Vascular structure and inflammation in a bi-ethnic South African population: The SABPA study

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Dissertation submitted in partial fulfilment of the requirements for the degree *Master of Science* in *Cardiovascular Physiology* at the

North-West University

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Graduation May 2018

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ACKNOWLEDGMENTS

I would like to express my sincerest thanks to the following people:

- Dr S Botha and Dr L Lammertyn for their unwavering support and motivation throughout the year. In addition, for their continued guidance, boundless knowledge and advice without which this MHSc would not be possible. Thank you for inspiring me throughout this year. I have great admiration for the both of you.
- All the participants for their time and willingness to participate in the SABPA study.
- All HART staff and students for their hard work and efforts in collecting data.
- To my mom, thank you for all the prayers, love and support throughout this project.
- Rohan, for inspiring and encouraging me every day.
- All my closest family and friends for always being there and supporting me during this year.

And lastly, a special word of thanks to the Lord for his grace and for giving me the opportunity and strength to follow my passion.

PREFACE

This dissertation for the MHSc study of Ms C Swart on “Vascular structure and inflammation in a bi-ethnic South African population: The SABPA study”, is submitted in fulfilment of the requirements for the degree *Master of Health Sciences in Cardiovascular Physiology* at the North-West University.

The dissertation is presented in article format and consists of one article (presented in Chapter 3), as approved by the North-West University’s guidelines for postgraduate studies.

The chapter outline of this dissertation is as follows:

Chapter 1: Background, literature review, motivation, aims and hypotheses

Chapter 2: Methodology chapter

Chapter 3: Article for publication

Chapter 4: Concluding remarks and findings

The relevant references are provided at the end of each chapter. The manuscript was prepared according to the author guidelines of the *International Journal of Cardiology* (which is summarised before the manuscript). In order to ensure uniformity of the dissertation, the Vancouver reference style was used throughout.

AUTHOR CONTRIBUTIONS

Ms C Swart

Responsible for conducting literature search; writing of the initial research proposal and ethics application. Writing the literature study; performing statistical analyses; as well as the design, planning and writing of the dissertation.

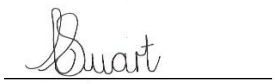
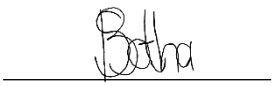
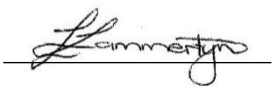
Dr S Botha

Supervised the writing of the proposal, ethics application, literature study and manuscript; collecting and interpretation of data, guidance regarding statistical analyses; initial planning and design of the dissertation.

Dr L Lammertyn

Co-supervised the writing of the proposal, ethics application, literature study and manuscript; collecting and interpretation of data, guidance regarding statistical analysis; initial planning and design of the mini-dissertation.

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

Ms C Swart | Dr S Botha | Dr L Lammertyn

SUMMARY

Background

South Africa is experiencing a high incidence of cardiovascular disease (CVD). In addition to modifiable risk factors, inflammation was found to be an independent risk factor for CVD development. Inflammatory markers such as interleukin-6 (IL-6), C-reactive protein (CRP) and soluble urokinase plasminogen activation receptor (suPAR) have been linked to the pathogenesis of CVD's, including atherosclerosis and coronary artery disease. Despite this reality, there is still a measure of uncertainty regarding the effect that inflammation could have on early changes in vascular structure, as measured by intima-media thickness (IMT) and cross-sectional wall area (CSWA). The central aim of this study was therefore to determine whether vascular structure relates to inflammation in South Africans over three years.

Objective

Our objectives for this study were to (i) assess the extent to which vascular structure (IMT and CSWA) and inflammation (suPAR, CRP and IL-6) changed over three years; and (ii) to explore whether three-year changes in vascular structure (IMT and CSWA) are associated with three-year changes in inflammation.

Methods

This MHSc study forms part of the Sympathetic Activity and Ambulatory Blood Pressure in Africans study (SABPA). A total of 303 participants at baseline (in 2008-2009) and at follow-up (in 2011-2012) were included. The participants were teachers from the North-West Province, South Africa, aged between 20 and 65 years. A validated questionnaire about demographic and lifestyle information was completed. Standardised methods were used to determine plasma levels of inflammatory markers (IL-6, CRP and suPAR) and to obtain cardiovascular, anthropometric and other biochemical measurements.

Results and conclusion

We found that IMT (5.13%, 95%CI: 3.75;6.51), CSWA (9.53%, 95%CI: 7.32;11.7), suPAR (4.93%, 95%CI: 0.81;9.06) and IL-6 (28.2%, 95%CI: 16.9;39.5) increased over the three years, while CRP decreased (3.31%, 95%CI: -16.0;9.42). After adjusting for conventional risk factors, percentage change in IMT inversely associated with percentage change in suPAR (β =-0.12, p=0.036; adjusted R2=0.16), while IMT did not associate with either CRP or IL-6. These results contradicted previous findings and warrant further investigation into the mechanisms linking vascular structure with inflammation, especially during the early stages of vascular structural change.

***Key words*:** C-reactive protein, inflammation, interleukin-6, intima-media thickness, South Africa, soluble urokinase plasminogen activator receptor

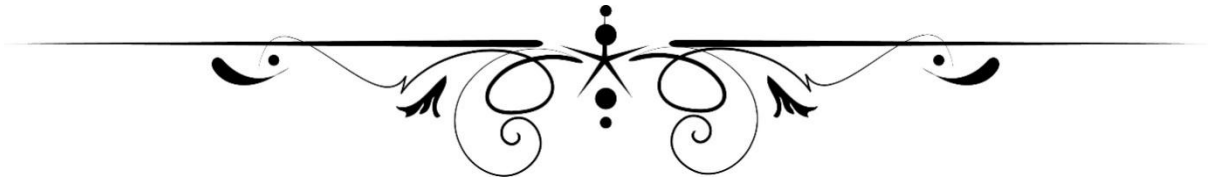
LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

ABPM	Ambulatory blood pressure monitoring
CRP	C-reactive protein
CSWA	Cross-sectional wall area
CVD	Cardiovascular disease
HDL	High-density lipoprotein cholesterol
HIV	Human immune deficiency virus
IL-6	Interleukin-6
IMT	Intima-media thickness
LDL	Low-density lipoprotein cholesterol
MHSc	Master of Health Sciences
SABPA	Sympathetic activity and Ambulatory Blood Pressure in Africans
SuPAR	Soluble urokinase plasminogen activator receptor
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor
VCAM-1	Vascular adhesion molecule-1

Chapter 1

Literature review, aim, objectives and hypotheses



1. Introduction and background

The prevalence of cardiovascular disease (CVD) [1] is increasing in sub-Saharan Africa. It is estimated that 7-10% of the total adult medical admissions to hospitals [2] and 9.4% of deaths in Africa are related to CVD [3]. South Africa in particular has a high incidence of CVD [4]. In 2003, Bradshaw *et al*. [5] reported that South Africans have higher age-standardised CVD mortality rates compared to first world countries. A 2017 report from Statistics South Africa indicated that heart disease was the fourth overall natural cause of death in South Africa during 2015, followed by the human immunodeficiency virus, which was ranked fifth [4].

Conventional risk factors such as smoking, obesity, alcohol abuse and lack of physical activity increase the risk for CVD [6-9]. In addition to these factors, it has been found that inflammation plays a role in the development of CVDs such as atherosclerosis and coronary artery disease [10, 11].

The term inflammation describes the immune state of a person where disease or infection is present [12]. The net inflammatory response is determined by the balance between anti- and pro-inflammatory molecules [13]. Inflammatory reactions can be activated during non-pathological conditions, resulting in low-grade inflammation [14]. Low-grade inflammation is characterised by increased levels of pro-inflammatory markers and is associated with the pathogenesis of atherosclerosis [15]. In fact, inflammatory reactions are up-regulated by interleukin-6 (IL-6), C-reactive protein (CRP) and soluble urokinase plasminogen activator receptor (suPAR) as the atherosclerotic process progresses [13, 16]. Also, it should be noted that an increase in plasma levels of inflammatory markers and progression of atherosclerosis is associated with progression in age [17, 18].

Atherosclerosis refers to a disease of the blood vessels where changes in vascular structure occur, leading to the formation of plaque that is accompanied by the narrowing of blood vessels [19]. The vascular structural changes found during atherosclerosis and other vascular diseases can be determined by measuring the intima-media thickness (IMT) and cross-

sectional wall area (CSWA) of the common carotid artery. Previous literature has shown associations between markers of vascular structure and several inflammatory markers [20, 21]. However, most of these associations were found in subjects with progressed atherosclerosis or other related diseases.

Literature on the relationship between vascular structural changes and inflammation during the early phases of vascular structural changes, where vascular changes have not yet progressed into atherosclerotic disease, is limited. Therefore, an investigation into the association between changes in vascular structure and inflammation will add to the paucity of data.

2. Processes involved in vascular structural changes

2.1 The vascular wall

An artery wall has three distinct layers, namely the tunica intima, tunica media and tunica externa (**Figure 1**). The tunica intima is the inner layer of the artery and consists of an endothelial lining, a surrounding layer of connective tissue and a number of elastic fibres [22]. The middle layer of the artery is known as the tunica media. This layer contains smooth muscle cells as well as loose connective tissue and collagen fibres that bind the tunica media to the tunica intima and externa [22]. Smooth muscle cells surround the endothelial lining of the blood vessel lumen. Thus, during contraction of the smooth muscle cells, the lumen diameter decreases (vasoconstriction) or *vice versa* (vasodilation) [22]. These processes play a distinct role in regulating total peripheral resistance and thus blood pressure [23]. The outer layer of the artery is referred to as the tunica externa and is a cover of connective tissue with collagen and elastic fibres [22].

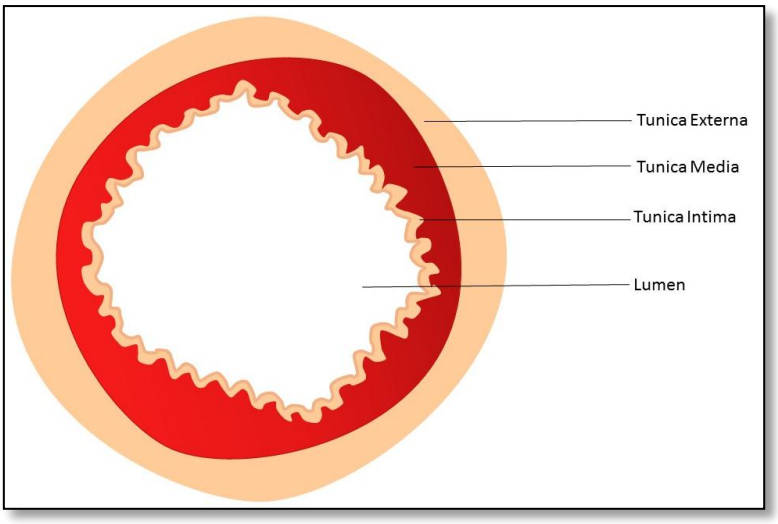


Figure 1 *Structure of the vascular wall*

2.2 Early changes in vascular structure

Healthy endothelial cells generally resist the adhesion of leukocytes [24]. However, during pro-inflammatory stimuli such as smoking, hypertension, obesity and hypercholesterolemia, changes within the vascular wall may occur [25], as summarised in **Figure 2**. During the early stages of vascular damage, low-density lipoprotein cholesterol inside the sub-endothelial layer becomes oxidised [19], which causes vascular endothelial cell dysfunction as well as the expression of chemokines and vascular adhesion molecules such as P-selectin and vascular cell adhesion molecule-1 [26-28]. In response, chemokines stimulate the migration of mononuclear leukocytes, also known as monocytes, into the intima of the vascular wall [15], where they differentiate into macrophages [19].

2.3 Progressed vascular structural changes

When the macrophages within the vascular wall endocytose accumulated oxidised low-density lipoprotein cholesterol [15, 24], these foam cells produce growth factors and more cytokines, which contribute to long-term vascular changes by causing a proliferation of vascular smooth muscle cells and increased plaque formation [15, 19]. At the same time, T helper cells are also recruited across the endothelial layer, which contributes to a further increase in the production of cytokines. These processes all lead to an inflammatory cascade [12, 19, 29], progressed

changes in vascular structure, and ultimately the development of CVDs such as coronary artery disease [30] and hypertension [23]. The measurement of changes in vascular structure, as quantified by an increase in intima-media thickness (IMT) [31] and cross-sectional wall area (CSWA) [32], may thus potentially act as markers of CVD risk prediction.

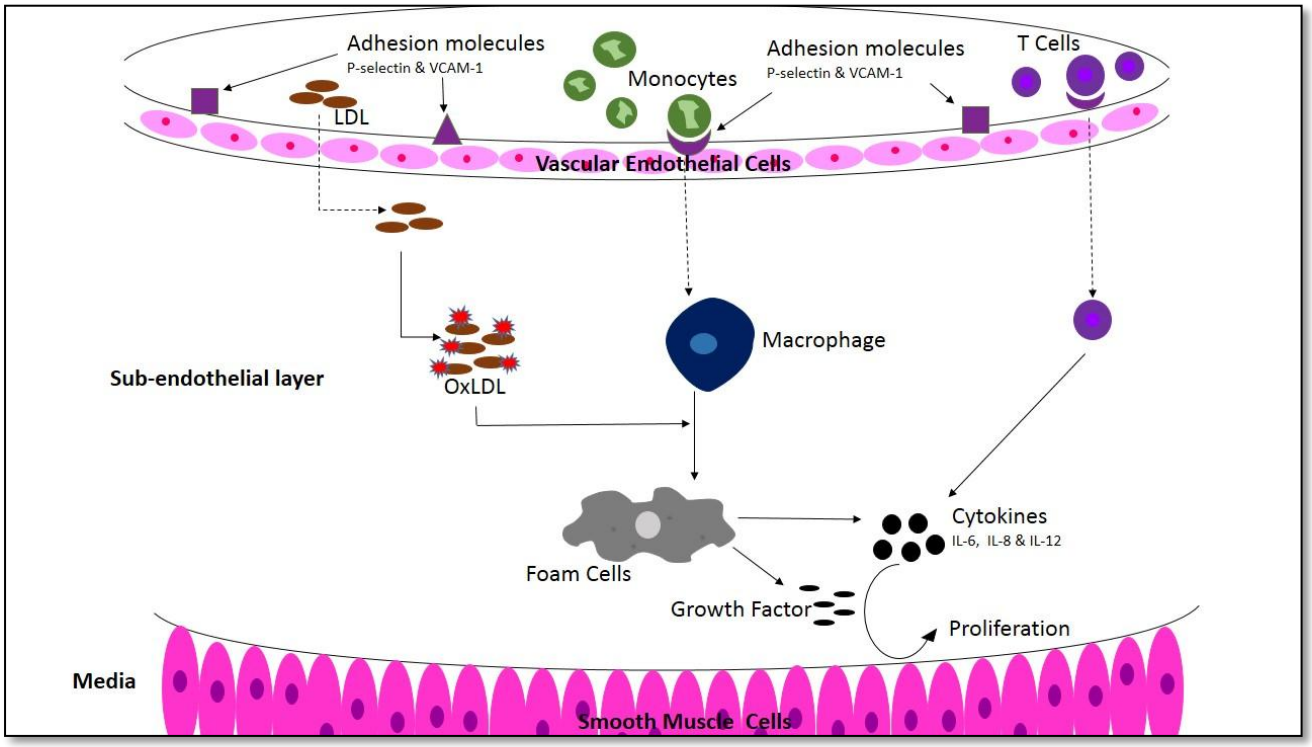


Figure 2 *Process of vascular structural changes. VCAM-1, vascular adhesion molecule-1; LDL, low-density lipoprotein; OxLDL, oxidized low-density lipoprotein; IL-6, interleukin-6; IL-8, interleukin-8 and IL-12, interleukin-12.*

An increase in IMT is a well-used indicator of vascular structural changes and can be measured relatively easy and noninvasively, also in large study samples [33]. According to the 2016 European guidelines on cardiovascular disease prevention in clinical practice, an IMT value of >0.9 mm or a focal increase in IMT of 0.50 mm or more can be regarded as a cardiovascular risk factor [34].

In addition to IMT, CSWA can also be calculated to confirm changes in vascular structure. Studies conducted in the United States of America found that both the lumen diameter and CSWA increase during the initial stages of atherosclerosis, an occurrence known as

compensatory enlargement. However, at later stages, thickening of the intimal layer with a subsequent increase in CSWA [36] and decrease in lumen diameter [37], may occur.

Increased IMT has been associated with traditional cardiovascular risk factors [38-41] and with the prevalence of atherosclerosis and coronary artery disease [42]. In a study completed early this year by Ibrahim and colleagues [43] it was reported that an IMT value of larger than 1 mm strongly associates with cardiovascular risk factors, including smoking, hypertension and diabetes mellitus. Longitudinal studies have also found that IMT can predict future cardiovascular events such as myocardial infarction and stroke within the general population [44-46]. A study conducted among 4 384 elderly participants from the Cardiovascular Health Study found that IMT improved ten year risk prediction for CVD and stroke more strongly than other traditional risk factors [47]. A study conducted in South Africa reported similar results, since increased IMT correlated with the prevalence of coronary artery disease in black men and women aged 30-70 years [48]. Nonetheless, both markers of vascular structure [42] and inflammation [13] have been associated with the development of CVD, which may suggest that a combination of these markers could serve as a strong predictor of CVD.

3. Biomarkers of inflammation related to vascular structural changes

Various biomarkers of inflammation have been proposed to relate to changes in vascular structure, including tumour necrosis factor-α [19], interleukin-8 [19], interleukin-12 [19], IL- 6 [19], CRP [19] and suPAR [14]. This section focuses on the latter three which were investigated in this study.

Interleukin-6 (IL-6)

IL-6 is a well-known pro-inflammatory cytokine [49] that was discovered in 1986 [50]. IL-6 forms part of a family of 20kD polypeptide cytokines [50] and has a short half-life of less than two hours [51]. In addition to type 1 macrophages, IL-6 is also produced by monocytes,

leukocytes, fibroblasts, T-cells, adipose cells and endothelial cells [52-55]. The production of IL-6 is regulated by a selection of signals. For example, T-cell mitogens induce IL-6 production in the presence of macrophages [56].

In addition, other stimuli such as infection and lifestyle factors lead to an increase in the synthesis of IL-6 [57-59]. For instance, lipopolysaccharides specifically increase the production of IL-6 in monocytes, while different types of cytokines such as interleukin-1, tumour necrosis factor-alpha and platelet-derived growth factor increase the expression of the IL-6 gene in various cells [56, 60]. It has also been found that smoking can lead to elevated levels of IL-6, as determined in a previous study where plasma IL-6 concentrations were 16% higher in smokers than in non-smokers [57, 58].

Even though most cytokines function through autocrine or paracrine mechanisms, the biological effect of IL-6 regularly takes place at sites away from its source, where the cytokine binds to a specific receptor complex found on target cells [61, 62]. After binding to its receptor, IL-6 can exert a number of functions depending on the target site. These functions include antiviral [63] and coagulation effects [64], proliferation of cells [65] and the promotion of CRP release from hepatocytes [66].

According to the literature, there is controversy regarding normal plasma levels of IL-6. Jones *et al*. [67] indicate that under normal conditions, circulating IL-6 levels vary between 1-5 pg/mℓ, while Alecu *et al*. [68] refer to normal IL-6 levels as 5-15 pg/mℓ. Nonetheless, elevated levels of IL-6 are considered as a strong predictor of CVD [69], of cardiovascular events such as unstable angina [70] and myocardial infarction [71], as well as of cardiovascular mortality [57].

With regards to vascular structure, it has been suggested that IL-6 directly contributes to the development of atherosclerosis by facilitating coagulant activity and increasing the expression of adhesion molecules and chemokines by endothelial cells [61]. Results from genetic studies showed positive associations between IL-6 and IMT [72, 73]. This was in line with a four-year American prospective study which reported an independent positive association between IL 6

and carotid atherosclerosis [74]. Contradicting results have, however, been reported where the association between IL-6 and IMT that existed disappeared when other conventional risk factors such as age, sex, hypertension and diabetes mellitus were taken into account [75-77].

C-reactive protein (CRP)

CRP is produced in the liver [78], mainly in response to stimulation by IL-6 [79-81]. Furthermore, other factors such as interleukin-1 and glucocorticoids act along with IL-6 to increase the production of CRP [82-84]. CRP, an acute phase protein [85], is a well-known and conventionally used biomarker of low-grade inflammation [74]. It is a calcium-dependent ligand binding protein [86], a member of the pentraxin family of plasma proteins [87] and has a long plasma half-life [88] of 19 hours [89]. Although CRP is sensitive to acute inflammatory stimuli, CRP concentrations are not easily influenced by diurnal rhythm [98]. According to Ridker *et al*. [88], subjects with CRP levels lower than 1 mg/ℓ are classified as having a low risk for future cardiovascular events, while subjects with CRP levels of higher than 3 mg/ℓ have a high risk for future cardiovascular events.

CRP was first discovered in 1930 in patients suffering from pneumonia [90] and 66 years later it was linked to the occurrence of coronary heart disease [91]. It is now known that the biological function of CRP includes the recognition and removal of pathogens by activating a classical pathway and resulting in a phagocytic cell response [87, 92, 93]. This occurs through the binding of plasma CRP to phosphocholine [94], ribonucleoprotein particles [95, 96] and phagocytic cell receptors [97].

Some controversy exists in the literature regarding the role of CRP in vascular structural changes and related CVD such as atherosclerosis and coronary artery disease [99]. It has been suggested that changes in vascular structure may not be directly related to CRP. A longitudinal study that investigated the link between inflammation and carotid atherosclerotic measures within an American population found no significant association between IMT and CRP [74]. Another prospective study reported similar results as their results indicated no

independent association between early progression of IMT and CRP in Europeans [100]. On the other hand, in 2003, Szmitko *et al*. [101] suggested that CRP has a direct effect on the pathogenesis of atherosclerosis and is a powerful predictor of vascular related mortality. This relationship was also highlighted by a cross-sectional study of 234 Swedish middle-aged men [75], that showed that CRP associated with IMT, independent of other inflammatory markers, cardiovascular risk factors and oxidative stress [75].

Possible factors that may explain this relationship between adverse changes in vascular structure and CRP have been proposed in the literature. In a genetics study, it was found that human recombinant CRP causes a reduction in nitric oxide release by down regulating the transcription of endothelial nitric oxide synthase and by destabilising endothelial nitric oxide synthase messenger ribonucleic acid [102]. In addition to these effects, CRP up-regulates adhesion molecules, stimulates the release of IL-6 and endothelin-1, and facilitates the uptake of low-density lipoprotein cholesterol by macrophages [103].

Soluble urokinase plasminogen activator receptor (suPAR)

The more novel and less-known marker, suPAR, was first identified in 1991 by Ploug *et al.* [104]. The protease enzyme, urokinase-type plasminogen activator (uPA), its receptor (uPAR), and associated inhibitors together form the uPA-uPAR system [105]. Upon activation, uPA binds to uPAR, causing the receptor to undergo conformational changes [106, 107]. This can cause uPAR, which is found in vascular endothelial cells and immunologically active cells such as T-lymphocytes and macrophages [12, 106, 108], to be hydrolysed by a process of “shedding” [109, 110], leading to the formation of its soluble form, suPAR [109, 110].

Urokinase-type plasminogen activator receptor and suPAR can be activated by inflammatory stimuli [12], including pro-inflammatory cytokines and growth factors such as interleukin-1 and vascular endothelial growth factor [111, 112]. After activation, suPAR can be found in various body fluids such as urine, blood and cerebrospinal fluid [113-117]. This molecule exerts a number of immunological functions related to processes that occur during changes in vascular

structure. For example, suPAR is involved in the modulation of cell adhesion molecules, signal induction through integrins, migration of monocytes, as well as the proliferation and remodelling of vascular tissue [12, 109, 118, 119]. Supporting this, a study conducted on uremic patients found that suPAR independently associated with IMT [120].

Soluble urokinase plasminogen activator receptors has a low clearance rate [121], are not influenced by circadian rhythm [105] and unlike CRP [122], it remains stable during an acute inflammatory stimuli [12]. However, there has been some controversy over the years regarding differences in suPAR concentrations under different conditions. According to Chavakis and colleagues [112], a normal plasma suPAR level ranges between 1-10 ng/mℓ, while another study reported a mean level of 2.74 ng/mℓ in apparently healthy Swedish participants, as compared to a higher concentration of 2.96 ng/mℓ in participants with carotid plaque [123].

Nonetheless, suPAR remains one of the more pronounced markers of low-grade inflammation [12]. Over the past few years, suPAR has also emerged as a stronger marker of CVD [124] and related mortality [125] as compared to CRP. Literature indicates that suPAR and CRP may represent different processes in vascular inflammation [111]. CRP relates strongly to anthropometric and lifestyle factors, while suPAR associates with endothelial dysfunction and progressed atherosclerosis [111]. In fact, a study conducted on stroke patients reported that suPAR was associated with a higher prevalence of carotid plaque and incidence of coronary artery disease [123].

4. Problem statement and motivation

The high incidence of CVD in South Africa warrants investigation into early structural changes in the vasculature and related risk factors to help identify “first responders” (factors contributing to CVD early on) as a measure to implement preventative strategies. Inflammation has been associated with changes in vascular structure and with the resultant development of CVD, including hypertension, atherosclerosis and coronary artery disease. Although a measure controversy still exists, it is clear from the literature that adverse structural changes in the

vasculature, as measured by an increase in IMT and CSWA, is related to adverse changes in the inflammatory profile, which can be measured by an increase in IL-6, CRP and suPAR. Most of these studies were performed on population groups outside of South Africa and focused on vascular changes during progressed atherosclerosis. Therefore, investigating the link between changes in vascular structure and inflammatory markers among South Africans, where vascular changes have not yet progressed to disease and cardiovascular events, may contribute to the current understudied literature.

5. Aim

The central aim of this study is to determine whether vascular structure relates to inflammation in South Africans over three years.

6. Objectives

Our objectives are to:

1. Assess the extent to which vascular structure (IMT and CSWA) and inflammation (suPAR), CRP and IL-6) changed over three years; and

2. Explore whether three-year changes in vascular structure (IMT and CSWA) are associated with three-year changes in inflammation.

7. Hypotheses

Based on the available literature, we hypothesise that:

1. Markers of vascular structure and inflammation will increase over three years; and
2. Adverse three-year changes in markers of vascular structure will associate with an increase in inflammatory markers in South Africans.

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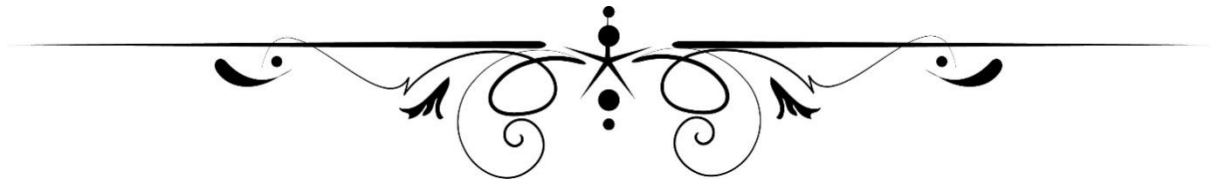
Chapter 2

Methodology



1. Study design and participants

This MHSc study made use of existing longitudinal data from the Sympathetic Activity and Ambulatory Blood Pressure in Africans (SABPA) study [1]. The original SABPA study was designed to investigate neural mechanistic pathways involved in emotional distress and vascular remodelling [1]. The central aim of this MHSc study is to determine whether a three-year change in vascular structure relates to inflammation in South Africans. To investigate this, we included 303 participants from the SABPA baseline (2008-2009) and follow-up (2011-2012) study. The participants were black and white teachers from the Dr Kenneth Kaunda Education district of the North-West Province South Africa (**Figure 1**). **Figure 2** indicates the study design, while **Table 1** lists the inclusion and exclusion criteria, together with relevant justifications, for both the original SABPA and this MHSc study.

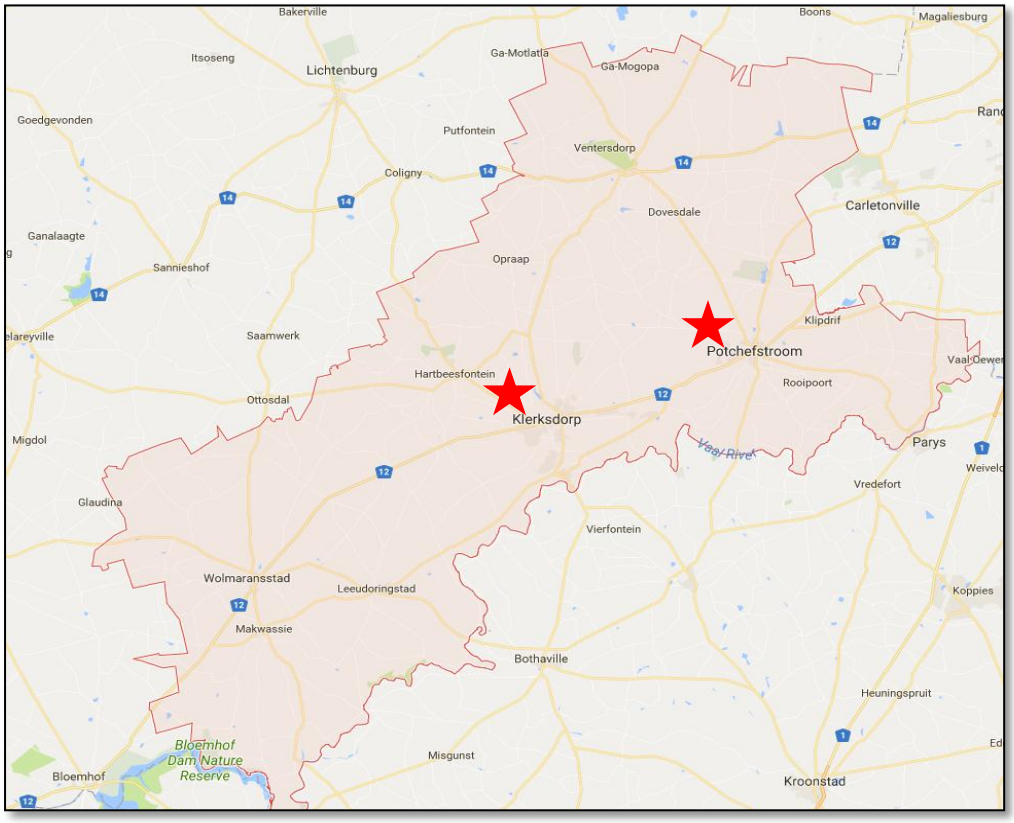


Figure 1 *Location of Potchefstroom and Klerksdorp within the Kenneth Kaunda Education district, North West Province, South Africa, where data collection took place.*

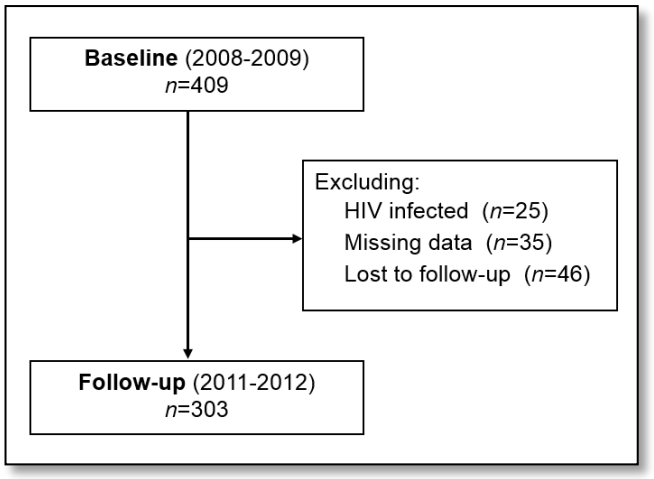


Figure 2 *Study population for this MHSc study*

Table 1 Inclusion and exclusion criteria

Inclusion criteria	**Justification**
Teachers from the Dr Kenneth Kaunda district, aged 20- 65 years.	To ensure socio-economic homogeneity in the study sample.
Exclusion criteria	
A tympanic temperature above 37.5°C.	Tympanic temperature above 37.5°C indicates systemic microbial infection.
Blood donors.	Blood donation in the preceding three months would exclude the participants from having blood drawn (due to risk of anaemia).
Vaccinations less than three months prior to data collection.	Vaccination artificially increases white blood cell counts.
Pregnant or lactating women	Pregnancy and lactation would cause disturbances in various (mostly biochemical) measures due to for instance changes in hormones and fluid volume that occur.
Individuals dependent on or abusing psychotropic substances.	Psychotropic drugs would cause disturbances in various (mostly biochemical) measures.
Participants infected with the human immune deficiency virus (HIV) *	The HIV virus has a known effect on the inflammatory process.
Missing data for main dependent and independent variables, at either baseline or follow-up, and participants lost to follow-up.*	To ensure comparable results as this MHSc study follows a longitudinal study design.

*Denotes additional exclusion criteria for this MHSc study that did not form part of the original exclusion criteria of the SABPA study but which were deemed relevant for this MHSc study.

Ethical considerations

Both the original SABPA study (NWU-0003607S6) and this MHSc study (NWU-00051-17-A1) were approved by the Ethics Committee of the North-West University, Potchefstroom Campus, South Africa and comply with the Declaration of Helsinki (revised 2004). For purposes of the original SABPA study, consent and cooperation agreements were obtained from the Department of Education of North-West, the South African Democratic Teacher Union and the headmasters of the respective schools.

2. Organisational procedures

For the original SABPA study, recruitment systematically took place over a three-month period prior to clinical assessments during baseline and follow-up. A standard participant information sheet was given to the participants during recruitment. A registered nurse and a trained field worker provided volunteers with a detailed description of the planned measurements, the protocol and expected outcomes in their home language, after which the prospective participants were given the opportunity to ask questions. When an individual indicated that he or she was interested in participating in the study, the nurse confirmed eligibility for inclusion in the main study by obtaining a brief medical history of each individual. The nature, benefits and risks of the study were explained again in detail to the included participants and their written informed consent was obtained before participation. Participants were made aware that participation in the research project is voluntary and that they are allowed to withdraw from the study at any stage. They were provided with the telephone number of the principle investigator, in case they had any additional enquiries.

Data for each participant was collected over a period of two days. Day one included fitting the ambulatory blood pressure measurement (ABPM) device on the participants at their place of work, where they continued with their normal activities. At the end of the working day, the participants reported to the Metabolic Unit Research Facility of the North-West University

where they slept over. This was done to make sure that participants were in a relaxed and calm environment. Upon arrival, the participants were reassured of the measures to follow and pre-HIV counselling was provided to each participant by a trained HIV counsellor. The participants also completed a socio-demographic health questionnaire.

On day two, the ABPM device were disconnected. Anthropometric measurements were done and resting fasting blood samples of 70 ml were then obtained by a registered nurse. This was followed by cardiovascular measurements that included resting blood pressure, pulse wave velocity and intima-media thickness (IMT) measurements. After completion of the cardiovascular measurements, post-HIV counselling were provided to each participant in private. Thereafter, participants were thanked for their participation and given a take-away breakfast and drink.

3. Methodology pertaining to this MHSc study

3.1 Questionnaire, anthropometric and physical activity measurements

Questionnaire

A validated questionnaire was completed by each participant to collect demographic (age, sex, race and medication use) and lifestyle (alcohol use and smoking habits) information, all of which may have an influence on the relationship that is being investigated [2-5].

Anthropometric measurement

Waist circumference was measured according to the prescribed standardised procedures from international standards for anthropometric assessment [6]. The measurement was taken in triplicate and the median reported. Obesity classification was done using waist circumference data and classified according to the World Health Organisation guidelines as a waist circumference of >102 cm for men and >88 cm for women [10]. Waist circumference was used due to the strong association between abdominal fat distribution and inflammation [7-9].

Physical activity

Physical activity data was obtained by an Actical (Mini Mitter Co., Inc., Bend, Canada) device that was fitted on the participant during baseline, and an Actiheart (CamNtech Ltd, Cambridgeshire, UK) device was used during follow-up measurements. Physical activity is known to influence inflammatory levels by initially increasing, for instance, IL-6 [11]. However, over the long term, physical activity has an anti-inflammatory [12-14] and anti-atherosclerotic effect [13].

3.2 Cardiovascular measurements

Vascular structural measurements

IMT is commonly used as the standard marker of vascular structural changes [15] due to its strong association with vascular risk factors and its ability to predict cardiovascular events [15-18]. We conducted ultrasound images of the left and right common carotid arteries from which carotid IMT and lumen diameter were determined. The Sonosite Micromaxx® ultrasound system (SonoSite Inc., Bothell, WA, USA) was used to capture ultrasonographs. These images were digitally analysed with Artery Measurement Systems automated software II v1.139 (Gothenburg, Sweden) to quantify IMT and luminal diameter (**Figure 3**). All IMT measurements were obtained at two optimal angles during baseline and follow-up. We used the mean IMT of the left, right, far and near carotid walls. To confirm structural changes, we also calculated CSWA by using the following formula: $CSWA = \pi(d/2 + IMTf)2 - \pi(d/2)2$, with d as the lumen diameter.

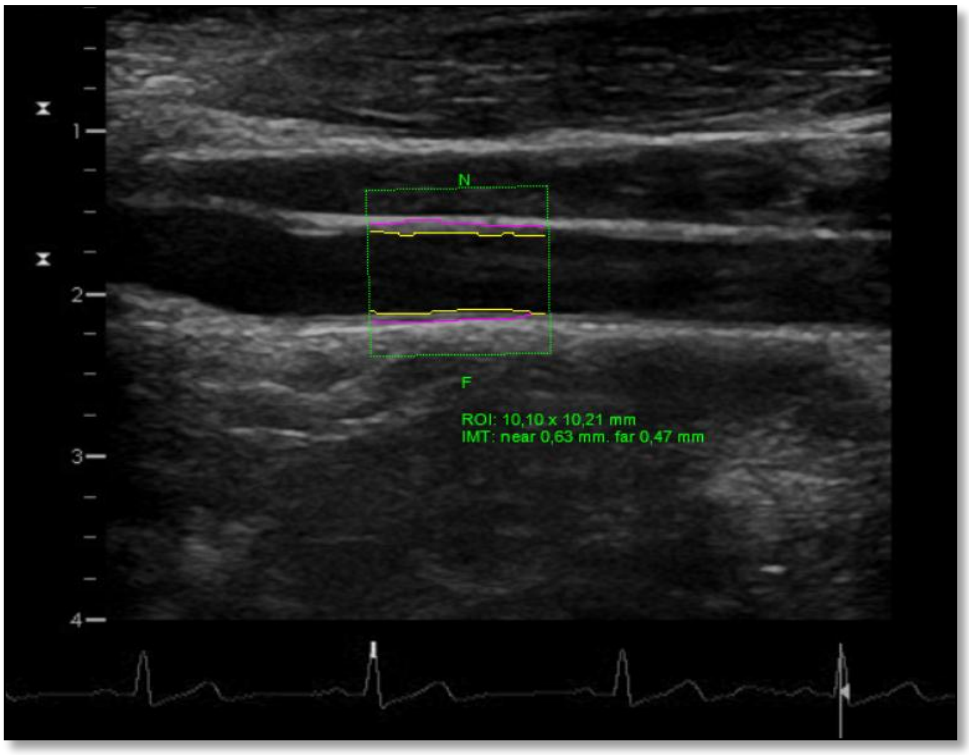


Figure 3 *Example of an image taken from the student's own right carotid artery, which was used to determine IMT*

Blood pressure measurements

The Sub-Committee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research recommend using 24-hour ambulatory blood pressure measurement for measuring blood pressure, because it was found to be a better predictor of cardiovascular disease (CVD) risk than office blood pressure measurements [19]. The CardioTens ABPM device (CE0120, Meditech, Hungary), validated by the British Hypertension Society, was used to obtain 24-hour ABPM. Measurements included systolic, diastolic, mean arterial blood pressure and pulse pressure. An appropriate-sized cuff was fitted to the participant's non-dominant arms and instructions were given to participants on how to optimise successful inflations across the 24-hour time period. The device was programmed to take blood pressure measurements every 30 minutes during the day (6am-10pm) and every 60 minutes (10pm-6am) during the night. Successful inflation rates of 70% or more were required for data to be valid. Each participant was also requested to complete an ambulatory diary card which records any abnormalities or activities throughout the 24-hour period. Data

was exported and analysed with CardioVisions version 1.15 software (Cardiovisions, Meditech, Hungary).

3.3 Blood sampling and biochemical analyses

Blood sampling

To obtain fasting blood samples, participants were asked to refrain from consuming any food or beverages, except water, after 10 pm on the night before the blood samples were collected. Blood samples were obtained by a registered nurse with a sterile winged infusion set. Standardised methods were followed for the preparation of the serum, plasma and sodium fluoride samples that were stored in a laboratory freezer at -80^{0}C until analysis.

Biochemical analyses

The suPARnostic® (ViroGates, Copenhagen, Denmark) test was used to measure EDTA plasma soluble urokinase plasminogen activator receptor (suPAR) levels. Serum levels of interleukin-6 (IL-6) were measured by a Quantikine high-sensitivity enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MN USA). C-reactive protein (CRP), serum high-density lipoprotein cholesterol, triglycerides, sodium fluoride glucose and gamma-glutamyltransferase were analysed with the Unicel DXC 800 (Beckman and Coulter, Germany) apparatus at baseline and Integra 400 (Roche, Switzerland) apparatus during follow-up. We analysed CRP with the particle enhanced turbidimetric assay method, gamma-glutamyltransferase with the enzyme rate method and high-density lipoprotein and triglycerides with the homogeneous enzymatic colorimetric assay method.

Power analysis

In an *a priori* power analysis, using the G* power v3.1.9.2 software, a sample size of 303 was computed as a function of the required power level. The preselected power was 95% at a significance level of α= 0.05 and medium effect size of d=0.5 for vascular structure and inflammation as the main outcome measures. The analysis calculated that a population

sample of 105 per group is needed. Our sample sizes of 303 was thus sufficient to test the hypotheses of this study with relevant statistical methods, as described in Chapter 3.

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Chapter 3

Article for publication

Summary of author instructions

JOURNAL DETAILS				
Title:	*International Journal of Cardiology*			
Impact factor:	6.189			
Publisher:	Elsevier			
Aim and scope: The IJC publishes reports of research that contributes to all aspects of cardiology including, cardio-metabolic, vascular and genetic research, as well as research that focuses on the prevalence of cardiovascular diseases.				
JOURNAL GUIDELINES				
Author guidelines:	https://www.elsevier.com/journals/international-journal-of-cardiology/0167-5273/guide-for-authors			
Title: **Abstract:**	Maximum of 25 words Maximum of 250 words		**Language:**	English, U.K. or American
Keywords:	3-6		**Spacing:**	Double
Tables and Figures:	Maximum of 4; double spacing; each on separate sheet; contain only horizontal lines.			
References:	± 50 references. Vancouver style of numbering should be used and all authors should be listed when six or fewer, when seven and more list only first three authors and add et al. Example of correct reference: De Soyza N, Thenabadu PN, Murphy ML, Kane JJ, Doherty JE. Ventricular arrhythmia before and after aortocoronary bypass surgery. Int J Cardiol 1981; 1:123-130.			
Sections:	Title page; Abstract; Introduction, Methods, Results, Discussion; Acknowledgements; Refrences			
Ethical considerations:	Manuscripts containing research done on human subjects must include a statement that assures that informed consent was obtained from each participant, as well as a statement that assures that the study done adheres to the Declaration of Helsinki.			

Vascular structure and inflammation in a South African population: The SABPA study

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[†]*This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation*

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Conflict of interest

The authors declare no conflict of interest.

Keywords: C-reactive protein, interleukin-6, inflammation, intima-media thickness, South Africa, soluble urokinase plasminogen activator receptor..

Abstract

Background: Inflammation contributes to the development of vascular diseases. However, the long-term effects thereof are unknown due to a lack of longitudinal studies on this topic, especially among South African populations. Therefore, we explored whether changes in vascular structure are associated with changes in inflammation.

Methods: We investigated 303 South African teachers aged 20-65 years at two intervals, three years apart. Standardised methods were used to determine carotid intima-media thickness (IMT) and cross-sectional wall area (CSWA) as measures of vascular structure, as well as the inflammatory markers soluble urokinase plasminogen activator receptor (suPAR), C-reactive protein (CRP) and interleukin-6 (IL-6) at baseline and follow-up.

Results: A three-year percentage increase was seen in IMT (5.13%, 95%CI: 3.75;6.51), CSWA (9.53%, 95%CI: 7.32;11.7), suPAR (4.93%, 95%CI: 0.81;9.06) and IL-6 (28.2%, 95%CI: 16.9;39.5), while CRP decreased (3.31%, 95%CI: -16.0;9.42) After adjusting for confounding factors, percentage change in IMT inversely associated with percentage change in suPAR (β=- 0.12, p=0.036; adjusted R^2=0.16) only.

Conclusion: Despite adverse vascular changes and a mixed inflammatory profile over the three years, change in inflammatory markers did not play an independent role in the adverse structural changes observed. Future studies are needed to investigate mechanisms that may link vascular structure to inflammation, especially in early disease stages within a South African context.

Word count: 211

Introduction

Cardiovascular disease (CVD) is the leading cause of disease-related mortality worldwide [1]. In addition to conventional and behavioural risk factors [2, 3], inflammation also plays a role in the enhancement of CVD development [4, 5]. During non-pathological conditions, a balance between anti- and pro-inflammatory molecules exists [6]. However, low-grade inflammation, as characterised by increasing levels of pro-inflammatory markers such as C-reactive protein (CRP) [7], interleukin-6 (IL-6) [8] and soluble urokinase plasminogen activator receptor (suPAR) [7], results in the development of endothelial dysfunction [9]. Such events may, in turn, contribute to an inflammatory cascade; evident by a further increase in inflammation [10]. Over time, changes in the vasculature occur, as measured by an increase in carotid intima-media thickness (IMT) and cross sectional wall area (CSWA), thereby leading to the development of atherosclerosis and other CVD's [11-14]. This ultimately increases the risk of cardiovascular-related events and mortality [15, 16].

Cross-sectional studies exist that indicate a positive association between vascular structural changes and several inflammatory markers among, for instance, Hungarian and Finnish populations [17, 18]. A previous study also reported adverse changes in IMT and CSWA, as well as inflammation such as IL-6 and tumour necrosis factor-α, in our population [19]. Regardless, longitudinal studies in the South African context that investigate the influence of such changes in inflammation on the development of vascular structural changes, remain limited. To address this lack of information, we explored whether changes in vascular structure are associated with changes in inflammation in a South African population.

Methods

Study design and participants

This study forms part of the Sympathetic Activity and Ambulatory Blood Pressure in Africans study (SABPA). A detailed protocol has been published elsewhere [20]. We examined 303 participants at baseline (in 2008-2009) and follow-up (in 2011-2012). Teachers from the North West Province, South Africa, aged between 20-65 years were included. Exclusion criteria were a tympanic temperature above 37.5°C, blood donators, those who had received vaccinations fewer than three months prior to data collection, pregnant and lactating women, participants dependent on psychotropic substances, as well as HIV infected individuals. We also excluded those that were lost to follow-up (n=46) and who had missing data at either baseline or follow-up (n=35).

Written informed consent was obtained from each participant prior to baseline and follow-up data collections. This study was approved by the Ethics Committee of the North-West University, Potchefstroom (NWU-00051-17-A1), and complies with the Declaration of Helsinki (as revised in 2004).

Questionnaire, anthropometric and physical activity measurements

A questionnaire was completed to obtain demographic (age, sex, race and medication use) and lifestyle (alcohol use and smoking habits) data. Waist circumference was measured according to prescribed standardised procedures [21] in triplicate, and the median was reported. Obesity classification, based on waist circumference, was done according to World Health Organisation guidelines as a waist circumference of >102 cm and >88 cm for men and women, respectively [22] indicate obesity. Physical activity data as obtained by means of an Actical (Mini Mitter Co., Inc.,Bend, Canada) device during baseline and an Actiheart (CamNtech Ltd, Cambridgeshire, United Kingdom) device during follow-up.

Cardiovascular measurements

We determined carotid intima-media thickness (IMT), lumen diameter and cross-sectional wall area (CSWA) as markers of vascular structure. Images of the left and right common carotid arteries were taken with the Sonosite Micromaxx® ultrasound system (SonoSite Inc., Bothell, WA, USA) and digitally analysed with Artery Measurement Systems automated software II v1.139 (Gothenburg, Sweden) to quantify IMT and lumen diameter. The mean IMT of the far and near walls on both sides were reported. CSWA was calculated as $CSWA = \pi(d/2 + IMTf)^2 - \pi(d/2)^2$, with d as the lumen diameter.

A validated ambulatory blood pressure measurement (ABPM) device (Cardiotens, CE0120, Meditech, Hungary) was used to obtain systolic, diastolic, pulse and mean arterial blood pressure. Blood pressure was taken at 30-minute intervals during the day (6am-10pm) and 60-minute intervals at night (10pm-6am). Successful inflation rates of 70% or more were required for a measurement to be regarded as adequate.

Biochemical analyses

Samples were obtained from fasting participants and stored at -80°C until analyses could be performed. Plasma suPAR levels were measured with the suPARnostic® (ViroGates, Copenhagen, Denmark) test. Serum levels of IL-6 were measured by a Quantikine high-sensitivity enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MN USA). The Unicel DXC 800 (Beckman and Coulter, Germany) apparatus was used to determine high-sensitivity CRP, serum high-density lipoprotein cholesterol, triglycerides, sodium fluoride glucose and gamma-glutamyltransferase at baseline, while the Integra 400 (Roche, Switzerland) apparatus was used to analyse these markers at follow-up. CRP was analysed with the particle-enhanced turbidimetric assay method, gamma-glutamyltransferase with the enzyme rate method and high-density

lipoprotein cholesterol and triglycerides with the homogeneous enzymatic colorimetric assay method.

Statistical analyses

Statistica version 13.0 (Dell software, Tulsa, OK) was used to perform statistical analyses. Normally distributed, continuous data was presented as arithmetic means ± standard deviation. Non-Gaussian data was log-transformed and presented as geometric means with 5^{th} and 95^{th} percentiles. Categorical data was presented as proportions. We additionally adjusted for mean arterial pressure with regards to IMT, CSWA and lumen diameter. Possible interactions of sex and race on the association of vascular structure with inflammatory markers were tested by means of multiple regression analyses. Differences in means between baseline and follow-up were determined with dependent *t*-tests for continuous, and with McNemar tests for categorical data. Relationships were established with Pearson correlations, and multiple linear regression analyses were performed to determine independent relationships. In all cases, the % change in IMT was regarded as the main dependent and the % change in inflammatory markers as the main independent variables in order to account for the regression to the mean. Covariates used for multiple regression models included race, sex, age, waist circumference, gamma-glutamyltransferase, glucose, triglyceride-to-high-density lipoprotein ratio, mean arterial pressure, self-reported tobacco and anti-inflammatory medication use. All measurements were two-tailed and a $p \leq 0.05$ was used to indicate statistical significance. To determine the extent to which inflammation contributed to a progression in IMT and CSWA, we classified groups according to changes in IMT and CSWA: Increased IMT and CSWA groups were based on whether IMT (n=199) or CSWA (n=98) increased in individuals from baseline to follow-up.

Results

The baseline and follow-up characteristics are described in **Table 1**. Of the 303 South Africans included, 49.5% were men and 39.9% were black. At follow-up, anthropometric measures, pulse pressure, triglycerides-to-high-density lipoprotein cholesterol ratio, total energy expenditure, as well as anti-hypertensive and statin medication use were all higher (all $p \leq 0.015$), while diastolic blood pressure, glucose, gamma-glutamyltransferase and tobacco use were lower (all $p \leq 0.001$) than at baseline.

With regards to vascular structure (IMT, CSWA and lumen diameter), all markers were higher at follow-up than at baseline (all $p > 0.001$; **Table 1**). This was confirmed after adjusting for baseline (**Table 2**) where all vascular structure markers showed a percentage increase over the three years (IMT=5.13%, 95%CI: 3.75;6.51; CSWA=9.53%, 95%CI: 7.32;11.7; LD=1.95, 95%CI: 1.30;2.61). Regarding the inflammatory markers, CRP was higher at follow-up than at baseline ($p<0.001$; **Table 1**). However, after we adjusted for baseline (**Table 2**), suPAR (4.93%, 95%CI: 0.81;9.06) and IL-6 (28.2%, 95%CI: 16.9;39.5) increased, while CRP decreased (3.31%, 95%CI: -16.0;9.42) over the three years. Similar results were found in both groups with increased IMT and CSWA.

In partial (**Table 3**) and multiple regression (**Table 4**) analyses, an inverse association was only observed between % change in IMT and % change in suPAR (r=-0.13; p=0.028 and β=-0.12, p=0.036; adjusted R^2=0.16) in the total group. No associations were obtained between any of the vascular structure markers and other inflammatory markers in the groups with increased IMT or CSWA.

With interaction testing, we tested for the effect of sex and race, respectively, on the association between % change in IMT and % change in inflammatory markers, but found no significant effect (**Table S1**). We also performed sensitivity analyses to test for the effect of statins and anti-

hypertensive medication use, respectively, on the association between IMT and inflammatory markers, but found no significant effect.

Table 1 Characteristics of the study population

	Baseline (*n*=303)	**Follow-up** (*n*=303)	***p***
Socio-demographic profile			
Race, black *n* (%)	121 (39.9)		
Sex, men *n* (%)	150 (49.5)		
Age (years)	46 ± 9.19	49 ± 9.19	--
Anthropometric measurements			
Waist circumference (cm)	92.2 (71.2;117)	95.7 (73.4;124)	<0.001
Obesity, *n* (%)*	130 (42.9)	155 (51.2)	<0.001
Cardiovascular measurements			
Systolic blood pressure (mmHg)	128 ± 14.8	128 ± 15.2	0.98
Diastolic blood pressure (mmHg)	80.0 ± 9.91	79.0 ± 9.99	< 0.001
Mean arterial pressure (mmHg)	96 ± 11.1	95.0 ± 11.3	0.099
Pulse pressure (mmHg)	48.0 ± 7.94	50.0 ± 8.31	0.001
Intima-media thickness (mm)†	0.67 ± 0.12	0.70 ± 0.11	<0.001
Cross sectional wall area (mm^2)†	13.8 ± 3.88	14.8 ± 3.83	<0.001
Lumen diameter (mm)†	5.87 ± 0.63	5.98 ± 0.65	<0.001
Inflammatory variables			
SuPAR (ng/mℓ)	2.43 (1.56;3.82)	2.42 (1.61;3.81)	0.87
C-reactive protein (mg/ ℓ)	2.78 (0.99;18.29)	1.88 (0.18;15.7)	<0.001
Interleukin-6 (pg/m ℓ)	1.03 (0.33;3.10)	0.98 (0.31;3.05)	0.40
Biochemical variables			
Glucose (mmol/ ℓ)	5.55 (4.50;7.30)	4.69 (3.02;7.11)	<0.001
Triglycerides-to-HDL ratio	1.34 ± 1.57	1.47 ± 1.55	0.015
Lifestyle variables			
Gamma-glutamyltransferase (U/ ℓ)	27.7 (9.00;122)	25.2 (8.40;119)	<0.001
Tobacco use, *n* (%)	47 (15.6)	42 (13.9)	<0.001
Total energy expenditure (kCal/day)	2804 (1769;4419)	3175 (1761;5945)	<0.001
Medication use			
Anti-hypertensive, *n* (%)	76 (25.1)	83 (27.4)	<0.001
Anti-inflammatory, *n* (%)	17 (5.61)	44 (14.5)	0.71
Statins, *n* (%)	11 (3.63)	34 (11.22)	<0.001

SuPAR, soluble urokinase plasminogen activator receptor; HDL, high-density lipoprotein cholesterol. Data are expressed as arithmetic mean ± standard deviation, geometric mean (5^{th} and 95^{th} percentile boundaries), or % of *n*. *p*-values for comparison between groups were obtained with dependent *t*-tests and McNemar tests. *Categorised according to waist circumference values. †Adjusted for mean arterial pressure.

Table 2 Three year % change in markers of vascular structure and inflammation

	Total group (*n*=303)	**Increased IMT** (*n*=199)	**Increased CSWA** (*n*=98)
Intima-media thickness (mm)	5.13 (3.75;6.51)	11.4 (10.2;12.5)	2.75 (8.40;11.1)
CSWA (mm^2)	9.53 (7.32;11.7)	18.0 (15.9;20.1)	18.3 (16.3;20.2)
Lumen diameter (mm)	1.95 (1.30;2.61)	2.51 (1.78;3.25)	2.80 (2.11;3.49)
SuPAR (ng/mℓ)	4.93 (0.81;9.06)	1.80 (-2.92;6.51)	2.98 (-1.78;7.75)
C-reactive protein (mg/ℓ)	-3.31 (-16.0;9.42)	-1.38 (-20.8;18.0)	-5.04 (-21.1;11.0)
Interleukin-6 (pg/mℓ)	28.2 (16.9;39.5)	21.6 (8.29;34.9)	24.0 (10.6;37.4)

CSWA, cross sectional wall area; suPAR, soluble urokinase plasminogen activator receptor; IMT, intima-media thickness. Data are expressed as arithmetic mean (95% confidence intervals).

Table 3 Relationship of % change in IMT and CSWA, respectively, with % change in inflammatory markers

	Total group		**Increased vascular structure**	
	r	***P***	***r***	***p***
IMT	*n*=295		*n*=199	
SuPAR (ng/mℓ)	-0.13	0.028	-0.08	0.26
C-reactive protein (mg/ℓ)	-0.01	0.83	-0.09	0.20
Interleukin-6 (pg/mℓ)	-0.04	0.53	-0.09	0.23
CSWA	*n*=295		*n*=98	
SuPAR (ng/mℓ)	-0.06	0.31	0.004	0.95
C-reactive protein (mg/ℓ)	-0.01	0.90	-0.08	0.25
Interleukin-6 (pg/mℓ)	0.01	0.84	0.07	0.33

IMT, intima-media thickness; CSWA, cross-sectional wall area; suPAR, soluble urokinase plasminogen activator receptor. *p*-values were obtained with partial correlations. Adjusted for race, sex, age, waist circumference and mean arterial pressure.

Table 4 Independent associations of % change in IMT and CSWA, respectively, with % change in inflammatory markers in the total group

	Adjusted R^2	β (95%CI)	*p*
IMT			
SuPAR (ng/mℓ)	0.16	-0.12 (-0.22;-0.01)	0.036
C-reactive protein (mg/ℓ)	0.15	-0.01 (-0.12;0.10)	0.89
Interleukin-6 (pg/mℓ)	0.15	-0.04 (-0.15;0.07)	0.52
CSWA			
SuPAR (ng/mℓ)	0.11	-0.03 (-0.09;0.03)	0.35
C-reactive protein (mg/ℓ)	0.11	0.003 (-0.02;0.02)	0.98
Interleukin-6 (pg/mℓ)	0.11	-0.09 (-0.22;0.05)	0.78

β, partial regression coefficient; 95% CI, 95% confidence intervals of β. IMT, intima-media thickness; suPAR, soluble urokinase plasminogen activator receptor; CSWA, cross-sectional wall area. Models include age, race, sex, waist circumference, glucose, high-density lipoprotein cholesterol, total energy expenditure, gamma-glutamyltransferase, tobacco use, anti-inflammatory medication use and mean arterial pressure.

Discussion

We investigated whether changes in vascular structure are associated with changes in inflammation over a three-year period. We found that the vascular structure markers IMT and CSWA, as well as the inflammatory markers suPAR and IL-6 increased, while CRP decreased over three years. Further, % change in IMT inversely associated with % change in suPAR only.

Even though percentage change in IMT, but not CSWA associated with percentage change in suPAR, this association was inverse, and neither IMT nor CSWA associated with either CRP or IL-6. This result was unexpected, since a 15-year study among men and women aged 45 to 68 years found that suPAR associated with an increase in carotid plaque and a higher prevalence of coronary artery disease [23]. This positive association with suPAR was further confirmed in other cross-sectional studies [24, 25]. However, it should be taken into account that these studies were conducted in subjects with progressed CVD [23] or related diseases [24, 25], which was not the case in our population sample. Participants in our population had relatively normal IMT values, seeing that only 4% of our participants had follow-up IMT values of >0.90 mm, which is the proposed cut point for sub-clinical IMT, as prescribed by the 2016 European guidelines on cardiovascular disease prevention in clinical practice [26].

Nonetheless, we expect that a positive association between vascular structural changes and suPAR may only become evident over the longer term, once vascular structure has progressed to the stage were atherosclerosis and other associated cardiovascular diseases have become evident. Our novel results, showing an inverse relationship between IMT and suPAR, underline the need for more research to determine the possible mechanism(s) behind this relationship, especially at early vascular disease stages. Other factors may also influence this relationship. For instance, both anti-inflammatory and antioxidant biomarkers [27] have been found to play a protective role during the early stages of cardiovascular disease, but literature investigating their effects on suPAR, and specifically their effect on the association between early changes in vascular structure and pro-inflammatory markers, is scarce.

The absence of associations of vascular structural markers with CRP and IL-6 supports previous findings. In another three-year study, which included 3 122 subjects from Europe, no association was found between early progression of IMT and CRP after adjusting for conventional risk factors [28]. A study conducted in the United States confirmed these results, as the relationship between IMT and CRP weakened after adjusting for risk factors such as smoking, blood pressure and diabetes mellitus [29]. With regards to IL-6, a stimulator of CRP production [30], a Rotterdam study with a sample size of 7 983 subjects aged 55 years and older, also found no independent association between IL-6 and IMT [31]. Similar results were obtained in an Australian study where IL-6 was only found to be a predictor of IMT when CRP, fibrinogen and monocyte count was included in the linear regression model. However, after adjusting for conventional risk factors, the association between IMT and IL-6 did not remain significant [32]. These findings indicate that neither CRP nor IL-6 have an independent effect on an increase in IMT, but may rather facilitate the effect that some conventional risk factors such as obesity, tobacco and alcohol use have on vascular structure.

Furthermore, it is known that suPAR and CRP may represent different processes in vascular inflammation [13]. SuPAR, which is secreted by smooth muscle cells and endothelial cells [35], plays a role as marker of inflammation in the vascular wall [13] and is regarded as a better marker of CVD [33] and cardiovascular-related mortality [34] when compared to CRP. CRP, on the other hand, originates from adipose tissue, is synthesised by the liver, and is therefore rather associated with adipose-related inflammation [13].

The strength of the present study is lodged in its longitudinal nature and the inclusion of data which are limited in the South African research setting. Even though this study may have been limited by the small sample size, power analyses proved this sample size to be sufficient. Our study design further contributed to this being a well-controlled group.

In conclusion, we found that vascular structure worsened over three years, which was related to changes in suPAR but not in CRP or IL-6 within this population. More in-depth research is

recommended to investigate and compare the specific mechanisms through which suPAR, CRP and IL-6, respectively or in combination, contribute to changes in vascular structure. Future studies should also investigate the effect of the relationship between pro- and anti-inflammatory markers on early changes in vascular structure in the South African population.

Acknowledgements

We are grateful towards the participants in this study, the research team, field workers and the North-West University, South Africa.

Financial disclosure

The SABPA study was financially supported by the Metabolic Syndrome Institute in France; as well as the North-West University, the Medical Research Council, the National Research Foundation, the PA & Alize Malan Trust, the North West Department of Education and Roche Diagnostics, South Africa.

Conflict of interest

The authors declare no conflict of interest.

Table S1 Effect of race and sex on the association between %change in IMT and baseline inflammatory markers in the total group

	Adjusted R^2	***β* (95%CI)**	***p***
Interaction with race			
SuPAR (ng/mℓ)	0.15	1.46 (-1.46;4.38)	0.34
C-reactive protein (mg/ℓ)	0.15	1.04 (-1.31;3.40)	0.82
Interleukin-6 (pg/mℓ)	0.14	1.03 (-1.35;3.41)	0.89
Interaction with sex			
SuPAR (ng/mℓ)	0.01	2.46 (-0.64;5.56)	0.050
C-reactive protein (mg/ℓ)	0.05	1.04 (-1.49;3.56)	0.88
Interleukin-6 (pg/mℓ)	0.01	1.22 (-1.15;3.58)	0.30

β, partial regression coefficient; 95%CI, 95% confidence intervals of *β*. IMT, intima-media thickness; suPAR, soluble urokinase plasminogen activator receptor.

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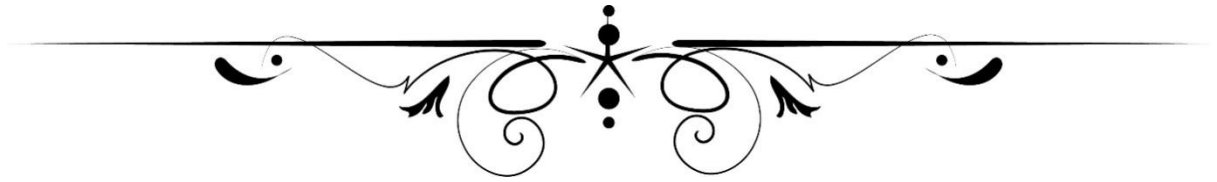
Chapter 4

Final remarks and recommendations for future studies



Introduction

This chapter presents a summary of the findings reported in the article in Chapter 3. Key findings of the article are discussed and compared to the literature. Thereafter, recommendations made for future studies investigating the link between early vascular structural changes and inflammation.

Summary of key findings and reflection on hypotheses

It has been shown that cardiovascular outcomes can occur in patients in whom the conventional risk factors were absent, suggesting that alternative mechanisms may also contribute towards CVD's [1]. Improvements in the field of vascular biology has shown a significant association between inflammation and CVD [2, 3]. The inflammatory markers interleukin-6 (IL-6), C-reactive protein (CRP) and soluble urokinase plasminogen activation receptor (suPAR) have been found to play a role in vascular changes as measured by intima-media thickness (IMT) and cross-sectional wall area (CSWA) [4-6]. However, most of these were cross-sectional studies conducted in areas other than South Africa. These studies also focused on vascular changes in participants with progressed atherosclerosis or related diseases. We therefore explored whether vascular structure relates to inflammation in South Africans over three years.

We **firstly** hypothesised that markers of vascular structure and inflammation will increase over three years. As expected, we found that IMT, CSWA, IL-6 and suPAR increased, but that CRP unexpectedly decreased. Our results confirm those of other longitudinal studies which also observed an increase in IMT [7], CSWA [7], IL-6 [7] and suPAR [8] over time. However, in contrast to our results, a previous study showed an increase in CRP over five years [8], while another study reported that 21% of their population sample had increased levels of CRP, while 27% showed a decrease after nine years [9]. The decrease in CRP levels in our population was an unexpected finding which cannot yet be explained and warrants further investigation.

We can, however, speculate that the acute nature of CRP [6] might explain why longer term increases in CRP were not found. Therefore, the first hypothesis is only partially accepted.

Secondly, we hypothesised that changes in vascular structure markers will associate with an increase in inflammatory markers in South Africans over three years, due to the important role of inflammation in vascular structural changes. Surprisingly, we only found an inverse association between IMT (but not CSWA) and suPAR (but not IL-6 or CRP) within the total group. These results both confirm and contradict previous findings. Preceding studies found a direct association between IMT and suPAR [10-12]. However, our population did not have progressed IMT values, which might also explain the lack of association between IMT and inflammation. This might suggest that suPAR will only associate with vascular changes once vascular damage is more progressed. Furthermore, a number of studies reported an association between IMT and IL-6 [13] and CRP [14], respectively, while our results are similar to other studies that found no independent association between IMT and IL-6 [15, 16] or CRP [17, 18]. This absence of an association between vascular structure markers and IL-6 and CRP may suggest that neither IL-6 nor CRP has an independent effect on vascular structure changes, but may rather assist the effect that some modifiable risk factors have on vascular structure. Our second hypothesis is thus rejected.

Strengths, limitations and recommendations for future studies

It is important to interpret the findings of this study in the context of its strengths, limitations and possible confounding factors.

This study was of longitudinal nature. We were able to account for regression to the mean by including baseline values during the calculation of percentage change in variables of interest. This added to the limited data available in the South African research setting, since most studies on this topic were conducted in a cross-sectional manner.

In addition, we included three different inflammatory markers. Previous studies have explored associations between inflammation and various diseases within different populations. However, they did not necessarily include all three of the markers (IL-6, CRP and suPAR), especially not where the association between inflammation and changes in vascular structure was explored.

The sample size included for this study was not very large (*n*=303); however, we did perform power analyses in advance which proved that this sample size was sufficient in order to test our hypotheses.

In addition, this was a well-controlled study. A detailed protocol and standardised methods were followed during both baseline and follow-up measurements to ensure comparability. Measurements were completed in a stable environment where conditions such as dietary intake, physical activity and room temperature were regulated during measurements. Furthermore, to avoid the effect of seasonal changes on biological markers, all measurements were conducted during the months of February and May and the stability of blood samples were assured by storing it at -80°C.

In order to account for other traditional confounding factors, we included race, sex, age, waist circumference, gamma glutamyltransferase (as indicator of alcohol use), glucose, triglycerides-to-high-density lipoprotein ratio, mean arterial pressure, self-reported tobacco and anti-inflammatory medication use.

Recommendations

While this was a longitudinal study, we recommend that prospective studies of longer than three years should be conducted to investigate the long-term cause-and-effect relationship between vascular structure and inflammation, especially in a South African setting, comparing populations with and without established cardiovascular diseases.

Future studies should also include other markers involved in inflammation (such as tumour necrosis factor-α, interleukin-8, amayloid protein A, white blood cell count and fibrinogen), as well as anti-inflammatory and anti-oxidant markers, in order to provide an even broader inflammatory profile. These approaches may help to explore the mechanisms and possible prognostic link between early changes in vascular structure and both a pro- and anti-inflammatory profile in a South African population.

Furthermore, using measurements that could quantify vascular function, such as pulse wave analyses, pulse wave velocity, and pulse pressure amplification, can contribute to our understanding of the link between inflammation and vascular structure

We further recommended that cut points for IL-6, CRP and suPAR should be explored, specifically in a South African population, to investigate whether these markers can assist in predicting cardiovascular risk.

Conclusion

In conclusion, we found that, although vascular structure worsened over three years, inflammation did not play a contributing role therein. Our results show that more in-depth research is needed within the South African population to investigate and compare the specific mechanisms through which suPAR, CRP and IL-6, respectively or in combination, contribute to both early and progressed changes in vascular structure. Such information may assist in the attempt to combat the already high CVD prevalence and may possibly help to lower cardiovascular morbidity and mortality risk in this population.

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8. Botha S, Fourie CM, Schutte R, Eugen-Olsen J, Schutte AE. Soluble urokinase plasminogen activator receptor and hypertension among black South Africans after 5 years. Hypertens Res 2015;38(6):439-444.
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11. Persson M, Östling G, Smith G, Hamrefors V, Melander O, Hedblad B, et al. Soluble Urokinase Plasminogen Activator Receptor. Stroke 2014;45(1):18-23.
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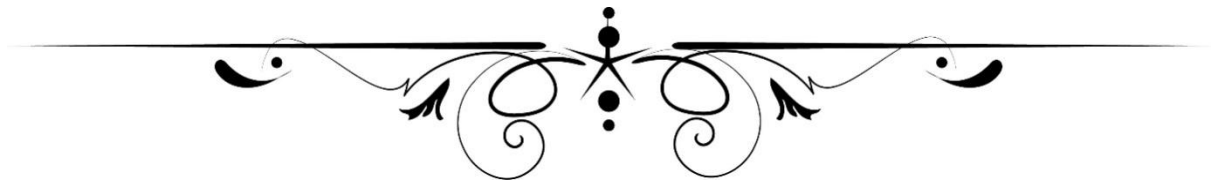
17. Lorenz MW, Karbstein P, Markus HS, Sitzer M. High-sensitivity C-reactive protein is not associated with carotid intima-media progression. Stroke 2007;38(6):1774-1779.
18. Sitzer M, Markus HS, Mendall MA, Liehr R, Knorr U, Steinmetz H. C-reactive protein and carotid intimal medial thickness in a community population. J Cardiovasc Risk 2002;9(2):97-103.

Appendix A

Approval from the Health Research Ethics Committee



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Institutional Research Ethics Regulatory Committee
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Email : Ethics@nwu.ac.za

ETHICS APPROVAL CERTIFICATE OF STUDY

Based on approval by **Health Research Ethics Committee (HREC)** on **20/06/2017**after being reviewed at the meeting held on **17/05/2017**, the North-West University Institutional Research Ethics Regulatory Committee (NWU-IRERC) hereby **approves** your study as indicated below. This implies that the NWU-IRERC 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: Exploring the associations of change in vascular structure and blood pressure with inflammation in a bi-ethnic South African population: The SABPA study
Study Leader/Supervisor: Dr S Botha
Student: C Swart-24177865

Ethics number:

N	W	U	-	0	0	0	5	1	-	1	7	-	A	1
Institution				Study Number						Year			Status	

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

Application Type: Single Study
Commencement date: 20/06/2017 **Risk:** Minimal

Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation.

Special conditions of the approval (if applicable):

- Translation of the informed consent document to the languages applicable to the study participants should be submitted to the HREC (if applicable).
- Any research at governmental or private institutions, permission must still be obtained from relevant authorities and provided to the HREC. Ethics approval is required BEFORE approval can be obtained from these authorities.

General conditions:

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

- *The study leader (principle investigator) must report in the prescribed format to the NWU-IRERC via HREC:*
  - *annually (or as otherwise requested) on the monitoring of the study, and upon completion of the study*
  - *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.*
- *Annually a number of studies may be randomly selected for an external audit.*
- *The approval applies strictly to the proposal as stipulated in the application form. Would any changes to the proposal be deemed necessary during the course of the study, the study leader must apply for approval of these amendments at the HREC, prior to implementation. Would there be deviated from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility the NWU-IRERC and HREC retains 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 HREC or that information has been false or misrepresented,*
    - *the required amendments, annual (or otherwise stipulated) report and reporting of adverse events or incidents was not done in a timely manner and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *HREC can be contacted for further information or any report templates via Ethics-HRECApply@nwu.ac.za or 018 299 1206.*

The IRERC 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 IRERC or HREC for any further enquiries or requests for assistance.

Yours sincerely

Prof LA Du Plessis
Digitally signed by Prof LA Du Plessis
Date: 2017.07.13 10:17:44 +02'00'

Prof Linda du Plessis
Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

Appendix B

Certificate of Language Editing

Declaration

This is to declare that I, Annette L Combrink, accredited language editor and translator of the South African Translators' Institute, have language-edited the mini-dissertation by

C Swart

with the title

Vascular structure and inflammation in a bi-ethnic South African population: The SABPA study

Prof Annette L Combrink
Accredited translator and language editor
South African Translators' Institute
Membership No. 1000356
Date: 16 November 2017

Appendix C

Turn-it-in Report

Carla Swart_24177865

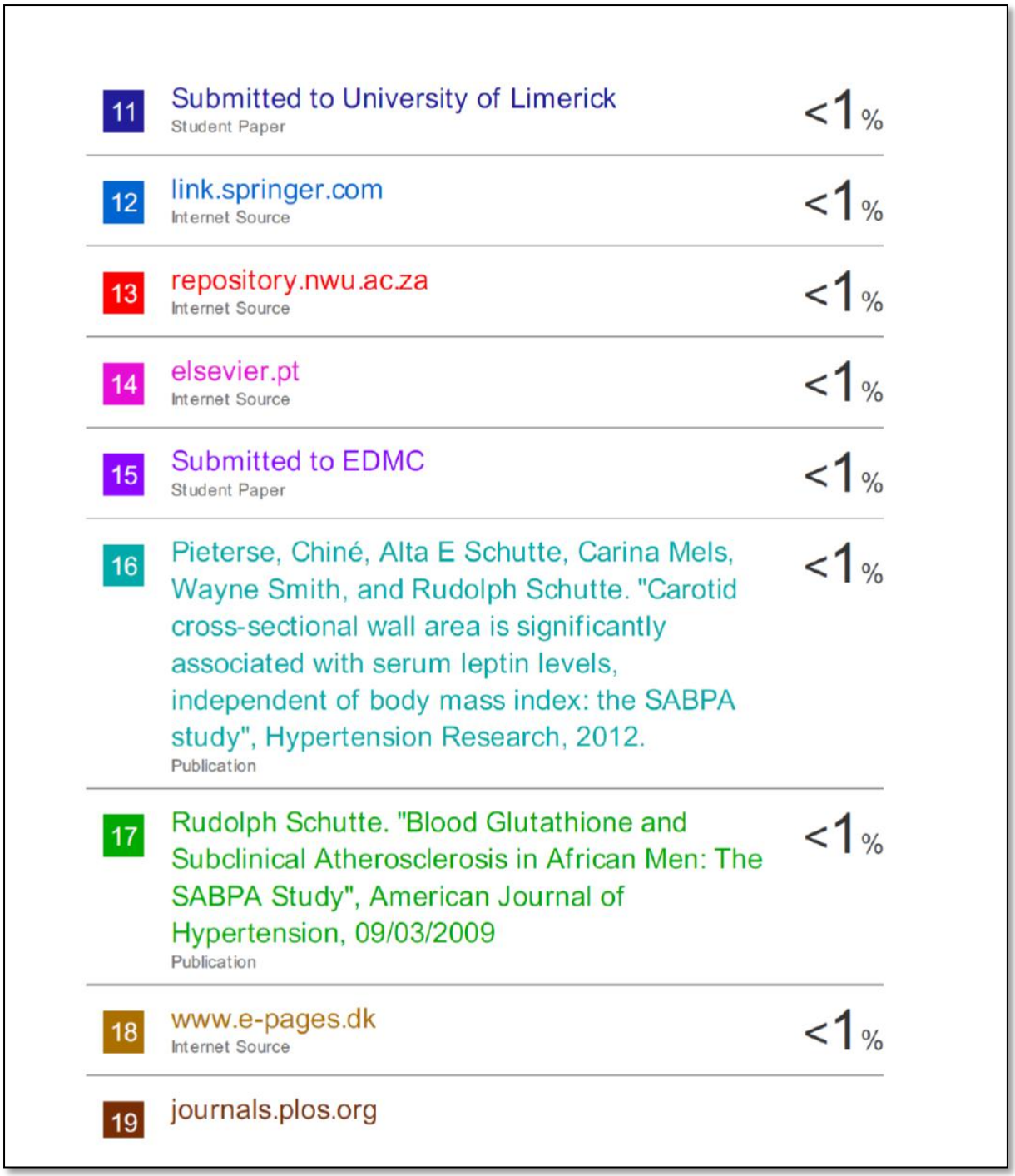
ORIGINALITY REPORT

20%	11%	9%	12%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to North West University Student Paper	10%
2	dspace.nwu.ac.za Internet Source	2%
3	Botha, Shani, Carla M.T. Fourie, Rudolph Schutte, Jesper Eugen-Olsen, Ronel Pretorius, and Aletta E. Schutte. "Soluble urokinase plasminogen activator receptor as a prognostic marker of all-cause and cardiovascular mortality in a black population", International Journal of Cardiology, 2015. Publication	1%
4	Botha, Shani, Carla MT Fourie, Rudolph Schutte, Jesper Eugen-Olsen, and Aletta E Schutte. "Soluble urokinase plasminogen activator receptor and hypertension among black South Africans after 5 years", Hypertension Research, 2015. Publication	1%
5	Submitted to University of Newcastle	

	Student Paper	<1%
6	LyngbÃ¦k, Stig, Jacob L. Marott, Thomas Sehestedt, Tine W. Hansen, Michael H. Olsen, Ove Andersen, Allan Linneberg, Steen B. Haugaard, Jesper Eugen-Olsen, Peter R. Hansen, and JÃ¸rgen Jeppesen. "Cardiovascular risk prediction in the general population with use of suPAR, CRP, and Framingham Risk Score", International Journal of Cardiology, 2012. Publication	<1%
7	Yehuda Shoenfeld. "Mechanisms of Disease: atherosclerosis in autoimmune diseases", Nature Clinical Practice Rheumatology, 02/2006 Publication	<1%
8	preview-jcmr-online.biomedcentral.com Internet Source	<1%
9	jap.physiology.org Internet Source	<1%
10	Matthias Barton. "Endothelium and Atherogenesis: Endothelial Therapy Revisited :", Journal of Cardiovascular Pharmacology, 11/2001 Publication	<1%



11	Submitted to University of Limerick Student Paper	<1%
12	link.springer.com Internet Source	<1%
13	repository.nwu.ac.za Internet Source	<1%
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15	Submitted to EDMC Student Paper	<1%
16	Pieterse, Chiné, Alta E Schutte, Carina Mels, Wayne Smith, and Rudolph Schutte. "Carotid cross-sectional wall area is significantly associated with serum leptin levels, independent of body mass index: the SABPA study", Hypertension Research, 2012. Publication	<1%
17	Rudolph Schutte. "Blood Glutathione and Subclinical Atherosclerosis in African Men: The SABPA Study", American Journal of Hypertension, 09/03/2009 Publication	<1%
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22	C Harris, H Demmelmair, A von Berg, I Lehmann, C Flexeder, B Koletzko, J Heinrich, M Standl. "Associations between fatty acids and low-grade inflammation in children from the LISAplus birth cohort study", European Journal of Clinical Nutrition, 2017 Publication	<1%
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33	Mok, C. C.. "Accelerated atherosclerosis, arterial thromboembolism, and preventive strategies in systemic lupus erythematosus", Scandinavian Journal of Rheumatology, 2006. Publication	<1%