

Exploring the links of cardiovascular structure and function with biomarkers related to vascular calcification: The African-PREDICT study

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Preface

The format of this dissertation was chosen and approved by the North-West University. This dissertation consists of a background and motivation, literature overview, methodology, a manuscript that will be submitted to a peer review journal and a concluding chapter which summarises the main findings of the study and recommendations for future studies

The layout of the dissertation is as follows:

Chapter 1: Literature review, motivation, aims, objectives and hypotheses

Chapter 2: Methodology

Chapter 3: Research manuscript

Chapter 4 Summary of main findings

References are provided at the end of each chapter according to the reference style recommended by the Hypertension Research journal. All figures used throughout this dissertation were produced by Servier Medical Art, available from <http://smart.servier.com/>

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The following statement from the co-authors confirms their individual involvement in this study and gives their permission that the relevant research article may form part of this dissertation.

Hereby, I declare that I approved the abovementioned dissertation and that my role in this study (as stated above) is representative of my contribution towards the research article and supervised Master's study. I also give my consent that this research article may be published as part of the dissertation of Ashleigh Craig.



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Summary

Motivation

Evidence linking the onset of vascular calcification via abnormal mineral metabolism as contributor to the development of cardiovascular disease (CVD) warrants exploring. Vascular calcification is linked to disease states including chronic kidney disease, hypertension and type 2 diabetes mellitus, especially in older populations, while less is known about the potential links of cardiac and arterial structure and function with markers related to vascular calcification in young black and white individuals with no apparent CVD.

Aim

To explore whether associations of left ventricular relative wall thickness and systolic function exists with biomarkers related to vascular calcification in young South Africans.

Methodology

This study formed part of the larger African prospective study on early detection and identification of cardiovascular disease and hypertension (African-PREDICT). Cross-sectional data of the first 400 participants which included black (n=160) and white (n=175) men and women after exclusion. Participants who presented with missing variables of interest were excluded from this study. This study obtained the appropriate ethical approval from the Health Research Ethics Committee of the North-West University (NWU-00048-17-S1). Anthropometric measures included body height, weight and waist circumference. Body mass index as well as body surface area were additionally calculated. Blood pressure was measured on the left arm in duplicate whilst participants remained in a rested seating position. The General Electric Vivid E9 device (GE Vingmed Ultrasound A/S; Hearten, Norway) and a 3-lead ECG was used to determine relative wall thickness. Stroke volume was determined and normalised for height in the power of 2.04 as the stroke volume index. By multiplying the stroke volume with heart rate, cardiac index was obtained. Additionally, fractional shortening as well as left ventricular ejection fraction was furthermore determined. We performed biochemical analyses which included a lipid profile (triglycerides, high density lipoprotein cholesterol, low density lipoprotein cholesterol and total cholesterol),

gamma glutamyl-transferase, cotinine, high sensitivity C-reactive protein, creatinine, alkaline phosphatase, and calcium. The ratio of total cholesterol to high density lipoprotein cholesterol was additionally calculated. Glutathione peroxidase, a marker of oxidative stress was determined in whole blood and one of the measurable, reactive oxygen species (serum peroxides) was determined in serum. We performed independent T-tests and Chi-square tests to compare means and proportions. Single and multiple regression analyses were performed to investigate the associations of cardiac structure (relative wall thickness) and function (ejection fraction, fractional shortening, systolic index and cardiac index) with markers of vascular calcification (alkaline phosphatase and calcium).

Results

When comparing the black and white groups, we found that the black group presented with higher blood pressure measures, relative wall thickness as well as alkaline phosphatase (all $p \leq 0.001$). In single and multivariate regression analyses, after adjusting for age, sex and body mass index (stroke index additionally adjusted for waist circumference), positive associations of relative wall thickness and alkaline phosphatase existed in the black group only (adj. $R^2=0.030$; $\beta=0.176$; $p=0.037$). Ejection fraction (adj. $R^2=0.083$; $\beta=-0.208$; $p=0.015$) and fractional shortening (adj. $R^2=0.103$; $\beta=-0.195$; $p=0.021$) associated inversely with alkaline phosphatase in the white group. Cardiac index associated inversely with calcium in both the black (adj. $R^2=0.096$; $\beta=-0.181$; $p=0.031$) and white (adj. $R^2=0.403$; $\beta=-0.141$; $p=0.021$) groups. Stroke index associated inversely with calcium in the black (adj. $R^2=0.165$; $\beta=-0.161$; $p=0.046$) and white (adj. $R^2=0.353$; $\beta=-0.147$; $p=0.019$) groups as well as alkaline phosphatase (adj. $R^2=0.354$; $\beta=-0.172$; $p=0.016$) in the white group only.

Conclusion

Our results indicate that in young apparently healthy populations, cardiac structure (relative wall thickness) and function (systolic function markers) associated with markers of vascular calcification (alkaline phosphatase and calcium). Thus, an altered mineral metabolism may contribute to early vascular calcification manifestations and promote premature cardiac compromise. The different associations seen in the black versus the white group may suggest different mechanisms at play for the onset of

vascular calcification in younger participants. These findings need to be confirmed in larger prospective studies.

Key Words: Alkaline phosphatase, calcium, cardiovascular disease, ethnicity, mineralisation, vascular calcification.

List of abbreviations

α	Alpha
African-PREDICT	African Prospective study on the Early Detection and Identification of Cardiovascular Disease and Hypertension
BMI	Body mass index
BSA	Body surface area
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
GGT	Gamma-glutamyltransferase
HIV	Human immunodeficiency virus
kg	Kilogram
m	Metre
mg/dL	Milligrams per decilitre
ml	Millilitre
mm	Millimetres
mmHg	Millimetres of mercury
mmol/L	Millimole per litre
n	Number of participants
NRF	National Research Foundation
PURE	Prospective Urban and Rural Epidemiology
SAFREIC	South African study on the Influence of Sex, age and Ethnicity on Insulin Sensitivity and cardiovascular function
SAMRC	South African Medical Research Council
SARChI	South African Research Chairs Initiative

SHIP	Strategic Health Innovation Partnerships
U/L	Units per litre
μmol/L	Micromoles per litre

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Chapter 1

Literature review and motivation of study

1.1 Introduction

There is undoubtedly an abundance of factors leading to cardiovascular morbidity and mortality, including vascular calcification.¹ Over several decades, the presence of vascular calcification namely ectopic calcification in the vasculature was seen as a passive degeneration of the inevitable aging process.^{1,2} Upon recent evidence, the competition between factors promoting vascular calcification and the inhibition of the mineralisation process were highlighted as the potential mechanisms initiating pathogenesis.¹

Black South Africans are subjected to early vascular alterations within the vasculature therefore increasing their susceptibility for blood vessel stiffening and resultant cardiac damage.³ Several markers such as alkaline phosphatase and circulating calcium were associated with vascular calcification.⁴⁻⁷ The interactions of cardiovascular structure and function with markers of vascular calcification will be discussed in detail in this literature review.

1.2 Cardiovascular structure and function

The functional role of the cardiovascular system is to maintain cellular homeostasis via the delivery of sufficient blood supply at a high pressure and constant flow to the peripherals.^{8,9} The various anatomical regions serve the left ventricle and tissues that are in need of blood. These regions include: (i) large elastic arteries such as the carotid and aorta; (ii) muscular arteries such as femoral and brachial and; (iii) arterioles.^{8,10,11}

The vascular wall consists of three concentric zones as depicted in Figure 1, namely: tunica intima, media and adventitia of which each layer consists of specialised cells that function interactively to maintain adequate blood distribution.^{8,12} The artery consists of two predominant supporting proteins (collagen and elastin) as well as smooth muscle tissue.¹³ The thick muscular layer within the artery allows for the transportation of blood ejected from the cardiac muscle.¹⁴ In the event where there is a reduction in elastin production, an increase in collagen and calcium deposits is inevitable.¹⁵ This tends to lead to an increase in the intima media thickness which could be characterised with the development of several cardiac diseases.¹⁵

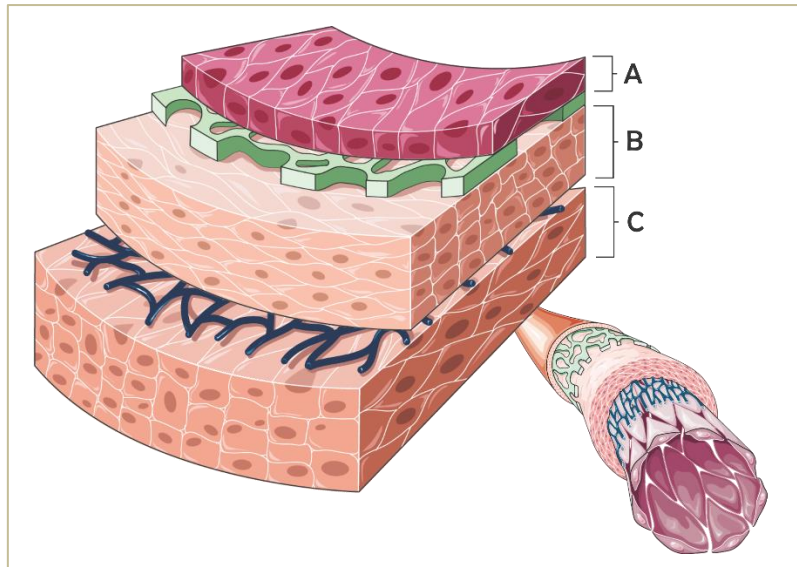


Figure 1. Cross sectional schematic view of the vascular wall (tunica intima, tunica media and tunica adventitia respectively). The vascular wall consists of three functional layers. The first layer (A) consists of the innermost endothelial layer and small amounts of connective tissue located just below the endothelium. The second layer (B) composed mainly of smooth muscle cells and elastin-rich extracellular matrix. The third layer (C) is largely comprised of collagen fibres yet fewer elastin fibres.¹⁰

The haemodynamic demands of the cardiovascular system requires the storage of energy in its elastance within the aorta during systole and its release during diastole.¹⁶ This known as the Windkessel model which aims to minimize cardiac workload as reflected in the high density of elastin in the arch.¹⁶ Energy stored within the aorta is lost due to an increase in arterial stiffness, as seen within calcified arteries.¹⁶ This tends to result in thoracic summation which causes overall detrimental effects such as increased systolic and pulse pressures.¹⁶ This leads to an elevation in cardiac workload, aiding in heart failure, diastolic dysfunction and left ventricular hypertrophy.¹⁶

With age, the elastic lamellae is subjected to a disruption and fragmentation as well as there is an alteration in the collagen-to-elastin ratio within the central arteries.¹⁷ This deterioration is therefore accelerated by the presence of several cardiovascular compromises such as diabetes mellitus, chronic kidney disease as well as the most reported compromise, hypertension.^{10,18} Vascular calcification can therefore occur in either the tunica intima, the tunica media or alternatively occurring in both layers simultaneously.¹⁸

1.3 Vascular calcification

1.3.1 Pathophysiological mechanisms

The deposition of the calcium phosphate mineral, namely hydroxyapatite in cardiovascular tissues is termed vascular calcification.¹⁹ For half a century, vascular calcification has been associated with a poor prognosis as a result of vascular disease.² The pathogenesis of calcification is characterised by the synthesis of densely structured bone modelling and physicochemical accumulation of minerals without cellular involvement.²⁰ Recent laboratory and clinical results revealed an increase in the recognition that vascular calcification is an active, regulated process which may be treatable and preventable.^{21,22} Vascular calcification and accompanied stiffness progress with the aging process.²³ However, the onset to vascular calcification may arise due to cardiovascular injury, disease or genetic deficiency that favours heterotopic mineral deposition.²³

The two most important arterial complications leading to cardiovascular events are intimal and medial calcification.²⁴ Intimal calcification is a fundamental part of atherosclerotic plaque development and serves as a strong predictor for the development of cardiovascular disease (CVD).^{25,26} Intimal calcification is a disorganised process including vascular smooth muscle cells, connective tissue, oxidised lipids and macrophages.²⁷ On the contrary, medial calcification can be seen as an organised mineral deposition seen along the elastic lamellae including vascular smooth muscle cells and elastin fibres.²⁷ Medial calcification is normally seen in the elderly as well as in individuals with chronic renal failure and diabetes mellitus.¹⁵ It is therefore of importance that the pathophysiology of vascular calcification is elucidated to assess potential contributing factors and clinical implications thereof, especially since medial calcification is known to reduce arterial compliance.²⁸

Furthermore, evidence supports that vascular calcification is a process similar to mineralisation in bone tissue.²³ Contractile cells such as vascular smooth muscle cells are located on the medial layer of the vascular wall.²⁹ Once triggered, these cells trans-differentiate into calcified vascular cells which no longer exhibit the phenotypic attributes responsible for normal smooth muscle cell contractility.²⁹ These cells can undergo further physiological alterations resulting in the cell entering a synthesis state with copious extracellular matrix protein production followed by mediated calcification

of the matrix vesicle.^{29,30} Pathological calcification can be seen as an analogue to bone mineralisation as both processes are characterised by vascular smooth muscle cells entering an osteoblast-like differentiated state.²⁹ Osteoblast-like cells are proficient in the production of bone matrix proteins such as type I collagen and osteopontin which may regulate the mineralisation process.³¹ This in turn initiates an increase in calcium and phosphorus production which accelerates the process of calcification leading to stiffening of the vessel.³²

Dysfunctional vascular smooth muscle cells facilitate the mechanisms that are involved in the pathogenesis of vascular calcification.¹⁶ The precise mechanism to which the vascular smooth muscle cells calcify is incompletely characterised. However, several studies have suggested that only certain pools of vascular smooth muscle cells have osteogenic potential.^{33,34} Vascular calcification may arise either due to reduced inhibition of mineralisation; a lack of certain matrix proteins (matrix GLA protein) or; collagen type I pyrophosphate expression (alkaline phosphatase).^{20,35,36}

1.4 Factors involved in calcification

Over the past decade, accumulating evidence points to the eminent role of increased alkaline phosphatase and calcium influx in the pathogenesis of CVD.^{37,38}

1.4.1 Alkaline phosphatase

Alkaline phosphatase has become an emerging marker of cardiovascular risk amongst the general population.³⁹ Alkaline phosphatase is present in most human tissues and is known to catalyse potent inhibitors thus prompting calcification.^{23,40} Serum alkaline phosphatase has shown to be a foretelling indicator for cardiovascular events in individuals with renal disease.^{41, 42} Additionally, elevated alkaline phosphatase levels have shown an association with cardiovascular events in individuals with normal kidney function.^{41,42}

In addition to assessing bone health, recent evidence suggests that alkaline phosphatase may also have value for predicting CVD outcomes.⁴³ Several prospective studies have proven that elevated alkaline phosphatase is independently associated with the presence of cardiac disease.^{4,44,45} The mechanism to which alkaline

phosphatase induces CVD is not quite clear. It is, however, possible that higher alkaline phosphatase may be linked to the development of vascular calcification.^{46,47} This could either occur directly through the hydrolysis of potent calcification inhibitors or indirectly as a replacement marker for other mediators of vascular calcification such as insufficient vitamin D metabolism.^{46,47}

Alkaline phosphatase has been associated with several inflammatory markers.³⁹ It is considered an inflammatory mediator due to the direct and significant association alkaline phosphatase displayed with C-reactive protein. This is possibly due to the potential common biological pathways they may share.³⁹

1.4.2 Calcium

Several factors actively regulate serum calcium levels such as parathyroid hormone, alkaline phosphatase and vitamin D.⁴⁸ The parathyroid glands control the calcium levels in the blood to support healthy bone and mineral homeostasis.⁴⁹ An expected heightened level of calcium may decrease circulating parathyroid hormone levels thus reducing the risk of CVD development.⁵⁰ The effects of calcium on the presence of vascular disease still warrants thorough investigation. However, with the process of calcification, a dysregulation of calcium is observed.⁵¹

A number of factors seem to actively control urinary excretion of calcium via the stabilisation of constant circulating calcium at normal concentrations. This is achieved by balancing the deposition of bone calcium with gastrointestinal absorption.⁵¹ Via the increase of parathyroid hormone secretion of which may be a result of insufficient vitamin D, a decrease in circulating calcium and phosphorus is inevitable.⁵² This will however, cause calcium reabsorption within the kidney to facilitate the conversion of vitamin D into its active form as well as to initiate bone reabsorption in order to increase serum calcium back to a normal concentration.⁵³

Cardiovascular risk stratification via a primary risk evaluation is the key step towards the goal of reducing cardiovascular mortality.⁵⁴ Due to traditional risk factor assessments displaying poorly to sensitivity to predict CVD outcomes as well as coronary heart disease presenting in asymptomatic patients, there is a constant need to improve risk stratification measures.⁵⁴ The National Cholesterol Education Program

has set aside specific guidelines to classify patients into different categorical groups based upon the presence of risk factors.⁵⁴ Intermediate-risk groups may be further stratified based on the presence of coronary artery calcium.⁵⁴ Coronary artery calcium plays a role in the development of coronary artery disease, occurs extensively in atherosclerotic coronary artery disease and is found completely absent in normal arteries.⁵⁵ Although numerous risk scores predict cardiovascular outcomes moderately well, there has been exploration for a better risk factor. This can therefore be accomplished using coronary artery calcium scoring.⁵⁴ Numerous studies have highlighted the prognostic value of coronary artery calcium score leading to a great deal of interest in this particular scoring stratification.⁵⁶⁻⁵⁹ Therefore, coronary artery calcium scoring may be a valuable non-invasive imaging modality for cardiovascular risk stratification in asymptomatic individuals.⁵⁴

1.4.3 Other factors

Parathyroid hormone is secreted or its release inhibited continuously to regulate bone and mineral metabolism to stimulate the conversion of vitamin D into its active form.⁶ However, several studies have emphasized parathyroid hormone not only functioning as a biomarker of vitamin D status but as an independent cardiovascular risk factor.^{6,38}

Increased left ventricular mass, a strong independent cardiovascular mortality predictor has been observed in several individuals with primary hyperparathyroidism.⁶⁰ Similarly, diastolic dysfunction is considered a CVD predictor.⁶⁰ Myocardial infarction, stroke or even cardiac death are all attributable to mitral annular calcification which is clearly demonstrated in almost all primary hyperparathyroidism individuals.⁶⁰ Therefore, abnormal parathyroid hormone exerts unwanted effects on the cardiovascular system leading to an overall greater left ventricular mass thus escalating the susceptibility of an individual to disease development.³⁸

Recent studies have shown that vitamin D deficiency has become a global health concern.^{62,64,65,66} Vitamin D insufficiency is a common finding amongst individuals with confirmed heart failure.⁶⁶ Evidence points to the level of vitamin D being inversely related to blood pressure and the risk of hypertension development.^{68,69} Low circulating vitamin D has been associated with increased renin-angiotensin-aldosterone activity resulting in arterial hypertension and myocardial hypertrophy.⁶⁷

Animal studies provide strong support for down-regulatory effects of vitamin D on renin expression and the renin-angiotensin-aldosterone system activity via its interaction with the vitamin D receptor.⁷⁰ Vitamin D hinders various aspects of inflammation leading to the onset of intimal and medial calcification.²⁴ Inflammatory signals aid in the presence of low circulating vitamin D.⁶⁷

1.5 Pathogenesis of cardiovascular disease

An epidemiological study revealed that large artery damage is the foremost contributory factor to the high cardiovascular mortality rate we see today.⁷¹ The most widespread complication is arterial occlusion and/or stiffness which is caused by an increase in calcium and extensive calcification.^{72,73} In the general populations as well as in individuals with some form of renal disease, the presence of arterial calcification is an independent predictive consequence of CVD.⁷⁴

A symptomatic CVD event generally occurs either through a flow-limiting disease that causes ischemia or through the formation of a thrombus on the existing atherosclerotic plaque as a result of rupture.⁷⁵ Although not everyone who has underlying plaque experiences a CVD event, prevention of cardiac morbidity and mortality lies in the detection and quantification of the presence of vascular disease.^{76,77}

1.5.1 Atherosclerosis

Nearly 100 years ago, fatty degeneration and vessel stiffening was termed atherosclerosis.⁷⁸ Atherosclerosis is a disease affecting medium and large-sized arteries characterised by inflammatory changes.⁷⁹⁻⁸¹ Atherosclerosis is the most imperative cause of CVD as seen in myocardial infarction, arterial aneurysm, stroke and heart failure.^{80,81} It can also be classified as the leading cause of chronic renal failure.^{80,81}

Atherosclerotic development is caused by a combination of various genetic, environmental and other factors.⁸² However, the aetiological factors resulting in atherosclerosis are not completely understood. Accumulation of lipid-laden foam cells within the intima layer of the artery is a representation of the fatty streak that is the earliest observable lesion of atherosclerosis.⁷⁹ Progressively, the fatty streak

advances into fibrous plaque (Figure 2), the hallmark of traditional atherosclerotic development.⁷⁹ This plaque can, however, evolve to such an extent to which it contains large amounts of lipids that over time become unstable, cause denudation of the endothelium and rupture.⁷⁹ Plaque rupture may result in thrombotic occlusion of the artery.⁷⁹

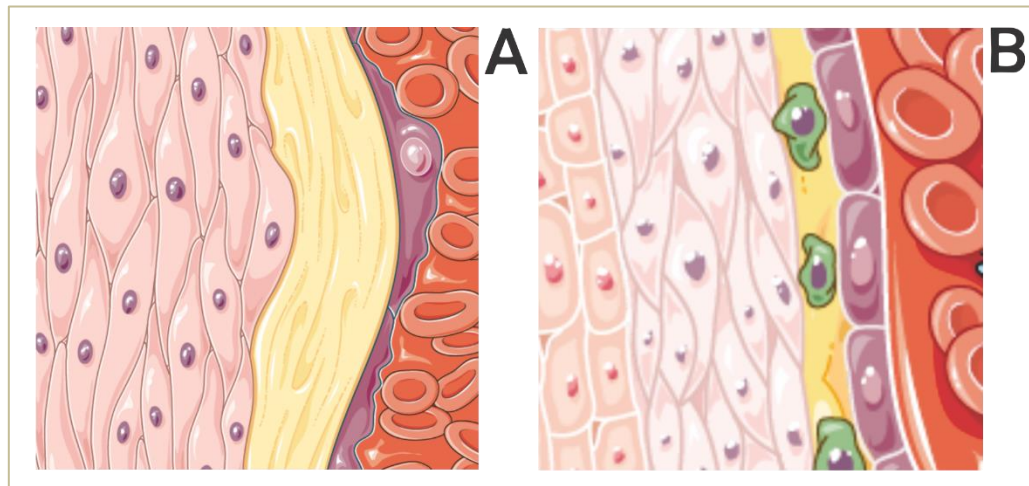


Figure 2. Normal (A) versus atherosclerotic endothelium (B). Atherosclerosis is characterised by the co-occurrence of fatty degeneration and stiffening of the arterial wall.⁷¹

Atherosclerotic lesions consist of several components: firstly, smooth muscle cells and macrophages; secondly, connective tissue matrix and extracellular lipids and thirdly, intracellular lipids that eventually cluster within the macrophages until they are converted into foam cells.⁷⁹ Atherosclerotic lesions develop as a result of various factors such as: inflammatory stimulus, various cytokines, smooth muscle cell proliferation, connective tissue matrix synthesis or the build-up of lipid and macrophages.⁷⁹

In its early stages, atherosclerosis is characterised by endothelial dysfunction.⁷⁹ This process is likely to have been initiated by unfavourable serum lipid profiles to which the endothelial cells respond via the increase in adhesion molecule frequency.⁷⁹ It is now widely accepted that the development of atherothrombosis is largely mediated by an inflammatory cascade.⁸³ Given the importance of this inflammatory cascade, the pathogenesis of atherosclerosis has profound clinical interest with focus directed on the presence of risk markers, one such predominant marker is C-reactive protein.⁷⁹ C-reactive protein levels remain an independent predictor for peripheral artery disease as well as atherosclerosis.⁸⁴

There have been some contradictory findings when it comes to the presence of vascular calcification and its effects on atherosclerotic plaque development. Some findings propose that the presence of calcification employs more of a biochemical stress on the newly formed plaque which is the predisposing factor leading to plaque rupture.⁸⁶ Alternate studies indicate that calcification could in fact exert potentially beneficial effects suggesting a protective mechanism that ultimately provides plaque stability and with time, decreases the risk of plaque rupture.⁸⁶ Some findings have also suggested that the dispersal of calcium within the vascular wall could be the determinant for plaque rupturing.^{75,87}

The degeneration and stiffening of the medial layer within the vascular wall results from the vascular smooth muscle cell degradation partly as a result of the aging process.⁷⁴ Elastic fibres also decrease due to degeneration; however, collagen fibres tend to increase in this instance.^{88,89}

1.5.2 Arterial Stiffness

The decrease in the contraction and expansion ability of the artery in response to a change in pressure is termed arterial stiffness.⁹⁰ Arterial stiffness has been known to run concurrently with several cardiovascular related diseases as well as for its implications in cardiac performance, arterial pressure and flow dynamics.⁹¹ Stiffening of the artery results in a rise in the workload of the left ventricle due to an increased systolic blood pressure as well as the development of left ventricular hypertrophy due to a reduction in diastolic blood pressure.⁹²

Several histological changes occur due to an increase in arterial stiffness. With an increase in arteriole pressure, a rise in transmural pressure is inevitable.⁹³ This results in the large artery elastic lamellae to stretch and therefore stiffen.⁹³ Due to the differing proportions of the collagen-to-elastin ratio as well as the vascular smooth muscle cells responsibility for varying responses, central elastic arteries are more likely to undergo stiffening with age compared to muscular arteries.⁹⁴⁻⁹⁶ Therefore, the presence of arterial stiffness can be considered an inevitable consequence of the aging process; however, the magnitude to which arterial stiffness presents itself could be relevant to the presence and extent of various cardiac complications.¹³

1.5.2.1 Intima media thickness

The carotid artery is an elastic artery to which an increase in carotid intima media thickness is most often observed.⁹⁷ This could be representative to intima layer thickening.⁹⁷ Although intimal thickening is known to progress with age, thickening of this layer could be a result from hyperplasia.^{75,98} On the other hand, the media layer may undergo thickening within itself which is attributable to the aging process.⁹⁹ However, these changes could be a result of separation within the elastin network rather than increased production of cells.⁹⁹

Previous findings from the prospective Rotterdam Study reported that the presence of carotid plaques, aortic calcium and an increased thickness of the intima media layer predicted the prevalence of myocardial infarction.¹⁰² More recently, it has been shown that carotid wall thickness is considered more of a delicate measure to the histological changes within the carotid arteries when compared to carotid intima media thickness.⁹⁸ Carotid intima media thickness is still seen as a marker of atherosclerosis and a predictor for atherosclerotic plaque build-up.^{101,102}

Oxidative stress, inflammation and an elevated lipid profile have all been associated with carotid intima media thickening.¹⁰²⁻¹⁰⁵ Schutte *et al.*, reported reduced blood glutathione levels associated with increased carotid intima medial thickening in hypertenives.¹⁰⁴ The increased thickening may be as a result of a decrease in antioxidant capacity.¹⁰⁶ Inflammation is also linked to carotid intima media thickening, the process of atherosclerosis including vascular calcification. In this regard, inflammation is a key aspect for atherosclerotic plaque rupture.¹⁰⁷

Hyperlipidemia and hypercholesterolemia are two conditions associated with intima medial thickening as seen in a general healthy ethnically diverse population.¹⁰⁸ Heightened triglycerides together with a low density to high density lipoprotein cholesterol ratio are strong precursors of advanced carotid intima media thickening.¹⁰³ Both *in vitro* and *in vivo* studies have shown that oxidised lipids facilitate the mineralisation process of vascular cells and inhibit mineralisation of bone cells.¹⁰⁹ Low density lipoproteins have shown to correlate with the progression in both coronary and aortic valve calcification as low density lipoproteins gather within the calcified aortic valve.¹⁰⁷

1.5.3 Cardiac Remodelling

The risk of resultant mortality due to the presence of cardiovascular events either infarction, heart failure or even stroke gradually increases with the presence of either concentric remodelling, concentric hypertrophy or even eccentric hypertrophy.¹¹¹ This increased risk is therefore associated with relative wall thickness.¹¹¹

1.5.3.1 Relative wall thickness

Measurements of left ventricular mass have been widely recognised for the use in assessing resultant cardiac organ injury.¹¹¹ However, cardiac injury may already be present in individuals with a normal left ventricular mass.¹¹¹ Thus, concentric remodelling is prominently detected by an abnormal relative wall thickness. Alterations in relative wall thickness may be an early form of cardiac adaptation to the detrimental high blood pressure that surrounds this form of remodelling.¹¹¹ A elevation in relative wall thickness (concentric remodelling) has been characterised by heightened peripheral resistance, lowered cardiac index and an increase in arterial stiffening.¹¹²

Cardiovascular events due to left ventricular systolic dysfunction are confirmed by high morbidity and mortality rates. Left ventricular hypertrophy is known to associate with several pathophysiological outcomes thus promoting myocardial electric instability as well as ventricular arrhythmias.¹¹³ These results are significantly present in all hypertensive individuals.¹¹³ However, left ventricular systolic dysfunction can be seen as an even stronger predictor of sudden death.¹¹³ Epidemiological reports have identified hypertension as a risk factor for heart failure.^{113,114} Hypertension may therefore lead to the development of left ventricular hypertrophy.¹¹² Hypertension can be seen as a subsequent factor in coronary artery disease progression of which the most common aetiology of left ventricular systolic dysfunction.¹¹² Reducing the cardiac afterload may improve the state of left ventricular systolic dysfunction but could result in hemodynamic deterioration. This was seen in patients with aortic stenosis.¹¹³

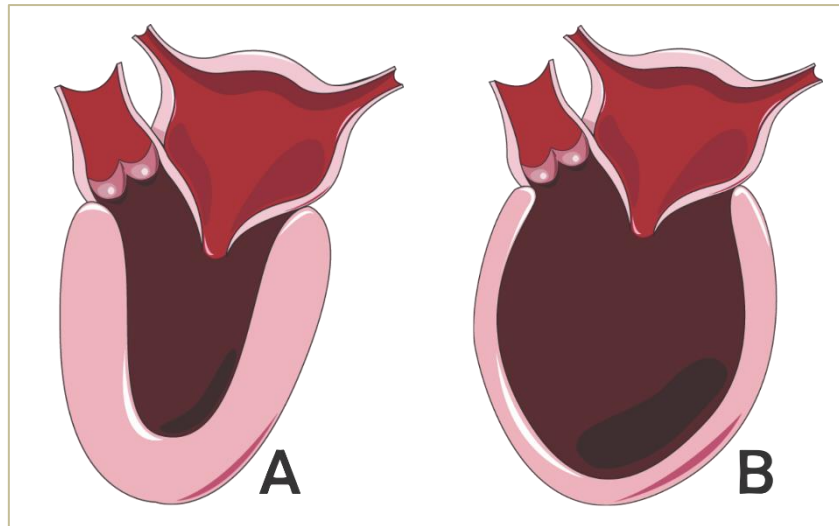


Figure 3 A schematic comparative illustration of (A) normal versus (B) left ventricular systolic dysfunction. Due to a rise in the volumetric load (preload) of the cardiac muscle, the cardiac muscle is now functioning at the limit of end diastolic volume. Thus, resulting in an alteration in the loading conditions and size of the ventricle. This is typically accompanied by eccentric hypertrophy due to an increase in the size of the left ventricle ultimately resulting in systolic dysfunction.

1.6 Factors contributing to vascular calcification

1.6.1 Renal function

As previously discussed, vascular calcification is symbolised by the conversion of the vascular smooth muscle cells into osteoblast-like cells as well as the construction of matrix vesicles thus resulting in mineral deposition.^{115,116} It is known that individuals with impaired renal function are at higher risk for cardiovascular events when compared to individuals with normal renal function.¹¹⁷ A major cause of mortality especially in patients with end-stage renal disease (ESRD), is CVD.^{118,119} Vascular calcification is present in almost all subjects over the age of 65 years, more frequent in diabetics and extremely common in ESRD individuals.¹⁶ Furthermore, a decrease in or an impairment of renal function has been known to contribute to the progression of carotid intima media thickening.¹²⁰

1.6.2 Oxidative stress and inflammation

The roles of oxidative stress and inflammation have been considered in the pathogenesis of hypertension.^{121,122} The importance of oxidative stress in cardiac events can be assessed solely on the fact that antioxidants prevent several pathophysiological processes such as cardiac hypertrophy and cardiac myocyte

apoptosis.¹²³ A number of researchers have explored the capability of antioxidants in the prevention of CVD.

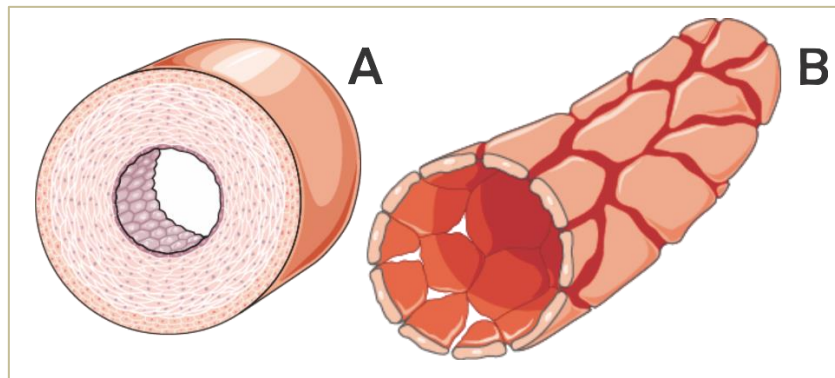


Figure 5. Illustrations of cardiac hypertrophy (A) and cardiac apoptosis (B) respectively.

Oxidative stress may be linked with early changes within the vasculature whereby the importance of the link between oxidative stress and cardiovascular markers needs to be explored. Evidence suggests that an increase in oxidative stress caused by the imbalance between oxidants and antioxidants favouring oxidants results in the disruption of redox signalling and control and/or molecular damage.¹²⁴ Oxidative stress has presented significantly as a non-traditional cardiovascular risk factor.¹²⁵ However, whether or not oxidative stress contributes solely to remodelling of the vasculature still warrants investigation.^{125,126}

1.6.3 Age, gender and ethnicity

The inevitable aging process renders prominent vascular damage.¹²⁷ With age, an accumulation of calcium within the vascular wall results in arteries becoming stiff.¹²⁸ This therefore results in a detrimental rise in pulse pressure which facilitates the progression of arterial remodelling that leads to the artery compensating for wall stress ultimately causing intimal or medial thickening.^{128,129}

In addition, gender is also considered a determinant of carotid intima media thickness.¹²⁸ Carotid intima medial thickening is independently associated with gender in which males showed a higher prevalence than females.¹²⁸ However, on the contrary to thought, women during menopause commonly present with higher arterial stiffening indices, as denoted by an elevated pulse wave velocity and augmentation index.¹²⁸

There is still much uncertainty when it comes to the ethnic differences in regard to the prevalence, progression and link of vascular disease. In South Africa, hypertension is considered one of the leading risk factors for cardiovascular mortality.¹³⁰ Studies have shown that hypertension indices are twice as high in black individuals when compared to their white counterparts.¹³¹ Similarly, results considering mortality rates have predicted that coronary heart disease is higher in black compared to white woman.¹³² Black individuals are more prone to developing acute myocardial infarction determined with a poor survival rate.¹³²

There are findings available suggesting that differences in the prevalence of coronary calcification exists amongst different ethnicities.¹³³ Black African men are more eligible to early vascular calcification and premature cardiac overload when compared to their white counterparts.¹³⁵ It is known that low circulating vitamin D, specifically 25(OH)D₃ is associated with arterial stiffness.¹³⁵ In this relation, it may contribute to the differing pulse wave velocity proportions that were observed in different ethnic societies.¹³⁵

1.6.4 Lifestyle

Gamma-glutamyltransferase (GGT) is a marker of liver function often linked to alcohol consumption. Coronary artery calcification, a strong precursor of atherosclerosis was found to correlate significantly with serum levels of GGT as well as with factors relating to coronary heart disease.¹³⁶ According to Atar *et al.*, serum GGT concentrations proved to be an independent marker of coronary artery calcification.¹³⁶ But findings from Ellison *et al.* reported no significant associations between alcohol consumption and atherosclerotic plaque development.¹³⁷

Furthermore, the association between cigarette smoking and coronary heart disease have been established in several publications.¹³⁸⁻¹⁴⁰ This association is said to be mediated via the physiological mechanisms such as lipid profile modifications, vascular calcification and inflammation.¹⁴¹ Smoking reportedly increases low density lipoprotein cholesterol and reduces high-density lipoprotein cholesterol.¹⁴² This alteration in lipid profile concentrations is said to potentially modify the mechanisms of vascular calcification.¹⁴² Therefore, lifestyle risk factors such as smoking and alcohol usage are major contributors to CVD development and have been widely researched.

1.7 Motivation

Previous reports have concluded that African populations may be predisposed to the process of vascular calcification due to altered bone and calcium metabolism, especially in older populations.¹⁴³ To the best of our knowledge, evidence exploring the onset of vascular calcification via abnormal mineral metabolism contributing to the development of CVD is limited. Furthermore, it is known that vascular calcification is linked to several disease states including chronic kidney disease, hypertension and type 2 diabetes mellitus, especially in older populations, while less is known about the potential links of cardiac and arterial structure and function with biomarkers related to vascular calcification in young black and white individuals with no apparent CVD.

1.8 Summary

A potential risk factor for CVD development is vascular calcification. Vascular calcification can be explored by determining the association between factors that are involved in the calcification process as well as markers related to cardiovascular structure and function.

There are numerous studies stating the impact of vascular calcification on cardiovascular structure and function. However, most studies have reported findings on older populations, individuals with diabetes mellitus, and/or renal impairments such as chronic kidney disease. The associations of vascular calcification markers such as alkaline phosphatase and calcium were significantly associated with the presence of several cardiovascular events leading to vascular disease. Nevertheless, there are limited studies addressing the onset of vascular calcification in a young generally healthy population.

In addition, black South Africans are in fact more susceptible to developing cardiovascular complications. The role of vascular calcification amongst this population still warrants exploring.

1.9 Aim

To explore whether associations of left ventricular relative wall thickness and systolic function exists with biomarkers related to vascular calcification in young South Africans.

1.10 Objectives-

In a study population of black and white men and women, our objectives are to:

- i. Compare markers of cardiac structure (relative wall thickness) and function (ejection fraction, fractional shortening, cardiac output, stroke volume) with vascular calcification markers (alkaline phosphatase and serum calcium) between our black and white groups;
- ii. Explore the associations of cardiovascular measures (ejection fraction, fractional shortening, stroke index, cardiac index and relative wall thickness) with markers of vascular calcification (alkaline phosphatase and calcium) and;
- iii. Explore if oxidative stress (glutathione peroxidase) and inflammation (c-reactive protein) contributes to the association of cardiovascular measures with markers of vascular calcification.

1.11 Hypotheses

We hypothesise that:

From our first objective:

- i. Markers of cardiac structure (relative wall thickness) and function (ejection fraction, fractional shortening, cardiac index and stroke index) will present higher in the black group compared to their white counterparts.
 - Alkaline phosphatase and serum calcium will present higher in the black group.

From our second objective:

- ii. Cardiovascular measures will associate adversely with markers of vascular calcification.

From our third objective:

- iii. Both oxidative stress and inflammation will contribute to the associations of cardiovascular structure and function with markers of vascular calcification.

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Chapter 2

Methodology

2.1 Introduction

Cardiovascular disease (CVD) is an ever-increasing phenomenon not only seen in South Africa but across the globe.¹ CVD is known to be accompanied by pathophysiological alterations within circulation, ultimately impairing haemodynamics.² The highest recorded blood pressure measures were that of populations who reside on the African continent.³ Premature CVD development is ever-increasing whereby the early detection, prevention and/or intervention may result in a reduction in resultant cardiac damage during adulthood.⁴ This chapter will therefore outline the specific methodology and justifications as to the anthropometric, cardiovascular, echocardiographic and biochemical analyses used in compiling the prevailing manuscript chapter to follow.

2.1.1 Study design and population demographics

This cross-sectional study forms part of the larger African prospective study on the early detection and identification of cardiovascular disease and hypertension (African-PREDICT). The aim of the prospective study is to identify premature CVD development in young South Africans. By understanding the pathophysiological implications of early disease development, prevention strategies can be implemented to possibly eradicate the early onset of cardiac compromise.

The African-PREDICT study is currently in its baseline phase with an aim to include 1200 participants from Potchefstroom, North West Province, South Africa (Figure 1). Recruitment, screening and assessment of normotensive, apparently healthy participants with equal sex distribution was conducted at the Hypertension clinic, located within the North-West University, Potchefstroom campus in addition to external locations.



Figure 1. Geographical location of Potchefstroom, North West Province, South Africa

The recruitment of participants takes place on a continuous basis until the full baseline sample has been reached. Recruitment took place via;

- (i) Active contact with field worker;
- (ii) Access through the workplace and;
- (iii) Advertisements by means of radio and alternative media.

This study will therefore contribute to the limited knowledge surrounding calcification within a generally healthy population with the aim to explore whether associations of left ventricular relative wall thickness and systolic function exists with biomarkers related to vascular calcification in young South Africans.

2.1.2 Organisational procedures

In this cross-sectional baseline study, we included the first 400 participants which included black (n=160) and white (n=175) men and women after the exclusion of participants who presented with missing variables of interest (n= 65).

The inclusion criteria for the African-PREDICT study are apparently healthy black and white men and women (20-30 years old) from the surrounding Potchefstroom area, with a blood pressure within normal range (<140/90 mmHg), no apparent CVD, no reported chronic illness/disease (HIV, diabetes mellitus, liver disease, cancer, tuberculosis or renal disease), not currently consuming any chronic medication nor pregnant or breastfeeding women.

This cross-sectional study as well as the larger prospective African-PREDICT study was approved by the Health Research Ethics Committee of the North-West University (Potchefstroom campus) and conformed to the ethical guidelines of the Declaration of Helsinki (revised in 2008) for investigation of human participants.

Participants were requested to fast for approximately 8 hours, preferably overnight before participation. Measures that require participant fasting seemingly avoid the variability of all biochemical parameters associating with meals. Therefore, a fasting measure will produce a more stable and reliable estimate.⁵ While some components of the lipid profile are not affected by food (total cholesterol and high-density lipoprotein), triglycerides in particular are.⁵ Plasma triglycerides are known to significantly increase following a meal and therefore a fasting phase evades the variability of triglyceride-meal association. Thus, a fasting triglyceride concentration delivers a steadier estimation for an individual's risk assessment.⁵

Participants were asked to arrive at the research facility at 08:00am and were shown around the research unit to ensure comfortability and familiarity after which each participant had the opportunity to solicit any questions. Written consent was obtained from each participant.

Following participant consent, each participant was escorted to a number of measurement stations that included anthropometric, cardiovascular, echocardiographic measures and biochemical analyses of which will be discussed in detail in the subsequent sections.

2.1.3 General health questionnaire

Data was obtained through the use of a general health questionnaire. The general health questionnaire can be seen as a self-administered screening tool used and designed to detect amongst others, any current disorders.⁶ Each participant completed the questionnaire prior to participation. The following information was gathered from the questionnaire: demographic (age and locality), self-reported alcohol and tobacco use, employment information as well as the use of medication. The questionnaires were completed on Apple iPads (Hon Hai Precision Industry Co., Ltd.) using a web-based program which took participants approximately 15 minutes to complete.

2.1.4 Socio-economic status

One of the most robust and reliable predictors of an individual's morbidity and mortality is an individual's socio-economic status.⁷⁻⁹ The socio-economic score of each participant was calculated using a point system that was adapted from Kuppuswamy's Socioeconomic Status Scale for a South African environment.¹⁰ Each participant was categorised into one of three categories; low, middle or high socio-economic groups. The most noteworthy influence socio-economic status has on specific markers of disease make its classification and capacity of critical importance.¹¹ It has been reported that Africans who are categorised into the low socio-economic group, have a higher tendency to lifestyle risks such as cigarette smoking and alcohol consumption.^{12, 13}

2.2 Anthropometric measures

Increased body size is known to associate with an elevated risk of cardiovascular morbidity and mortality as seen in numerous populations.¹⁴ We therefore used standard procedures to obtain each participant's height (m) (SECA 213 Portable Stadiometer; SECA, Hamburg, Germany), body weight (kg) (SECA 813 Electronic Scales; SECA, Hamburg, Germany) and waist circumference (Lufkin Steel Anthropometric Tape; W606PM; Lufkin; Apex; USA). All anthropometric procedures were performed according to specific guidelines set out by the International Society for the Advancement of Kinanthropometry (ISAK) in order to describe the body

composition our study population.¹⁵ All measurements were performed in a private room to ensure the privacy of each participant.

Body mass index (BMI) of each participant was calculated using each participant's height and weight.

Formula 1: $BMI = mass (kg) / height^2(m)$

Since obesity has become a critical challenge seen in public health across the globe, the application of using BMI has gained increased popularity and has been directly linked to an individual's health risk and mortality rates in several populations.¹⁶ The link between increased BMI and CVD risk was shown to be independent of age, sex and ethnicity. However, reports from cross-sectional as well as prospective epidemiological surveys have acknowledged that the cut-off values for BMI provided by the World Health Organisation did not adequately reflect an individual's overweight or obesity status.^{17, 18} Interest in the calculation of an individual's body surface area (BSA) (m²) dates back decades.¹⁹ At present the use of calculating BSA has been deemed useful in numerous medical fields from determining treatment dosages to the calculation of glomerular filtration rate.^{20,21} Therefore, for each of our study participants, BSA was calculated with the use of the Mosteller formula.²²

Formula 2: $BSA = height (m) \times weight (kg) / 3600$ ²²

2.2.1 Physical activity

Lifestyle modifications, including cessation of smoking and alcohol intake, transition to healthy dietary intake and reduced sedentary behaviour promote effective reduction in CVD risk.²³ Physical activity in particular, is known for its beneficial effects on CVD risk and all-cause mortality.²³ Participants were equipped with an ActiHeart physical activity monitor (CamNtech Ltd., England, UK). This compact, chest-worn monitoring device recorded heart rate, inter-beat-interval and physical activity in one combined unit in which each participant's activity energy expenditure was determined. The ActiHeart device was worn for a maximum of 7 days. The effects of physical activity on markers of vascular disease including serum lipoproteins, coronary artery calcium, systemic inflammation and carotid intima media thickness have been widely

published.²³ The importance of acquiring a measure of physical activity and activity energy expenditure should be appreciated for its importance in the increasing rate of non-communicable diseases, in particular CVD.²⁴

2.3 Blood pressure measures

Brachial blood pressure was measured using a Dinamap® Procare 100 Vital Signs Monitor (GE Medical Systems, Milwaukee, USA) whilst participants remained in a seated resting state, with their left arms supported at heart level. Clinic blood pressure measures were obtained in duplicate. Systolic blood pressure, diastolic blood pressure and heart rate were captured from each measurement. Mean arterial pressure was determined using the following formula:

Formula 3: *Mean arterial pressure = Diastolic blood pressure + (0.4 × pulse pressure)²⁵*

The categorization of normotensive or pre-hypertensive (SBP<140 and DBP<90mmHg) participants was based on a mean of four blood pressure measures in one day. Guidelines as set out by the American Society of Hypertension and the International Society of Hypertension were utilized in the determination of hypertensive, pre-hypertensive or normotensive participants.²⁶

2.4 Echocardiography and carotid ultrasound

Echocardiography is a non-invasive technique that allows for the evaluation of cardiac structure and function. Thus, allowing for the determination of cardiac defects, structural or functional compromise which is usually accompanied by the presence of CVD.

The General Electric Vivid E9 device (GE Vingmed Ultrasound A/S, Horten, Norway), a 2.5-3.5 MHz transducer and a 3 lead ECG was used to obtain a standard transthoracic echocardiography procedure. All standard transthoracic echocardiography procedures were performed by a medical clinical technologist, registered with the Health Professions Council of South Africa (HPCSA).

Participants were asked to remain in a partial left decubitus position with their heads on a modestly elevated examination table. This brings the heart forward towards the chest wall and closer to the transducer. Standardised methods were used to ensure high quality recordings according to guidelines set out by the American Society of Echocardiography and the European Association of Echocardiography.^{27, 28}

The magnitude of the cardiac chamber and ventricular function presents among the most clinically important and most frequently measured tasks of echocardiography.²⁷ Recommendations for chamber quantification include a series of measurements for the assessment of left ventricular alterations typically seen when the left ventricle adapts to the detrimental effects of CVD.²⁹ By using the measurements of left ventricular wall thickness, concentric remodelling; concentric hypertrophy and eccentric left ventricular hypertrophy can be assessed as the left ventricle responds to high blood pressure.²⁹ Concentric remodelling is therefore detected by abnormal measures of relative wall thickness with normal ranges of left ventricular mass.²⁹ This is the first sign of cardiac adaptation to elevated blood pressure.²⁹ We therefore determined relative wall thickness using the following formula:

Formula 4:

$$\text{Relative wall thickness} = (2 \times \text{posterior wall thickness}) / (\text{left ventricular internal diameter at end diastole})^{27}$$

A non-invasive assessment of the quantity of blood (ml) pumped by the left ventricle upon each contraction is known as stroke volume (mL/beat). We determined and normalised stroke volume for height in the power of 2.04 as stroke index.^{30, 31} Three factors that are taken into consideration when determining stroke volume are preload, contractility and afterload of the heart. Furthermore, changes in stroke volume may be early indicators of changes within blood volume and myocardial contractility. Thus, these changes will therefore occur earlier than changes in cardiac output.

We multiplied the stroke volume with heart rate, to obtain cardiac output.³²

Formula 5: *Cardiac output = stroke volume × heart rate*³²

Cardiac output is the primary determinant of oxygen transport within the cardiovascular system.³³ The foremost function of the cardiovascular system is to distribute oxygen around the body to meet metabolic demands of the tissues. We therefore see it being reasonable to measure cardiac output in the pursuit to prove cardiovascular insufficiency.³³

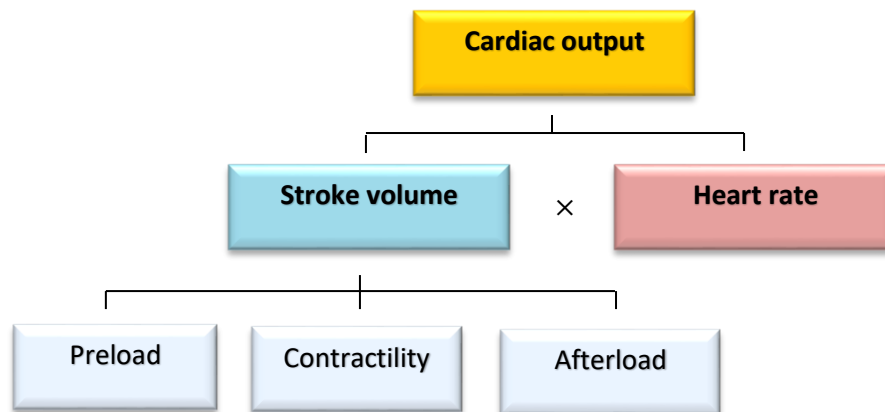


Figure 2 Diagrammatic illustration of the link between stroke volume, heart rate and cardiac output

Upon each contraction of the cardiac muscle, the magnitude of blood being ejected out of the left ventricle is measured.³⁴ This is noted as a percentage or referred to as left ventricular ejection fraction which enables the identification of the onset of heart failure amongst other cardiac compromises (Figure 3).³⁴ We calculated the left ventricular ejection fraction using left ventricular end-diastolic and end-systolic volume estimates derived from acquired 2D images according to the biplane method. A low ejection fraction is the earliest sign of resultant cardiac damage whereby the cardiac muscle does not pump a sufficient amount of blood around the body upon each contraction.³⁴ Furthermore, a low ejection fraction may result in a rapidly elevated heart rate which results in the cardiac muscle pumping ineffectively.

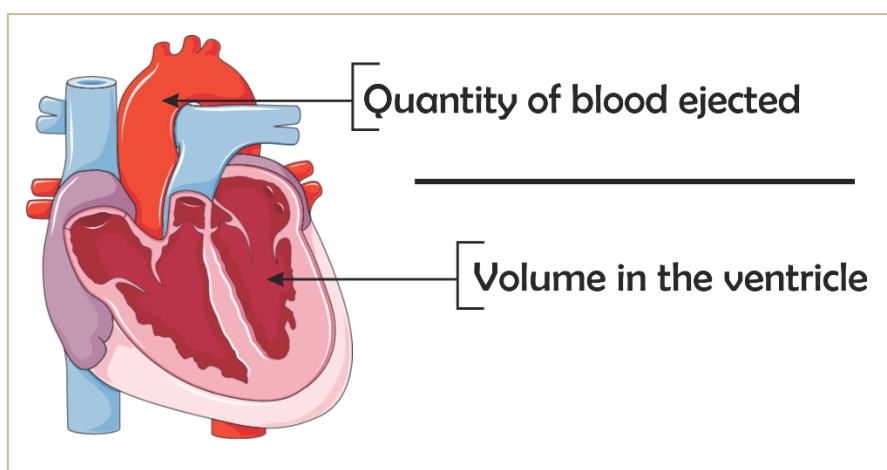


Figure 3 Graphic illustration of ejection fraction. The left ventricle can be considered the cardiac muscle's foremost propelling chamber that ejects oxygenated blood through the ascending aorta. Therefore, ejection fraction is determined in the left ventricle and expressed as a percentage. Thus, ejection fraction is the quotient of blood ejected through the ascending aorta by the total volume of blood in the ventricle.

It is well established that the normal functional ability of the cardiac muscle including its sophisticated orientation of fibres is essential for systolic function. When the mechanisms of the myocardium are interrupted, possibly due to several pathologies, the ultimate result is a detrimental effect on the left ventricle. The impairment of the longitudinal functionality of the left ventricle is an early marker of left ventricular dysfunction which is accompanied by a reduction in ejection fraction.³⁴ Therefore, fractional shortening is a relatively easy measure of left ventricular systolic function. The recorded measures illustrate the size of reduction of the left ventricle. Using standard methods endocardial fractional shortening (fractional shortening) was determined.^{32, 35}

Over the years, with the use of clinical trials, carotid intima media thickness was proven for outcomes that support the role of intima media thickness measures for predicting cardiovascular events.³⁶ Thus, in the left common carotid artery and in the internal carotid (General Electric Vivid E9, GE Vingmed Ultrasound A/S, Horten, Norway), carotid intima media thickness was derived. Digitised images were imported in the Artery Measurement System software for steadfast analyses (Gustavsson, Sweden).³⁶

2.5 Biochemical analyses

Biological samples (serum plasma, whole blood and urine) were prepared according to standard procedures and stored in cryovials in bio-freezers at -80°C until analysis. Biochemical analysis was done using standardised methodology by internationally recognised biochemical procedures, by a qualified biochemist using calibrated instruments.

For the determination of serum alkaline phosphatase, a colorimetric assay in accordance with standardised methods was used. In the presence of zinc and magnesium ions, p-nitrophenyl phosphate is cleaved by phosphatase into phosphate and p-nitrophenol. The resultant p-nitrophenol is furthermore directly proportional to the catalytic activity of alkaline phosphatase. Additionally, for the quantitative determination of serum calcium, calcium ions readily react with 5-nirto-5'methyl-BAPTA (NM-BAPTA) to form a complex. This complex formation takes place under alkaline conditions. The newly formed complex reacts with EDTA and the change in absorbance is noted as directly proportional to the concentration of calcium (Cobas Intgra 400 plus Roche, Basel Switzerland).

It is well established that hyperlipidemia is a growing problem and a potential risk factor for various metabolic and cardiovascular ailments.^{37, 38} It has been documented that changes in lipid levels such as high cholesterol and low-density lipoproteins are one of the most imperative and prominent factors in the aetiology of CVD.³⁹

Low-density lipoproteins are usually prominent in the arterial endothelium which, when elevated, accumulate in the intimal space where oxidised.⁴⁰ Thus, lipid profiles (triglycerides, high-density lipoproteins, low density lipoproteins and total cholesterol) were determined in serum (Cobas Integra 400 plus Roche, Basel Switzerland).

Increased oxidative stress are known to impair endothelial function thus increasing systemic pro-inflammatory and fibrogenic factors possibly triggering a sequence of mechanisms involved in the development of CVD.⁴⁰ C-reactive protein has emerged as a strong predictor of CVD risk in some studies thus prompting some to suggest that C-reactive protein should be a routine clinical assessment measure. Systemic inflammation as well as oxidative stress are considered to be non-traditional risk

factors for the development of CVD.⁴⁰ Moreover, oxidative stress may induce and result from, endothelial dysfunction and injury due to the endothelium being a source and a target of oxidants.⁴⁰ Reactive oxygen species, namely serum peroxides (BioTek, Winooski, VT, USA) as well as glutathione peroxidase (Randox, Crumlin, UK on Cobas Integra 400 plus Roche, Basel Switzerland) were measured.⁴¹ Additionally, high sensitive C-reactive protein were determined in serum (Cobas Integra 400 plus Roche, Basel Switzerland).

Blood serum levels are used to determine cystatin-C and creatinine concentrations determined in serum (Cobas Integra 400 plus Roche, Basel Switzerland). Cystatin-C and serum creatinine were then used to determine an estimated glomerular filtration rate (eGFR). With the use of the Chronic Kidney Disease Epidemiology (CKD-EPI) formula, estimated glomerular filtration rate (eGFR) was calculated. By multiplying the serum creatinine (mg/dl) with 88.4, serum creatinine is converted to the unit $\mu\text{mol/l}$ and used in the following calculation in the determination of eGFR:⁴²

Formula 6: $eGFR = (130 \text{ if female and } 135 \text{ if male}) \times \min(Scr / k, 1)^\alpha \times \max \min(Scr / k, 1)^{\alpha - 0.601} \min(Cys / 0.8, 1)^{-1.328} \times 0.993^{Age} \times 1.018 [\text{if female}] \times 1.159 [\text{if black}]$

Where *Scr* is serum creatinine; *Cys* is cystatin-C; *k* = 61.88 for females and 79.85 for males; α = -0.248 for females and -0.207 for males.

Lifestyle risk factors such as diet, cigarette usage, and alcohol consumption have been known to strongly influence the progression of CVD and affect several pathways such as oxidative stress and endothelial dysfunction. A 5-year prospective study showed 24% of black South Africans developed hypertension within 5 years, especially due to modifiable risk factors including alcohol and cigarette abuse.⁴³

Gamma glutamyl-transferase (GGT) is an enzyme located on the outer surface of the membranes and is involved in the clinical use as a test of alcohol abuse.⁴⁴ Furthermore, it is also well known that cotinine (a stable metabolite of nicotine) increased the risk of coronary heart disease. Thus, both GGT (Cobas Integra 400 plus Roche, Basel Switzerland) and serum cotinine levels were determined. Serum cotinine

levels were determined with Chemiluminescence method on the Immulite (Siemens, Erlangen, Germany).

2.6 Statistical analyses

We performed our statistical analyses using IBM®, SPSS® version 24 (IBM Corporation, Armonk, New York). We tested all variables used in the statistical analysis for normality by visual inspection of histograms and also reviewing the coefficients of skewness and kurtosis. In the event of non-Gaussian distribution, we performed a logarithmic transformation for each skewed variable (glucose, cotinine, gamma glutamyltransferase, low-density lipoprotein cholesterol, triglycerides, reactive oxygen species, glutathione peroxidase, and c-reactive protein). Data was expressed as mean $\pm$ standard deviation if normally distributed and as geometric mean with 5th and 95th percentile boundaries for logarithmic transformed variables.

We performed a comparison between the ethnic groups by the use of independent T-tests. Chi-square tests were conducted to compare proportions between groups. Pearson- and partial correlations were conducted to explore the relationship between variables associated with cardiac structure (relative wall thickness) and function, (ejection fraction, fractional shortening, cardiac index and stroke index) with markers of vascular calcification (alkaline phosphatase and calcium). Independent associations between cardiovascular structure and function with markers of vascular calcification were done by means of multiple linear regression analyses after adjusting for covariates, which included age, sex, body mass index, systolic blood pressure, estimated glomerular filtration rate and activity energy expenditure.

We furthermore performed a sensitivity analyses to assess the contribution of markers of inflammation (C-reactive protein) and oxidative stress (glutathione peroxidase) on the associations of cardiac structure (relative wall thickness) and function (ejection fraction, fractional shortening, cardiac index and stroke index) with markers of vascular calcification (calcium and alkaline phosphatase). Several multiple regression models were built without taking markers of oxidative stress and inflammation into account. We then added either a marker of oxidative stress, inflammation or both to test whether the associations remained robust.

2.7 Student contribution

I was involved in the initial screening phase of the African-PREDICT study. In terms of the African-PREDICT initial screening, I was responsible for urine analysis, cholesterol and glucose testing as well as blood pressure measures and blood grouping. I am currently involved in the Exercise; Arterial Modulation and Nutrition in Youth South Africa (EXAMIN Youth SA) study in which I am responsible and competent in pulse wave analysis with the use of the validated, oscillometric Mobil-o-Graph monitor (I.E.M. GmbH, Germany) with integrated ARCSolver software.

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Chapter 3

Research article

Summary of the instructions for the author

This article followed the specific guidelines as set out by the *Hypertension Research* journal specified below. A full list of details regarding the author's instructions are available at: http://mc.manuscriptcentral.com/societyimages/htr/HR_GTA.pdf

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Cardiovascular structure and function adversely relate to vascular calcification markers in young adults: The African-PREDICT study

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Abstract

Objectives: Vascular compromise is known to impact on cardiac structure and function in elderly and diseased populations. We aimed to explore whether associations of left ventricular relative wall thickness and systolic function exists with biomarkers related to vascular calcification in young healthy South Africans.

Methods: We included 160 black and 175 white men and women between 20–30 years old. We assessed blood pressure measures, echocardiography measures (relative wall thickness and systolic function). Biochemical analyses included amongst others alkaline phosphatase and calcium.

Results: Blood pressure measures (all $p \leq 0.001$) presented higher in the black group compared to their white counterparts. Although intima media thickness was found comparable between the groups, relative wall thickness present higher in the black group. Furthermore, higher levels of alkaline phosphatase were also evident in the black group ($p < 0.001$). Single, partial and multiple regression analyses indicated that only in the black group, relative wall thickness was independently associated with alkaline phosphatase (adj. $R^2 = 0.030$; $\beta = 0.176$; $p = 0.037$) whereas in white group, systolic function markers including cardiac (adj. $R^2 = 0.403$; $\beta = -0.141$; $p = 0.021$) and stroke (adj. $R^2 = 0.353$; $\beta = -0.147$; $p = 0.019$) indices inversely associated with calcium. Furthermore, stroke index associated inversely with alkaline phosphatase (adj. $R^2 = 0.354$; $\beta = -0.172$; $p = 0.016$) in the white group only.

Conclusion: Our results suggest that even at young ages, early vascular calcification mediators may contribute to impaired cardiac load and could prematurely escalate in future cardiac compromise.

Keywords: Alkaline phosphatase, calcium, cardiovascular disease, ethnicity, mineralisation, vascular calcification

3.1 Introduction

Over several decades, the presence of vascular calcification was seen as a passive degenerative process associated with aging.^{1,2} Reports increasingly recognise vascular calcification as an active, regulated process which may be modifiable.^{3, 4} Vascular calcification in particular is an important cause of decreased vascular compliance, partly due to age.⁵ Vascular calcification denotes the deposition of calcium phosphate mineral (hydroxyapatite) in various cardiovascular tissues including arteries, heart valves and cardiac muscle.⁶ Several factors have been linked to increased prevalence of vascular calcification including increased oxidative stress, inflammation, diabetes mellitus and hypertension.⁷

Experimental studies have reported vascular calcification to also associate with elastin degradation which may in turn initiate smooth muscle cell and dermal fibroblast conversion into osteoblast-like cells.⁸⁻¹⁰ When smooth muscle cells gain osteoblast-like characteristics, an increased amount of alkaline phosphatase is expressed.^{11, 12} Biomarkers of mineral metabolism namely alkaline phosphatase, calcium, parathyroid hormone and serum phosphates have been known to associate with adverse cardiovascular effects.^{13, 14} In older individuals (above 60 years of age) gradually enlarged deposits of calcium occur within almost all major arteries,¹⁵ which may result in a reduction in aortic and arterial compliance. Reduced aortic and arterial compliance increase the after-load on the heart and may consequently contribute to adverse alterations in cardiac structure and function.¹⁶ Furthermore, previous findings from the prospective Rotterdam Study reported that the presence of aortic calcium, carotid plaques and an increased intima media thickness predicted the incidence of myocardial infarction.¹⁷

Vascular compromise is known to impact on cardiac structure and function especially in the elderly and diseased populations. The links of cardiac structure and function with particular markers related to vascular calcification including alkaline phosphatase and calcium in young healthy individuals is unclear. We therefore aimed to explore whether associations of left ventricular relative wall thickness and systolic function exist with biomarkers related to vascular calcification in young South Africans.

3.2 Methodology

This study formed part of the larger African prospective study on early detection and identification of cardiovascular disease and hypertension (African-PREDICT). Inclusion criteria were apparently healthy black and white men and women (20-30 years old) from the surrounding Potchefstroom area, with a blood pressure within normal range ($<140/90$ mmHg), no apparent cardiovascular disease (CVD), no reported chronic illness/disease (human immunodeficiency virus (HIV), diabetes mellitus, liver disease, cancer, tuberculosis or renal disease), not currently consuming any chronic medication as well as not pregnant nor breastfeeding. In this cross-sectional study, we included black ($n=160$) and white ($n=175$) men and women after the exclusion of participants who presented with missing variables of interest ($n=61$).

This study was approved by the Health Research Ethics Committee of the North-West University (Potchefstroom campus) and conformed to the ethical guidelines of the Declaration of Helsinki (revised in 2008) for investigation of human participants.

Anthropometric measures and physical activity

Body height (m) (SECA 213 Portable Stadiometer; SECA, Hamburg, Germany), weight (kg) (SECA 813 Electronic Scales; SECA, Hamburg, Germany) and waist circumference (cm) (Lufkin Steel Anthropometric Tape; W606PM; Lufkin; Apex; USA) were obtained using a standard protocol.¹⁸ Body mass index (BMI) ($\text{weight (kg)} / \text{height (m}^2\text{)}$) and body surface area (BSA) (m^2) were additionally calculated.¹⁹ Activity energy expenditure was determined for each participant using the ActiHeart device (CamNtech Ltd., England, UK) and indexed by weight, expressed as kCal/kg/day. The ActiHeart device was worn for a maximum of 7 days.

Blood pressure measures

Duplicate brachial blood pressure measurements were taken on both arms of the participants with a 5-minute resting interval while they remained in a rested seating position with the approximately sized GE Critikon latex-free Dura-Cuff. Blood pressure measures were obtained using Dinamap® ProCare 100 Vital Signs Monitor (GE Medical Systems, Milwaukee, USA). A mean of the left arm clinical blood pressure

measures aided in obtaining systolic blood pressure, diastolic blood pressure and heart rate. Mean arterial pressure was calculated using the following formula, (diastolic blood pressure) / (0.4*pulse pressure).²⁰

Echocardiography and carotid ultrasound

A standard transthoracic echocardiography procedure was performed for each participant using the General Electric Vivid E9 device (GE Vingmed Ultrasound A/S; Horten, Norway), a 2.5–3.5 MHz transducer and a 3-lead ECG. To determine relative wall thickness (RWT), the following calculation was used: (2*posterior wall thickness) / (left ventricular internal diameter at end diastole).²¹ Stroke volume (mL/beat) was determined and normalised for height in the power of 2.04 as the stroke index.^{22,23} By multiplying the stroke volume with heart rate, cardiac output was calculated and indexed by height to the power 1.83 and expressed as (L/min/m^{1.83}).²⁴ Using standard methods, endocardial fractional shortening (also fractional shortening) was determined.^{24,25} Left ventricular ejection fraction was calculated using left ventricular end-diastolic and end-systolic volume estimates derived from acquired 2D images according to the biplane method.²⁶

Carotid intima media thickness was derived from the use of B-mode ultrasonography in which the walls of the left common carotid artery were measured (General Electric Vivid E9, GE Vingmed Ultrasound A/S, Horten, Norway). Digitised images were imported in the Artery Measurement System software (Gustavsson, Sweden) for determining the intima media thickness of the near and far walls of both carotid arteries.²⁷ The intima media thicknesses were combined into a mean for further statistical analysis.

Biochemical analyses

Participants were required to fast approximately 8 hours prior to the collection of biological samples. Biological samples (serum plasma, whole blood and urine) were prepared according to standard procedures and stored at –80°C until analysis.

Basic serum analyses included lipids (triglycerides, high density lipoprotein cholesterol, low density lipoprotein cholesterol and total cholesterol), gamma

glutamyltransferase, high sensitivity C-reactive protein, creatinine, alkaline phosphatase, and calcium (Cobas Integra 400 plus Roche, Basel Switzerland). The ratio of total cholesterol to high density lipoprotein cholesterol was additionally calculated. Serum cotinine levels were determined with a chemiluminescence method on the Immulite (Siemens, Erlangen, Germany). Plasma glucose was determined from blood processed in sodium fluoride plasma (Siemens, Erlangen, Germany). The intra-assay variability and inter-assay variability of all variables were below 10%. Cotinine presented with 10.7% low concentration and 5.5% high concentration intra-assay variation.

Glutathione peroxidase, a marker of oxidative stress was determined in whole blood (Randox, Crumlin, UK on Cobas Integra 400 plus Roche, Basel Switzerland) with an intra-assay variability and inter-assay variability below 10%. One of the measurable, reactive oxygen species serum peroxides was also determined in serum (BioTek, Winooski, VT, USA) with an intra-assay variability of 9.68% and inter-assay variability of 10.2%.

Questionnaires

General health questionnaires were used to evaluate demographics (age and locality), self-reported cigarette and alcohol usage, employment information, skill level, household income as well as medication usage. The socio-economic status of each participant was calculated using a point system that was adapted from Kuppuswamy's Socioeconomic Status Scale for a South African environment.²⁸ Each participant's socio-economic score was categorised into one of three categories. Skill level, education and household income were categorised into low, middle and high socio-economic groups.

Statistical analyses

For database management and statistical analyses, IBM®, SPSS® version 24 (IBM Corporation, Armonk, New York) was used. We tested for an interaction of ethnicity on the association between our markers of cardiovascular structure and function (intima media thickness, relative wall thickness, ejection fraction, fractional shortening, cardiac index and stroke index) and vascular calcification (alkaline phosphatase and

calcium). Variables were tested for normality using the Kolmogorov-Smirnov test and QQ-plots. Non-Gaussian variables were logarithmically transformed. Data was expressed as mean $\pm$ standard deviation if normally distributed and as geometric mean with 5th and 95th percentile boundaries for logarithmic transformed variables.

For comparisons between ethnic groups, independent T-tests were used. Chi-square tests were conducted to compare proportions between groups. The correlation of cardiac structure and function with markers of vascular calcification were explored using Pearson- and partial correlations. Standard multiple regression analyses were conducted in which each dependent variable was separately tested for its association with markers of vascular calcification. Covariates considered for entry in the multiple regression models included age, body mass index, systolic blood pressure, estimated glomerular filtration rate (eGFR) and activity energy expenditure.

Sensitivity analyses were performed to assess the contribution of markers of inflammation (C-reactive protein) and oxidative stress (glutathione peroxidase) to the associations of the relative wall thickness, intima media thickness, ejection fraction, fractional shortening cardiac index and stroke index with markers of vascular calcification (calcium and alkaline phosphatase).

3.3 Results

We found significant interactions of ethnicity on the associations of markers of cardiac structure (relative wall thickness) and function (ejection fraction, cardiac index and stroke index) with markers of vascular calcification (calcium and alkaline phosphatase), as seen in Supplementary Table 1.

The characteristics of the study population stratified by ethnicity are presented in Table 1. Despite the mean ages of the black group being younger than their white counterparts ($p=0.001$), the black group presented with higher relative wall thickness ($p<0.001$) and blood pressure measures (all $p\leq 0.001$), but lower cardiac and stroke indices (both $p\leq 0.001$) than the white group. Intima media thickness, ejection fraction and fractional shortening were all similar between black and white groups. The black group presented with higher levels of alkaline phosphatase ($p<0.001$) compared to the white group, whereas calcium levels were comparable between these groups.

TABLE 1: General characteristics of the study population stratified according to ethnicity

	Black (n= 160)	White (n= 175)	p value
Age (years)	24.31 ± 3.38	25.49 ± 2.83	0.001
Sex male (%)	46.25	41.14	
Body composition			
Height (cm)	164.40 ± 7.95	172.06 ± 8.59	<0.001
Weight (kg)	65.76 ± 13.21	76.72 ± 20.00	<0.001
Waist circumference (cm)	77.18 ± 10.86	81.86 ± 15.00	0.001
Body mass index (kg/m ²)	24.47 ± 5.47	25.76 ± 5.71	0.036
Body surface area (m ²)	1.72 ± 0.18	1.90 ± 0.27	<0.001
Cardiovascular measures			
Left intima media thickness (mm)	0.44 ± 0.07	0.44 ± 0.07	0.422
Systolic blood pressure (mmHg)	120 ± 12	115 ± 13	0.001
Diastolic blood pressure (mmHg)	80 ± 9	77 ± 8	<0.001
Mean arterial pressure (mmHg)	95 ± 9	92 ± 9	<0.001
Relative wall thickness (cm)	0.36 ± 0.07	0.33 ± 0.06	<0.001
Systolic function			
Ejection fraction (%)	68.20 ± 6.60	67.29 ± 6.56	0.205
Fractional shortening (%)	38.27 ± 5.27	37.85 ± 5.09	0.462
Cardiac index (L/min/m ^{1.83})	5.12 ± 1.22	5.80 ± 1.43	<0.001
Stroke index (ml/m ^{2.04})	67.33 ± 15.76	78.63 ± 18.80	<0.001
Biochemical measures			
Calcium (mmol/L)	2.31 ± 0.15	2.31 ± 0.15	0.801
Alkaline phosphatase (U/L)	72.64 ± 20.97	62.38 ± 19.14	<0.001
Glucose (mmol/L)	3.73 ((2.76 – 5.25)	4.43 (3.49 – 5.53)	0.003
Total cholesterol (mmol/L)	3.84 ± 0.84	4.76 ± 1.02	<0.001
Low density lipoprotein cholesterol (mmol/L)	2.29 (1.26 – 3.87)	2.95 (1.73 – 4.80)	<0.001
Triglycerides (mmol/L)	0.77 (0.40 -1.58)	0.97 (0.44 – 2.16)	<0.001
Reactive oxygen species (mg/L H ₂ O ₂)	179.39 (94.12 – 328.32)	164.55 (83.64 – 371.71)	0.052
Glutathione peroxidase (U/L)	18.47 (14.83 – 20.66)	19.80 (17.56 – 21.78)	<0.001
C-Reactive Protein (mg/L)	1.28 (0.14 – 9.34)	1.07 (0.12 – 40.73)	0.221
eGFR (ml/min/1.73m ²)	120.90 ± 13.90	106.64 ± 14.23	<0.001
Lifestyle risk			
Cotinine (ng/ml)	4.95 (1 – 321.74)	2.71 (1 – 256.45)	0.013
Gamma-glutamyl transferase (U/L)	26.13 (10.51 – 89.87)	17.63 (6.98 – 51.88)	<0.001
Activity Energy Expenditure (kCal/Kg/day)	6.86 ± 2.81	5.90 ± 2.87	0.002
Socio-economic score	17.71 ± 5.68	25.01 ± 5.02	<0.001

Values are arithmetic mean ± standard deviation or geometric mean (5th and 95th percentiles) for logarithmically transformed variables. Abbreviations: eGFR – estimated glomerular filtration rate.

In single and partial regression analyses (adjusted for age, sex and body mass index), relative wall thickness associated positively with alkaline phosphatase in the black group only ($r=0.173$; $p=0.030$). In the white group, diastolic blood pressure ($r=0.184$; $p=0.016$) and mean arterial pressure ($r=0.164$; $p=0.031$) positively associated with alkaline phosphatase. Cardiac index inversely associated with calcium ($r=-0.190$; $p=0.013$) and alkaline phosphatase ($r=-0.150$; $p=0.050$) in the white group, as well as with calcium ($r=-0.197$; $p=0.014$) in the black group. Additionally, an inverse association existed between stroke index and calcium in the black ($r=-0.185$; $p=0.020$) and white ($r=-0.190$; $p=0.012$) group. In the white group only, stroke index was inversely associated with alkaline phosphatase ($r=-0.204$; $p=0.007$).

TABLE 2: Partial correlations of cardiovascular measures with markers of vascular calcification of the study population stratified by ethnicity

	Calcium (mmol/L)				Alkaline Phosphatase (U/L)			
	Black (n= 160)		White (n= 175)		Black (n= 160)		White (n= 175)	
Cardiovascular structure & function								
Left intima media thickness (mm)	r= 0.083;	p= 0.299	r= 0.084;	p= 0.272	r= −0.109;	p= 0.175	r= −0.077;	p= 0.317
Systolic blood pressure (mmHg)	r= 0.060;	p= 0.456	r= 0.114;	p= 0.136	r= −0.024;	p= 0.764	r= 0.094;	p= 0.220
Diastolic blood pressure (mmHg)	r= 0.122;	p= 0.126	r= 0.092;	p= 0.228	r= −0.150;	p= 0.061	r= 0.184;	p= 0.016
Mean arterial pressure (mmHg)	r= 0.076;	p= 0.344	r= 0.075;	p= 0.331	r= −0.112;	p= 0.164	r= 0.164;	p= 0.031
Relative wall thickness (cm)	r= 0.049;	p= 0.542	r= −0.056;	p= 0.466	r= 0.173;	p= 0.030	r= 0.079;	p= 0.301
Ejection fraction (%)	r= −0.170;	p= 0.033	r= −0.012;	p= 0.878	r= 0.018;	p= 0.826	r= −0.187;	p= 0.014
Fractional shortening (%)	r= −0.191;	p= 0.017	r= −0.016;	p= 0.838	r= 0.006;	p= 0.941	r= −0.176;	p= 0.021
Cardiac index (L/min/m ^{1.83})	r= −0.197;	p= 0.014	r= −0.190;	p= 0.013	r= 0.017;	p= 0.835	r= −0.150;	p= 0.050
Stroke volume index (ml/m ^{2.04}) *	r= −0.185;	p= 0.020	r= −0.190;	p= 0.012	r= −0.056;	p= 0.484	r= −0.204;	p= 0.007
Biochemical measures								
Glucose (mmol/L)	r= −0.012;	p= 0.881	r= 0.107;	p= 0.164	r= 0.074;	p= 0.359	r= 0.029;	p= 0.706
Total cholesterol (mmol/L)	r= 0.330;	p<0.001	r= 0.218;	p= 0.004	r= −0.048;	p= 0.548	r= −0.002;	p= 0.978
Low density lipoprotein cholesterol (mmol/L)	r= 0.291;	p<0.001	r= 0.196;	p= 0.010	r= 0.051;	p= 0.524	r= −0.043;	p= 0.579
Triglycerides (mmol/L)	r= 0.228;	p= 0.004	r= 0.100;	p= 0.191	r= 0.045;	p= 0.577	r= 0.104;	p= 0.175
eGFR (ml/min/1.73m ²)	r= −0.274;	p= 0.001	r= −0.055;	p= 0.470	r= 0.172;	p= 0.031	r= −0.137;	p= 0.072
Reactive oxygen species (mg/L H ₂ O ₂)	r= −0.128;	p= 0.110	r= −0.143;	p= 0.061	r= 0.046;	p= 0.571	r= 0.048;	p= 0.533
Glutathione peroxidase (U/L)	r= −0.193;	p= 0.016	r= 0.278;	p<0.001	r= −0.215;	p= 0.007	r= −0.056;	p= 0.464
C-Reactive Protein (mg/L)	r= 0.005;	p= 0.949	r= −0.074;	p= 0.332	r= 0.104;	p= 0.193	r= 0.184;	p= 0.015
Lifestyle risk								
Cotinine (ng/ml)	r= −0.080;	p= 0.322	r= −0.157;	p= 0.040	r= 0.047;	p= 0.561	r= −0.049;	p= 0.524
Gamma-glutamyl transferase (U/L)	r= 0.175;	p= 0.029	r= 0.025;	p= 0.745	r= 0.070;	p= 0.382	r= 0.231;	p=0.002
Activity Energy Expenditure (kCal/Kg/day)	r= −0.015;	p= 0.850	r= 0.080;	p= 0.301	r= 0.081;	p= 0.317	r= 0.027;	p= 0.729
Socio-economic score	r= −0.036;	p= 0.651	r= 0.092;	p= 0.228	r= −0.167;	p= 0.037	r= −0.087;	p= 0.254

Adjusted for age, sex and body mass index. *Stroke index additionally adjusted for waist circumference. Abbreviations: n – number of participants, eGFR – estimated glomerular filtration rate.

In multiple regression analysis (Table 3), we found a consistent positive association between relative wall thickness and alkaline phosphatase in the black group only (adj. $R^2=0.030$; $\beta=0.176$; $p=0.037$). Cardiac index inversely associated with calcium in both the black (adj. $R^2=0.096$; $\beta=-0.181$; $p=0.031$) and white (adj. $R^2=0.403$; $\beta=-0.141$; $p=0.021$) groups. BMI contributed to the association between cardiac index and calcium in both black (adj. $R^2=0.096$; $\beta=0.294$; $p=0.003$) and white (adj. $R^2=0.403$; $\beta=0.491$; $p<0.001$) groups. Furthermore, stroke index associated inversely with calcium in both the black (adj. $R^2=0.165$; $\beta=-0.161$; $p=0.046$) and white groups (adj. $R^2=0.353$; $\beta=-0.147$; $p=0.019$). In the white group only, stroke index associated inversely with alkaline phosphatase (adj. $R^2=0.354$; $\beta=-0.172$; $p=0.016$). Both sex and waist circumference contributed to the association between stroke index and calcium in both the black (sex: $\beta=0.365$; $p<0.001$; waist circumference: $\beta=-0.253$; $p=0.027$) and white (sex: $\beta=0.309$; $p=0.001$; waist circumference: $\beta=-0.368$; $p<0.001$) groups as well as to the association between stroke index and alkaline phosphatase in the white group only (sex: $\beta=0.301$; $p=0.001$; waist circumference: $\beta=-0.253$; $p=0.027$).

TABLE 3: Standard multiple regression analyses of cardiovascular measures with markers of vascular calcification

	Calcium (mmol/L)			Alkaline Phosphatase (U/L)		
	Adjusted R ²	Std β (95 % CI)	p value	Adjusted R ²	Std β (95 % CI)	p value
Relative wall thickness (mm)						
Black (n=160)	−0.001	0.050 (−0.119; 0.218)	0.561	0.030	0.176 (0.011; 0.340)	0.037
White (n=175)	0.019	−0.074 (−0.226; 0.078)	0.338	0.008	0.060 (−0.114; 0.233)	0.498
Ejection fraction (%)						
Black (n=160)	0.042	−0.125 (−0.289; 0.040)	0.136	0.025	−0.120 (−0.177; 0.153)	0.888
White (n=175)	0.058	0.002 (−0.147; 0.151)	0.982	0.083	−0.208 (−0.375; −0.041)	0.015
Fractional shortening (%)						
Black (n=160)	0.047	−0.148 (−0.312; 0.016)	0.077	0.024	−0.025 (−0.189; 0.140)	0.769
White (n=175)	0.081	0.001 (−0.146; 0.149)	0.987	0.103	−0.195 (−0.360; −0.030)	0.021
Cardiac index (L/min/m^{1.83})						
Black (n=160)	0.096	−0.181 (−0.345; −0.017)	0.031	0.070	−0.021 (−0.181; 0.139)	0.793
White (n=175)	0.403	−0.141 (−0.260; −0.021)	0.021	0.385	−0.105 (−0.238; 0.028)	0.120
Stroke index (ml/m^{2.04})						
Black (n=160)	0.165	−0.161 (−0.319; −0.003)	0.046	0.146	−0.061 (−0.216; 0.094)	0.440
White (n=175)	0.353	−0.147 (−0.271; −0.024)	0.019	0.354	−0.172 (−0.311; −0.032)	0.016

Variables included in the models were: age, sex, body mass index, systolic blood pressure, total cholesterol to high density lipoprotein cholesterol ratio, gamma-glutamyl transferase, activity energy expenditure and estimated glomerular filtration rate. In the models with stroke index as a dependent variable, waist circumference was additionally added as a covariate. Abbreviations: n – number of participants

Sensitivity analysis

To investigate whether our associations of markers of cardiac structure (relative wall thickness) and function (ejection fraction, fractional shortening, cardiac index and stroke index) with vascular calcification markers (alkaline phosphatase and calcium) are dependent on oxidative stress (glutathione peroxidase) and/or inflammation (C-reactive protein), we included both variables in the same multiple regression model (Supplementary Table 3). By doing so, all significant associations as reported in the previous multiple regression analyses persisted, and neither glutathione peroxidase nor C-reactive protein were significant in these models.

3.4 Discussion

Our study explored the links between markers of cardiac structure and function with vascular calcification in a young and healthy cohort. We found adverse associations between markers of systolic function with both alkaline phosphatase and calcium in both of our study groups. We also found relative wall thickness to associate with alkaline phosphatase in the black group only.

Both relative wall thickness and alkaline phosphatase presented higher in the black study group when compared to their white counterparts. Although not abnormally high, increased relative wall thickness is known to indicate potential concentric remodelling or hypertrophy.²⁹ Additionally, vascular calcification is known to drastically reduce aortic and arterial elastance therefore impairing cardiovascular hemodynamics ultimately resulting in vascular compromise.³⁰ As a result of the latter, a reduction in coronary perfusion, the development of left ventricular hypertrophy and adverse remodelling of left ventricular geometry is inevitable.³¹ Thus, the independent relationship of relative wall thickness with alkaline phosphatase, may suggest premature cardiovascular compromise in black South Africans. Furthermore, this association may contribute to the high cardiovascular risk known to be present in black population groups.³²

Findings from previous South African studies indicated early cardiovascular deterioration in black South African populations.^{32, 33} However in the present study, associations of markers of systolic function with vascular calcification markers were

indicated in both the black and white study groups. Furthermore, previous findings have also concluded that black populations may in fact be predisposed to the development of vascular calcification.^{32, 33} It is worthwhile mentioning that in both the above mentioned South African studies, both sick and healthy participants were included. Thus, in accordance to the inclusion criteria as outlined in the African-PREDICT study, this may be the underlying factor as to why we see these findings in both our study groups.

Many theories have been proposed to explain the pathogenesis of vascular calcification. However, a complete and satisfactory explanation as to the possible trigger to this manifestation amongst an apparently healthy population is lacking. The independent inverse association seen between markers of systolic function and both alkaline phosphatase and calcium seen in both study groups may suggest the early onset of vascular calcification amongst our younger study participants. In disease states such as chronic kidney disease, vascular calcification plays a role to facilitate renal insufficiency thus resulting in systolic dysfunction; however, the evidence supporting this notion in a generally healthy cohort with a normal kidney function is stunted.^{34,35}

In both the black and white study groups, ejection fraction as well as both cardiac and stroke indices associated inversely with calcium. Higher levels of calcium may promote a decline in systolic function markers. However, systolic function is typically preserved until a much later age. This may suggest that early changes in calcium mineralisation could contribute to long term changes in left ventricular systolic function. Our results further suggest that male sex and waist circumference (abdominal obesity) could be mediating these associations.

At a molecular level, recent insights into the factors regulating mineralisation provide greater appreciation of its complexity. The process of mineralisation is limited to tissues that express both type 1 collagen and alkaline phosphatase.³⁶ These components adhere as mineral deposits in the process of calcific atherosclerotic development.³⁷ Thus, in the event of co-expression of these proteins, ectopic calcification is induced. Ectopic mineralisation is confined to adverse calcification taking place in the blood vessel walls. Membrane bound alkaline phosphatase will thus reduce inhibitor pyrophosphate expression and contribute to hydroxyapatite

formation.³⁸ However, due to the presence of ectopic calcification, phosphates are readily taken up by means of a positive feedback mechanism resulting in osteoblast conversion as well as the onset of vascular calcification.^{39, 40} These newly formed cells may be accountable for the pathological calcification therefore increasing the risk of CVD and mortality as seen in the black South African population.^{41, 42} Although the above-mentioned mechanism is only speculative, the association between relative wall thickness and alkaline phosphatase in our healthy cohort, corroborates the possibility for alterations in cardiac structure thus resulting in cardiovascular compromise.

Whether the associations reported are merely an indication of the environmental or traditional risk factors contributing to the development of CVD, or whether black South African populations are more susceptible to the early onset of adverse mineralisation remain unclear. We can however conclude that our findings are independent of inflammation (C-reactive protein) and/or oxidative stress (glutathione peroxidase). Furthermore, the association between a marker of cardiovascular alteration (relative wall thickness) and a marker of vascular calcification (alkaline phosphatase) amongst this population may require imperative intervention to reduce the ever-increasing tendency of cardiovascular morbidity and mortality resulting from vascular calcification.

The findings of our study should be interpreted within the context of its strengths and limitations. The essential aim of the longitudinal African-PREDICT study is to comprehend the premature pathophysiological alterations in young individuals as part of hypertension development. Our study is the first to explore the links between cardiac structure and function with markers of vascular calcification in a young healthy cohort. This study was well designed and performed under controlled conditions. Although coronary artery calcium scoring remains a robust measure for ectopic calcification, our study population was young and therefore coronary artery calcium scoring could not be justified. Reliable biomarkers of mineralisation were used instead to contribute to our understanding in the potential early changes in alkaline phosphatase and calcium levels that could contribute to cardiac compromise. Due to the fact that this was a cross-sectional study, cause and effect cannot be implied. Furthermore, the study

population cannot be regarded as a representative of the general South African population.

In conclusion, we observed adverse associations between markers of cardiac structure and function with markers of vascular calcification in young healthy individuals. Our results suggest that in young adulthood, cardiac structure and function could be compromised due to potential adverse mineralisation mediated by male sex and abdominal obesity. These findings are clinically relevant and need confirmation in larger prospective studies.

Conflict of interest

The authors report that they have no conflict of interest.

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SUPPLEMENTARY TABLE 1: Interactions of ethnicity on the associations of markers of cardiovascular structure and function

	Calcium (mmol/L)	Alkaline phosphatase (U/L)
	Ethnicity	Ethnicity
Intima media thickness (mm)	p= 0.290	p= 0.779
Relative wall thickness (mm)	p<0.001	p= 0.001
Ejection fraction (%)	p= 0.223	p= 0.020
Fractional shortening (%)	p= 0.461	p= 0.074
Cardiac index (L/min/m ^{1.83})	p<0.001	p<0.001
Stroke index (ml/m ^{2.04})	p<0.001	p<0.001

SUPPLEMENTARY 2: Pearson correlation of cardiovascular measures with markers of vascular calcification of the study population stratified by ethnicity

	Calcium (mmol/L)				Alkaline phosphatase (U/L)			
	Black (n= 160)		White (n= 175)		Black (n= 160)		White (n= 175)	
Age (years)	r= -0.214;	p= 0.007	r= -0.020;	p= 0.788	r= -0.135;	p= 0.088	r= -0.005;	p= 0.953
Height (cm)	r= -0.029;	p= 0.718	r= 0.072;	p= 0.341	r= 0.048;	p= 0.544	r= 0.251;	p= 0.001
Weight (kg)	r= -0.094;	p= 0.236	r= -0.021;	p= 0.786	r= 0.116;	p= 0.143	r= 0.405;	p<0.001
Waist circumference (cm)	r= -0.096;	p= 0.228	r= 0.006;	p= 0.935	r= 0.139;	p= 0.080	r= 0.423;	p<0.001
Body mass index (kg/m ²)	r= -0.077;	p= 0.331	r= -0.059;	p= 0.441	r= 0.087;	p= 0.272	r= 0.378;	p<0.001
Body surface area (m ²)	r= -0.101;	p= 0.204	r= 0.006;	p= 0.933	r= 0.103;	p= 0.193	r= 0.415;	p<0.001
Left intima media thickness (mm)	r= 0.096;	p= 0.226	r= 0.115;	p= 0.131	r= -0.104;	p= 0.192	r= -0.105;	p= 0.165
Systolic blood pressure (mmHg)	r= 0.056;	p= 0.481	r= 0.125;	p= 0.098	r= 0.036;	p= 0.648	r= 0.344;	p<0.001
Diastolic blood pressure (mmHg)	r= 0.085;	p= 0.288	r= 0.091;	p= 0.230	r= -0.135;	p= 0.088	r= 0.356;	p<0.001
Mean arterial pressure (mmHg)	r= 0.043;	p= 0.586	r= 0.091;	p= 0.233	r= -0.078;	p= 0.328	r= 0.372	p<0.001
Relative wall thickness (cm)	r= 0.055;	p= 0.489	r= -0.063;	p= 0.404	r= 0.188;	p= 0.018	r= 0.129;	p= 0.090
Ejection fraction (%)	r= -0.147;	p= 0.063	r= -0.040;	p= 0.630	r= 0.028;	p= 0.723	r= -0.235;	p= 0.002
Fractional shortening (%)	r= -0.166;	p= 0.036	r= -0.051;	p= 0.506	r= 0.022;	p= 0.778	r= -0.222;	p= 0.003
Cardiac output (L/min/m ^{1.83})	r= -0.200;	p= 0.011	r= -0.170;	p= 0.025	r= 0.051;	p= 0.524	r= 0.143;	p= 0.059
Stroke volume index (ml/m ^{2.04})	r= -0.162;	p= 0.040	r= -0.113;	p= 0.136	r= 0.013;	p= 0.869	r= 0.114;	p= 0.134
Glucose (mmol/L)	r= -0.028;	p= 0.727	r= 0.121;	p= 0.111	r= 0.059;	p= 0.456	r= 0.045;	p= 0.552
Total cholesterol (mmol/L)	r= 0.264;	p= 0.001	r= 0.192;	p= 0.011	r= -0.038;	p= 0.632	r= 0.038;	p= 0.038
Low density lipoprotein cholesterol (mmol/L)	r= 0.232;	p= 0.003	r= 0.187;	p= 0.013	r= 0.061;	p= 0.447	r= 0.090;	p= 0.238
Triglycerides (mmol/L)	r= 0.182;	p= 0.021	r= 0.093;	p= 0.220	r= 0.079;	p= 0.323	r= 0.235;	p= 0.002
eGFR (ml/min/1.73m ²)	r= -0.179;	p= 0.023	r= -0.090;	p= 0.234	r= 0.211;	p= 0.007	r= -0.109;	p= 0.150
Reactive oxygen species (mg/L H ₂ O ₂)	r= -0.145;	p= 0.067	r= -0.191;	p= 0.011	r= -0.007;	p= 0.932	r= -0.011;	p= 0.881
Glutathione peroxidase (U/L)	r= -0.208;	p= 0.008	r= 0.276;	p<0.001	r= -0.254;	p= 0.001	r= -0.101;	p= 0.185
C-Reactive Protein (mg/L)	r= -0.065;	p= 0.413	r= -0.108;	p= 0.154	r= 0.066;	p= 0.404	r= 0.266;	p<0.001
Cotinine (ng/ml)	r= -0.031;	p= 0.689	r= -0.139;	p= 0.066	r= 0.100;	p= 0.210	r= 0.032;	p= 0.670
Gamma-glutamyl transferase (U/L)	r= 0.139;	p= 0.079	r= 0.041;	p= 0.590	r= 0.118;	p= 0.139	r= 0.426;	p<0.001
Activity Energy Expenditure (kCal/Kg/day)	r= -0.026;	p= 0.747	r= 0.034;	p= 0.654	r= 0.029;	p= 0.714	r= -0.102;	p= 0.180
Socio-economic score	r= -0.127;	p= 0.110	r= 0.055;	p= 0.472	r= -0.204;	p= 0.010	r= -0.088;	p= 0.247

Abbreviations: n – number of participants, eGFR – estimated glomerular filtration rate.

SUPPLEMENTARY 3: Standard multiple regression analyses of cardiovascular measures with markers of vascular calcification

	Calcium (mmol/L)			Alkaline Phosphatase (U/L)		
	Adjusted R ²	Std β (95 % CI)	p value	Adjusted R ²	Std β (95 % CI)	p value
Relative wall thickness (mm)						
Black (n=160)	-0.010	0.064 (-0.109; 0.237)	0.465	0.026	0.210 (0.042; 0.378)	0.015
White (n=175)	0.037	-0.036 (-0.194; 0.122)	0.653	0.037	0.040 (-0.130; 0.209)	0.645
Ejection fraction (%)						
Black (n=160)	0.032	-0.129 (-0.299; 0.040)	0.134	0.018	-0.023 (-0.192; 0.146)	0.789
White (n=175)	0.057	-0.023 (-0.179; 0.133)	0.775	0.095	-0.219 (-0.383; -0.054)	0.009
Fractional shortening (%)						
Black (n=160)	0.036	-0.154 (-0.323; 0.016)	0.075	0.017	-0.036 (-0.205; 0.133)	0.675
White (n=175)	0.084	-0.026 (-0.179; 0.128)	0.743	0.177	-0.204 (-0.366; -0.042)	0.014
Cardiac index (L/min/m^{1.83})						
Black (n=160)	0.088	-0.181 (-0.346; -0.017)	0.031	0.060	-0.031 (-0.196; 0.134)	0.712
White (n=175)	0.389	-0.148 (-0.274; -0.022)	0.021	0.378	-0.109 (-0.245; 0.027)	0.116
Stroke index (ml/m^{2.04})						
Black (n=160)	0.151	-0.206 (-0.365; -0.047)	0.011	0.120	-0.086 (-0.247; 0.075)	0.293
White (n=175)	0.377	-0.195 (-0.322; -0.069)	0.003	0.363	-0.165 (-0.303; -0.026)	0.020

Variables included in the models were: age, sex, body mass index, systolic blood pressure, total cholesterol to high density lipoprotein cholesterol ratio, gamma-glutamyl transferase, activity energy expenditure, estimated glomerular filtration rate, C-reactive protein and glutathione peroxidase. In the models with stroke index as a dependent variable, waist circumference was additionally added as a covariate. Abbreviations: n – number of participants

Chapter 4

Summary of main findings

4.1 Introduction

This is a summative chapter that will include an elaborate interpretation and discussion of the main findings of this study. A comparison in light of the original hypotheses as set out in Chapter 1 is made with the results of this study as well as with existing literature, conclusions are drawn. This is followed by recommendations for future research regarding the link between cardiovascular structure and function and associated vascular calcification markers.

4.2 Summary of main findings

In this section, the main findings of this study will be addressed according to the original hypotheses. All hypotheses made were initially set out for a study population (n=400) including black and white men and women from South Africa.

Hypothesis 1: Markers of cardiac structure (relative wall thickness) and function (ejection fraction, fractional shortening, cardiac index and stroke index) will present higher in the black group compared to their white counterparts and alkaline phosphatase and serum calcium will present higher in the black group.

We partially accepted our first hypothesis as we demonstrated that relative wall thickness and alkaline phosphatase presented higher in the black group compared to the white group. Cardiac and stroke indices presented lower in the black group whilst ejection fraction, fractional shortening and serum calcium did not significantly differ between the groups.

Hypothesis 2: Cardiovascular measures will associate adversely with markers of vascular calcification.

Our second hypothesis was also partially accepted. Ejection fraction and fractional shortening did not associate adversely with calcium in the white group. Therefore, we partially accepted this hypothesis due to cardiac index, stroke index and relative wall thickness being the only cardiovascular measures to adversely associate with markers of vascular calcification (alkaline phosphatase and calcium). This adverse association of cardiac and stroke indices with alkaline phosphatase was evident in both the black

and white groups of our study whilst the adverse association between relative wall thickness and alkaline phosphatase was only evident in the black group.

Hypothesis 3: Both oxidative stress and inflammation will contribute to the associations of cardiovascular structure and function with markers of vascular calcification.

We performed a sensitivity analysis to determine if the associations of oxidative stress (glutathione peroxidase) and inflammation (C-reactive protein) altered the associations found between our markers of cardiovascular structure and function and vascular calcification markers. Due to the unchanged significant associations that persisted, we rejected our third hypothesis.

4.3 Comparison to relevant literature

When the results from this study are compared with results from other population groups, it is evident that certain findings confirm and others contradict previous observations. The confirming findings were that alkaline phosphatase presents higher in the black population when compared to their white counterparts. Elevated levels of alkaline phosphatase are known to associate with adverse mineral metabolism resulting in the likely onset of vascular calcification. Our study therefore confirmed the findings reported by Kruger *et al.* of higher levels of alkaline phosphatase in black populations.¹

An alteration in relative wall thickness, the first sign of cardiac remodelling, typically seen in concentric remodelling has been associated with the progression of vascular calcification. London *et al.* reported that relative wall thickness associated with arterial remodelling, although these findings were in patients with end stage renal disease, the associations were positive and independent.² Our study confirmed the positive independent association of relative wall thickness with alkaline phosphatase, a known marker of vascular calcification, in the black group.

Findings from both the Prospective Urban and Rural Epidemiology (PURE) as well as the South African study on the Influence of sex, Age and Ethnicity on Insulin Sensitivity and Cardiovascular function (SAFREIC) reported that black populations were in fact

predisposed to early vascular alterations typically seen with the presence of vascular calcification.^{1, 3} However, the contrary findings of this study included the observed associations between markers of cardiovascular structure and function with markers of vascular calcification in both the black and white groups of our study. This possibly suggests that both study groups are equally susceptible to the development of vascular calcification through the associations as outlined in Chapter 3 or possibly as a result of the initial inclusion criteria of the African-PREDICT study whereby apparently healthy individuals from both black and white ethnicities were recruited.

4.4 Discussion of main findings

Mounting evidence on the development of vascular calcification especially in a South African setting is ever-increasing. In the black group of our study, relative wall thickness was found significantly higher and an independent association persisted with alkaline phosphatase, which may be indicative of increased risk for vascular calcification according to several mechanisms as outlined in the aforementioned chapter.

Previous studies conducted have indicated increased vascular calcification in black individuals. Furthermore, reports have shown that black populations are also at risk for earlier development of hypertension and arterial stiffening. Although our study supports this concept and showed that the black group of our study may be in line to the development of vascular calcification possibly through altered mineral metabolism, we also showed associations of systolic markers with vascular calcification markers in the white group as well. Thus, showing in both the black and white study groups long-term systolic dysfunction typically seen in the presence of vascular stiffening or calcification is likely to develop.

Although the findings cannot be expanded to the whole South African population, it aids in the knowledge of non-communicable disease development especially in a South African context. Furthermore, providing a reference for forthcoming studies to explore other potential contributing factors as well as a reference to follow up phases of the African-PREDICT study.

4.5 Limitations, chance and confounders

It is essential to reflect on certain factors that may have influenced the results of our study. This therefore includes the methodology which was applied, our statistical analyses as well as the interpretation of results.

The cross-sectional design of this study only identifies the existing state of health and associations found, and therefore cannot imply causality. Furthermore, a single measurement of alkaline phosphatase and serum calcium may not reflect the long-term status, whereas intima media thickness, relative wall thickness and systolic dysfunction progress over time.

Ethnicity interacted significantly on the relationship of markers of cardiovascular structure and function with vascular calcification markers. Therefore, our study population was divided into black and white groups. The group sizes differed, with the black group being fewer than the white group. A power analysis was performed using the G* Power 3 statistical analysis program (see *output in* Table 4). Firstly, we computed the required effect size via independent T-tests which detected an alpha level of 0.05 and total sample size of (n=235). An effect size of 0.5 was calculated and used in the compromise analysis to detect implied alpha and power. Our power analysis indicated an alpha error probability of 0.05 and power at 99.53%. Our sample sizes were therefore sufficient to test our hypotheses.

Table 4: Power analysis report

T-tests – Difference between 2 independent means (two groups)		
Analysis: Post Hoc – Compute achieved power		
Input:	Tail(s)	= 2
	Effect size d	= 0.5
	α error probability	= 0.05
	Sample size group 1	= 160
	Sample size group 2	= 175
Output:	Noncentrality parameter δ	= 4.5711620
	Critical t	= 1.9671134
	Df	= 333
	Power (1- β error probability)	= 0.9953113

We did not additionally stratify our study population by sex, however, sex was considered as a covariate throughout our models. Additionally, the presence of arterial

calcium prompting arterial calcification could not be assessed since coronary artery calcium score, which is widely used as a measure, was not available in this study since our study participants were young. However, the associations of alkaline phosphatase and serum calcium could be indicative of the risk for the development of vascular calcification.^{4, 5} All participants were recruited from the surrounding Potchefstroom area, North West Province which included both rural and urban communities, as such this cannot be representative of the general African population. This study was well designed and followed a concise protocol. Furthermore, this study was carried out under strict controlled conditions.

Regarding the results of our study, the possibility of chance should be taken into consideration. Notwithstanding using uni- and multivariate regression analyses, there is a possibility that associations reported may be due to chance. Thus, these adjustments may have caused an over- or underestimation of the associations observed by markers of cardiovascular structure and function and vascular calcification markers. Although we described persistent findings taking various confounding variables into consideration and reported the same findings.

4.6 Conclusion

In conclusion, our study indicates that in young healthy individuals (20-30 years old) cardiac structure (relative wall thickness) and function (cardiac index and stroke index) associated with markers of vascular calcification (alkaline phosphatase and calcium). The associations merely describe different mechanisms between the black and white groups which may be attributable to the resultant effect of calcium regulation and bone metabolism. The present study provides relevant knowledge of altered mineralisation and the resultant effects on cardiac structure and function. Causality should be investigated in prospective and experimental studies.

4.7 Recommendations

Comparatively larger population samples are needed to explore the link of cardiovascular structure and function and the prevalence of vascular calcification. A non-invasive computed tomography (CT) scan of the cardiac muscle could possibly be utilised to calculate coronary artery calcium score, however more appropriate in

high risk individuals. Additionally, clinical or epidemiological studies may be emphasised to explore the various possible mechanisms, the foremost earliest risk factors which may shed light on this untimely cardiovascular burden in South African populations. New knowledge on such vascular calcification contributors would be invaluable in applying cardiovascular prevention programmes in young adults.

References

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3. Gafane LF, Schutte R, Kruger IM, Schutte AE. Large artery stiffness and carotid intima-media thickness in relation to markers of calcium and bone mineral metabolism in African women older than 46 years. *J Hum Hypertens* 2015; 29:152-158.
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Appendix A: Ethics approval for the African-PREDICT study.



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**Faculty of Health Sciences
Health Sciences Ethics Office for Research,
Training and Support
Health Research Ethics Committee (HREC)**

Tel: 018-285 2291
Email: Wayne.Towers@nwu.ac.za

6 September 2016

Prof AE Schutte
HART

Dear Prof Schutte

APPROVAL OF YOUR AMENDMENT REQUEST BY THE HEALTH RESEARCH ETHICS COMMITTEE (HREC) OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00001-12-A1

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

Study title: African Prospective Study for the Early Detection and identification of Cardiovascular Disease and Hypertension (African-PREDICT Study)

Study leader/supervisor: Prof AE Schutte

You are kindly informed that your application to amend the single study was reviewed at the meeting held on 11/05/2016 of the HREC, Faculty of Health Sciences, and was approved on 06/09/2016.

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-HRECAppl@nwu.ac.za.

Yours sincerely

A handwritten signature in black ink, appearing to read 'Wayne Towers'.

Dr Wayne Towers
HREC Chairperson

A handwritten signature in black ink, appearing to read 'Minrie Greeff'.

Prof Minrie Greeff
Ethics Office Head

Current details: (13210572) C:\Users\13210572\Documents\HREC\HREC - Letter templates\HREC Approval Letter - June 2016.docm
15 June 2016

File reference: 9.1.5.3

Ethics approval for this current study.



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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 **12/06/2017** 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 links of cardiovascular structure and function with biomarkers related to vascular calcification: The African-PREDICT study

Study Leader/Supervisor: Prof R Kruger

Student: A Craig-27751023

Ethics number:

N	W	U	-	0	0	0	4	8	-	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: 12/06/2017

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):

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

- x 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.
- x Annually a number of studies may be randomly selected for an external audit.
- x 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 deviation from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.
- x The date of approval indicates the first date that the study may be started.
- x 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.
- x HREC can be contacted for further information or any report templates via Ethics-HRECAApply@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
10:17:12 +02'00'

Prof Linda du Plessis

Chair NWU Institutional Research Ethics Regulatory Committee (IRERC)

Appendix B: Confirmation of language editing of this dissertation.

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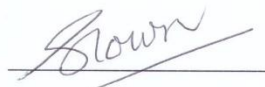
FACILITATION OF TUTOR SYSTEMS championssun@gmail.com

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This letter serves to confirm that I, Jennifer Jean Brown (ID: 5403110091080) herewith declare that I was solely responsible for the language editing undertaken on the following dissertation authored by Miss Ashleigh Craig (student number: 27751023) and co-authored by Professors R Kruger and CMC Mels at the North-West University (Potchefstroom campus).

Title: *Exploring the links of cardiovascular structure and function with biomarkers related to vascular calcification: The African-PREDICT study.*

Yours Sincerely,


Jennifer J. Brown
BA English (UNISA)

14/11/2017
Date

Appendix C: Turn it in originality report.



Digital Receipt

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

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Appendix D: Solemn declaration and permission to submit.



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Higher Degrees Administration

SOLEMN DECLARATION AND PERMISSION TO SUBMIT

1. Solemn declaration by student

I, Ashleigh Craig

declare herewith that the thesis/dissertation/mini-dissertation/article entitled (**exactly as registered/approved title**), Exploring the links of cardiovascular structure and function with biomarkers related to vascular calcification: The African-PREDICT study

which I herewith submit to the North-West University, Potchefstroom Campus, is in compliance /partial compliance with the requirements set for the degree:

Master of Health Sciences in Cardiovascular Physiology

is my own work, has been language-edited in accordance with the requirements and has not already been submitted to any other university.

I understand and accept that the copies that are submitted for examination become the property of the University.

LATE SUBMISSION: If a thesis/dissertation/mini-dissertation/article of a student is submitted after the deadline for submission, the period available for examination is limited. No guarantee can therefore be given that (should the examiners' reports be positive) the degree will be conferred at the next applicable graduation ceremony. It may also imply that the student would have to re-register for the following academic year.

Signature of student

University number

27751023

Signed on this 31 day of October of 20 17

2. Permission to submit and solemn declaration by supervisor/promoter

- The undersigned declares that:

the student is hereby granted permission to submit his/her mini-dissertation/ dissertation or thesis:

Yes ☒ No ☐

- that the student's work has been tested by me for plagiarism (for example by TurnItIn) and a satisfactory report has been obtained:

Yes ☒ No ☐

Signature/Supervisor/Promoter

Date

31/10/2017

Original details: Marietjie Ackermann(10512187) R/Support doc's\SOLEMN DECLARATION AND PERMISSION TO SUBMIT.docm 28 july 2017
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1