Myeloperoxidase and the vasculature in young adults: The African-PREDICT study

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Dissertation accepted in fulfilment of the requirements for the degree Master of Health Science in Cardiovascular Physiology at the North-West University

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Preface

The following dissertation '*Myeloperoxidase and the vasculature in young adults: The African-PREDICT study*' forms part of the requirements for the degree Master of Health Sciences in Cardiovascular Physiology at the North-West University, Potchefstroom Campus.

The dissertation follows the article format approved by the North-West University and consists of the following chapters:

Chapter 1: Background, motivation and literature overview

Chapter 2: Methodology

Chapter 3: Research manuscript

Chapter 4: Final remarks and recommendations for future studies

The manuscript (Chapter 3) will be submitted to the journal *Biomarkers* and is therefore presented in the prescribed format of the journal. Referencing throughout the dissertation is consistent with the authors' instructions of the aforementioned journal, and respective references are listed at the end of each chapter.

Author contributions

Ms A Brelage: Assisted with data collection by performing cardiovascular measurements and laboratory tasks within the African-PREDICT study. Gathered literature for the background and motivation, wrote the research proposal and ethics application, performed the statistical analyses, interpreted the results, planned, and wrote the dissertation.

Prof CMC Mels: Supervisor for the dissertation. Assisted with the data collection, made recommendations regarding the writing of the proposal and ethics application, planning of the manuscript as well as the statistical analyses, and interpretation of the data.

Prof R Kruger: Co-supervisor of the dissertation. Assisted with the collection of data, the writing of the proposal, ethics application, literature study and manuscript as well as the interpretation of the data.

Prof AE Schutte: Principal investigator of the African-PREDICT study and co- supervisor for the dissertation. Assisted with the collection of the data, the writing of the proposal, ethics application, literature study and manuscript as well as the interpretation of the data.

Prof W Smith: Assisted with collection of the data, the writing of the manuscript, and interpretation of the data regarding the microvasculature.

The individual involvement of the co-authors is confirmed in this statement, giving their permission that the research article may form part of this dissertation.

Prof CMC Mels

Prof R Kruger

Prof AE Schutte

Prof W Smith

Summary

Background and motivation

Myeloperoxidase (MPO) is an enzyme with both pro-inflammatory and pro-oxidative functions. Previous studies in the United States have found MPO levels to be higher in African American than in white groups, but studies examining MPO levels in South Africa are scant. Recent findings indicated that increased circulating levels of MPO are linked to hypertension and stroke, especially in older populations.

Early microvascular changes may aid in the prediction of cardiovascular disease (CVD). The retina is a unique site to investigate microvascular changes with non-invasive techniques, such as the Retinal Vessel Analyzer. Retinal vessel calibres can be determined from retinal fundus images to determine the central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE). In South African populations, CRVE was found to be wider and CRAE narrower in black adults when compared with white adults. Arterial narrowing is commonly associated with hypertension, whereas venular widening is associated with incident stroke. Retinal arterial narrowing is a difficult measure to estimate precisely and therefore a summary measure, the ratio of CRAE and CRVE, the arterio-to-venous ratio (AVR) was proposed. Arterio-to-venous ratio can be used as an index of the severity of arteriolar narrowing.

Whether there are potential associations between MPO levels and the microvasculature among young black and white individuals with no apparent CVD still needs to be investigated. The African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT) allows us to investigate the association between the microvasculature and MPO in young participants.

Aim

To determine whether measures of the retinal microvasculature associate with MPO in young, bi-ethnic South African adults.

Methods

We included the first 577 participants of the African-PREDICT study, aged 20-30 years, with complete retinal vessel calibre data at baseline, namely black (n=284) and white (n=285) men and women. Participants who presented with missing data for MPO (n=5) and those using anti-inflammatory medication (n=3) were excluded from this study. The final group consisted of 569 participants.

Data on anthropometric measures including body height, weight, and waist circumference were collected, and body mass index was calculated. Clinic blood pressure was measured on the left arm in duplicate while participants remained in a rested seated position. The Retinal Vessel Analyzer was used to capture retinal images. The images were analysed to calculate CRAE and CRVE. Biochemical analyses included MPO, the lipid profile (triglycerides, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and total cholesterol), gamma-glutamyltransferase (GGT), cotinine, high sensitivity C-reactive protein (CRP), white blood cell count, creatinine and serum peroxides, as an indicator of reactive oxygen species (ROS).

Statistical analyses included independent T-tests and Chi-square tests to compare means and proportions. Single, partial and multiple regression analyses were performed to investigate the associations between the retinal vessel calibres and MPO while adjusting for age, waist circumference, systolic blood pressure, total energy expenditure (TEE), white blood cell count, GGT, HDL-C, cotinine and glucose.

Results

Groups were divided based on interactions found for sex on the associations between CRVE and MPO (p=0.027) and between AVR and MPO (p=0.027), along with evidence in the literature reporting on different MPO levels and retinal microvascular calibres; found in black and white groups. No significant differences were found between black and white men (p=0.71) or women (p=0.95) when comparing MPO levels between the groups. When comparing black and white groups, CRAE and AVR (all $p<0.05$) were lower in the black men and women, and black women had a wider CRVE than white women (p=0.018). Only in white men a consistent positive association was found between CRVE and MPO (adj. R^2=0.25; β=0.19; p=0.032) in unadjusted, partially adjusted and fully adjusted models. In black women, a positive association was found between CRAE (adj. R^2=0.29; β=0.17; p=0.026) and AVR (adj. R^2=0.052; β=0.18; p=0.041) with MPO in unadjusted, partially adjusted and fully adjusted models.

Conclusion

In conclusion, our results suggest the involvement of MPO in retinal microvascular changes as early as in young adulthood. This finding seems to be dependent on ethnicity and sex.

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List of abbreviations

ºC	Degrees Celsius
α	Alpha
β	Beta
ACR	Albumin-to-creatinine ratio
African-PREDICT	African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension
AVR	Arterio-venous ratio
BMI	Body mass index
Br^-	Bromide
CI	Confidence interval
Cl^-	Chloride
CRAE	Central retinal artery equivalent
CRP	C-reactive protein
CRVE	Central retinal vein equivalent
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
EDTA	Ethylene-diamine-tetraacetic acid
ExAMIN Youth	Exercise, Arterial Modulation and Nutrition in Youth South Africa study
GGT	Gamma-glutamyltransferase
GPx-3	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HART	Hypertension in Africa Research Team
HDL-C	High-density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HOCl	Hypochlorous acid
HOBr	Hypobromous acid
HOSCN	Hypothiocyanous acid
HREC	Health Research Ethics Committee
kCal/kg/day	Kilocalorie/kilogram/day

Kg	Kilogram
LDL-C	Low-density lipoprotein cholesterol
m^2	Square metre
mg	Milligram
mg/L	Milligram per litre
mg/mmol	Milligram per millimole
MHSc	Master of Health Sciences
mmHg	Millimetres of mercury
mmol/L	Millimole per litre
MPO	Myeloperoxidase
MU	Measuring unit
N	Number of participants
ng/ml	Nanogram per millilitre
NRF	National Research Foundation
NWU	North-West University
POLA	Pathologies Oculaires Lie´es a` l'Age
PWA	Pulse wave analysis
PWV	Pulse wave velocity
REDCap	Research Electronic Data Capture
ROS	Reactive oxygen species
SABPA	Sympathetic activity and Ambulatory Blood Pressure in Africans
SAMRC	South African Medical Research Council
SARChi	South African Research Chairs Initiative
SBP	Systolic blood pressure
SCN	Thiocyanate
TEE	Total energy expenditure
U/L	Units per litre
WC	Waist circumference

Chapter 1

Background, motivation and literature overview

Background and motivation

1. The prevalence of cardiovascular disease

Cardiovascular disease (CVD) is the leading cause of death worldwide (Roth *et al*, 2017) with hypertension being the leading risk factor that contributes to the development of CVD (Stanaway *et al.*, 2018). The prevalence of hypertension differs significantly between ethnic groups, with African Americans exhibiting the highest risk of developing hypertension compared to all other ethnicities in the United States (Mozaffarian *et al.*, 2015). In South Africa, the prevalence of hypertension is increasing, thereby increasing the risk of CVD development, especially among urban black Africans (Day *et al.*, 2018).

2. The retinal microvasculature

The retinal vessel calibres are possible indicators of early microvascular changes (Liew and Wang, 2011), characterised by retinal arteriolar narrowing and retinal venular widening (Flammer *et al.*, 2013). These changes may be related to the development of arterial hypertension (Liew and Wang, 2011). Systemic microvascular changes include the altered wall-to-lumen ratio of larger arterioles, vasomotor tone abnormalities and network rarefaction that will lead to disturbed tissue perfusion and an increased chance for susceptibility to ischemia (Yannoutsos *et al.*, 2014). It has been established that the retinal vasculature shares numerous anatomical and physiological features with other vascular beds such as the cerebral and coronary vasculature (Flammer *et al.*, 2013). This may indicate that retinal arterial abnormalities may possibly reflect structural or physiological microvascular changes occurring in other organ systems (Rizzoni *et al.*, 2009).

2.1. *The anatomy and physiology of the retinal microvasculature*

The circulation of the eye is divided into four different parts: the anterior part of the eye where the ciliary body is found, the retina, choroid and the optic nerve head (Figure 1).

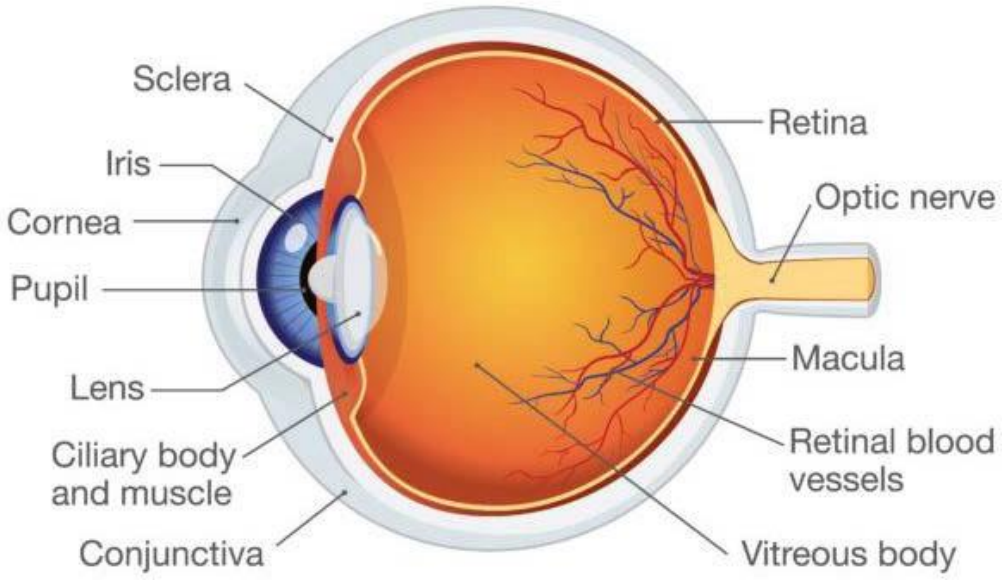

Figure 1: Structures of the eye (Helmenstine, 2019).

The retina represents a unique site whereby direct visualization of hypertension-related microvasculature changes can be observed (Liew *et al.*, 2008, Flammer *et al.*, 2013). The retinal microvasculature comprises three anatomically and functionally distinct segments: arterioles, capillaries and venules (Vitiello *et al.*, 2014, Pober and Sessa, 2015). By using the Dynamic Vessel Analyzer (Figure 2) (Werkmeister *et al.*, 2015), vessel calibres can be determined from retinal fundus images (Liew *et al.*, 2008). The central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) can be determined and thereafter the ratio of CRAE and CRVE, the arterio-venous ratio (AVR), is calculated (Knudtson *et al.*, 2003).

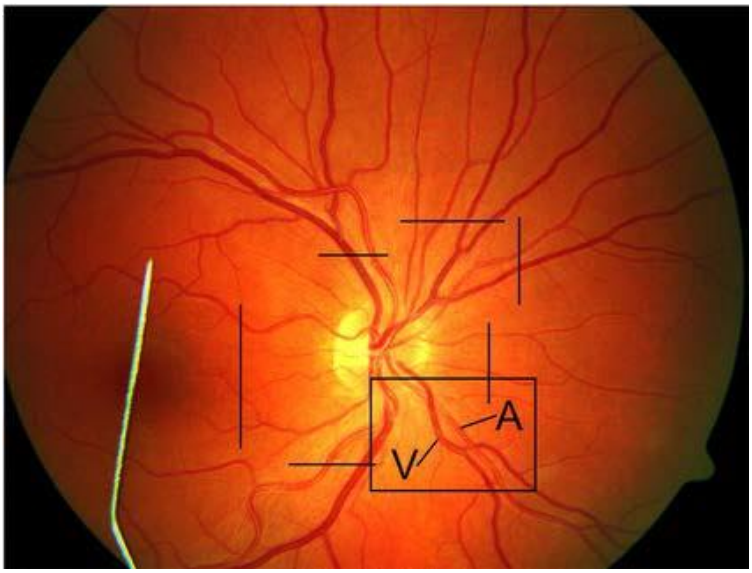

Figure 2: A colour image with a selection of vessel segments between 0.5 and 1.0 optic disc diameters from the outer margin of the optic disc to determine the central retinal vein and artery equivalent (Werkmeister *et al.*, 2015).

2.2. *Cardiovascular pathophysiology and the retinal microvasculature*

The retina is a unique site for studying hypertension-related microvasculature changes (Liew *et al.*, 2008, Flammer *et al.*, 2013). Microvascular changes can be characterised by arterial narrowing (Wong and McIntosh, 2005) and venular widening (Baker *et al.*, 2008). Arterial narrowing is associated with hypertension, which is regarded as a reduced AVR, narrower CRAE and a wider or unchanged CRVE (Wong *et al.*, 2006), whereas widening of the retinal venular calibres have previously been reported to independently predict incident stroke and inflammation (Baker *et al.*, 2008). The early microvascular changes may aid in the prediction of CVD (Witt *et al.*, 2006). Numerous studies have evaluated the associations of CVD risk factors (such as elevated blood pressure) with retinal microvascular calibres (Ikram *et al.*, 2004, Klein *et al.*, 2006a, Wong *et al.*, 2006). Both the Rotterdam study (Ikram *et al.*, 2004) and the Multi-Ethnic Study of Atherosclerosis (Wong *et al.*, 2006) found that narrower arterial calibre was associated with, amongst others, higher systolic blood pressure, current alcohol

consumption and a higher body mass index (BMI). A wider venular calibre associated with higher levels of C-reactive protein (CRP), current cigarette smoking, a higher BMI, higher levels of glucose, total cholesterol, triglyceride and low-density lipoprotein cholesterol (LDL-C) and lower levels of high- density lipoprotein cholesterol (HDL-C). The Beaver Dam Eye Study also found a positive association between a wider venular calibre and CRP, while controlling for age, current cigarette smoking and diabetes (Klein *et al.*, 2006a). The latter indicated that the retinal calibres might play an independent role in predicting CVD (Klein *et al.*, 2006a).

A cross-sectional analysis, conducted in 396 participants aged 50-85 years, established that retinal vascular calibre changes are associated with a range of systemic vascular diseases including coronary artery disease and hypertension (Klein *et al.*, 2006b). Data from the Beaver Dam Eye Study found that participants with the largest venular diameters exhibited the highest levels of inflammatory and endothelial dysfunction markers, suggesting that retinal venular dilation occurs during active inflammation (Klein *et al.*, 2006b). The associations were based on the inclusion of various inflammatory markers which included white blood cell count, serum albumin, CRP, interleukin-6 , tumour necrosis factor α and serum amyloid A - and endothelial dysfunction markers such as immunoglobulin G antibodies, serum soluble intercellular adhesion molecule-1 and serum soluble E-selectin to examine the relationship of systemic markers of inflammation and endothelial dysfunction to retinal vessel calibres (Klein *et al.*, 2006b).

These findings were consistent with a recent study, which indicated that the systemic inflammatory process appears to be the pathophysiological link for the interaction between small and large artery dysregulation (Anyfanti *et al.*, 2017). Furthermore, an increase in peripheral vascular resistance, which is associated with the narrowing of the systemic

microcirculation arterioles, is a distinctive characteristic of hypertension (Klein *et al.*, 2006b, Liew *et al.*, 2008). Studies have confirmed that the narrowing of retinal arteries are not only related to chronic exposure to hypertension but, may also predict the development of hypertension (Ikram *et al.*, 2013).

It is important to investigate both modifiable (obesity, smoking, physical inactivity, etc.) and non-modifiable (sex, ethnicity, age, etc.) risk factors of hypertension, seeing that the prevalence of hypertension in South Africa is increasing. Previous findings have indicated that 1 out of 2 adults over the age of 15 are hypertensive (Demographic, 2016, Steyn *et al.*, 2008). The Sympathetic activity and Ambulatory Blood Pressure in Africans (SABPA) Prospective Cohort study was conducted in 409 participants aged between 20 and 65 years and it was found that black participants presented with smaller AVR and wider venular calibres when compared with white participants (Lammertyn *et al.*, 2015). In young (20-30 years) healthy black and white cohort, from the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension - (African-PREDICT) it was confirmed that also in young healthy adults, black ethnicity was independently and negatively associated with retinal arterial calibre (Strauss *et al.*, 2016). Data from longitudinal cohort studies confirmed that retinal arterial narrowing and retinal venular widening are related to chronic hypertension and may also precede the development of hypertension (Wong *et al.*, 2004, Ding et al., 2014).

3. Myeloperoxidase

3.1. *The physiological role of myeloperoxidase*

Haemocytoblasts, multipotential haematopoietic stem cells located in bone marrow, are involved in the formation of all blood and immune system cells (Figure 3) (Orkin, 2000). Myeloperoxidase (MPO) is an enzyme synthesised in bone marrow during myeloid

differentiation in both the promyelocytes and promyelomonocytes (Koeffler *et al.*, 1985). The synthesis of MPO ceases in fully differentiated myeloid cells (Koeffler *et al.*, 1985). Myeloperoxidase is expressed mainly in the azurophilic granules of polymorphonuclear neutrophils, and to a lesser extent, in monocytes and macrophages (Arnhold and Flemmig, 2010, Anatoliotakis N, 2013) and is secreted during leukocyte activation (Hasanpour *et al.*, 2016).

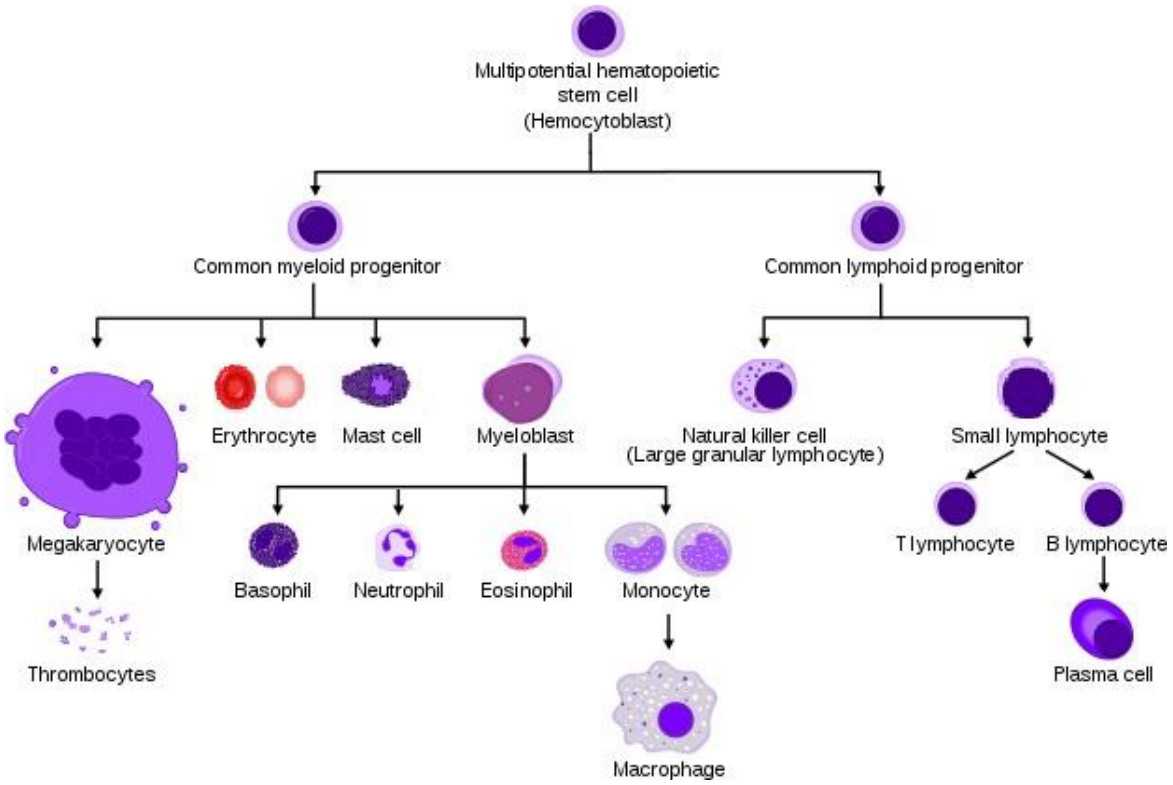

Figure 3: Haematopoiesis - the production of blood cells and platelets from haematopoietic stem cells (haemocytoblasts) in the bone marrow (Anon, 2015).

The main beneficial function of MPO is the direct defence mechanism against pathogens and bacteria (Arnhold and Flemmig, 2010, Nussbaum C, 2013, Gaul DS, 2017, Strzepa *et al.*, 2017). Previous studies confirmed that the combination of MPO, its substrate hydrogen peroxide (H_2O_2), and a halide or a pseudohalide (Figure 4) includes a very powerful antimicrobial system (M., 2014, Odobasic *et al.*, 2016, Tian *et al.*, 2017). The antimicrobial system exerts either stimulatory (platelets, mast cell secretion, activation of proteases such as

collagenase and gelatinase) or inhibitory effects (matrix metalloproteinases) against pathogens and bacteria (Klebanoff, 2005).

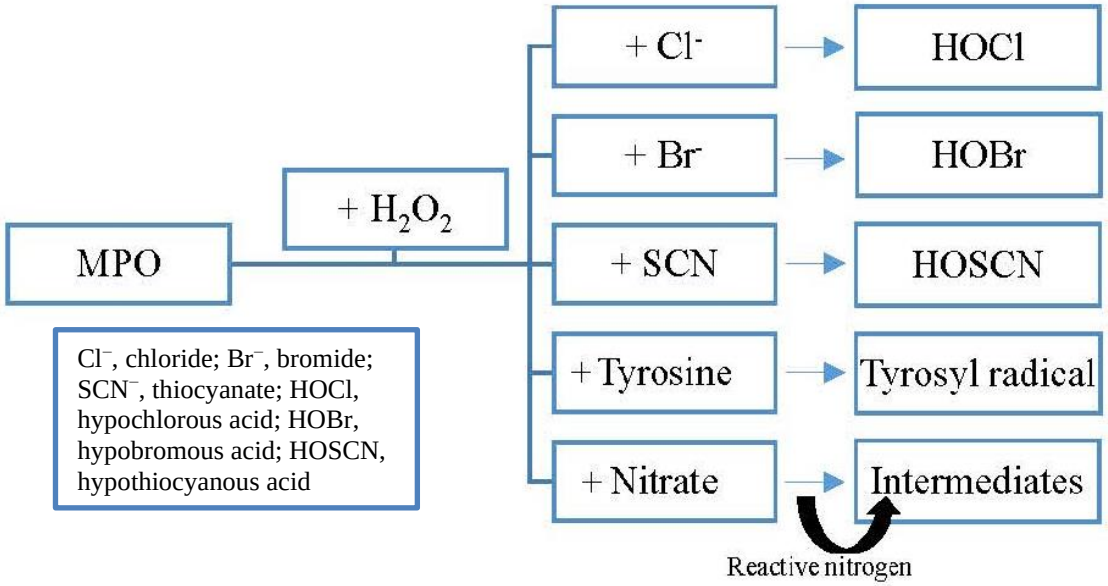

Figure 4: In the presence of hydrogen peroxide and chloride, bromide, thiocyanate, tyrosine or nitrite, myeloperoxidase catalyses the formation of hypochlorous, hypobromous and hypothiocyanous acids, tyrosyl radical and reactive nitrogen (adapted from Odobasic *et al*., 2016).

Systemic inflammation is considered to be a non-traditional risk factor for the development of CVD (Cottone *et al*, 2008). Another beneficial function of MPO includes the involvement of MPO in anti-inflammatory processes (Strzepa *et al.*, 2017). This includes the induction of reactive oxygen species-dependent apoptosis in neutrophils, MPO binding to surface epitopes of apoptotic cells and the production of regulatory molecules inside phagosomes of macrophages to release pro-inflammatory mediators (Loria *et al.*, 2008, Arnhold and Flemmig, 2010). Once neutrophils are secreted from peripheral blood, they accumulate in inflamed tissue to regulate the inflammatory process (Arnhold and Flemmig, 2010). The redundant neutrophils become apoptotic in the inflamed tissue. Rapid clearance of apoptotic

neutrophils by macrophages triggers the later cells to release anti-inflammatory mediators to the site of inflammation (Arnhold and Flemmig, 2010).

3.2. *Cardiovascular pathophysiology and myeloperoxidase*

Myeloperoxidase plays a critical role in the direct defence system by producing hypochlorous acid (HOCl) which contributes to killing pathogens (Odobasic *et al.*, 2016). However, MPO can be released from neutrophils causing oxidative damage to host tissues (Anatoliotakis N, 2013).

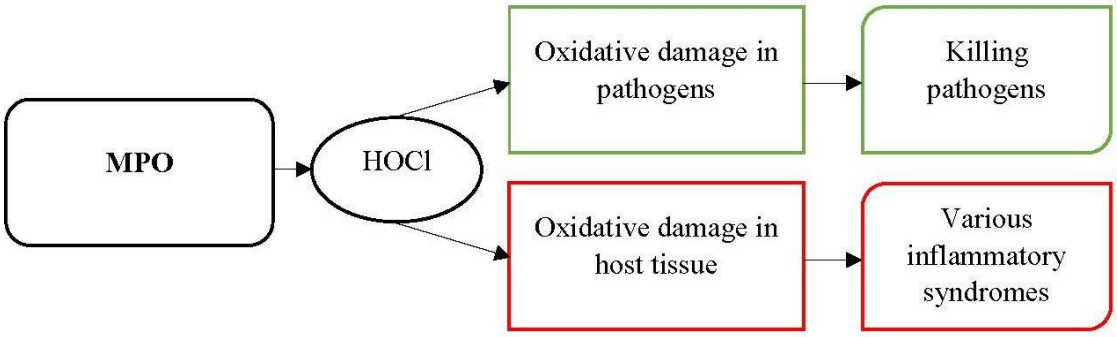

Figure 5: Schematic representation of the different roles of MPO and HOCl. Oxidative damage in pathogens can be caused inside the phagosome of the neutrophil or in host tissue outside the neutrophil. (adapted from Odobasic *et al*., 2016).

Elevated levels of MPO contribute to adverse cardiovascular manifestations (such as atherosclerosis) (Anatoliotakis N, 2013). The latter adverse manifestations may be due to the actions of MPO related to endothelial dysfunction. Myeloperoxidase acts as a leukocyte-derived mediator, which affects vascular nitric oxide bioavailability *in vivo* and is therefore associated with endothelial dysfunction (Vita JA, 2004, Nicholls SJ, 2005, Karakas M, 2012, Rudolph TK, 2012, Anatoliotakis N, 2013). The production of chlorinating and nitrating

reactive species by the action of MPO during vascular inflammatory responses may inactivate nitric oxide with subsequent increased vasoconstriction (Vita JA, 2004, Nicholls SJ, 2005). Myeloperoxidase also impairs endothelial and myocardial humeral and structural integrity, which contributes to the development of CVD (Nussbaum C, 2013). Elevated MPO was previously found to be implicated in multiple cardiovascular conditions including atherosclerosis, myocardial infarction and atrial fibrillation (Pulli B, 2013) (Nicholls SJ, 2005). Myeloperoxidase is expressed in high levels at atherosclerotic lesions, generating the end product hypochlorous acid and catalysing oxidative reactions within the arterial wall (Klebanoff, 2005).

4. Oxidative stress

Oxidative stress is the imbalance between reactive nitrogen and oxidant species (ROS) and the antioxidant defence capacity, favouring the oxidants, leading to molecular damage and/or altered redox signalling and regulation (Jones, 2006). Increased levels of oxidative stress are associated with the development of CVD (Touyz and Briones, 2011). The hierarchical oxidative stress model was presented in four tiers (Figure 6) (Eiserich *et al.*, 1998). During the first oxidative stress response tier, the activation of antioxidant enzymes, namely heme oxygenase-1 and catalase, takes place. During the second tier, the activation of the p38 mitogen-activated protein kinases and Jun kinase cascades are seen and the third tier is mediated by mitochondrial perturbation and leads to cytotoxic effects (Xiao *et al.*, 2003). This model may possibly suggest that oxidative stress occurs before the onset of inflammation and finally toxicity (Figure 6).

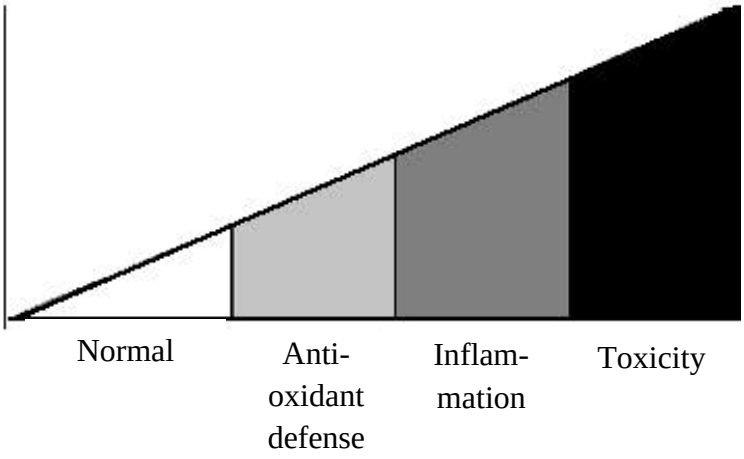

Figure 6: Schematic presentation to explain the hierarchical oxidative stress model in response to redox cycling chemicals (adapted from Xiao *et al.*, 2003).

Myeloperoxidase plays an essential role in producing oxidants and studies have indicated that MPO promotes oxidative stress in various inflammatory diseases (Nicholls SJ, 2005, Davies *et al.*, 2008). Myeloperoxidase has been associated with oxidative stress in, amongst others, rheumatoid arthritis, Parkinson disease, and Alzheimer disease (Davies *et al.*, 2008). Hydrogen peroxide is used by MPO to catalyse the production of hypochlorous acid, a powerful toxin (Kettle and Winterbourn, 1997). Chlorinated tyrosines, a specific biomarker of hypochlorous acid, has been identified at inflammatory sites and it was found that MPO contributes to protein damage in atherosclerosis (Hazen and Heinecke, 1997) and atrial fibrillation (Rudolph *et al.*, 2010).

Additionally, MPO may also cause oxidative damage to host tissue upon its release into the extracellular space during neutrophil activation (Karakas M, 2012, Delporte C, 2013, Ali M, 2016). In a previous study done on obese women, it was indicated that MPO levels were higher in women with preeclampsia when compared with normal pregnant or non- pregnant women (Shukla and Walsh, 2015). In this current study, three possible mechanisms were proposed to explain the link between oxidative stress, increased blood pressure and MPO (Figure 7).

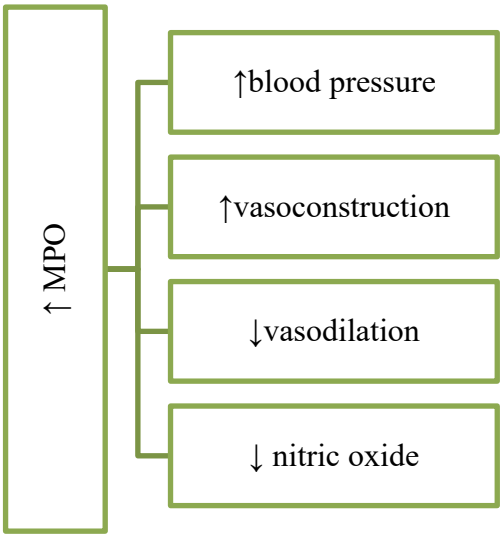

Figure 7: A schematic representation of the three possible mechanisms to explain the link between oxidative stress, increased blood pressure and elevated levels of myeloperoxidase.

In the presence of oxidative stress, elevated levels of MPO are associated with an elevation in blood pressure (Shukla and Walsh, 2015). The elevation in blood pressure is caused by a decrease in nitric oxide bioavailability, inhibition of prostacyclin synthase; thus, reducing the availability of prostacyclins (vasodilator), or by stimulating the induction of cyclooxygenase-2 to increase thromboxane (vasoconstrictor) (Shukla and Walsh, 2015). By reducing the bioavailability of the vasodilator nitric oxide, elevated levels of MPO may lead to hypertension (Shukla and Walsh, 2015).

Hypertension has previously been associated with both structural and functional changes of the retinal microvasculature (Yannoutsos *et al.*, 2014, Smith *et al.*, 2016), characterised by retinal venular narrowing and arteriolar widening (Liew *et al.*, 2008). In addition, it is known that when MPO is elevated, it exerts adverse effects on the vasculature (Kalász *et al.*, 2015). However, information with regard to MPO and the microvasculature is scant. Even more so in young disease-free populations.

Studies investigating the association between the different retinal microvascular calibres and oxidative stress are limited. The Pathologies Oculaires Lie´es a` l'Age (POLA) Study conducted an analysis, which included patients 60 years and older, to investigate the association between antioxidant enzyme activity and inflammatory markers with retinal vascular calibres (Daien *et al.*, 2013). In retinal arteries, it has been suggested that antioxidant enzyme activity (glutathione peroxidase (GPx-3)) may play a protective role against oxidative damage related to increased oxidative stress which may also lower the risk of developing CVD (Daien *et al.*, 2013, Buijsse *et al.*, 2012). Wider retinal arteriolar calibre was associated with higher activity of the antioxidant enzyme, GPx-3, after adjusting for age and sex (Swart et al., 2019). The activity of the antioxidant enzyme, GPx-3, plays an important protective role in the regulation of oxidative stress. However, an association was found between an insufficient GPx-3 activity and increased levels of ROS and reduced nitric oxide bioavailability (Wolin, 2011). The latter may suggest that the retinal microvasculature is sensitive to systemic oxidative stress, independent of known CVD risk factors (Daien *et al.*, 2013). The POLA study suggested that a wider retinal arteriolar calibre indicated a lower risk for developing CVD, whereas a wider venular calibre indicated a higher risk for developing CVD (Daien *et al.*, 2013).

5. Confounding factors of myeloperoxidase levels and the retinal microvascular calibres

5.1. *Age, sex and ethnicity*

Retinal vessel calibres have been examined in participants aged 49 years and above and it was found that retinal vessel calibres (both CRAE and CRVE) decrease with increasing age (Leung *et al.*, 2003). It has been found that older people (75-84 years) with or without higher blood pressure have narrower arterial diameters (Wong *et al.*, 2003), indicating the age-related link of retinal vessel changes. However, less is known about MPO levels in younger populations

with regards to sex and ethnic variation. Myeloperoxidase is released by polymorphonuclear neutrophils during the aging process (Mohàcsi *et al.*, 1996) and neutrophilic MPO activity was found to be higher in women, while serum MPO levels increased with age both in men and women (Hoy *et al.*, 2001b).

In South Africa, the SABPA study found black participants to present with smaller AVR and wider retinal venular calibres when compared with white participants (Lammertyn *et al.*, 2015), whereas the African-PREDICT study found black ethnicity to be independently and negatively associated with retinal arterial calibre (Strauss *et al.*, 2016). The Dallas Heart Study found that MPO levels were higher in African Americans when compared to white individuals (Khine *et al*., 2017). This finding suggested that MPO might be a risk factor that contributes to ethnic disparities in peripheral vascular diseases between African Americans and other ethnic groups (Chen *et al*, 2019).

5.2. *Smoking*

Only a few studies have examined the association between smoking and the retinal microvasculature. A cross-sectional study conducted on 2335 participants, aged 49 years and older, found associations of cigarette smoking with wider venular calibre and, to a lesser extent, wider arteriolar calibre (Kifley *et al.*, 2007). This may suggest long-term effects of smoking on venular calibre that may contribute to associations between smoking and CVD, even when adjusting for age, sex, and systolic blood pressure, amongst other confounding factors.

Cigarette smoking affects the leukocyte count and it was found that leukocytes are activated in smokers, demonstrating an effect of nicotine on superoxide anion generation by human neutrophils (Owasoyo *et al.*, 1988). Cigarette smoking is a major modifiable risk factor for cardiovascular disease, and its effects on large-vessel atherosclerosis and thrombosis are well known (Bøttcher and Falk, 1999). A positive association was found between smoking and

increased levels of MPO in men (Hoy *et al.*, 2001a). Smoking will therefore be taken into consideration as a possible confounder for the association between the retinal microvasculature and MPO.

5.3. *Obesity and dyslipidaemia*

Studies reported associations of larger venular calibre with obesity (greater BMI and waist-hip ratio) and dyslipidaemia (higher levels of plasma triglycerides and LDL-C and lower levels of HDL-C) (Ikram *et al.*, 2004, Nguyen and Wong, 2006). The Blue Mountains Eye Study found that a larger retinal venular calibre may predict the incidence of obesity over a five-year period, suggesting that diminished microvascular function may play a role in the pathogenesis of obesity and inflammation (Wang *et al.*, 2006). Myeloperoxidase, as an early biomarker of inflammation, is associated with CVD risk in obese children at prepubertal ages (Olza *et al.*, 2012).

6. Problem statement

Very limited information is available regarding the potential association between MPO and the retinal microvasculature. Although both MPO and retinal microvascular changes were indicated as being independently associated with the development of CVD, it is unknown whether retinal microvascular calibres and MPO are already linked in younger populations that are prone to the development of early vascular aging as previously confirmed in the African-PREDICT study (Breet *et al.*, 2017). Black South Africans may exhibit attenuated microvascular function – more so than whites (Wentzel *et al.*, 2018, Pienaar *et al.*, 2014). The narrowing of central retinal arteriolar calibres and widening of the central retinal venular calibres may precede clinical manifestations of CVD (Pienaar *et al.*, 2014) and may predict future cardiovascular complications (Wong *et al.*, 2002). Therefore, it is important to investigate the possible role of MPO and its association with microvascular calibres.

To the best of our knowledge no previous studies have investigated these factors in relation to one another in young populations. Also, limited information exists on ethnic and sex differences in young adults regarding MPO levels in populations worldwide. Based on these gaps in the literature, we therefore investigated the relationship between the microvasculature and MPO in young adults in an attempt to gain a better understanding of the early phases of cardiovascular disease development.

7. Aim

The aim of this study is to determine whether measures of the retinal microvasculature associate with MPO in a young bi-ethnic sample of South African adults.

8. Objectives

In a cross-sectional analysis of 577 black and white adults (age 20-30 years), we propose:

i. To compare MPO levels along with retinal microvascular calibres (CRAE, CRVE and AVR) between young black and white adults.
ii. To determine whether the retinal microvascular calibres are associated with MPO in young black and white adults.

9. Hypotheses

Based on the literature, we hypothesize that:

i. Myeloperoxidase levels will be higher, CRVE will be wider and CRAE will be narrower in black adults when compared to white adults.
ii. CRAE will associate negatively with MPO in young adults.
iii. CRVE will associate positively with MPO in young adults.

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Chapter 2

Methodology

1. Research design

This Master of Health Science (MHSc) study is embedded in the African Prospective study on the Early Detection and Identification of Cardiovascular disease and Hypertension (African-PREDICT) (Schutte *et al.*, 2019). The African-PREDICT study is a prospective study to longitudinally characterise and monitor the early stages of hypertension development in apparently young healthy black and white individuals.

This MHSc study used cross-sectional data of the first consecutive 577 participants of the African-PREDICT study, to determine the relationship between myeloperoxidase (MPO) and the retinal microvascular calibres in young South African adults.

2. Participant recruitment and selection

Participants were recruited for the African-PREDICT study from Potchefstroom and surrounding areas in the North West Province, South Africa. Recruitment of participants took place from 2012-2017 until the target of the full baseline sample was reached (N=1202). Participants were included on a voluntary basis provided they met the inclusion criteria. A research nurse was appointed to manage the recruitment of participants and to act as a gatekeeper between the principal investigator and the participants. Participants were invited to participate through various approaches such as active contact via field workers, direct recruitment at the workplace, and advertisements by means of radio, notice boards and local newspapers. To distribute participants into equal groups, they were stratified into different ethnic, sex and socio-economic groups. Participants that did not meet the inclusion criteria during screening were provided with feedback, counselling and referral to the necessary health services as appropriate. In this cross-sectional study we included 577 participants with complete retinal vessel calibre data. We excluded participants with missing data for MPO (n=5) and those using anti-inflammatory medication (n=3). The final group consisted of 569

participants: black (n=284) and white (n=285) men and women. The participants of the African-PREDICT study included either black or white, men and women, between the ages of 20-30 years (Schutte *et al.*, 2019). The participants included apparently healthy individuals with normotensive office blood pressure (<140/90 mmHg) who were not infected with the human immunodeficiency virus or any other previous diagnosis of a chronic disease. Individuals from low, middle and high socioeconomic status groups were specifically included.

3. Informed consent

Prior to participation, all procedures were explained to the participants verbally, where after they were afforded the opportunity of asking any questions, and if they wished to voluntarily participate, written informed consent was obtained.

4. Organisational procedures

Participants that met the required inclusion criteria during the screening stage were then invited to voluntarily take part in the research measurements. Participants arrived at the Hypertension Research and Training Clinic at 8:00 in the morning. They were requested to fast for 8 hours prior to participation. It was previously found that a fasting measure would produce a more stable and reliable result, avoiding the variability of all biochemical parameters (Bansal *et al.*, 2007).

Once the procedures had been explained, participants gave written informed consent. Thereafter biological sampling took place – the participants were requested to provide a spot urine sample and blood samples. Data on anthropometry and cardiovascular measurements were then collected. After completion of fasting-dependent procedures, the participants were provided with a light meal (excluding caffeine). When all measurements were completed at approximately 13:00, the participants received a grocery voucher as token of appreciation for

their participation and transport was provided to the participants to return home.

4.1. Questionnaires

General health and demographic questionnaires, which involved demographic and employment information and alcohol and tobacco use, were completed online. The questionnaires were completed with the help of the research nurse or a research assistant.

4.2. Anthropometric and physical activity measurements

Obesity is a major risk factor for cardiovascular disease (CVD) development (Klein *et al.*, 2004). Over the last 3 decades the prevalence of obesity has rapidly increased worldwide (Peters *et al.*, 2018), where South Africa has the highest prevalence of obesity in sub-Saharan Africa (Anon., 2017). To define body composition, a trained researcher used standard procedures to obtain height (Figure 1a) (SECA 213 Portable Stadiometer; SECA, Hamburg, Germany), weight (Figure 1b) (SECA 813 Electronic Scales; SECA, Hamburg, Germany) and waist circumference (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA). Body mass index (weight (kg) / height (m^2)) was calculated, along with the measurement of waist circumference, reflecting abdominal adiposity (Zhu *et al.*, 2002).

All anthropometric measurements were performed according to guidelines as described by the International Society for the Advancement of Kinanthropometry (Stewart *et al.*, 2011). Three measurements were performed, and the median was then used. The privacy of the participant was always taken into consideration; therefore, the measurements were done in a private temperature-controlled room.

Figure 1: Anthropometric measurements of (a) height and (b) weight.

Physical inactivity is a major risk factor for CVD (Artinian *et al.*, 2010). To evaluate physical activity, participants were fitted with an ActiHeart monitor (Figure 2) (CamNtech Ltd., England, UK) to record heart rate, inter-beat-interval and physical activity. The ActiHeart device was worn for a maximum of seven consecutive days to record total energy expenditure (TEE). Appropriate levels for TEE were determined according to each participant's personal information such as age, weight, height and sex.

Figure 2: Fitting of the ActiHeart monitor to record heart rate, inter-beat-interval and physical activity.

4.3. Blood pressure measurements

The main aim of a blood pressure measurement is to identify any changes from the normal blood pressure values, which may indicate disease such as hypertension (Khawaja *et al.*, 2010). To effectively diagnose and manage hypertension, accurate measurements of blood pressure is critical (Daskalopoulou *et al.*, 2015). There are different blood pressure measurements that can be performed for clinical use. According to the criteria of the British Hypertension Society, the Dinamap ProCare monitor is recommended for clinical use in an adult population (Reinders *et al.*, 2006, de Greeff *et al.*, 2007). Hence, we used the Dinamap ProCare 100 Vital Signs Monitor (GE Medical Systems, Milwaukee, USA) with appropriately sized cuffs to measure the brachial blood pressure (Figure 3).

Figure 3: Brachial blood pressure measurement on the participant's left arm with the 100 Vital Signs Monitor.

Prior to the measurement being performed, participants were requested to not have smoked, exercised or eaten at least 30 minutes preceding the measurement. Duplicate systolic blood

pressure, diastolic blood pressure and heart rate measurements of the participants were taken on the left and right arm after they had been seated calmly for 5 minutes. For the purposes of this study we made use of the second left arm blood pressure measurement.

4.4. Retinal microvascular imaging and measurements

The retina represents a unique and non-invasive site whereby direct visualization of hypertension-related microvasculature changes can be observed (Liew *et al.*, 2008, Flammer *et al.*, 2013, Al-Fiadh *et al.*, 2014).

Prior to the measurement the research nurse determined the intraocular pressure in each eye with the Tonometer - TonopenAvia (Reichert technologies) and Ocu-film sleeve. A local anaesthetic, Novesin Wander (0.4% eye drop) was instilled before this measurement. If the eye pressure exceeded 24 mmHg, no further eye measurements were performed. An eye pressure above 24 mmHg is generally associated with intraocular hypertension or glaucoma (Mao *et al.*, 1991). Thirty minutes prior to the retinal measurement, a drop of Tropicamide (1% Alcon) was administered in the right eye to induce mydriatic conditions. To avoid inducing angle-closure glaucoma, an estimation of the depth of the angle of the anterior chamber was done by the research nurse prior to administering the Tropicamide. The Retinal Vessel Analyzer (Imedos, Jena, Germany), fitted with a Zeiss Fundus camera FF-450 plus at a 50° angle, was used to capture monochrome and colour retinal images (using Visualis 2.81 software). The analyses of the images were done using VesselMap2 software, and in cases where the image quality was not sufficient, the colour image was used. The vessel trunks that were set within 0.5 and 1.0 optic disc diameters from the outer margin of the optic disc were marked as either an artery or a vein. Thereafter the central retinal artery equivalent (CRAE) and central retinal vein equivalent (CRVE) were calculated using revised formulas proposed by Knudtson (Figure 4) (Knudtson *et al.*, 2003). Only the 6 largest artery and 6 largest vein segments were included in the calculation. Both CRAE and CRVE were measured in

measuring units (MU), where 1 MU is equivalent to 1 μM if the dimensions of the eye were comparable to the normal Gullstrand eye. The arterio-venous ratio (AVR) was calculated as CRAE/CRVE. The reproducibility of the analysis was computed previously in a randomly selected cohort (Malan *et al.*, 2015).

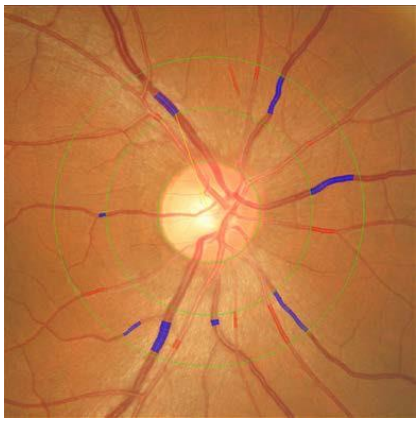

Figure 4: A monochrome image with a selection of vessel segments between 0.5-1.0 optic disc diameters from the outer margin of the optic disc.

An eye patch was provided after the measurement to all participants due to pupil dilation and to reduce excessive exposure to sunlight. Transport was provided to take the participants home (to ensure that participants did not drive themselves). If the researcher observed any abnormal findings, these results were noted, and the participant was referred to an ophthalmologist for further professional testing.

4.5. Blood sampling and biochemical analyses

A selection of cardiovascular biomarkers was studied to investigate the development of hypertension in this population. Early in the morning, before 9:30, venous blood samples were collected from the brachial vein branches, using a sterile needle, by a qualified nurse and in a temperature controlled private room. This is an invasive procedure. However, it carries

minimal risk to the participant. All participants were aware of the procedure prior to giving informed consent. The samples were prepared according to standardised protocols and stored at -80°C until the time of analysis.

Serum samples were used to measure MPO as part of the MILLIPEX MAP Human Cardiovascular Disease Panel 2 (Merck Millipore, Darmstadt, Germany) multiplex immunoassay on a Luminex 200™ system (Luminex, Austin, TX, US) (Figure 5).

Figure 5: Luminex 200^{TM} System - used to measure myeloperoxidase from serum samples.

Various other cardiovascular disease risk markers were measured and included serum high sensitivity C-reactive protein (CRP), total cholesterol, low-density lipoprotein- cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglycerides, gamma-glutamyltransferase (GGT), creatinine and sodium fluoride plasma glucose levels (Cobas Integra 400plus, Roche, Basel, Switzerland) (Figure 6a). Cotinine was determined with a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Whole ethylene-diamine-tetraacetic acid (EDTA) blood samples were used to obtain full blood counts (Coulter AcT 5 diff Analyzer, Beckman Coulter, California, United States) (Figure 6b). Albumin and creatinine were analysed in spot urine samples (Cobas Integra 400plus, Roche, Basel,

Switzerland) (Figure 6a) and the albumin-to-creatinine ratio (ACR) was calculated. Serum peroxides, as an indicator of reactive oxygen species (ROS), were determined using a high-throughput spectrophotometric assay and analysed on a Synergy HT microplate reader (BioTek, Winooski, VT, USA). Reactive oxygen species were reported in units, where 1mg H_2O_2/L was equivalent to one unit (Hayashi *et al.*, 2007).

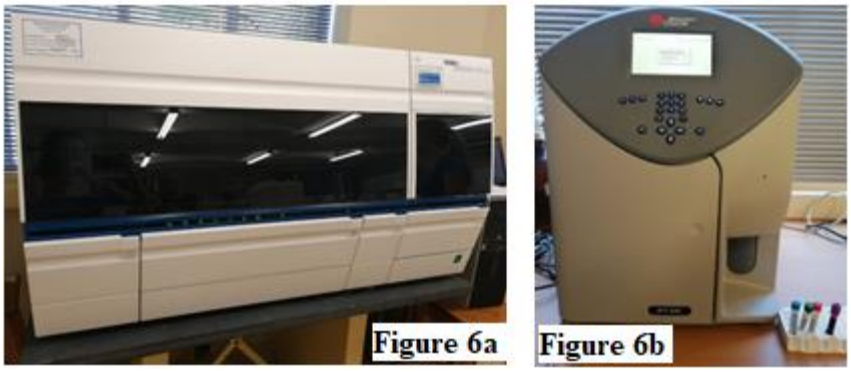

Figure 6: (a) Cobus Integra 400 plus - used for biochemical analyses and **(b)** the Coulter Act 5 diff Analyser – used for full blood count.

All biochemical analyses were performed with calibrated instruments by a trained biochemist. Laboratory personnel wore the necessary personal protective clothing while performing these analyses and all biological waste was disposed of according to the regulations of the North-West University (NWU).

5. Data handling

The African-PREDICT study uses the Research Electronic Data Capture (REDCap)system to capture data – see: http://project-redcap.org. REDCap is an electronic database software for collecting and tracking information and data from research studies. The database is password protected and access is strictly controlled. Following ethical approval, the student received a password protected dataset, compiled by the data manager with only the data variables applicable to this study.

6. Statistical analyses

Data analysis was done using Statistica version 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) and GraphPad Prism version 5 (GraphPad Software Inc., CA, USA) was used to prepare graphs. The detailed statistical methods used are provided in Chapter 3 as part of the research manuscript.

7. Ethical considerations

Prior to the conduction of data collection, the Health Research Ethics Committee of NWU approved the project in 2012 (NWU-00001-12-A1). The application for ethical approval, for this study was approved by the Health Research Ethics Committee of the North-West University, Potchefstroom Campus (NWU-00070-18-S1). This study also conformed to the ethical guidelines of the Declaration of Helsinki (revised in 2008) for investigation on human participants.

8. Student contributions

Although I was not involved in the initial screening phase of the African-PREDICT study, I have been involved in the first follow-up phase of the study and gained experience in several cardiovascular measurements in different population studies (as set out below). This is done as part of the postgraduate student training programme, and as indicated, includes measurements beyond those used in this dissertation.

8.1. Responsibilities as research assistant

During the follow-up phase I, as research assistant, was responsible for contacting the participants to schedule their appointments and for making the necessary arrangements regarding transport and accommodation.

8.2. Laboratory contributions

Additionally, I contributed to laboratory work where I was responsible for various tasks in the laboratory regarding the blood and urine samples. Once blood collection was completed, I was involved in ensuring that blood samples were centrifuged and aliquoted into individually marked cryovial tubes and correctly stored in -80°C biofreezers.

8.3. Blood pressure and physical activity measurement

I was also responsible for the application of the ambulatory blood pressure device and for downloading the data to compile the reports. Before the device could be fitted, I had to prepare the participant for the procedure by explaining the application of the device. The electrocardiogram stickers were placed on the participant's chest, a blood pressure cuff was placed around their non-dominant arm and a bag and belt was fitted around their waist to hold the device (Figure 7a). Before the first measurement could commence, the device was connected and programmed. The device was worn for 24 hours in order to have a minimum of 20-day and 7-night inflations. Along with the device, I explained the diary card that contained any information that could possibly affect the blood pressure measurements. The diary card was only completed when a participant did light physical activity, experienced any symptoms that were possibly related to blood pressure or when they experienced any stress. Along with this, I also attached the ActiHeart monitor (Figure 7b) to record heart rate, inter-heart beat variability and their physical activity over a 7-day period.

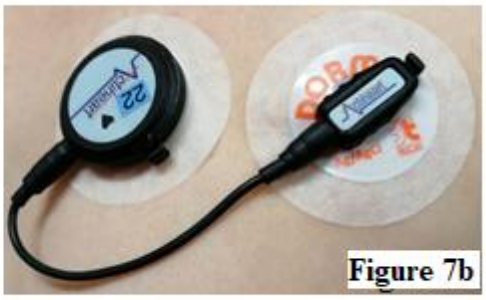

Figure 7: (a) CardioXplore 24h blood pressure device and **(b)** the ActiHeart physical activity monitor.

8.4. Pulse wave analysis and pulse wave velocity

I was involved in numerous studies measuring pulse wave analysis (PWA) and pulse wave velocity (PWV) with, amongst other devices, the SphygmoCor and Mobil-o-Graph.

In the EndoAfrica Study I was involved with PWA (Figure 8a) using the SphygmoCor® XCEL device (AtCor Medical Pty. Ltd., Sydney, Australia) to determine carotid-femoral PWV (Figure 8b) non-invasively, as a marker of arterial stiffness, according to the manufacturer's instructions. The download and capturing of the PWA and PWV data were also some of my responsibilities.

Figure 8: (a) The measurement of PWA and **(b)** PWV using the SphygmoCor® XCEL device.

I was also involved in the Exercise; Arterial Modulation and Nutrition in Youth South Africa (EXAMIN Youth SA) study in which I was responsible for pulse wave analysis on children (Figures 9a & b) with the use of the validated, oscillometric Mobil-o-Graph monitor (I.E.M. GmbH, Germany) with integrated ARCSolver software.

Figure 9: (a) The placement of the appropriately sized blood pressure cuff for **(b)** the PWA measurement with the Mobil-o-Graph monitor.

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Chapter 3

Research Manuscript

Summary of author instructions

This manuscript follows the specific guidelines as set out by the journal, *Biomarkers*, specified below.

1. The cover page should include the authors' details.
2. The structured abstract should be no more than 200 words with 5-10 keywords.
3. The clinical significance of the manuscript is a mandatory section.
4. Please supply all details required by your funding and grant-awarding bodies.
5. Include a disclosure statement to acknowledge any financial interest.
6. Figures should be high quality.
7. Tables should present new information.
8. Use SI units (non-italicized).

Myeloperoxidase and retinal microvascular calibres in young adults: The African-PREDICT study

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Abstract

Purpose: Increased levels of myeloperoxidase (MPO) have both pro-inflammatory and pro-oxidative functions previously linked to cardiovascular mortality. Towards understanding the role of MPO in early development of cardiovascular disease, we evaluated MPO and its associations with retinal microvasculature in young people.

Materials and methods: We included 284 black and 285 white men and women aged 20-30 years. Retinal images were captured to calculate the central retinal artery and vein equivalents (CRAE and CRVE). The arterio-venous ratio (AVR) was calculated as CRAE/CRVE. Biochemical analyses included analyses of serum MPO, C-reactive protein and peroxides.

Results: Myeloperoxidase levels did not differ between black and white men ($p=0.71$) or women ($p=0.95$). Both CRAE and AVR presented lower in both black sexes (white groups all $p<0.05$). In black women, CRVE was larger ($p=0.018$) than in white women. Unadjusted, partially and fully adjusted models, CRVE associated positively with MPO ($\beta=0.19$; $p=0.032$) in white men; CRAE positively with MPO ($\beta=0.17$; $p=0.026$) in black women.

Conclusion: Young adults showed an independent positive association between retinal venular calibre and MPO, only in white men, underlining the potential pro- oxidative role of MPO. Black women, showed a positive association between retinal arteriolar calibre and MPO, requiring further investigation.

Keywords: Oxidative stress, inflammation, myeloperoxidase, retinal microvasculature, central retinal artery equivalent, central retinal vein equivalent, young adults.

Introduction

Myeloperoxidase (MPO) is an enzyme mainly stored in neutrophils (Klebanoff, 2005) and contributes to innate human defence mechanisms under normal physiological conditions (Arnhold and Flemmig, 2010). Myeloperoxidase was associated with oxidative stress and inflammation and may reflect endothelial dysfunction in older patients with known cardiovascular disease (CVD) (Vita *et al.*, 2004). During inflammation, MPO is released into the extracellular fluid where it produces chlorinating and nitrating reactive species (Arnhold and Flemmig, 2010), potentiating further inflammation. Myeloperoxidase and its by-products contribute to tissue damage through the inactivation of nitric oxide and subsequently contributes to vasoconstriction (Arnhold and Flemmig, 2010, Ali M, 2016). Due to the pro-inflammatory and pro-oxidative functions of MPO, adverse effects on the structural integrity of the arterial wall may develop, confirming the mechanistic link between MPO and CVD (Rudolph TK, 2012).

Elevated circulating MPO levels were found to be associated with CVD (Heslop *et al.*, 2010, Anatoliotakis N, 2013) and may assist in CVD risk stratification (Schindhelm *et al.*, 2009). Higher levels of MPO were found to be more prevalent in African American than in white groups in the United States (Chen *et al.*, 2011, Khine *et al.*, 2017). It was indicated in coronary artery disease patients that elevated MPO levels might predict cardiovascular mortality (Zhang *et al.*, 2001, Scharnagl *et al.*, 2014). However, in young healthy individuals elevated levels of MPO are uncommon (Hoy *et al.*, 2001) and it is unknown whether retinal vascular calibres are associated with MPO in young populations.

The retina is a unique site where the microcirculation can be directly visualized (Campbell Matthew *et al.*, 2018), and provide the possibility of detecting retinal microvasculature changes, including arteriolar narrowing and venular widening (Liew *et al.*, 2008). Retinal

venular widening has been associated with inflammation, endothelial dysfunction, atherosclerosis and stroke (Ikram *et al.*, 2013, Al-Fiadh *et al.*, 2014), and retinal arteriolar narrowing with hypertension (Klein *et al.*, 2007, Shukla and Walsh, 2015).

Since previous studies linked both MPO and retinal vessel calibres to inflammation and oxidative stress, it is hypothesised that MPO may link positively with retinal venular widening and negatively with arteriolar narrowing. We therefore aimed to determine whether associations exist among the retinal microvascular calibres (central retinal arteriolar calibre (CRAE), central retinal venular calibre (CRVE) and arterio-venous ratio (AVR)) and MPO in young black and white adults.

Materials and methods

Study design and participant selection

This study formed part of the African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension (African-PREDICT) (Schutte *et al.*, 2019), conducted in Potchefstroom and surrounding areas in the North West Province, South Africa. The participants included apparently healthy black and white men and women (20-30 years of age) with a clinic brachial blood pressure of <140 and 90 mmHg. Individuals with any chronic disease such as human immunodeficiency virus, self-reported diabetes mellitus, liver disease, cancer, tuberculosis or renal disease, those using any chronic medication or pregnant and breastfeeding women were excluded. We included 577 participants with complete retinal vessel calibre data and excluded participants with missing MPO data (n=5) and those using anti-inflammatory medication (n=3). The final group consisted of 569 participants. This study complied with the criteria of the Declaration of Helsinki and was approved by the Health Research Ethics Committee of North-West University.

Organisational procedures

Participants arrived at the Hypertension Research and Training Clinic at 8:00 in the morning. They were requested to fast for 8 hours prior to participation. Once the procedures were explained, participants gave written informed consent. Thereafter biological sampling took place, and spot urine and blood samples were collected. Afterwards we collected data on anthropometry and cardiovascular measurements. After completion of fasting-dependent procedures, the participants were given a light meal (excluding caffeine).

Questionnaires

General health and demographic questionnaires were completed online and involved demographic and employment information and alcohol and tobacco use.

Anthropometry and physical activity monitoring

Body height (SECA 213 Portable Stadiometer; SECA, Hamburg, Germany), weight (SECA 813 Electronic Scales; SECA, Hamburg, Germany) and waist circumference (Lufkin Steel Anthropometric Tape; W606PM; Lufkin, Apex, USA) were obtained using standard procedures (Stewart *et al.*, 2011) and body mass index (BMI) was calculated. Participants were fitted with an ActiHeart physical activity monitor (CamNtech Ltd., England, UK) for a maximum of seven consecutive days to record total energy expenditure (TEE).

Blood pressure measurements

Brachial clinic blood pressure was measured using the Dinamap Procare 100 Vital Signs Monitor (GE Medical Systems, Milwaukee, USA) with an appropriately sized cuff. Participants were requested not to have smoked, exercised or eaten at least 30 minutes preceding the measurements. Duplicate systolic blood pressure, diastolic blood pressure and

heart rate measurements were taken on the left and right arm after the participants had been seated calmly for 5 minutes. We made use of the second systolic blood pressure reading of the left arm.

Retinal microvascular measurements

Thirty minutes prior to the retinal measurement, a drop of Tropicamide (1% Alcon) was administered in the right eye of participants to induce mydriatic conditions. The Retinal Vessel Analyzer (Imedos, Jena, Germany), fitted with a Zeiss Fundus camera FF-450 plus at a 50° angle, was used to capture monochrome and colour retinal images (using Visualis 2.81 software). The analyses of the images were performed using VesselMap2 software, and in cases where the image quality was insufficient, the colour image was used. The vessel trunks that were set within 0.5 and 1.0 optic disc diameters from the outer margin of the optic disc were marked either as an artery or a vein. Thereafter CRAE and CRVE were calculated using revised formulas proposed by Knudtson (Knudtson *et al.*, 2003). Only the 6 largest artery and 6 largest vein segments were included in the calculation. Both CRAE and CRVE were measured in measuring units (MU), where 1 MU is equivalent to 1 μM if the dimensions of the eye were comparable to the normal Gullstrand eye. The AVR was calculated as CRAE/CRVE. The reproducibility of the analysis was computed previously in a randomly selected sample (Malan *et al.*, 2015).

Blood sampling and biochemical analyses

All samples were prepared according to standardised protocols and stored at –80°C until the time of analysis. Serum samples were used to measure MPO as part of the MILLIPEX MAP Human Cardiovascular Disease Panel 2 (Merck Millipore, Darmstadt, Germany) with a multiplex immunoassay on a Luminex 200™ system (Luminex, Austin, TX, US). Various other markers were also measured including serum high sensitivity C-reactive protein (CRP),

total cholesterol, low-density lipoprotein-cholesterol (LDL-C), high- density lipoprotein-cholesterol (HDL-C), triglycerides, gamma-glutamyltransferase (GGT), creatinine and sodium fluoride plasma glucose levels (Cobas Integra 400 plus, Roche, Basel, Switzerland). Cotinine was determined by applying a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Whole EDTA blood samples were used to obtain full blood counts (Coulter AcT 5 diff Analyzer, Beckman Coulter, California, United States). Albumin and creatinine were analysed in spot urine samples (Cobas Integra 400plus, Roche, Basel, Switzerland) and the albumin-to-creatinine ratio (ACR) was calculated. Serum peroxides, hereafter referred to as reactive oxygen species (ROS), were determined using a high-throughput spectrophotometric assay and analysed on a Synergy HT microplate reader (BioTek, Winooski, VT, USA). Reactive oxygen species were reported in units, where 1 mg H_2O_2/L was equivalent to one unit (Hayashi *et al.*, 2007).

Statistical analyses

Data analysis was done using Statistica version 13.3 (TIBCO Software Inc., Palo Alto, CA, USA) and GraphPad Prism version 5 (GraphPad Software Inc., CA, USA) was used to prepare graphs. Descriptive statistics were used to assess whether the data was normally distributed via visual inspection of histograms and Q-Q plots. Logarithmic transformations were necessary for BMI, waist circumference, TEE, ACR, white blood cell count, CRP, GGT and MPO. If data was normally distributed, it was expressed as mean ± standard deviation and as geometric mean with 5^{th} and 95^{th} percentile boundaries for logarithmically transformed variables. We investigated the interactions of sex on the associations between retinal vascular markers (CRAE, CRVE and AVR) and MPO. Chi- square tests were used to compare proportions of categorical variables while independent T-tests were used to compare continuous variables between groups. The correlations of CRAE, CRVE and AVR with MPO

were explored using Pearson and partial correlations (adjusted for age, waist circumference and systolic blood pressure). Multi-variable adjusted regression analyses included the retinal vessel calibres (either CRAE, CRVE or AVR) and MPO while adjusting for age, waist circumference, systolic blood pressure, TEE, white blood cell count, GGT, HDL-C, cotinine and glucose. When CRAE was selected as dependent variable, CRVE was included as a covariate and vice versa (Liew *et al.*, 2007).

Results

We found significant interactions of sex on the associations between CRVE and MPO (p=0.027) and between AVR and MPO (p=0.027) (Supplementary table 1). Based on the aforementioned interactions and the literature (Chen *et al.*, 2011, Khine *et al.*, 2017), we stratified groups according to sex and ethnicity, as presented in Table 1.

Although all participants were between the ages of 20 and 30 years, the black men (p=0.001) and women (p=0.017) were younger than the white groups. The black men presented with lower BMI and waist circumference (p<0.001) than white men, whereas black women had higher BMI (p=0.001) and waist circumference (p=0.032) than white women. Glucose levels presented higher in white men and women (both p<0.001). Overall, the black groups had a more favourable lipid profile (all p<0.001) than the white groups, despite the HDL-cholesterol that presented lower (p<0.001) in the black women compared to white women. However, MPO levels did not differ between black and white men (p=0.71), or between black and white women (p=0.95).

Systolic blood pressure (SBP) (p=0.77), diastolic blood pressure (DBP) (p=0.44) and mean arterial pressure (MAP) (p=0.71) were similar among the men, but black women presented with higher SBP, DBP and MAP (all p≤0.001) than white women. Both CRAE (both p≤0.043) and AVR (both p≤0.003) were lower in the black men and women than in the white groups.

In black women, CRVE was larger (p=0.018), with no differences noted in the men (p=0.22).

Table 1: Characteristics of the study population

	Men			Women		
	Black	**White**	***P-value***	**Black**	**White**	***P-value***
N	109	124		175	161	
Age, years	24.2 ± 3.30	25.5 ± 2.94	0.001	24.7 ± 3.36	25.5 ± 2.76	0.017
Anthropometric measurements						
Body mass index, kg/m^2	21.4 (17.4; 27.9)	27.1 (20.5; 36.4)	<0.001	25.9 (17.9; 39.7)	24.0 (18.8; 33.9)	0.001
Waist circumference, cm	73.2 (63.1; 92.4)	90.0 (74.5; 117)	<0.001	78.2 (63.0; 103)	75.5 (63.4; 99)	0.032
Biochemical measurements						
Myeloperoxidase, ng/ml	106 (34.3; 366)	102 (36.5; 319)	0.71	104 (39.2; 345)	103 (39.9; 286)	0.95
Total cholesterol, mmol/L	3.82 ± 0.87	4.94 ± 1.22	<0.001	3.87 ± 0.85	4.76 ± 0.95	<0.001
HDL-cholesterol, mmol/L	1.33 ± 0.36	1.13 ± 0.30	<0.001	1.28 ± 0.34	1.62 ± 0.42	<0.001
LDL-cholesterol, mmol/L	2.33 ± 0.78	3.40 ± 1.18	<0.001	2.46 ± 0.83	2.89 ± 0.80	<0.001
Triglycerides, mmol/L	0.89 ± 0.51	1.36 ± 1.06	<0.001	0.75 ± 0.29	1.03 ± 0.61	<0.001
Glucose, mmol/L	4.17 ± 0.94	5.07 ± 0.65	<0.001	4.34 ± 0.77	4.67 ± 0.81	<0.001
C-reactive protein, mg/L	0.67 (0.10; 5.94)	0.94 (0.10; 8.08)	0.050	1.75 (0.20; 11.9)	1.06 (0.13; 11.0)	0.001
Reactive oxygen species, Units*	156.4 ±47.4	138.6 ± 40.4	0.017	231.8 ± 71.5	207.6 ± 104	0.060
White blood cell counts, $x10^9/L$	4.82 (3.20; 8.30)	5.60 (3.6; 9)	<0.001	5.20 (2.60; 8.60)	5.67 (3.60; 9.20)	0.018
Albumin-to-creatinine ratio, mg/mmol	4.08 (1.41; 68.2)	3.34 (1.41; 17.8)	0.17	7.59 (1.48; 155)	4.64 (1.58; 110)	0.92

Cardiovascular measurements						
Systolic blood pressure, mmHg	122 ± 12	122 ± 9	0.77	113 ± 11	108 ± 11	0.001
Diastolic blood pressure, mmHg	81 ± 9	80 ± 9	0.44	78 ± 8	75 ± 8	<0.001
Mean arterial pressure, mmHg	98 ± 9	97 ± 8	0.71	92 ± 8	89 ± 8	<0.001
Central retinal artery equivalent, MU	157 ± 13	160 ± 11	0.043	156 ± 11	162 ± 12	<0.001
Central retinal vein equivalent, MU	249 ± 19	246 ± 16	0.22	252 ± 17	248 ± 18	0.018
Arterio-venous ratio	0.63 ± 0.05	0.65 ± 0.05	0.003	0.62 ± 0.04	0.66 ± 0.05	<0.001
Lifestyle measurements						
Total energy expenditure, kCal/kg/day	2180 (1803; 2712)	2568 (1920; 3380)	<0.001	2116 (1557; 3157)	2105 (1626; 3170)	0.85
Cotinine, ng/ml	107 ± 145	72.7 ± 128	0.06	28 ± 74.7	24.1 ± 69.2	0.63
Gamma-glutamyl transferase, U/L	27.0 (13.0; 82.8)	25.8 (10.8; 64.5)	0.57	22.6 (10.3; 59.7)	14.3 (7.0; 35.9)	<0.001

Values are arithmetic mean ± standard deviation, geometric mean (5th to 95th percentile interval), or number of participants (%).
Abbreviations: n, number of participants; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Reactive oxygen species was available for only 374 participants.

In unadjusted (Figure 1 and Supplementary table 2), partially adjusted (Table 2) and fully adjusted (Table 3) linear regression analyses, CRAE (adj. R^2=0.29; β=0.17; p=0.026) and AVR (adj. R^2=0.052; β=0.18; p=0.041.)

associated positively with MPO only in black women. In white men, a positive association was found between CRVE and MPO (adj. R^2=0.25; β=0.19; p=0.032). We found no other associations between retinal vessel calibres and MPO in the other groups

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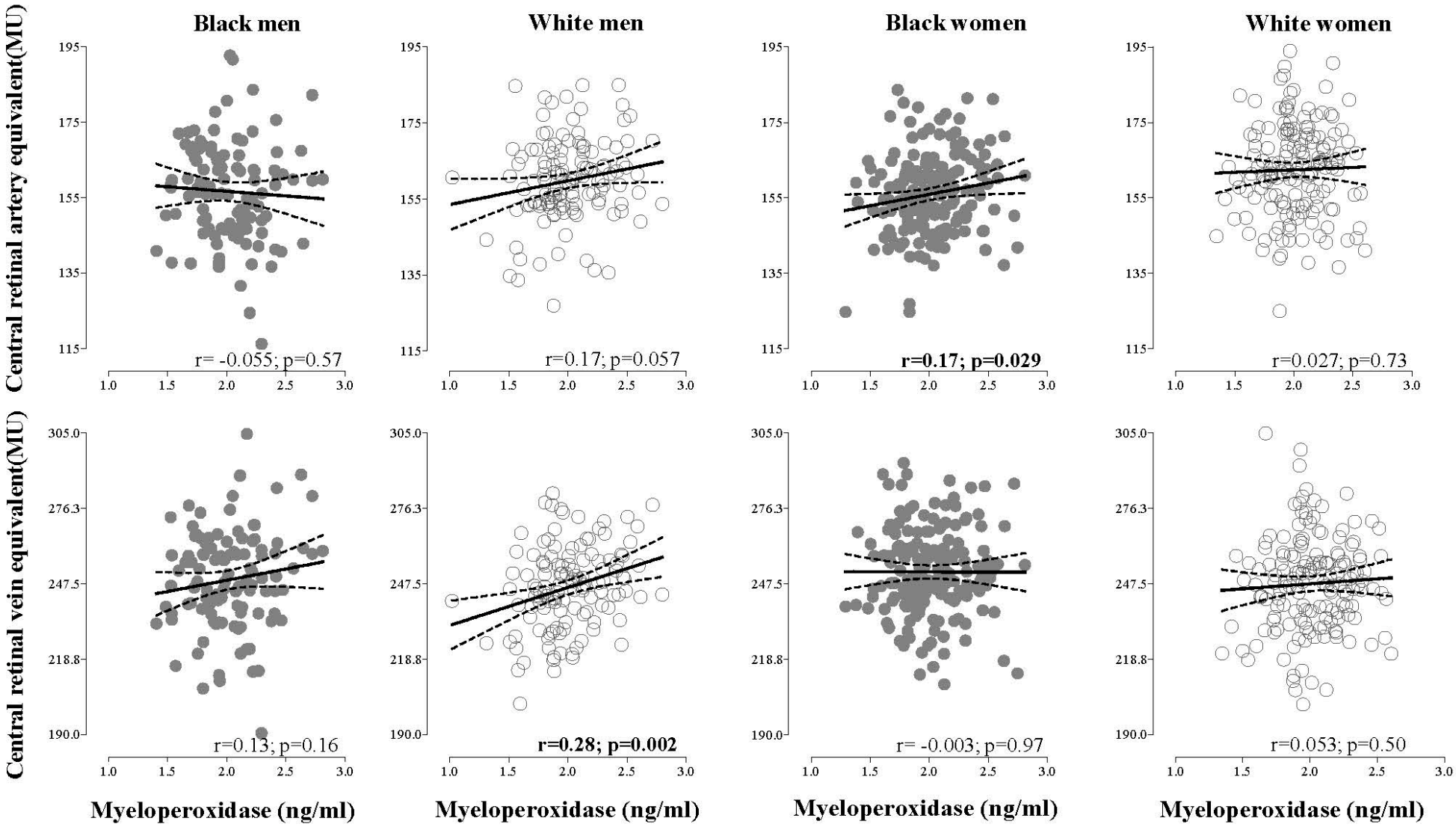

Figure 1: Scatterplots representing the linear relationship between retinal vessel calibres and MPO.

Table 2: Partial correlations between retinal vessel calibres and myeloperoxidase in black and white men and women.

	Myeloperoxidase (ng/ml)	
	Men	
	Black	**White**
	N=109	**N=124**
Central retinal artery equivalent, MU	r= -0.068; p=0.49	r=0.16; p=0.073
Central retinal vein equivalent, MU	r=0.14; p=0.15	**r=0.28; p=0.002**
Arterio-venous ratio	r= -0.19; p=0.052	r= -0.11; p=0.24
	Women	
	Black	**White**
	N=175	**N=161**
Central retinal artery equivalent, MU	**r=0.17; p=0.027**	r=0.057; p=0.48
Central retinal vein equivalent, MU	r= -0.025; p=0.74	r=0.048; p=0.55
Arterio-venous ratio	**r=0.18; p=0.019**	r= -0.017; p=0.98

Adjusted for age, waist circumference and systolic blood pressure. Bold values indicate $p<0.05$.

Table 3: Summary of forward stepwise multiple regression analyses of retinal microvascular calibres and myeloperoxidase

	Black men (n=109)		White men (n=124)		Black women (n=175)		White women (n=161)	
	Central retinal artery equivalent (MU)							
Adjusted R^2	0.28		0.26		0.29		0.40	
	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
MPO, ng/ml	-	-	-	-	**0.17 (0.022; 0.32)**	**0.026**	-	-
CRVE, MU	**0.39 (0.19; 0.58)**	**<0.001**	**0.47 (0.30; 0.64)**	**<0.001**	**0.39 (0.25; 0.54)**	**<0.001**	**0.56 (0.42; 0.70)**	**<0.001**
Age, years	-	-	0.16 (-0.017; 0.33)	0.081	-	-	-	-
WC, cm	-	-	-	-	**-0.19 (-0.34; -0.031)**	**0.020**	-	-
Glucose, mmol/L	-0.15 (-0.35, 0.037)	0.12	-	-	-	-	-	-
WBC count, $x10^9$/L	-0.19 (-0.37; 0.004)	0.059	-	-	-	-	-	-
SBP, mmHg	**-0.28 (-0.47; 0.082)**	**0.007**	**-0.24 (-0.42; -0.070)**	**0.008**	**-0.20 (-0.36; -0.050)**	**0.012**	**-0.32 (-0.45; -0.18)**	**<0.001**
	Central retinal vein equivalent (MU)							
Adjusted R^2	0.28		0.25		0.19		0.34	
MPO, ng/ml	0.18 (-0.10; 0.37)	0.067	**0.19 (0.020; 0.37)**	**0.032**	-	-	-	-
CRAE, MU	**0.48 (0.29; 0.67)**	**<0.001**	**0.41 (0.24; 0.59)**	**<0.001**	**0.40 (0.24; 0.56)**	**<0.001**	**0.61 (0.47; 0.76)**	**<0.001**
Age, years	-	-	-	-	-0.14; -0.30; 0.019)	0.087	-	-
GGT, U/L	**0.22 (0.030; 0.41)**	**0.026**	-	-	-	-	-	-
Glucose, mmol/L	**0.21 (0.025; 0.40)**	**0.029**	-	-	-	-	-0.11 (-0.25; 0.026)	0.12
Cotinine, ng/ml	-	-	0.15 (-0.026; 0.32)	0.10	-	-	-	-
SBP, mmHg	-	-	-	-	-	-	**0.20 (0.054; 0.35)**	**0.008**
	Arterio-venous ratio							
Adjusted R^2	0.093		00.062		0.052		0.067	
MPO, ng/ml	-0.18 (-0.39; 0.031)	0.098	-	-	**0.18 (0.01; 0.35)**	**0.041**	-	-
Age, years	-	-	0.17 (-0.020; 0.37)	0.082	-	-	-	-
WC, cm	-0.21 (-0.42; 0.003)	0.057	-	-	**-0.20 (-0.37; -0.033)**	**0.021**	-	-
Glucose, mmol/L	**-0.22 (-0.44; 0.011)**	**0.042**	-	-	-	-	-	-
SBP, mmHg	-	-	**-0.26 (-0.45; -0.062)**	**0.011**	-	-	**-0.35 (-0.51; -0.19)**	**<0.001**

Variables included in the models were age, waist circumference, cotinine, gamma-glutamyltransferase, glucose, high-density lipoprotein cholesterol, systolic blood pressure, myeloperoxidase, total energy expenditure, white blood cell counts. When CRAE was selected as dependent variable, CRVE was included as a covariate and vice versa. *Abbreviations*: n, number of participants; CI, confidence interval; β, beta; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; GGT, gamma-glutamyltransferase; MPO, myeloperoxidase; WC, waist circumference; WBC, white blood cell; SBP, systolic blood pressure. Bold values denote $p<0.05$

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Sensitivity analyses

Myeloperoxidase is known as both an inflammatory and oxidative stress marker (Anatoliotakis N, 2013). Therefore, to clarify the nature of MPO (inflammatory or oxidative stress related) in the positive association between CRVE and MPO found in white men, MPO was firstly substituted with CRP, a marker of inflammation (Supplementary table 3). No association was evident between CRVE and CRP in this analysis. Thereafter, MPO was replaced with ROS as main independent variable in multiple regression analyses (Supplementary table 4), and a positive association between CRVE and ROS was found in white men (adj. R^2=0.23; β=0.21; p=0.047) only. When evaluating MPO by including ROS/CRP in the same model, no significant associations were seen in any of the groups between measures of the retinal microvascular and MPO or ROS/CRP.

Discussion

We investigated associations between retinal microvascular calibres and the pro-inflammatory and pro-oxidative biomarker, MPO, in young black and white adults. In white men, retinal venular calibre associated independently and positively with MPO, although neither MPO levels nor the retinal venular calibres differed between black and white men. Both the above mentioned and the sensitivity analyses suggests the involvement of MPO as pro-oxidant in retinal microvascular changes. In black women, the retinal arteriolar calibre and the arterio-venous ratio were found to be independently and positively associated with MPO.

Our first main finding is the positive association between the retinal venular calibre and MPO observed in white men. To our knowledge, previous studies have not yet investigated the association between retinal venular calibres and MPO, but the associations between other

inflammatory markers (CRP) and antioxidant enzyme activity (superoxide dismutase and glutathione peroxidase) with retinal venular calibres have been established (Daien *et al.*, 2013). Myeloperoxidase relates to CVD via its effects through inflammation (Kimak *et al.*, 2018) and oxidative stress (Anatoliotakis N, 2013). In this study, when MPO was replaced with the inflammatory marker CRP, CRP did not associate with CRVE, despite higher levels of CRP in the white men compared to those in black men. In further support of a pro-oxidative role of MPO, we found oxidative stress to be associated with CRVE when MPO was replaced with serum peroxides in the multiple variable adjusted regression model. This potential mechanism may explain our finding and is in agreement with the literature indicating a role of elevated MPO to promote oxidative stress and endothelial dysfunction (Vita JA, 2004, Nicholls SJ, 2005, Karakas M, 2012, Rudolph TK, 2012, Anatoliotakis N, 2013). Myeloperoxidase exerts its effects on the heart and vessels through the direct effects of reactive species on the arterial wall, and the oxidative damage may lead to endothelial dysfunction (Anatoliotakis N, 2013).

The underlying pathophysiological mechanism for an association between CRVE and endothelial dysfunction remains unclear. It has been speculated that endothelial dysfunction and inflammatory processes play essential roles in the development of wider retinal venular and arteriolar calibres in patients with diabetes (Klein *et al.*, 2006a). We also found that despite the young age of the white men, they presented with a profile that may reflect a higher metabolic risk, including higher waist circumference, total cholesterol and CRP when compared with black men. A wider retinal venular calibre was also previously associated with a higher risk for developing cardiovascular disease and is known to independently predict incident stroke (Baker *et al.*, 2008, McGeechan *et al.*, 2009). This may help to explain the positive association we found between a wider retinal venular calibre and increased MPO levels in white men only.

We also found an independent positive association between the retinal arteriolar calibre and MPO in the black women. A study conducted in patients aged ≥ 60 years suggested that a positive association between the retinal arteriolar calibre and the antioxidant, glutathione peroxidase, was cardioprotective, but they also found a wider arteriolar calibre to be associated with smoking (Daien *et al.*, 2013). In addition, a wider CRAE has previously been associated with diabetes (Nguyen *et al.*, 2008, Ikram *et al.*, 2013) and heart failure, independent of traditional cardiovascular risk factors, including coronary artery disease, diabetes or hypertension (Phan *et al.*, 2015a). The potential mechanism that may explain the positive association between the retinal arteriolar calibre and MPO in a young healthy population remains unclear and needs to be investigated further. An argument that may serve as a possible explanation for our finding is that early retinal arteriolar widening may lead to the development of inflammation and retinal microvascular damage, which can progress to retinopathy, as commonly seen with incident diabetes (Yau *et al.*, 2012, Phan *et al.*, 2015b).

A narrower retinal arteriolar calibre is traditionally associated with hypertension (Klein *et al.*, 2006a, Liew *et al.*, 2008, von Hanno *et al.*, 2014), incident stroke and coronary heart disease (Wong *et al.*, 2006). Widening in the retinal arteriolar calibre has been reported to indicate a lower CVD risk (Daien *et al.*, 2013). However, the positive association between the retinal arteriolar calibre and MPO in our study formed part of the same regression model where the retinal arteriolar calibre was also inversely associated with systolic blood pressure and waist circumference, which confirms previous studies reporting inverse associations between CRAE and blood pressure (Klein *et al.*, 2006b), as well as CRAE and waist circumference (Yatsuya *et al.*, 2010). Our study is the first to report a positive association of CRAE with MPO (while confirming other established associations).

The results of our study should be interpreted within the context of the strengths and limitations. Our study had a cross-sectional design and was not representative of the entire South African population. Due to its design, causality cannot be inferred. A strength of our study is the inclusion of detailed measures of the retinal microvasculature - and young healthy individuals allowing us to investigate the results without the influence of cardiovascular pathologies and related inflammatory conditions. However, some of our findings may have been influenced by unknown confounding factors.

In conclusion, in young, apparently healthy participants, we found an independent positive association between the retinal venular calibre and MPO, only in white men, underlining the potential pro-oxidative role of MPO. In black women, the retinal arteriolar calibre was positively associated with MPO and may suggest early retinal microvascular changes. The evaluation of retinal imaging along and MPO levels, at subclinical level, may contribute to risk stratification in young, apparently healthy individuals.

Clinical significance

The investigation of MPO as both an oxidative stress and pro-inflammatory marker is of clinical importance as it was found to be involved in, amongst others, atherosclerosis, stroke, coronary heart disease and hypertension. Considering that this study was conducted in a young and apparently healthy population, the associations we observed between MPO and retinal vessel markers further support the clinical potential of MPO as a marker of subclinical cardiovascular risk.

Retinal imaging is a convenient and non-invasive tool for evaluating potential early cardiovascular manifestations. Our study found that retinal calibres associated independently with MPO in young adults. These findings suggest that, at subclinical level, the evaluation of both retinal imaging and MPO levels may contribute to risk stratification in individuals free

from overt CVD.

Conflicts of interest

The authors report that they have no conflict of interest.

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Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors; therefore, the NRF does not accept any liability in this regard.

Supplementary tables

Supplementary table 1: Interaction terms of sex on the relationship between retinal microvascular markers and myeloperoxidase.

	Myeloperoxidase * sex (n=569)
Central retinal artery equivalent, MU	0.82
Central retinal vein equivalent, MU	**0.027**
Arterio- venous ratio	**0.027**

Supplementary table 2: Pearson correlations between measures of the retinal microvasculature and myeloperoxidase.

	Myeloperoxidase (ng/ml)	
	Men	
	Black N=109	**White N=124**
Central retinal artery equivalent, MU	r= -0.055; p=0.57	r=0.17; p=0.057
Central retinal vein equivalent, MU	r=0.13; p=0.16	**r=0.28; p=0.002**
Arterio- venous ratio	r= -0.17; p=0.079	r= -0.089; p=0.33
	Women	
	Black N=175	**White N=161**
Central retinal artery equivalent, MU	**r=0.17; p=0.029**	r=0.027; p=0.73
Central retinal vein equivalent, MU	r= -0.003; p=0.97	r=0.053; p=0.50
Arterio- venous ratio	**r=0.16; p=0.032**	r= -0.035; p=0.66

Supplementary table 3: Summary of forward stepwise multiple regression analyses of retinal microvascular calibres and C-reactive protein.

	Black men (n=109)		White men (n=124)		Black women (n=175)		White women (n=161)	
	Central retinal artery equivalent (MU)							
Adjusted R^2	0.28		0.26		0.29		0.42	
	β (95% CI)	P-value	*β (95% CI)*	P-value	*β (95% CI)*	P-value	*β (95% CI)*	P-value
CRP mg/L	-	-	**-0.17 (-0.34; -0.004)**	**0.048**	-	-	**-0.15 (-0.28; -0.022)**	**0.024**
CRVE, MU	**0.39 (0.19; 0.58)**	**<0.001**	**0.46 (0.29; 0.63)**	**<0.001**	**0.39 (0.25; 0.54)**	**<0.001**	**0.57 (0.43; 0.70)**	**<0.001**
WC, cm	-	-	-	-	**-0.17 (-0.32; -0.011)**	**0.038**	-	-
Glucose, mmol/L	-0.15 (-0.35, 0.037)	0.12	-	-	-	-	-	-
WBC count, $x10^9$/L	-0.19 (-0.37; 0.004)	0.059	-	-	-	-	-	-
SBP, mmHg	**-0.28 (-0.47; 0.082)**	**0.007**	**-0.20 (-0.37; -0.034)**	**0.021**	**-0.22 (-0.37; -0.059)**	**0.008**	**-0.31 (-0.44; -0.18)**	**<0.001**
	Central retinal vein equivalent (MU)							
Adjusted R^2	0.26		0.22		0.21		0.36	
CRP mg/L	-	-	-	-	**0.16 (0.004; 0.31)**	**0.047**	**0.15 (0.007; 0.29)**	**0.041**
CRAE, MU	**0.46 (0.28; 0.66)**	**<0.001**	**0.41 (0.24; 0.59)**	**<0.001**	**0.40 (0.24; 0.56)**	**<0.001**	**0.63 (0.49; 0.78)**	**<0.001**
Age, years	-	-	-	-	-0.15; -0.31; 0.005)	0.060	-	-
GGT, U/L	**0.22 (0.034; 0.42)**	**0.026**	-	-	-	-	-	-
Glucose, mmol/L	0.19 (0.004; 0.38)	0.053	-	-	-	-	-0.13 (-0.27; 0.008)	0.067
Cotinine, ng/ml	-	-	0.16 (-0.016; 0.34)	0.077	-	-	-	-
SBP, mmHg	-	-	-	-	-	-	**0.20 (0.054; 0.35)**	**0.008**
	Arterio-venous ratio							
Adjusted R^2	0.072		0.064		0.028		0.015	
CRP mg/L	-	-	-0.18 (-0.37; 0.013)	0.071-	-	-	**-0.20 (-0.36; -0.040)**	**0.016**
WC, cm	**-0.22 (-0.44; 0.011)**	**0.043**	-	-	**-0.19 (-0.36; -0.018)**	**0.033**	-	-
Glucose, mmol/L	**-0.20 (-0.41; 0.013)**	**0.070**	-	-	-	-	0.13 (-0.030; 0.29)	0.12
SBP, mmHg	-	-	**-0.21 (-0.41; -0.023)**	**0.031**	-	-	**-0.34 (-0.50; -0.18)**	**<0.001**

Variables included in the models were age, waist circumference, cotinine, gamma-glutamyltransferase, glucose, high-density lipoprotein cholesterol, systolic blood pressure, C-reactive protein, total energy expenditure, white blood cell counts. When CRAE was selected as dependent variable, CRVE was included as a covariate and vice versa. *Abbreviations*: n, number of participants; CI, confidence interval; β, beta;

CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; GGT, gamma-glutamyltransferase; WC, waist circumference; WBC, white blood cell; SBP, systolic blood pressure; CRP, C-reactive protein. Bold values denote $p<0.05$

Supplementary table 4: Summary of forward stepwise multiple regression analyses of retinal microvascular calibres and reactive oxygen species.

	Black men (n=109)		White men (n=124)		Black women (n=175)		White women (n=161)	
	Central retinal artery equivalent (MU)							
Adjusted R^2	0.24		0.23		0.25		0.40	
	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value	β (95% CI)	P-value
CRVE, MU	**0.38 (0.16; 0.60)**	**<0.001**	**0.46 (0.26; 0.66)**	**<0.001**	**0.39 (0.21; 0.58)**	**<0.001**	**0.56 (0.41; 0.71)**	**<0.001**
SBP, mmHg	**-0.26 (-0.48; -0.044)**	**0.022**	**-0.22 (-0.42; -0.012)**	**0.042**	**-0.22 (-0.42; -0.031)**	**0.025**	**-0.32 (-0.47; -0.17)**	**<0.001**
WC, cm	-	-	-	-	-0.17 (-0.35; 0.035)	0.11	-	-
	Central retinal vein equivalent (MU)							
Adjusted R^2	0.25		0.23		0.18		0.33	
ROS, Units	-	-	**0.21 (0.007; 0.42)**	**0.047**	-	-	-	-
CRAE, MU	**0.47 (0.26; 0.68)**	**<0.001**	**0.44 (0.24; 0.64)**	**<0.001**	**0.43 (0.24; 0.62)**	**<0.001**	**0.62 (0.45; 0.78)**	**<0.001**
SBP, mmHg	-	-	-	-	-	-	**0.20 (0.031; 0.36)**	**0.022**
GGT, U/L	**0.22 (0.012; 0.44)**	**0.043**	-	-	-	-	-	-
Glucose, mmol/L	0.19 (-0.022; 0.40)	0.084	-	-	-	-	-	-
	Arterio-venous ratio							
Adjusted R^2	0.070		0.038		0.023		0.11	
SBP, mmHg	-	-	-0.23 (-0.45; 0.002)	0.056	-0.19 (-0.39; 0.024)	0.090	**-0.35 (-0.53; -0.16)**	**<0.001**
WC, cm	-0.23 (-0.47; 0.005)	0.060	-	-	-	-	-	-
Glucose, mmol/L	-0.20 (-0.44; 0.038)	0.10	-	-	-	-	-	-

Variables included in the models were age, waist circumference, cotinine, gamma-glutamyltransferase, glucose, high-density lipoprotein cholesterol, systolic blood pressure, re3active oxygen species, total energy expenditure, white blood cell counts. When CRAE was selected as dependent variable, CRVE was included as a covariate and vice versa. *Abbreviations*: n, number of participants; CI, confidence interval; β, beta; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; GGT, gamma-glutamyltransferase; WC, waist circumference; WBC, white blood cell; SBP, systolic blood pressure; ROS, reactive oxygen species. Bold values denote p<0.05

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Chapter 4

Final remarks, conclusion and recommendations for future studies

1. Introduction

This chapter provides an overview of the main findings and conclusions regarding the manuscript titled, *Myeloperoxidase and the vasculature in young adults: The African-PREDICT Study*. A comparison is drawn between the original hypotheses as set out in Chapter 1, the results of this study as well as with existing literature. This is followed by the limitations and strengths of the study as well as recommendations for future research regarding myeloperoxidase (MPO) and the microvasculature.

The aim of this study was to determine whether measures of the microvasculature associate with MPO in young South African adults.

2. Summary of main findings

The main findings of this study will be addressed in accordance with the original hypotheses. All hypotheses made were at first set out for a study population (n=577) that included young and apparently healthy black and white men and women from South Africa.

Retinal microvascular changes can be characterised by arterial narrowing (Wong and McIntosh, 2005) and venular widening (Baker *et al.*, 2008).

Seeing that previous studies have linked both MPO and retinal vessel calibres to inflammation and oxidative stress, it was hypothesised that MPO levels will be higher in black adults when compared with white adults, and that the central retinal venular calibre (CRVE) will be wider in black adults when compared with white adults whereas the central retinal arteriolar calibre (CRAE) will be narrower in black adults when compared with white adults. It was also hypothesised that CRAE will associate negatively with MPO in young adults and that CRVE will associate positively with MPO in young adults.

A summary of the main findings of the research article (Chapters 3) is as follows:

Hypothesis 1: MPO levels will be higher in black adults when compared with white adults and CRVE will be wider in black adults when compared with white adults whereas CRAE will be narrower in black adults when compared with white adults. MPO levels did not differ between ethnic groups (men and women, respectively). Central retinal arteriolar calibre was narrower in black adults (men and women, respectively) when compared with whites. Central retinal venular calibre was wider in black women - than in white women (but did not differ in men). Therefore, the first hypothesis is partially accepted.

Hypothesis 2: Central retinal arteriolar equivalent will associate negatively with myeloperoxidase in young adults.

In unadjusted, partially adjusted and fully adjusted models, CRAE and AVR were shown to have a positive association with MPO only in black women. Therefore, the second hypothesis is rejected.

Hypothesis 3: Central retinal venular equivalent will associate positively with myeloperoxidase in young adults.

A positive association was shown between CRVE and MPO, but only in white men. Therefore, we partially accept this hypothesis.

Discussion of main findings and comparison with the literature

When our results were compared with data from other studies, it became evident that certain findings confirm previous observations while others differed from it.

The association between the central retinal arteriolar calibre and myeloperoxidase.

Generally, retinal arterial narrowing is associated with hypertension (Figure 1), which is regarded as a smaller AVR and narrower CRAE, while CRVE is wider or remained unchanged (Wong *et al.*, 2006). In previous studies (Ikram *et al.*, 2004) (Wong *et al.*, 2006), a narrower retinal arterial calibre was associated with higher systolic blood pressure, current alcohol consumption and a higher body mass index (BMI) which indicated that the retinal calibres may play an independent role in predicting CVD.

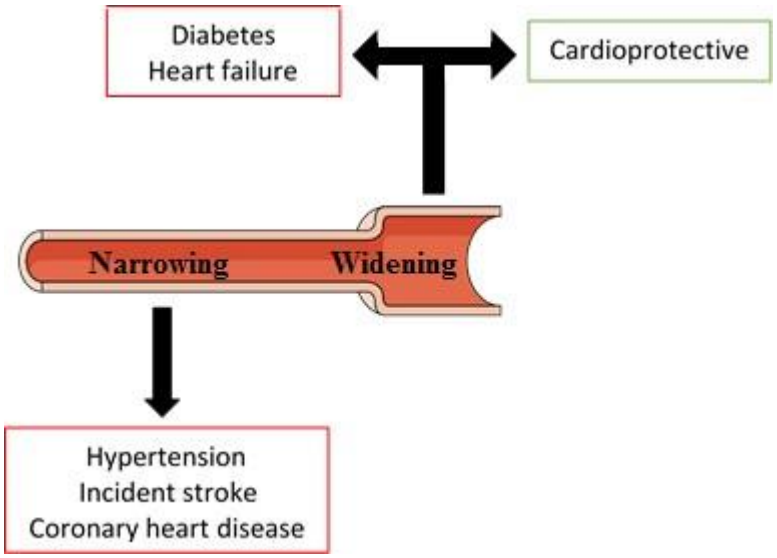

Figure 1: A schematic representation of the generally known associations between arteriolar narrowing and arteriolar widening (Wong *et al.*, 2006, Daien *et al.*, 2013).

However, the focus of our study is on MPO and its association with retinal microvascular calibres in young adults and is the first to report a positive association of CRAE with MPO. It is important to note that the positive association between the retinal arteriolar calibre and MPO in our study formed part of the same regression model in which the retinal arteriolar calibre was also inversely associated with systolic blood pressure and waist circumference. These

inverse associations are in agreement with previous studies that reported inverse associations between arterial narrowing and blood pressure (Klein *et al.,* 2006), as well as retinal arterial narrowing and waist circumference (Yatsuya *et al.,* 2010).

A study conducted to investigate the association between antioxidant enzyme activity and inflammatory markers with retinal vascular calibres (Daien *et al.,* 2013) found that arterial widening is associated with higher activity of the antioxidant enzyme, GPx-3, after adjusting for age and sex. They suggested that the retinal microvasculature is sensitive to systemic oxidative stress, independent of known CVD risk factors (Daien *et al.,* 2013). It was speculated that a wider retinal arteriolar calibre indicated a lower risk for developing CVD (Figure 1) (Daien *et al.,* 2013). The mechanism to explain the positive association between the retinal arteriolar calibre and MPO in the black women remains unclear and needs to be further investigated.

The association between the central retinal venular calibre and myeloperoxidase

Retinal venular widening has been associated with inflammation, endothelial dysfunction, atherosclerosis and stroke (Figure 2) (Ikram *et al.,* 2013, Al-Fiadh *et al.,* 2014). In previous studies, a widening of CRVE was associated with, amongst others, higher levels of C-reactive protein (CRP), current cigarette smoking, a higher BMI, higher levels of glucose and cholesterol, higher plasma triglycerides, plasma LDL- cholesterol and lower levels of HDL-cholesterol.

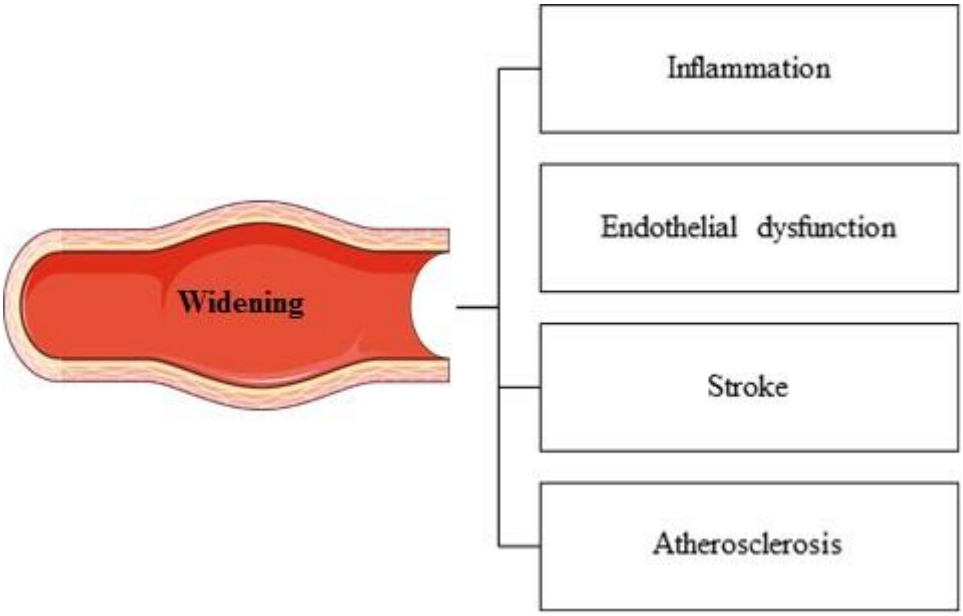

Figure 2: A schematic representation of previous associations with central retinal venular calibre widening Ikram *et al.*, 2013, Al-Fiadh *et al.*, 2014.

Myeloperoxidase is known as both an inflammatory and oxidative stress marker (Anatoliotakis N, 2013). To better clarify our result of a positive association between retinal venular widening and MPO, we performed a sensitivity analysis.

When including CRP as main independent variable in the multiple regression analysis instead of MPO, no association was evident between CRVE and CRP. However, when we replaced MPO with reactive oxygen species (ROS) as main independent variable in multiple regression analyses, we found a positive association between CRVE and ROS in white men only. This suggests that MPO may be considered a pro-oxidative rather than a pro-inflammatory marker in this cohort.

Limitations

A limitation of this study is the cross-sectional design. A further limitation is that it is difficult to explain why only certain groups (white men and black women) exhibited clear associations

between retinal microvascular calibres and MPO, whereas no such associations existed in the other groups (black men and white women). This may be due to the study population being young and apparently healthy or that MPO and the retinal microvascular calibres are influenced by other factors we might not have considered. Only the first 577 participants had retinal microvascular calibre data available for this study. Additional retinal microvascular measurements, which include wall-to-lumen ratio, measured using scanning laser Doppler flowmetry, will hold advantages as this would provide further insight into microvascular changes that occur over a longitudinal period.

Recommendations

- Since the associations between the retinal vessel calibres and MPO still remain unexplained, longitudinal studies examining the retinal microvascular changes and the changes in MPO levels may provide clarity on these associations and the potential mechanisms thereof.
- The association between the wall-to-lumen ratio needs to be investigated further in terms of the association with MPO, as it may clarify in more detail the potential vascular role of MPO.
- A randomised larger population which represents the entire South African population group is needed to clarify whether our results are widely applicable.

Conclusion

An independent positive association was found between the retinal venular calibre and MPO, only in white men, which may highlight the potential pro-oxidative role of MPO. The retinal arteriolar calibre was positively associated with MPO, in black women, and may suggest early retinal microvascular changes. It should be considered that this study was done in young healthy

participants, and therefore results may not yet be as prominent as it would be in older or unhealthy patients, but it does indicate that clear associations are already present at young ages.

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Appendix A

Author instructions

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This article followed the specific guidelines as set out by the *Biomarker* journal specified below. A full list of details regarding the author's instructions is available at: https://www.tandfonline.com/action/authorSubmission?journalCode=ibmk20&page=instruction s.

1. The cover page should include the authors' full names, affiliations, postal addresses, telephone numbers and email addresses. Where available, include ORCiDs and social media handles (Facebook, Twitter or LinkedIn). Indicate the corresponding author, with their email address.
2. A structured abstract of no more than 200 words should cover (in the following order) the purpose of the article, its materials and methods (the experimental system and procedures used), the results and conclusions. Include 5-10 keywords.
3. The clinical significance of the manuscript is a mandatory section and should include a short paragraph or bulleted list of no more than 100 words.
4. Please supply all details required by your funding and grant-awarding bodies.
5. Include a disclosure statement to acknowledge any financial interest or benefit that has arisen from the direct applications of your research.
6. Supplemental material can be a video, dataset, file set, sound file or anything which supports (and is pertinent to) your paper.
7. Figures should be high quality (1200 dpi for line art, 600 dpi for grayscale and 300 dpi for colour, at the correct size) and should be supplied in one of our preferred file formats: EPS, PS, JPEG, GIF, or Microsoft Word (DOC or DOCX).
8. Tables should present new information rather than duplicating what is in the text. Readers should be able to interpret the table without reference to the text. Please supply editable files.
9. Use SI units (non-italicized).

Appendix B

Approval from the Health Research Ethics Committee

Private Bag X1290,
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Tel: 018 299-1111/2222
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Web: http://www.nwu.ac.za

Research Ethics Regulatory Committee
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ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North West University Health Research Ethics Committee (NWU-HREC) on 09/08/2018, the NWU Health Research Ethics Committee hereby approves your study as indicated below. This implies that the North-West University Research Ethics Regulatory Committee (NWU-RERC) grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Myeloperoxidase and the vasculature in young adults: The African-PREDICT study.
Study Leader/Supervisor (Principal Investigator)/Researcher: Prof CMC Mels
Student: A Brelage

Ethics number:

Institution Study Number Year Status

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

N W U - 0 0 0 7 0 - 1 8 - A 1

Application Type: Single study
Commencement date: 2018/08/09
Expiry date: 2019/08/31

Risk: Minimal

Approval of the study is initially provided for a year, after which continuation of the study is dependent on receipt and review of an annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The study leader/supervisor (principle investigator)/researcher must report in the prescribed format to the NWU-HREC:*
  - *annually (or as otherwise requested) on the monitoring of the study, whereby a letter of continuation will be provided, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form.*

Should any amendments to the proposal be deemed necessary during the course of the study, the study leader/researcher must apply for approval of these amendments at the NWU-HREC, prior to

implementation. Should there be any deviations from the study proposal without the necessary
approval of such amendments, the ethics approval is immediately and automatically forfeited.

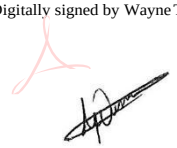
- *Annually a number of studies may be randomly selected for an external audit.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-RERC and NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion*
    of the study;
  - *to ask further questions, seek additional information, require further modification or monitor* the conduct of your research or the informed consent process;
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-HREC or*
      that information has been false or misrepresented;
    - *submission of the annual (or otherwise stipulated) monitoring report, the required* amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and / or
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information or any report templates via* [*Ethics*- HRECApply@nwu.ac.za](mailto:HRECApply@nwu.ac.za) or 018 299 1206.

Special in process conditions of the research for approval (if applicable): None

The NWU-HREC would like to remain at your service as scientist and researcher and wishes you well with your study. Please do not hesitate to contact the NWU-HREC or the NWU-RERC for any further enquiries or requests for assistance.

Yours sincerely

Digitally signed by Wayne Towers



Prof Wayne Towers

Appendix C

Turn it in Report

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ORIGINALITY REPORT

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13	edoc.ub.uni-muenchen.de Internet Source	1%

Appendix D

Certificate of Language Editing

13 August 2019

I, Ms Cecilia van der Walt, hereby declare that I took care of the editing of the dissertation of Ms Annica Brelage titled *Myeloperoxidase and the Vasculature in Young Adults: The African-PREDICT study*.

MS *CECIUA* VAN DER WALT

BA *(Cum I.tJude)*
THED *(Cum I.tJude),*
Plus Language editing and translation at Honours level *(Cum I.tJude),* Plus Accreditation with SATI for Afrikaans and translation Registration number with SATI: 1000228

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