, Hungary) were used for the collection of 24-hour blood pressure measurements programmed to take recordings every 30 minutes during the day (6 am to 10 pm) and every hour during the night (10 pm to 6am). The ambulatory blood pressure monitoring (ABPM) device was fitted to each participant at approximately the same time every day (late morning), using an appropriate sized cuff. Only participants with >70% of valid blood pressure measurements, >20 day measurements and >7 night measurements were included in the final statistical analysis. Pulse wave velocity (PWV) was captured using the SphymoCor XCEL device (SphygmoCor EXCEL, AtCor Medical, Australia). PWV was captured at the femoral and carotid arterial pulse points. The femoral artery wave form was captured via an appropriate size cuff placed around the thigh, and the carotid arterial wave form was captured simultaneously via applanation tonometry.

Retinal vessel calibres were determined by performing retinal imaging with a Dynamic Vessel Analyser (Plus 12100003 with Zeiss Fundus Camera FF 450 Plus Myriatic, Imendos, Jena, Germany). As far as probable the right eye was selected. Participants were not in fasting state at the time of the measurement, but did not eat, drink, smoke or perform exercise an hour prior to the measurement. Mydriasis was induced 30 minutes prior to the measurements being taken. Optic disc-centred colour images were used to determine retinal vessel calibres. In the event that the colour image was of insufficient quality, the grey-scale image was used. First-order Artery or vein vessel segments located within the inner zone of a measuring ring (0.5 – 1.0 optic disc diameters from the margin of the optic disc) were manually delineated using VesselMap 2 software. The software then determined the central retinal artery or vein equivalent (CRAE and CRVE) using the Knudtson variation of the Parr-Hubbard formula²³. The arterio-venous ratio (AVR) was also automatically calculated (CRAE/CRVE). Vessel calibres were expressed in measuring units (MU) where 1 MU is equivalent to 1µM in the normal Gullstrand eye.

4.3.2.3 Biochemical Measurements

Participants were divided into two groups: smoking and non-smoking. The smoking group consisted of heavy smokers, moderate smokers, light smokers and those individuals exposed to second-hand smoking. The cut-off points for serum cotinine were <10 ng/ml for non-smokers, 10-100 ng/ml for second-hand smokers or light smokers, 101-300 ng/ml for

moderate smokers and 300 ng/ml for heavy smokers. Serum cotinine was measured using Chemiluminescence method of the Immulite (Siemens, Erlangen, Germany). If cotinine values were indicated as <10, they were changed to 1 and If values were indicated as >500, they were changed to 500. Cobas Integra 400plus (Roche, Basel Switzerland) was used to measure serum high sensitivity C-reactive protein, low-density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, creatinine, and urinary albumin and creatinine (all with an intra and inter-assay variability of <10%). Serum reactive oxygen species were analysed using the Synergy H4 hybrid microplate reader (BioTek, Winooski, VT, USA) with an intra and inter-assay variability of 9.68% and 10.20% respectively.

4.3.2.4 Statistical Analyses

A power analysis for linear regression was calculated by using G*Power 3.1.9 to determine whether our cross-sectional sample size was sufficient to address the proposed hypothesis. Using the linear multiple regression: Fixed model, R^2 deviation from zero procedure, we determined that a sample size of 64 is sufficient to achieve 80% power at a probability of $\alpha=0.05$. All statistical analyses were performed using Statistica 12 (StatSoft, Inc., Tulsa, OK, USA, 2015). Normal distribution of the variables was tested prior to any statistical analyses being performed. Variables that did not fulfil these criteria were logarithmically transformed (reactive oxygen species, cotinine, urinary albumin creatinine ratio and C-reactive protein).

We categorised participants according to their smoking status using the serum cotinine levels. Smokers were classified as individuals who currently smoked (active smokers), past smokers and secondary smokers. Non-smokers were those individuals who never smoked and those with cotinine values of less than 1ng/ml. Secondary smokers and active smokers were identified by cotinine levels between 1-30 ng/ml and >30ng/ml, respectively.

The association of AVR and PWV pulse wave velocity with ROS was tested for interaction of ethnicity by introducing appropriate interaction terms by means of ANCOVA analyses. Two-way ANOVA was performed to compare means and Chi-square tests to compare proportions among groups. Pearson and partial correlations were done to explore the relationships of AVR, PWV with ROS. In partial correlations, the adjustments were made for age, gender and body mass index. PWV was additionally adjusted for mean arterial pressure (MAP). Independent associations were determined by performing forward stepwise multiple regression analysis. Variables included in the multiple regression models were age, gender, body mass index (BMI), systolic blood pressure (SBP), MAP, C-reactive protein

(CRP), total cholesterol and ROS. If the CRVE was the main independent then CRAE was adjusted for and vice versa ²⁴.

4.4 Results

Interaction terms were introduced for the main effects of ethnicity on the associations of AVR and PWV with ROS. We found an interaction of ethnicity with PWV ($F(131)=6.00$; $p<0.001$) in smokers only and therefore stratified the cohort according to ethnicity.

The general characteristics of the study population are presented in table 1. The mean ages of black and white non-smokers were similar ($p=0.14$). Among smokers the mean age in black individuals was approximately two years younger than white individuals ($p<0.001$). The CRAE was lower in black non-smokers compared to that in white non-smokers ($p<0.001$), whereas the CRVE was comparable between groups regardless of smoking status. The AVR was lower in black non-smokers compared to the white non-smokers ($p=0.001$). Blood pressure was comparable between all groups. ROS and urinary albumin-to-creatinine-ratio (UACR) (all $p<0.05$) were higher in black than in white non-smokers with no difference in the smoking groups. CRP also did not differ between black and white non-smokers. Glucose was higher in white non-smokers ($p<0.0001$) as well as in white smokers ($p<0.0001$) compared to their black counterparts. Total cholesterol was higher among both black and white smokers ($p<0.0001$) compared to non-smokers.

In single regression analysis (table S1), ROS correlated inversely with AVR ($p=0.002$) and positively with CRVE ($p=0.014$) in black smokers only. ROS also correlated inversely with PWV in white non-smokers ($p=0.041$). In partial correlations (after adjustments for age, gender, BMI and additionally for MAP when assessing PWV) (table 2), the negative association of ROS with AVR remained in black smokers ($p=0.011$). A positive correlation of ROS with PWV emerged in black smokers ($p=0.042$), and a positive association between ROS and SBP in white smokers ($p=0.027$). The positive association of ROS and CRP was evident only in white non-smokers, black smokers and white smokers (all $p<0.05$).

In multiple regression analysis (table 3) we observed an independent association of CRAE (adj. $R^2=0.18$, $\beta=-0.25$; $p=0.034$) and AVR (adj. $R^2=0.15$, $\beta=-0.37$; $p=0.002$) with ROS in black smokers only. PWV independently associated with ROS (adj. $R^2=0.35$, $\beta=0.24$; $p=0.042$) in black smokers only. No association of PWV with ROS was evident in white smokers. No association of CRAE, CRVE, AVR or PWV with ROS was observed in non-smokers. (table S2).

Table 4.1: General characteristics of the study population stratified by smoking status

	Non-smokers		Smokers	
	(n=237)		(n=145)	
	Black	White	Black	White
	(n=108)	(n=129)	(n=78)	(n=67)
Age (years)	24.9 ± 3.31 ^a	25.4 ± 2.83 ^c	23.6 ± 3.42 ^{abc}	25.6 ± 2.88 ^b
Gender (men/women)	31/77	46/83	53/25	32/35
Body composition				
Body mass index (kg/m ²)	25.7 ± 5.45 ^a	25.2 ± 5.10 ^b	22.7 ± 4.74 ^{abc}	27.0 ± 6.46 ^c
Body surface area (m ²)	1.74 ± 0.19 ^a	1.87 ± 0.23 ^{ac}	1.69 ± 0.17 ^{bcd}	1.96 ± 0.31 ^{bd}
Microvascular variables				
Central retinal artery equivalent (MU)	156 ± 12.2 ^{ab}	162 ± 12.3 ^{ac}	157 ± 12.7 ^c	161 ± 11.6 ^b
Central retinal vein equivalent (MU)	245 ± 17.3	246 ± 17.5	247 ± 20.9	245 ± 13.7
Artery-to-vein ratio	0.64 ± 0.049 ^{ab}	0.66 ± 0.049 ^{ac}	0.64 ± 0.062 ^c	0.66 ± 0.047 ^b
Macrovascular variables				
Systolic blood pressure (mmHg)	117 ± 9.19	117 ± 9.59	118 ± 9.57	119 ± 8.75

Diastolic blood pressure (mmHg)	70 ± 5.05	69 ± 5.53	70 ± 7.43	70 ± 6.89
Pulse pressure (mmHg)	47 ± 7.08	48 ± 7.66	48 ± 6.39	49 ± 6.66
Mean arterial pressure (mmHg)	85 ± 5.83	85 ± 6.16	85.71 ± 7.64	86.3 ± 6.88
Pulse wave velocity (m/s)	6.40 ± 0.89	6.19 ± 0.79	6.58 ± 0.92	6.44 ± 1.01
Biochemical variables				
Reactive oxygen species (mg/L H ₂ O ₂)	192 (182–204) ^{ab}	165 (152–177) ^a	171 (156–187)	160 (144–175) ^b
Urinary albumin-to-creatinine ratio (mg/g)	0.94 (0.45–0.63) ^{ab}	0.38 (0.34–0.45) ^a	0.45 (0.37–0.53)	0.37 (0.30–0.45) ^b
C-reactive protein (mg/L)	1.57 (1.25–1.97)	1.12 (0.88–1.44)	1.02 (0.74–1.39)	1.0 (0.72–1.39)
Cotinine (ng/ml)	–	–	32.1 (18.3–56.2) ^a	8.48 (4.39–14.8) ^a
Total Cholesterol (mmol/L)	3.88 ^{ab}	3.80 ^c	4.74 ^a	4.69 ^{bc}
Glucose (mmol/L)	4.09 ± 0.79 ^{abce}	4.64 ± 0.97 ^b	3.55 ± 0.82 ^{ade}	4.83 ± 0.50 ^{cd}

Values are expressed as mean ± standard deviation and geometric mean (95% confidence interval). Superscript letters (a–e) depicts statistical significance for p≤0.05.

Table S1: Bivariate correlations of reactive oxygen species with cardiovascular, biochemical and body composition measures in black and white non-smokers and smokers

	Reactive oxygen species (mg/L H ₂ O ₂)			
	Non-smokers		Smokers	
	(n=237)		(n=145)	
	Black (n=108)	White (n=129)	Black (n=78)	White (n=67)
Central retinal artery equivalent (MU)	r=−0.04; p=0.70	r=0.0081; p=0.93	r=−0.14; p=0.24	r=0.068; p=0.59
Central retinal vein equivalent (MU)	r=−0.08; p=0.42	r=−0.032; p=0.72	r=0.29; p=0.014	r=0.13; p=0.30
Artery-to-vein ratio	r=0.038; p=0.71	r=0.024; p=0.79	r=−0.37; p=0.002	r=−0.044; p=0.73
Pulse wave velocity (m/s)	r=−0.15; p=0.12	r=−0.18; p=0.041	r=−0.12; p=0.28	r=−0.0093; p=0.94
Age (years)	r=0.17; p=0.074	r=0.031; p=0.73	r=−0.15; p=0.19	r=−0.14; p=0.24
Body mass index (kg/m ²)	r=0.23; p=0.019	r=0.20; p=0.025	r=0.39; p<0.0001	r=0.0048; p=0.97
Systolic blood pressure (mmHg)	r=−0.19; p=0.053	r=−0.12; p=0.16	r=0.023; p=0.84	r=0.14; p=0.27
C-reactive protein (mg/L)	r=0.31; p=0.001	r=0.52; p<0.0001	r=0.50; p<0.0001	r=0.55; p<0.0001

Table 4.2: Adjusted correlations of reactive oxygen species with cardiovascular and biochemical measures in black and white non-smokers and smokers

	Reactive oxygen species (mg/L H ₂ O ₂)			
	Non-smokers		Smokers	
	(n=237)		(n=145)	
	Black (n=108)	White (n=129)	Black (n=78)	White (n=67)
Central retinal artery equivalent (MU)	r=−0.0084; p=0.93	r=0.051; p=0.53	r=−0.10; p=0.50	r=−0.028; p=0.89
Central retinal vein equivalent (MU)	r=0.047; p=0.62	r=0.052; p=0.52	r=0.17; p=0.26	r=0.12; p=0.56
Artery-to-vein ratio	r=0.12; p=0.26	r=0.003; p=0.98	r=−0.31; p=0.011	r=−0.10; p=0.43
Pulse wave velocity (m/s)	r=−0.006; p=0.95	r=−0.032; p=0.73	r=0.24; p=0.042	r=0.074; p=0.57
Systolic blood pressure (mmHg)	r=−0.003; p=0.97	r=0.007; p=0.94	r=0.18; p=0.12	r=0.28; p=0.027
C-reactive protein (mg/L)	r=0.14; p=0.16	r=0.45; p<0.0001	r=0.37; p=0.001	r=0.58; p<0.0001

Adjustments were applied for age, gender and body mass index. Pulse wave velocity was additionally adjusted for mean arterial pressure

Table 4.3: Forward stepwise multiple regression analyses of cardiovascular measures with reactive oxygen species in black and white smokers

	Black (n=78)		White (n=67)	
	Central retinal artery equivalent (MU)			
	Adjusted R ² =0.18		Adjusted R ² =0.25	
	Standardized beta (95% CI)	p-value	Standardized beta (95% CI)	p-value
Reactive oxygen species (mg/L H ₂ O ₂)	−0.25 (−17.92 to −17.48)	0.034	–	–
Central retinal vein equivalent (MU)	0.38 (−0.01 to 0.47)	0.002	0.48 (0.19 to 0.63)	<0.0001
C-reactive protein (mg/L)	0.18 (0.77 to 3.0)	0.24	–	–
Age (years)	–	–	−0.11 (−0.68 to −0.24)	0.30
Systolic blood pressure (mmHg)	−0.22 (−0.51 to −0.07)	0.055	−0.35 (−0.68 to −0.24)	0.003
	Central retinal vein equivalent (MU)			
	Adjusted R ² =0.21		Adjusted R ² =0.25	
Reactive oxygen species (mg/L H ₂ O ₂)	0.21 (24.5 to 25.0)	0.12	–	–
Central retinal artery equivalent (MU)	0.33 (0.32 to 0.76)	0.005	0.46 (0.33 to 0.77)	0.0001
C-reactive protein (mg/L)	0.22 (7.25 to 7.75)	0.11	–	–
Systolic blood pressure (mmHg)	–	–	0.23 (0.1 to 0.64)	0.092

Gender	–	–	0.15 (3.94 to 4.44)	0.25
Age (years)	–0.13 (–1.03 to –0.55)	0.27		
<i>Artery-to-vein ratio</i>				
	Adjusted R ² =0.15		Adjusted R ² =0.15	
Reactive oxygen species (mg/L H ₂ O ₂)	–0.37 (–0.35 to 0.09)	0.002	–	–
Systolic blood pressure (mmHg)	–0.12 (–0.22 to 0.22)	0.30	–0.42 (–0.24 to 0.24)	0.0006
Age (years)	–	–	–0.14 (–0.24 to 0.24)	0.24
<i>Pulse wave velocity (m/s)</i>				
	Adjusted R ² =0.35		Adjusted R ² =0.12	
Reactive oxygen species (mg/L H ₂ O ₂)	0.24 (0.014 to 0.47)	0.042	–	–
Age (years)	0.36 (0.161 to 0.55)	0.0007	0.23 (–0.16 to 0.32)	0.054
Gender	0.34 (0.097 to 0.59)	0.008	0.35 (0.46 to 0.94)	0.005
Body mass index (kg/m ²)	–0.36 (–0.593 to –0.13)	0.003	–	–
Mean arterial pressure (mmHg)	0.13 (–0.08 to 0.34)	0.23	–	–

Abbreviations: CI- confidence interval. Variables included in the model were age, gender BMI, SBP, CRP, total cholesterol and ROS. MAP was used in the PWV model instead of SBP. If the CRVE was the main independent, then CRAE was adjusted for and vice versa.

Variables that did not enter the model are indicated with “–” or omitted if applicable for both groups.

Table S2: Forward stepwise multiple regression analyses of cardiovascular measures with reactive oxygen species in black and white non-smokers

	Black (n=108)		White (n=129)	
	Central retinal artery equivalent (MU)			
	Adjusted R ² =0.30		Adjusted R ² =0.30	
	Standardized beta (95% CI)	p-value	Standardized beta (95% CI)	p-value
Reactive oxygen species (mg/L H ₂ O ₂)	–	–	–	–
Central retinal vein equivalent (MU)	0.39 (0.1 to 0.44)	<0.0001	0.49 (0.19 to 0.51)	<0.0001
Systolic blood pressure (mmHg)	–0.22 (–0.51 to –0.074)	0.055	–0.40 (–0.73 to –0.29)	<0.001
Gender	0.20 (5.0 to 5.5)	0.09	0.15 (3.73 to 4.07)	0.11
Body mass index (kg/m ²)	–0.20 (–0.67 to –0.23)	0.08	0.12 (0.11 to 0.47)	0.19
C-reactive protein (mg/L)	0.16 (3.4 to 3.8)	0.13	–	–
	Central retinal vein equivalent (MU)			
	Adjusted R ² =0.23		Adjusted R ² =0.26	
Reactive oxygen species (mg/L H ₂ O ₂)	–0.11 (–12.86 to –12.48)	0.27	–	–
Central retinal artery equivalent (MU)	0.42 (0.27 to 0.57)	<0.0001	0.52 (0.37 to 0.65)	<0.0001
C-reactive protein (mg/L)	0.12 (3.85 to 4.21)	0.19	0.13 (3.6 to 3.9)	0.14

Systolic blood pressure (mmHg)	−0.13 (−0.42 to −0.051)	0.19	0.21 (0.19 to 0.57)	0.033
Age (years)	−0.11 (−0.77 to −0.41)	0.22	0.11 (0.54 to 0.86)	0.16
Body mass index (kg/m ²)	–	–	−0.19 (−0.90 to −0.46)	0.063
Artery-to-vein ratio				
	Adjusted R ² =0.022		Adjusted R ² =0.066	
Reactive oxygen species (mg/L H ₂ O ₂)	–	–	–	–
Gender	–	–	0.12 (−0.11 to 0.33)	0.30
Body mass index (kg/m ²)	−0.18 (−0.37 to 0.02)	0.081	0.21 (−0.014 to 0.44)	0.068
Systolic blood pressure (mmHg)	–	–	−0.42 (−0.68 to −0.17)	0.001
C-reactive protein (mg/L)	–	–	−0.11 (−0.32 to 0.075)	0.27
Pulse wave velocity (m/s)				
	Adjusted R ² =0.23		Adjusted R ² =0.33	
Reactive oxygen species (mg/L H ₂ O ₂)	–	–	–	–
Age (years)	0.22 (0.044 to 0.40)	0.016	0.13 (−0.014 to 0.28)	0.079
Gender	0.14 (−0.077 to 0.35)	0.21	0.24 (0.085 to 0.40)	0.003
Body mass index (kg/m ²)	−0.24 (−0.47 to −0.032)	0.035	−0.20 (−0.365 to −0.035)	0.019

Mean arterial pressure (mmHg)	0.25 (0.15 to 0.35)	0.016	0.49 (0.314 to 0.66)	<0.0001
C-reactive protein (mg/L)	-0.14 (-0.342 to 0.062)	0.18	–	–

Abbreviations: CI – confidence interval. Variables included in the models were age, gender BMI, SBP, CRP, total cholesterol and ROS. MAP was used in the PWV model instead of SBP. If the CRVE was the main independent then CRAE was adjusted for and vice versa. Variables that did not enter the model are indicated with “–” or omitted if applicable for both groups.

4.5 Discussion

We demonstrated independent and adverse associations of CRAE, AVR and PWV with ROS in black smokers. These associations were absent in black non-smokers and the white groups. Our study, which focused on a young population that showed no advanced cardiovascular dysfunction, however, highlights the potential of early vascular changes mediated by oxidative stress especially among black smokers.

Nicotine (the addictive component in cigarette smoke) has been linked to various harmful cardiovascular effects including acute increases in blood pressure, heart rate, cardiac output^{25,26}, myocardial contractility²⁷, vasoconstriction^{26,28}, chronic lipolysis²⁹ and endothelial cell injury or toxicity³⁰. Cotinine is used as a marker of nicotine exposure, both in smokers and non-smokers, as it is one of the main metabolites of nicotine with a longer half-life than its precursor³¹. Our study described higher cotinine levels in black smokers, indicating the possibility of higher frequency tobacco use or potentially a slower clearance rate of cotinine¹⁹ as described by the slower oxidative metabolism of nicotine to cotinine, via cytochrome P450 in black populations³². Slower nicotine metabolism indicates that levels of nicotine in the body stay higher for a longer period of time, permitting a longer timeframe for nicotine to interact with nicotinic receptors throughout the body³³.

A number of studies investigated the influence of cigarette smoking on cardiovascular function but these studies did not include microvascular parameters (alterations which may reflect early vascular changes¹²) or consider the potential contribution of oxidative stress in smokers in relation to micro- and macrovascular function^{21, 34, 35}. Measures of retinal artery and vein calibres have been shown to associate with systemic cardiovascular risk factors or diseases such as hypertension, diabetes, stroke and coronary heart disease (CHD)³⁶⁻⁴⁰. Both CRAE and CRVE have been shown to be wider among current and past smokers and this effect was greater in venules^{3, 41}, this could be due to reduced oxyhemoglobin and tissue hypoxia, nicotine-induced alterations in vessel auto-regulation and secondary polycythemia⁴². In contrast, both CRAE and CRVE did not differ between smokers and non-smokers in the present study. This could be because our study population was young and have not yet shown progressive cardiovascular dysfunction and duration of smoking may also not have been long enough. We also did not report on pack years which may have given more insight to the results. Despite this reason, our study demonstrates a potential mechanism by which smoking may contribute to altered retinal vessel calibres. The negative independent association of CRAE with ROS in black smokers, may aid in the speculation that black smokers are at a higher risk for CVD. A smaller CRAE has been shown to be associated with increased risk of hypertension⁴³ and increased ROS levels may also contribute to hypertension in the black

population ²⁰. Indeed, the black South African population were shown to be more at risk for developing hypertension ^{44, 45}. The negative association between CRAE and ROS in black smokers suggests that smoking may contribute to the risk of hypertension in the black population via an oxidative stress related mechanism at the level of the microvasculature.

ROS also associated positively with PWV in black smokers only. This positive association is in line with the available literature, but adds information on this link in young healthy smokers from South Africa. Both acute and chronic smoking was shown to induce oxidative stress, altering vascular tone and increasing arterial stiffness ⁴. However, in our study this was evident only in black smokers. A number of studies demonstrated an acute increase in arterial stiffness after the use of tobacco products as measured by augmentation index, carotid-femoral pulse wave velocity, brachial-radial pulse wave velocity or carotid-radial pulse wave velocity ^{13, 46, 47}. Free radicals found in components of cigarette smoke reduce the nitric oxide generation or bioavailability ⁴⁸ due to increased arterial ROS production – an important functional component of arterial stiffness ⁴⁹. Despite the levels of ROS not being elevated, our result therefore describes oxidative stress to be a possible link between tobacco use and increased arterial stiffness, especially among the young normotensive black population.

The above associations were only observed in the black smokers. In a study by Fearheller et al., (2011) showed that black individuals have increased oxidative stress compared to their white counterparts. In this study the ROS levels do not differ between blacks and whites. This could indicate that ethnic differences might be playing a role, which also was partially supported by the interaction of ethnicity as previously described. Further to this Fearheller et al., (2011) showed that the black race is an independent risk factor for enhanced oxidative stress and inflammation ⁵⁰.

Cigarette smoke induces oxidative stress by activating the endothelium through the induction of adhesion molecule expression as well as macrophages and platelets ³⁵. Platelet activation is one of the major factors by which cigarette smoke mediates the pathogenesis of cardiovascular diseases, which is related to endothelial dysfunction ^{2, 51, 52}. Smoking-related endothelium dysfunction results in the reduction of nitric oxide release which normally inhibits platelet activation ⁵². This reduction of nitric oxide levels within the cells result in the loss of function of smooth muscle cells in the blood vessel media ³⁵. Cigarette smoking is associated with evidence of chronic inflammation ⁵³. In response to cigarette smoke exposure, endothelial cells release inflammatory and proatherogenic cytokines ³⁵. An increase in smoking-induced oxidative stress (ROS), decreases the production of NO and increases platelet function. All of the above increase vascular tone; thus leading to the increase of arterial stiffness ⁴. Elevated stiffness and pulse pressure may stimulate hypertrophy, remodeling or rarefaction in the

microcirculation ⁵⁴. Since the major pathophysiology processes in most patients develop long before cardiovascular disease is detected, simple investigations able to identify the early vascular remodeling process leading to disease would be of significant value for timely intervention and prevention ¹².

This was a well-designed and controlled study which included the use of the golden standard carotid-femoral PWV for the determination of arterial stiffness ⁵⁵ along with well-validated methods for quantifying cotinine. This was also the first study to our knowledge describing oxidative stress as mediator of both microvascular and large artery compromise in black smoking individuals. The limitations of this study should also be noted. This was a cross-sectional analysis, limiting our ability to judge the temporal sequence of the associations reported. The types of tobacco products used or their cotinine concentration was not taken into consideration and this might have influenced the serum cotinine concentration. Since we only described reactive oxygen species as measure of oxidative stress, more studies are encouraged to investigate whether anti-oxidant capacity or other pathways of oxidative stress are more involved in smoking-induced cardiovascular compromise.

In conclusion, we demonstrated independent adverse associations of oxidative stress (ROS) with micro- and macrovascular measures in a young and apparently healthy black cohort of smokers. Our results suggest that cigarette smoking modifies the relationship between ROS and vascular function and may contribute to a potential acceleration of increasing cardiovascular morbidities, especially in a population subjected to the burden of early onset vascular remodeling.

4.6 References

1. Erhardt L. Cigarette smoking: an undertreated risk factor for cardiovascular disease. *Atheroscler* 2009; **205**: 23–32.
2. Benowitz NL. Cigarette smoking and cardiovascular disease: pathophysiology and implications for treatment. *Prog Cardiovasc Dis* 2003; **46**: 91–111.
3. Kifley A, Liew G, Wang JJ, Kaushik S, Smith W, Wong TY, et al. Long-term effects of smoking on retinal microvascular caliber. *Am J Epidemiol* 2007; **166**: 1288–1297.
4. Doonan RJ, Hausvater A, Scallan C, Mikhailidis DP, Pilote L, Daskalopoulou SS. The effect of smoking on arterial stiffness. *Hypertens Res* 2010; **33**: 398–410.
5. Baldassarre D, Castelnovo S, Frigerio B, Amato M, Werba JP, De Jong A, et al. Effects of timing and extent of smoking, type of cigarettes, and concomitant risk factors on the association between smoking and subclinical atherosclerosis. *Stroke* 2009; **40**: 1991–1998.
6. Chen Z, Boreham J. Smoking and cardiovascular disease. *Semi Vasc Med* 2002; **2**: 243–252.
7. Szczech R, Hering D, Narkiewicz K. Smoking and cardiovascular risk: new mechanisms and further evidence for a ‘guilty’ verdict. *J Hypertens* 2004; **22**: 31–34.
8. Benowitz NL, Burbank AD. Cardiovascular toxicity of nicotine: Implications for electronic cigarette use. *Trends Cardiovasc Med* 2016; **26**: 515–523.
9. Isik B, Ceylan A, Isik R. Oxidative stress in smokers and non-smokers. *Inhal Toxicol* 2007; **19**: 767–769.
10. Leung H, Wang JJ, Rochtchina E, Tan AG, Wong TY, Klein R, et al. Relationships between age, blood pressure, and retinal vessel diameters in an older population. *Invest Ophthalmol Vis Sci* 2003; **44**: 2900–2904.
11. Smith W, Wang JJ, Wong TY, Rochtchina E, Klein R, Leeder SR, et al. Retinal Arteriolar Narrowing Is Associated With 5-Year Incident Severe Hypertension. The Blue Mountains Eye Study *Hypertens* 2004; **44**: 442–447.
12. Daien V, Carriere I, Kawasaki R, Cristol JP, Villain M, Fesler P, et al. Retinal Vascular Caliber Is Associated with Cardiovascular Biomarkers of Oxidative Stress and Inflammation: The POLA Study. *PLoS One* 2013; **8**: e71089. doi: 10.1371/journal.pone.0071089.
13. Lemogoum D, Van Bortel L, Leeman M, Degaute J-P, Van De Borne P. Ethnic differences in arterial stiffness and wave reflections after cigarette smoking. *J Hypertens* 2006; **24**: 683–689.
14. Pasupathi P, Saravanan G, Farook J. Oxidative stress bio markers and antioxidant status in cigarette smokers compared to nonsmokers. *J Pharm Sci Res* 2009; **1**: 55–62.
15. Prabha V, Buhutkar P, Bhutkar M, Sivagami G. A comparative study of arterial stiffness indices between smokers & non smokers. *Al Ameen J Med Sci* 2014; **7**: 207–212.

16. Rehill N, Beck CR, Yeo KR, Yeo WW. The effect of chronic tobacco smoking on arterial stiffness. *Br J Clin Pharmacol* 2006; **61**: 767–773.
17. Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H, Kannel WB. Prediction of coronary heart disease using risk factor categories. *Circ* 1998; **97**: 1837–1847.
18. Onat A. Risk factors and cardiovascular disease in Turkey. *Atheroscler* 2001; **156**: 1–10.
19. Perez-Stable EJ, Herrera B, Jacob III P, Benowitz NL. Nicotine metabolism and intake in black and white smokers. *JAMA* 1998; **280**: 152–156.
20. Kruger R, Schutte R, Huisman H, Van Rooyen J, Malan N, Fourie C, et al. Associations between reactive oxygen species, blood pressure and arterial stiffness in black South Africans: the SABPA study. *J Hum Hypertens* 2012; **26**: 91–97.
21. Zatu MC, Van Rooyen JM, Schutte AE. Smoking and vascular dysfunction in Africans and Caucasians from South Africa. *Cardiovasc J Afri* 2010; **22**: 18–24.
22. Flisher AJ, Parry CD, Muller M, Lombard C. Stages of substance use among adolescents in Cape Town, South Africa. *J Subst Use* 2002; **7**: 162–167.
23. Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. *Curr Eye Res* 2003; **27**: 143–149.
24. Liew G, Sharrett AR, Kronmal R, Klein R, Wong TY, Mitchell P, et al. Measurement of retinal vascular caliber: issues and alternatives to using the arteriole to venule ratio. *Invest Ophthalmol Vis Sci* 2007; **48**: 52–57.
25. Benowitz NL, Porchet H, Sheiner L, Jacob P. Nicotine absorption and cardiovascular effects with smokeless tobacco use: comparison with cigarettes and nicotine gum. *Clin Pharmacol Ther* 1988; **44**: 23–28.
26. Benowitz NL, Jacob P, Jones RT, Rosenberg J. Interindividual variability in the metabolism and cardiovascular effects of nicotine in man. *J Pharmacol Exp Ther* 1982; **221**: 368–372.
27. Bayer F, Bohn I, Strauer B. The contraction of left ventricle behaviour under nicotine exposure. *Therapy Week* 1985; **35**: 1968–1974.
28. Waeber B, Schaller M, Nussberger J, Bussien J-P, Hofbauer KG, Brunner HR. Skin blood flow reduction induced by cigarette smoking: role of vasopressin. *Am J Physiol Heart Circulatory Physiol* 1984; **247**: 895–901.
29. Hellerstein MK, Benowitz N, Neese R, Schwartz J, Hoh R, Jacob 3rd P, et al. Effects of cigarette smoking and its cessation on lipid metabolism and energy expenditure in heavy smokers. *J Clin Invest* 1994; **93**: 265–272.
30. Pittilo M. Cigarette smoking, endothelial injury and cardiovascular disease. *Int J Exp Pathol* 2000; **81**: 219–230.

31. Velloso J, Khalil N, Fonseca L, Brunetti IL, Oliveira OMMF. Does cotinine act upon reactive oxygen species and peroxidases? *Eclética Química* 2007; **32**: 65–70.
32. Benowitz NL, Perez-Stable EJ, Fong I, Modin G, Herrera B, Jacob P. Ethnic differences in N-glucuronidation of nicotine and cotinine. *J Pharmacol Exp Ther* 1999; **291**: 1196–1203.
33. Hoffman AC. The health effects of menthol cigarettes as compared to non-menthol cigarettes. *Tobacco Induc Dis* 2011; **9**: 1–9.
34. Papathanasiou G, Mamali A, Papafloratos S, Zerva E. Effects of smoking on cardiovascular function: the role of nicotine and carbon monoxide. *Health Sci J* 2014; **8**: 274–290.
35. Messner B, Bernhard D. Smoking and cardiovascular disease mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol* 2014; **34**: 509–15.
36. Wong TY, Shankar A, Klein R, Klein BE, Hubbard LD. Prospective cohort study of retinal vessel diameters and risk of hypertension. *BMJ* 2004; **329**: e79. doi: 10.1136/bmj.38124.682523.55.
37. Wong TY, Mitchell P. Hypertensive retinopathy. *N Eng J Med* 2004; **351**: 2310–2317.
38. 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**: 59–80.
39. Wong T, Hubbard L, Klein R, Marino E, Kronmal R, Sharrett A, et al. Retinal microvascular abnormalities and blood pressure in older people: the Cardiovascular Health Study. *Br J Ophthalmol* 2002; **86**: 1007–1013.
40. Ikram MK, Witteman JC, Vingerling JR, Breteler MM, Hofman A, de Jong PT. Retinal vessel diameters and risk of hypertension the rotterdam study. *Hypertens* 2006; **47**: 189–194.
41. Ikram MK, de Jong FJ, Vingerling JR, Witteman JC, Hofman A, Breteler MM, et al. Are retinal arteriolar or venular diameters associated with markers for cardiovascular disorders? The Rotterdam Study. *Invest Ophthalmol Vis Sci* 2004; **45**: 2129–2134.
42. Wimpissinger B, Resch H, Berisha F, Weigert G, Schmetterer L, Polak K. Response of retinal blood flow to systemic hyperoxia in smokers and nonsmokers. *Graefe's Arch Clin Exp Ophthalmol* 2005; **243**: 646–652.
43. 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**: 207–215.
44. Opie LH, Seedat YK. Hypertension in sub-Saharan African populations. *Circ* 2005; **112**: 3562–3568.
45. Seedat Y, Seedat M, Hackland D. Prevalence of hypertension in the urban and rural Zulu. *J Epidemiol Community Health* 1982; **36**: 256–261.

46. Mahmud A, Feely J. Effect of smoking on arterial stiffness and pulse pressure amplification. *Hypertens* 2003; **41**: 183–187.
47. Berlin I, Cournot A, Renout P, Duchier J, Safar M. Peripheral haemodynamic effects of smoking in habitual smokers. A methodological study. *Eur J Clin Pharmacol* 1990; **38**: 57–60.
48. Ambrose JA, Barua RS. The pathophysiology of cigarette smoking and cardiovascular disease: An update. *J Am Coll Cardiol* 2004; **43**: 1731–1737.
49. Wilkinson IB, Franklin SS, Cockcroft JR. Nitric oxide and the regulation of large artery stiffness from physiology to pharmacology. *Hypertens* 2004; **44**: 112–116.
50. Feairheller DL, Park JY, Sturgeon KM, Williamson ST, Diaz KM, Veerabhadrapa P, et al. Racial differences in oxidative stress and inflammation: in vitro and in vivo. *Clin Transl Sci* 2011; **4**: 32–37.
51. Law MR, Morris J, Wald NJ. Environmental tobacco smoke exposure and ischaemic heart disease: an evaluation of the evidence. *BMJ* 1997; **315**: 973–980.
52. Prasad D, Kabir Z, Dash A, Das B. Smoking and cardiovascular health: a review of the epidemiology, pathogenesis, prevention and control of tobacco. *Indian J Med Sci* 2009; **63**: 520–533.
53. Salahuddin S, Prabhakaran D, Roy A. Pathophysiological Mechanisms of Tobacco-Related CVD. *Glob Heart* 2012; **7**: 113–20.
54. Mitchell GF, Vita JA, Larson MG, Parise H, Keyes MJ, Warner E, et al. Cross-Sectional Relations of Peripheral Microvascular Function, Cardiovascular Disease Risk Factors, and Aortic Stiffness. The Framingham Heart Study. *Circ* 2005; **112**: 3722–3738.
55. Huybrechts SA, Devos DG, Vermeersch SJ, Mahieu D, Achten E, de Backer TL, et al. Carotid to femoral pulse wave velocity: a comparison of real travelled aortic path lengths determined by MRI and superficial measurements. *J Hypertens* 2011; **29**: 1577–1582.

CHAPTER 5

GENERAL FINDINGS AND CONCLUSIONS

5.1 Introduction

In this concluding chapter, a summary of the key findings from the research article are detailed. The results from the article are also debated, interpreted, explained and matched to relevant literature. The hypotheses will be accepted or rejected. Conclusions are drawn and recommendations are made to researchers investigating the associations of ROS with vascular function in smokers and non-smokers from a South African population.

5.2 Summary of Main Finding and Reflection of Initial Hypotheses

An independent correlation of CRAE, AVR and PWV with ROS was observed in black smokers only. These associations were not evident in black non-smokers and white smokers and non-smokers.

Hypothesis 1: *Smokers will have higher levels of ROS when compared with non-smokers.*

This hypothesis is rejected as we found ROS levels to be similar between smokers and non-smokers. In a study by Tavailani et al. (2012) they found that smokers (67 years of age) had increased oxidative stress when compared with non-smokers. They showed that SOD activity is expressively lower in smokers. This decrease in activity was explained as being the response to augmented ROS generation which, with severe or chronic exposure to oxidant conditions, may be insufficient to get rid of high ROS levels ¹.

Comparable ROS levels noted in our smoking and non-smoking groups could be due to the fact that these study subjects are still young (20-30 years of age), our ROS test only measures one form of ROS, it is possible that other measures of oxidative stress not measured in this study may be higher. Oxidative stress has been reported to increase in elderly subjects ² and with pathophysiological onsets of CVD. Duration of smoking could also have an impact on ROS accumulation. We have not reported on pack years, which might have explained the groups' smoking habits and whether the ROS levels are indeed similar due to similar amounts of cigarette-smoking exposure and duration.

Hypothesis 2: *Smokers will present with adverse micro- and macrovascular parameters when compared with non-smokers.*

CRVE was comparable between groups regardless of smoking status. However, AVR was lower in black non-smokers compared to white non-smokers. CRAE was lower in black non-smokers compared to white non-smokers and these values were comparable between

smokers. Macrovascular parameters (including PWV) were comparable between groups regardless of smoking status. This hypothesis is therefore rejected.

Kifley et al., (2007) showed that past and current smokers have wider CRVE and that current smokers have wider CRAE. They found no major difference in AVR between smoking subgroups. Retinal venular dilation could reveal accumulative lifetime exposure to risk factors such as smoking ³.

In this current study blood pressure was comparable between groups. The occurrence of high blood pressure is increased in individuals who smoke 15 or more cigarettes a day ⁴ and Groppe et al., (1992) found that in chronic smokers there is a persistent increase in blood pressure and also an increase in blood pressure variability ⁵. PWV did not differ between smokers and non-smokers. Mahmud et al. (2003) showed that cigarette smoking increases AIx (augmentation index) and PWV, indicating an increase in arterial stiffness ⁶. However, in our study the participants are young and do not present with adversely high PWV.

Hypothesis 3: ROS will adversely relate to micro- and macrovascular markers in smokers only.

This hypothesis is partially accepted because ROS correlated negatively with AVR, CRAE and positively with PWV in black smokers only. Oxidative stress is believed to have a part in the development of vascular dysfunction ⁷ and may have a central role to the atherogenic process ^{7,8}. ROS such as superoxide anion have been shown to be elevated in hypercholesterolemia, hypertension and cigarette use ^{9,10}. Acute and prolonged smoking has been presented to cause oxidative stress, modifying vascular tone and elevating arterial stiffness ¹¹.

5.3 Discussion of Main Findings

Oxidative stress has been shown to be involved in the pathogenesis of numerous CVDs such as hypercholesterolemia, atherosclerosis, diabetes, hypertension and heart failure ¹². Seeing that there are independent associations of CRAE, AVR and PWV with ROS in black smokers only, the concern is whether the black smokers from our study are at risk for future cardiovascular events.

Though it is challenging to infer the results to the general black population of South Africa, the findings of this study offer an initial point for larger-scale prospective studies with a study population involving randomly selected participants in order to limit or address the early development of CVD in this population. The negative association between ROS and CRAE

observed in black smokers possibly point to early vascular modifications that may already be present at a fairly young age. This stresses the significance of smoking cessation in the interest of lowering oxidative stress and possibly reduce early microvascular changes in this population group. Smaller CRAE is a cardiovascular risk factor¹³. Black non-smokers already have smaller CRAE than white non-smokers. In the black smokers there is a negative association between ROS and CRAE, which if the non-smoking black individuals already have smaller CRAE, could suggest risk in the black population as a whole.

Oxidative stress is thought to be involved in the development of vascular dysfunction. Arterial stiffness is one of the most significant manifestations of aging and vascular diseases¹⁴,¹⁵. Black smokers in this study population show an association between PWV and ROS and they might have an increased chance of developing arterial stiffness and ROS might be the driving force behind this development. This population has both micro- and macrovascular dysfunctions (at a relatively young age), which may result in early onset of various cardiovascular diseases.

5.4 Chance and Confounding

It is important to report on potential determinants that might have affected the results of this study. With regard to the methodology, this was a cross-sectional study; hence one cannot deduce causality. The results acquired stemmed from a specific target population and do not necessarily mirror the status of the entire black and white populations of South Africa. The number of participants included in this study provided satisfactory statistical power.

Furthermore, the number of black (n=78) and white (n=67) smokers in this study population was moderately small, yet associations acquired were independent of significant covariates and confounders and power calculations indicated that a sample group of 64 is sufficient for analysis of our posed hypotheses. The types of tobacco products used by the participants and their cotinine concentration was not taken into account and this might have had an impact on the serum concentration of cotinine. We also did not take into consideration racial differences in the metabolism of nicotine and cotinine, although the interaction of race did highlight this difference between groups. In this study only peroxides were used as a measure of ROS.

5.5 Conclusions

ROS is negatively associated with CRAE, AVR and positively associated with PWV in a relatively young black smoking population, but not in a white smoking population. This

suggests that some of the vascular changes occurring in the black population later may in part be mediated by ROS found in cigarette smoke and other cardiovascular risk factors. Furthermore, the inverse association observed between ROS, CRAE and AVR and the positive association between ROS and PWV in black smokers might indicate that these individuals are at risk for developing arterial stiffness prematurely and potential future cardiovascular risk. It is therefore vital to highlight the importance of smoking cessation in this population and by so doing eliminate or limit the increased tendency of cardiovascular morbidity and mortality in blacks.

5.6 Recommendations

The following recommendations are intended for future studies which may focus on the topic of smoking, oxidative stress and cardiovascular disease in humans:

- ❖ It would be of interest to quantify the enzymes involved in nicotine metabolism along with the entire redox signalling system to describe the underlying mechanisms and phenotypes for black versus white individuals.
- ❖ Larger cohort population studies, longitudinal studies and prospective experimental intervention studies are required to define the cause of this cardiovascular burden.

The recommendations that follow are proposed to mend cardiovascular health of the South African population, especially black individuals:

- ❖ Smoking cessation programs should be encouraged where the central role of government is essential. Research indicates that the finest way for people to stop smoking is through evidence-based smoking cessation technologies and programmes. Such services include, but are not limited to, prescription drugs, insurance programmes, nicotine over-the-counter products, and quit lines. Smokers who take part in cessation programmes are more probable to successfully quit smoking (described as abstinence for six months or more) than those who try to quit on their own.
- ❖ A number of factors (whether inherent, modifiable or genetic) contribute to adverse cardiovascular function and even though human physiology is intricate and intersected, it is recommended that early interventions be employed to address the early onset of cardiovascular diseases in the black population.

5.7 References

1. Tavilani H, Nadi E, Karimi J, Goodarzi MT. Oxidative stress in COPD patients, smokers, and non-smokers. *Respiratory Care* 2012; **57**: 2090–2094.
2. Andriollo-Sanchez M, Hininger-Favier I, Meunier N, Venneria E, O'Connor J, Maiani G, et al. Age-related oxidative stress and antioxidant parameters in middle-aged and older European subjects: the ZENITH study. *Eur J Clin Nutr* 2005; **59**: 58–62.
3. Kifley A, Liew G, Wang JJ, Kaushik S, Smith W, Wong TY, et al. Long-term effects of smoking on retinal microvascular caliber. *Am J Epidemiol* 2007; **166**: 1288–1297.
4. Bowman TS, Gaziano JM, Buring JE, Sesso HD. A prospective study of cigarette smoking and risk of incident hypertension in women. *J Am Coll Cardiol* 2007; **50**: 2085–2092.
5. Groppelli A, Giorgi DM, Omboni S, Parati G, Mancia G. Persistent blood pressure increase induced by heavy smoking. *J Hypertens* 1992; **10**: 495–499.
6. Mahmud A, Feely J. Effect of smoking on arterial stiffness and pulse pressure amplification. *Hypertens* 2003; **41**: 183–187.
7. Nedeljkovic Z, Gokce N, Loscalzo J. Mechanisms of oxidative stress and vascular dysfunction. *Postgrad Med J* 2003; **79**: 195–200.
8. Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. *Cardiovasc Res* 1999; **43**: 652–671.
9. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. *J Clin Invest* 1993; **91**: 2546–2551.
10. Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griending KK, et al. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 1996; **97**: 1916–1923.
11. Cacciola RR, Guarino F, Polosa R. Relevance of endothelial-haemostatic dysfunction in cigarette smoking. *Curr Med Chem* 2007; **14**: 1887–1892.
12. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000; **87**: 840–844.
13. Ikram MK, Ong YT, Cheung CY, Wong TY. Retinal vascular caliber measurements: clinical significance, current knowledge and future perspectives. *Ophthalmol* 2012; **229**: 125–136.
14. Kawamoto R, Ninomiya D, Kusunoki T, Kasai Y, Ohtsuka N, Kumagi T. Oxidative stress is associated with increased arterial stiffness in middle-aged and elderly community-dwelling persons. *J Clin Gerontol Geriatrics*. 2016; **2016**: 1-5

15. Paravicini TM, Touyz RM. Redox signaling in hypertension. *Cardiovasc Res* 2006; **71**: 247–58.

ANNEXURES

Journal of Hypertension Research: Instructions to authors

The journal publishes papers reporting original clinical or experimental research that contribute to the improvement of knowledge in the field of hypertension and related cardiovascular diseases. The journal accepts original articles, reviews, correspondence and commentaries.

Preparation of Manuscript

All papers must be written in brief English but must contain satisfactory detail to demonstrate how the results were obtained. Manuscripts must be double-spaced with wide margins.

Manuscripts must enclose a statement stating that all human studies have been reviewed by the appropriate ethics committee or it must be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study.

Cover Letter

The covering letters should state that the manuscript has not been submitted for publication elsewhere while it is being considered by Hypertension Research. The name, full postal address and fax number of the corresponding author must clearly be stated. The authors are welcome to offer suggestions of suitable expert reviewers.

Conflict of Interest

In order to be transparent and to assist reviewers evaluate any potential bias, Hypertension Research needs authors of all submitted papers to state any conflict of interest (COI) with regards to the submitted work. Authors submitting their manuscripts using the journal's online manuscript tracking system are needed to make their declaration as part of this process and to stipulate the competing interests in cases where they exist.

Sections of Manuscript

Generally, all manuscripts must be divided in to the following sections:

Title Page

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

Abstract and Keywords

An abstract should not contain more than 250 words. It must be understandable to readers prior to them reading the whole article, and abbreviations and reference citations within the abstract must be avoided. The abstract must outline the purpose of the study, the basic procedures and the most significant conclusions.

Three to five keywords, which may or may not appear in the title, should be given in alphabetical order below the abstract, each separated by a comma (,).

Introduction

The introduction must not be a review of the literature. It must give a short, clear interpretation of the background and motives for undertaking the study.

Methods

This part of the manuscript must contain satisfactory details so that all experimental procedures can be reiterated by others in combination with cited references. This section may be divided into subheadings to help the reader. Names of products and manufacturers must be included only if other sources are judged unsatisfactory, giving both the company name and city. Generic names of drugs should be used.

Unique experimental procedures must be described in great detail, but published procedures must be referred to by literature citation of the original article and published modifications. Use of standard abbreviations and SI units of measurement (according to the Systeme International d'Unites) is encouraged. Measurements that are not currently converted to SI units in biomedical applications are blood and oxygen pressures, enzyme activity, H+

concentration, temperature, and volume. Abbreviations, if used, should be defined on their first appearance in the text.

Results

The representation of results should not just repeat data that appear in tables and figures and, similarly, the same data must not be displayed in both tables and figures. The results section must be brief and follow a reasonable sequence. If the paper describes a complex series of experiments, it is acceptable to explain the protocol/experimental design before presenting the results. Do not discuss the results or draw any conclusions in this section. This section may be divided into subheadings to help the reader. Large datasets or other cumbersome data relevant to the manuscript may be submitted as supplementary information.

Discussion

Do not reiterate the results, but discuss their significance alongside the background of current knowledge, and identify clearly those aspects that are unique. The final paragraph must highlight the key conclusion(s), and provide some suggestion of the direction future research should take. This section may be divided into subheadings to help the reader.

Acknowledgments

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

References

Authors are accountable for the accuracy of the references. All authors must be quoted. In the text of the manuscript, references to the literature must be numbered successively and indicated by a superscript. Each reference must be numbered independently and listed at the end of the manuscript. Only articles that have been published or accepted and waiting for publication (listed as 'in press' following digital object identifier number) must be in the reference list. Reference to 'unpublished data' and 'personal communications' should not appear in the list but should be cited in the text parenthetically only (e.g. Smith A, 2007, unpublished data). Written proof for 'personal communication' and preprint for 'in press' may

be requested for review. Abstracts may be cited only if they are the sole sources, and must be identified in the reference list as '(Abstract)'. The names of journals cited should be abbreviated (without full stops) according to the 'International List of Periodical Title Word Abbreviations (Chemical Abstracts Service, Columbus, Ohio, USA, 1970)'.

Example of References

Journal article

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

Price RA, Curry N, McCann KE, Fielding JL, Lockett M, Abercrombie E. Analysis of obesity in twins. *Hum Hered* 1989; **39** (Suppl 1): 121–135.

Feig SA, Lenarsky C, Moss T, Gallardo RL, Juneja HS, Gordon MY. Bone marrow transplantation for neuroblastoma. *Exp Hematol* 1985; **13**: 362 (abstract 102).

Journal article –online only

Jelinic P, Stehle JC, Shaw P. The testis-specific factor CTCFL cooperates with the protein methyltransferase PRMT7 in H19 imprinting control region methylation. *PLoS Biol* 2006; **4**: e355. doi:10.1371/journal.pbio.004035.

Journal article -in press

Davis ID, Chen Q, Morris L, Quirk J, Stanley M, Tavarnesi ML, Parente P, Cavicchiolo T, Hopkins W, Jackson H, Dimopoulos N, Tai TY, MacGregor D, Browning J, Svobodova S, Caron D, Maraskovsky E, Old LJ, Chen W, Cebon J. Blood dendritic cells generated with Flt3 ligand and CD40 ligand prime CD8+ T cells efficiently in cancer patients. *J Immunother* (in press).

Journal article -e-pub ahead of print

Tian X-P, Long L-J, Li S-M, Zhang J, Xu Y, He J, Li J, Wang F-Z, Li W-J, Zhang C-S, Zhang S. *Pseudonocardia antitumoralis* sp. nov., a new deoxynyboquinone-producing actinomycete isolated from a deep-sea sedimental sample in South China Sea. *Int J Syst Evol Microbiol* (e-pub ahead of print 25 May 2012; doi:10.1099/ijs.0.037135-0).

Book

Safar ME, O'Rourke MF (eds). Handbook of Hypertension, 23rd edn. Elsevier: Edinburgh, UK, 2006, 503–516.

Chapter in a Book

Morrow DA, Gersch BJ, Braunwald E. Chronic coronary artery disease. In Zipes DP, Libby P, Bonow RO, Braunwald E (eds), Heart Disease, 7th edn. WB Saunders: Philadelphia, PA, USA, 2005, 1281–1341.

Electronic Material

National Institutes of Health. Genome-Wide Association Studies (GWAS). <http://grants.nih.gov/grants/gwas/index.htm>. Accessed 4 January 2007.

Tables

Tables should be labelled consecutively as Table 1, Table 2, etc. Each table must be saved in a separate file, numbered and titled, and cited in the text. Reference to table footnotes should be made by means of Arabic numerals. Tables should not duplicate the content of the text. They should consist of at least two columns; columns should always have headings. Tables should have a brief footnote that identifies all abbreviations used. Authors should make sure that the data in the tables are consistent with those cited in the relevant places in the text, totals add up correctly, and percentages have been calculated correctly. Tables should be supplied as separate electronic files (as Word or Excel file formats).

Figures

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