Indices of calcium metabolism and their relationships with arterial structure and function in African women: The PURE study

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Preface

The article-format has been chosen for this dissertation. This is the format approved and recommended by the North-West University. The dissertation consists of a motivation, literature overview, a manuscript to be submitted to a peer reviewed journal, namely *Atherosclerosis* and a concluding chapter which summarises the main findings and recommendations.

The layout of the dissertation is as follows:

Chapter 1: Background and motivation

Chapter 2: Broad literature study and detailed aim and objectives

Chapter 3: Research article consisting of author's instructions for the journal *Atherosclerosis,* an abstract, introduction, materials and methods, results, discussion, conclusion and acknowledgements.

Chapter 4: Discussion of main findings, limitations, conclusion and recommendations.

References are provided at the end of each chapter according to the Vancouver referencing style.

Contributions of the authors

The following researchers contributed to the article:

Miss LF Gafane

Responsible for conducting the literature search. The candidate performed all statistical analyses, designed, wrote and compiled the manuscript. The candidate is also experienced with the detailed methodology of performing brachial and central blood pressures, and large artery stiffness measurements, using the Sphygmocor.

Prof AE Schutte

Supervisor

Supervised all stages of compiling the manuscript, was responsible for collection of data and gave general professional input.

Prof R Schutte

Co-supervisor

Provided recommendations on statistical analyses, writing of the manuscript and interpretation of results.

This is a statement from the authors confirming their individual contribution to the study and their permission that the manuscript may form part of this dissertation.

Prof AE Schutte **Prof R Schutte**

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Summary

Indices of calcium metabolism and their relationships with arterial structure and function in African women: The PURE study

Motivation

The burden of cardiovascular diseases (CVD) is increasing in developing countries worldwide, but even more so in sub-Saharan Africa. Due to rapid urbanisation, black populations experience lifestyle changes (e.g. unhealthy diet, increased access to alcohol and tobacco) that predispose them to increased obesity and cardiovascular risk. In this study, attention will be given to cardiovascular alterations, specifically arterial calcification, in lean and overweight/obese women nearing or already experiencing menopause. These include elevated blood pressure, large artery stiffness (indicated by increased central pulse pressure (cPP)) and carotid intima-media thickness (CIMT). Other factors linked to arterial calcification include the level of obesity as well as low bone mineral density.

Ectopic calcification plays a significant role in cardiovascular morbidity and mortality, especially in renal failure patients, osteoporotic and elderly women. Factors contributing to the development and progression of arterial calcification include calciotropic hormones and altered bone metabolism, particularly in older postmenopausal women. This is due to the lack of protective effects of oestrogen against vascular alterations and bone loss after menopause. Previous studies have shown that increased bone resorption indicated by elevated levels of c-telopeptide of type I collagen (CTX), parathyroid hormone (PTH), low 25-hydroxycholecalciferol ($25(OH)D_3$) and parathyroid hormone to 25-hydroxycholecalciferol ratio ($PTH:25(OH)D_3$) are independently linked to arterial stiffening, CIMT and vascular calcification. Knowledge on the contribution of altered bone metabolism and associated calciotropic hormones on cardiovascular health in Africans is limited. Previous studies on ectopic calcification in South Africans focused on men and renal failure patients. This study

will explore the possible role of altered calcium regulation and bone metabolism in the development of arterial calcification and CVD in older African women.

Aim

The aim of this study was to investigate the associations of brachial and central pressures and CIMT with PTH, PTH:25(OH)D_3 and CTX, a marker of bone resorption, in lean and overweight/obese African women older than 46 years.

Methodology

This sub-study forms part of the Prospective Urban Rural Epidemiology (PURE) study. A total of 434 urban and rural women older than 46 years were included in the study. Women infected with the human immunodeficiency virus (HIV) were excluded from the study. The study was reviewed and approved by the Ethics Committee of the North-West University (Potchefstroom campus) and all participants signed an informed consent form prior to enrolment into the project. Field workers administered demographic, general health and physical activity questionnaires in the participants' home language. Anthropometric measurements included weight, height and waist circumference, while body mass index (BMI) was calculated in kg/m^2. Cardiovascular measurements included brachial and central systolic blood pressure (SBP), brachial diastolic blood pressure (DBP), brachial and central pulse pressure (PP) as well as CIMT and carotid cross-sectional wall area (CSWA). Blood pressure measurements were performed on the right arm with the participant in the sitting position. Blood was drawn after an overnight fasting period. We performed biochemical analyses from serum and plasma samples for follicle stimulating hormone (FSH), PTH, 25(OH)D_3, and CTX. HIV testing was performed according to standardised procedures. Since interactions existed for BMI with regards to associations of CIMT and cPP with PTH:25(OH)D_3, the study population was divided into the lean (BMI <25 kg/m^2) and overweight/obese (BMI ≥25 kg/m^2) groups. We performed independent T-tests to compare means and used the chi-square test to compare proportions. Single and multiple regression

analyses were performed to investigate the associations of markers of vascular structure and function with CTX and calciotropic hormones.

Results

In this study, 90% of the women displayed an FSH concentration exceeding the cut-off value of 35 mIu/mL, indicating a postmenopausal state. When comparing lean and overweight/obese African women, we found that lean women had higher levels of CTX and $25(OH)D_3$ (both $p<0.001$), while the overweight/obese group was older ($p=0.007$) and presented with higher PTH and PTH:$25(OH)D_3$ levels (both $p<0.001$). Brachial and central pressures did not differ between the groups ($p\geq0.23$), except for DBP being higher in the overweight/obese group ($p=0.017$). Overweight/obese women had higher CIMT ($p<0.001$) and CSWA ($p=0.001$) as compared to their lean counterparts. A larger proportion of lean women smoked (63%) and self-reported on alcohol use (37%) than overweight/obese women (41% and 18%, respectively) (both $p<0.001$). Forty-one percent of overweight/obese women used antihypertensive medication, opposed to 25% in the lean group ($p=0.001$).

In multivariate regression analyses, an independent positive association existed between CIMT and PTH:$25(OH)D_3$ ($R^2=0.22$; $\beta=0.26$; $p=0.003$) in lean women. In the overweight/obese group independent positive associations were confirmed between brachial SBP and PTH ($p=0.013$) and CTX ($p=0.038$), and between DBP and PTH ($p=0.030$). Brachial PP and central SBP remained positively associated with CTX ($p=0.016$ and $p=0.024$, respectively), while cPP was independently associated with PTH:$25(OH)D_3$ ($R^2=0.20$; $\beta=0.17$; $p=0.017$) and CTX ($R^2=0.20$; $\beta=0.17$; $p=0.025$).

Conclusion

Our results indicate that in older African women, large artery structure and function are associated with calciotropic hormones and bone resorption, suggesting that altered bone metabolism and associated calciotropic hormones play a role in the development of vascular calcification. The different associations in lean and overweight/obese women suggest

different mechanisms at work regarding arterial calcification in states of low and high adiposity. These findings need confirmation in larger prospective and experimental studies.

Key words: Parathyroid hormone, 25-hydroxycholecalciferol, c-telopeptide of type I collagen, carotid intima-media thickness, arterial stiffness, pulse pressure, postmenopausal women

Opsomming

Afrikaanse titel: **Merkers van kalsium metabolisme en die verwantskappe daarvan met arteriële struktuur en funksie in swart vrouens: Die PURE studie**

Motivering

Kardiovaskulêre siektes is wêreldwyd aan die toeneem veral in ontwikkelende lande, en selfs meer so in Sub-Sahara Afrika. As gevolg van verstedeliking ondervind swart populasiegroepe veranderinge in lewensstyl (byvoorbeeld ‘n ongesonde dieet, asook toenemende beskikbaarheid van alkohol en tabak). Dit stel hulle tot groterwordende mate bloot aan risiko vir die ontwikkeling van obesiteit en kardiovaskulêre siekte. In hierdie studie word aandag gegee aan kardiovaskulêre veranderinge, spesifiek metings van arteriële kalsifisering, in skraal en oorgewig/obese vrouens wat bykans of alreeds menopouse ervaar. Dit sluit in verhoogde bloeddruk, arteriële styfheid (soos aangedui deur ‘n toename in sentrale polsdruk (sPD) en verdikking van die arteriële wand van die karotis arterie (CIMT)). Ander faktore wat met arteriële kalsifisering verband hou sluit in die mate van obesiteit asook lae beenmineraaldigtheid.

Ektopiese kalsifisering speel `n belangrike rol in kardiovaskulêre morbiditeit en mortaliteit, veral in nierversakingspasiënte, sowel as in ouer vroue wat aan osteoporose ly. Faktore wat bydra tot die ontwikkeling van arteriële kalsifisering sluit in kalsiotropiese hormone sowel as veranderende beenmetabolisme, veral in ouer postmenopousale vroue. Dit is te wyte aan gebrekkige estrogeen beskerming teen vaskulêre veranderinge en beenverlies na menopouse. Vorige studies het getoon dat beenresorpsie, wat aangedui word deur verhoogde vlakke van c-telopeptied van tipe 1 kollageen (CTX), asook paratiroïedhormoon (PTH), lae 25-hidroksiecholekalsiferol ($25(OH)D_3$) en die paratiroïedhormoon tot 25-hidroksiecholekalsiferol verhouding ($PTH:25(OH)D_3$) onafhanklik verband hou met arteriële styfheid, CIMT en vaskulêre kalsifisering. Kennis met betrekking tot die bydrae van veranderde beenmetabolisme en geassosieerde kalsiotropiese hormone en kardiovaskulêre

gesondheid in swart populasies, is beperk. Vorige studies van ektopiese kalsifisering in Suid Afrikaners was toegespits op mans en nierversakingspasiënte. Hierdie studie sal die moontlike rol van veranderde kalsiumregulering en beenmetabolisme in die ontwikkeling van arteriële kalsifisering en kardiovaskulêre siekte in ouer swart vroue ondersoek.

Doel

Die doel van hierdie studie is om die verwantskappe van brachiale en sentrale drukke, asook CIMT met PTH, PTH:25(OH)D_3 en CTX, `n merker van beenresorpsie, te ondersoek in skraal en oorgewig/obese swart vroue ouer as 46 jaar.

Metode

Hierdie substudie vorm deel van die *Prospective Urban Rural Epidemiology* (PURE) studie. ‘n Totale groep van 434 landelike en verstedelikte vroue ouer as 46 jaar, is ingesluit in die substudie. Vroue geïnfekteer met die menslike immuniteitsgebrekvirus (MIV) is uitgesluit. Die studie is deur die Etiekkomitee van die Noordwes-Universiteit (Potchefstroomkampus) goedgekeur en al die deelnemers het `n ingeligte toestemmingsvorm onderteken voordat hulle aan die studie deelgeneem het. Met die hulp van veldwerkers het deelnemers `n demografiese, algemene gesondheids- en fisieke aktiwiteitsvraelys in die deelnemers se huistaal voltooi. Antropometriese metings het gewig, lengte en middelomtrek ingesluit, en liggaamsmassa-indeks (LMI) is bepaal in kg/m². Kardiovaskulêre metings het ingesluit brachiale en sentrale sistoliese bloeddruk (SBD), diastoliese bloeddruk (DBD), brachiale en en sentrale polsdruk (PD) sowel as CIMT en die dwarsdeursnee van die karotiswand. Bloeddrukmetings is uitgevoer op die regterarm met die deelnemer in sittende posisie. Bloed is getrek nadat proefpersone oornag gevas het. Biochemiese analises is uitgevoer deur van van serum- en plasmamonsters gebruik te maak. Analises vir follikel stimulerende hormoon (FSH), PTH, 25(OH)D_3, en CTX is uitgevoer. MIV toetsing is uitgevoer volgens standaard-prosedures. As gevolg van die interaksie van LMI met betrekking tot die assosiasie van CIMT en sPD met PTH:25(OH)D_3 is die studie populasie verdeel in skraal (LMI <25 kg/m^2)

en oorgewig/obese groepe (LMI ≥25 kg/m^2). Ons het onafhanklike T-toetse uitgevoer om gemiddelde te vergelyk, en chi-kwadraat toetse is gebruik om proporsies te vergelyk. Enkel en meervoudige regressie analises is uitgevoer om die assosiasies tussen merkers van vaskulêre struktuur en funksie met CTX en kalsiotropiese hormone te bepaal.

Resultate

In hierdie studie het 90% van die vrouens ‘n FSH-vlak getoon bokant die afsnywaarde van 35 mIU/mL, wat ‘n postmenopousale toestand aandui. ‘n Vergelyking tussen skraal en oorgewig/obese swart vroue het getoon dat skraal vroue hoër vlakke van CTX en 25(OH)D_3 (beide p <0.001) het, terwyl die oorgewig/obese groep hoër vlakke van PTH en PTH:25(OH)D_3 (beide p<0.001) getoon het. Brachiale en sentrale drukke het nie tussen die groepe verskil nie (p≥ 0.23), behalwe diastoliese bloeddruk wat hoër in die oorgewig/obese groep was (p=0.017). Oorgewig/obese vroue het hoër metings van CIMT en dwarsdeursnee van die karotisarterie getoon (p≤0.001) in vergelyking met hul skraal eweknieë. Meer skraal vroue het gerook (63%) en het alkoholgebruik gerapporteer (37%) vergeleke met oorgewig/obese vroue (41% en 18%, respektiewelik)(beide p<0.001). Een-en-veertig persent van oorgewig/obese vroue het antihipertensiewe medikasie gebruik, teenoor 25% in die skraal groep (p=0.001).

Meervoudige regressie analises het ‘n onafhanklike positiewe assosiasie tussen CIMT en PTH:25(OH)D_3 (R^2=0.22; β=0.26; p=0.003) in skraal vroue aangetoon. In die oorgewig/obese groep is onafhanklike positiewe assosiasies bevestig tussen brachiale SBD en PTH (p=0.013) en CTX (p=0.038), en tussen DBD en PTH (p=0.030). Brachiale PD en sentrale SBD was positief gekorreleer met CTX (p=0.016 en p=0.024, respektiewelik), terwyl cPD onafhanklik korreleer met PTH:25(OH)D_3 (R^2=0.20; β=0.17; p=0.017) en CTX (R^2=0.20; β=0.17; p=0.025).

Gevolgtrekking

Ons resultate dui aan dat in ouer swart vroue, arteriële struktuur en funksie geassosieer word met kalsiotropiese hormone en beenresorpsie, wat aandui dat veranderde beenmetabolisme en die geassosieerde kalsiotropiese hormone `n rol speel in die ontwikkeling van vaskulêre kalsifisering. Die verskillende assosiasies in skraal en oorgewig/obese vroue dui daarop dat verskillende meganismes werksaam is met betrekking tot arteriële kalsifisering in toestande van lae en verhoogde vetsugtigheid. Hierdie bevindinge moet bevestig word in groter longitudinale en eksperimentele studies.

Sleutelwoorde: paratiroïedhormoon, 25-hidroksiecholekalsiferol, c-telopeptied van klas I kollageen, karotis intima-media dikte, arteriële styfheid, polsdruk, postmenopousale vroue

List of abbreviations

$25(OH)D_3$:	25-hydroxycholecalciferol
$1,25(OH)_2D_3$:	Calcitriol
AGEs:	Advanced glycation products
AI:	Augmentation index
ALP:	Alkaline phosphatase
BMD:	Bone mineral density
BMI:	Body mass index
CAC:	Coronary artery calcium
CIMT:	Carotid intima-media thickness
CKD:	Chronic kidney disease
CVD:	Cardiovascular diseases
CWT:	Carotid wall thickness
cm:	Centimetres
CrCl:	Creatinine clearance
CRP:	C-reactive protein
CSWA:	Cross-sectional wall area
CTX:	C-telopeptide type I collagen crosslinks
DBP:	Diastolic blood pressure
ECF:	Extracellular fluid
EDTA:	Ethylenediaminetetraacetic acid
FSH:	Follicle stimulating hormone
g/L:	Grams per litre
GFR:	Glomerular filtration rate
GGT:	Gamma glutamyl transferase
GSH:	Glutathione
HbA1c:	Glycosylated haemoglobin
HDL:	High density lipoprotein

HIV:	Human immunodeficiency virus
HSMCs:	Human smooth muscle cells
s-ICAM1:	Soluble intercellular adhesion molecule type 1
kg/m^2:	Kilograms per meter squared
kg:	Kilograms
LDL:	Low density lipoprotein
mg/L:	Milligrams per litre
mL/min:	Millilitres per minute
mm:	Millimetres
mmHg:	Millimetre mercury
mmol/L:	Millimole per litre
ng/mL:	nanograms per millilitre
NTX:	N-telopeptide of type I collagen crosslink
OPN:	Osteopontin
PP:	Pulse pressure
PTH:	Parathyroid hormone
PURE:	Prospective Urban and Rural Epidemiology
PWV:	Pulse wave velocity
RAAS:	Renin angiotensin aldosterone system
ROS:	Reactive oxygen species
SBP:	Systolic blood pressure
SD:	Standard deviation
TC:	Total cholesterol
U/L:	Units per litre
WHO:	World Health Organisation
VSMCs:	Vascular smooth muscle cells
WC:	Waist circumference

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Background and motivation

1. General introduction

The burden of cardiovascular diseases (CVD) is devastating and increasing in developing countries within Sub-Saharan Africa [1]. This major health problem is especially evident among urbanised black South Africans experiencing a health and nutritional transition [2, 3]. When focusing on the vascular system, some of the risk factors for cardiovascular morbidity and mortality include, but are not limited to vascular calcification, arterial stiffening and hypertension [4-6]. The focus of this dissertation is to improve our understanding of some of the factors involved in arterial calcification, including, calciotropic hormones and bone resorption in black South African women. This study will explore how these factors relate to measures of arterial function and structure such as brachial and central blood pressure (BP), brachial and central pulse pressure (PP) and carotid intima-media thickness (CIMT).

Vascular calcification can be defined as extracellular calcium deposition in the arterial wall in the form of hydroxyapatite and is frequently observed in patients with hypertension, chronic renal failure and diabetes [7-10]. Arterial stiffening and calcification accompany ageing in the healthy population [11, 12]. In postmenopausal women, development of arterial calcification has been linked to altered bone mineral metabolism, characterised by high bone resorption and associated calciotropic hormones, which predisposes this population to CVD [13, 14]. C-telopeptide of type I collagen (CTX) is marker of bone resorption and it will be included in the present study.

Deviation from normal levels of calciotropic hormones can result in alterations leading to arterial stiffness, hypertension and atherosclerosis [15-17]. The African population is at high risk of developing CVD as a result of early vascular alterations, and low renin status which can subsequently result in a high prevalence of cardiovascular morbidity and mortality [18-20]. Other factors that influence the relationships between vascular calcification and CVD include

increased adiposity, smoking and alcohol consumption [21-23]. These contributing factors, as well as other known confounders, will be taken into consideration when exploring the associations of brachial and central blood pressures, pulse pressures and CIMT with calciotropic hormones and CTX in the present study.

2. Motivation and problem statement

To combat the increasing burden of hypertension and its associated cardiovascular morbidities in South Africans, which affects more women than men [19, 24]; it is vital to clarify mechanisms involved such as vascular calcification. Arterial calcification is now regarded as one of the reasons for increased cardiovascular mortality in renal failure patients [25]. In addition, vascular calcification forms part of the ageing process and can be accelerated by disruption of the balance between inhibition and promotion of calcification that is observed in the elderly and postmenopausal women [7, 26]. Metabolic disorders associated with diabetes and obesity that can lead to inflammation has also been linked to vascular mineralisation [27].

Premenopausal women are generally regarded to be at a lower cardiovascular risk; however, this changes during menopause when the protective oestrogen levels are decreased [28]. As a result postmenopausal women are predisposed to overall increases in cardiovascular morbidity and mortality [29]. The prevalence of vascular calcification in osteoporotic and postmenopausal women has been associated with atherosclerosis and arterial stiffening [30-32]. Low bone mineral density (BMD) and an increase in bone resorption, frequently observed in older postmenopausal women, are associated with an increase in arterial calcium deposits [13]. Loss of oestrogen decreases renal calcium reabsorption, resulting in increased parathyroid hormone (PTH) secretion, accelerating bone resorption in order to correct blood calcium levels [33]. However, PTH has been identified as an independent predictor of vascular calcification development in renal failure patients [16, 34].

Previous studies on vascular calcification in South Africans were performed mostly in men and indicated that normotensive and hypertensive African men had an increased risk of arterial calcification [35, 36]. However, Freercks *et al*. reported a low prevalence of coronary calcification in black South African adults on dialysis, suggesting that black race provides some form of protection against coronary calcification [37]. However, Sliwa *et al*. reported low prevalence of coronary artery diseases, but a high prevalence of hypertensive heart disease [38]. Additionally, Schutte *et al*. found a relationship between large artery stiffness and alkaline phosphatase (ALP), a promoter of calcification [36]. This evidence indicates that vascular mineralisation may currently be a factor in the development of CVD in Africans, and the present study will elaborate on the contribution of calciotropic hormones and altered bone mineral metabolism.

Kruger *et al*. found that black South African women presented with low dietary calcium intake and low 25-hydroxycholecalciferol ($25(OH)D_3$), resulting in elevated circulating PTH. Consequently, increased bone resoption was also observed in this group, which predisposes these women to bone fractures [39]. The present study will specifically focus on the same African women described by Kruger *et al*. against their portrayed altered bone metabolism and disorders of calciotropic hormones, which are known as contributing factors for arterial calcification.

New insights on how bone metabolism and calciotropic hormones relate to vascular structure and function could help to prevent further complications that could result from arterial calcification, such as atherosclerosis and arteriosclerosis. Additionally, the findings may reveal areas for further research into the increasing prevalence of CVD in Africans. Therefore, the motivation for this study is to add to the existing knowledge by reporting on the associations of measures of vascular structure and function that include brachial and central pressures and

carotid wall thickness with parathyroid hormone to 25-hydroxycholecalciferol ratio (PTH:25(OH)D_3) ratio and bone resorption in African women older than 46 years.

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Chapter 2

Literature study

CONTENTS

2.1. Introduction

Vascular calcification is an emerging risk factor for cardiovascular morbidity and mortality [1], and it can be defined as ectopic deposition of calcium phosphate crystals in the vessel wall [2]. Initially, it was thought to be a passive degenerative process that forms part of ageing [1]. However, advances in research have now established that vascular calcification is an actively regulated process and is associated with bone mineral metabolism and calciotropic hormones [3, 4].

Calciotropic hormones such as parathyroid hormone (PTH) and vitamin D are associated with increased cardiovascular risk and have a role in the development of arterial calcification especially in postmenopausal women experiencing increased bone resorption [5, 6]. PTH and 25-hydroxycholecalciferol ($25(OH)D_3$) have been independently linked with arterial stiffness, intima-media thickening and elevated blood pressure; there is however, limited evidence on these relationships in the African population [5, 7, 8]. The interactions of markers of vascular structure and function with factors involved in calcification including markers of bone mineral metabolism and associated calciotropic hormones, will be discussed in detail in this literature review.

2.2. Vascular structure

The arterial system is physiologically designed to transfer blood at an optimum high pressure in a continuous stream to the peripheral vasculature for efficient tissue perfusion [9]. Different types of arteries exist to accomplish this function, including the large elastic arteries such as the aorta and common carotid arteries [10], and muscular arteries such as the femoral and brachial arteries [11, 12].

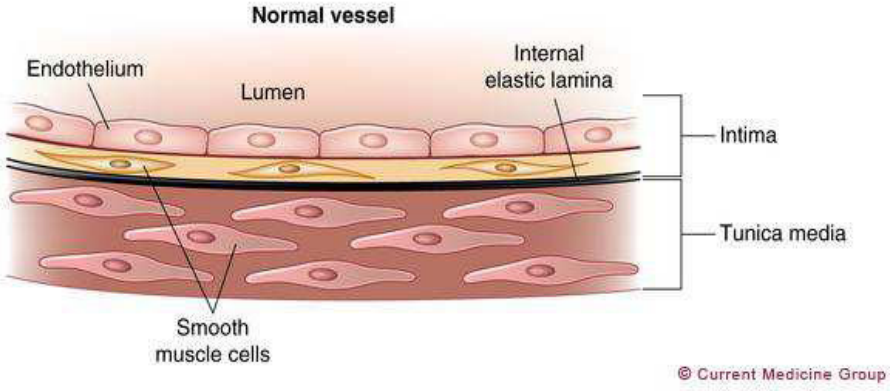

Figure 1: Structure of the vessel wall (adapted from Falik *et al*., 2009) [13]**.**

The vascular wall consists of three functional layers, namely the intima, media, and adventitia [13] (Figure 1). The first layer is the intima, which consists of the innermost endothelial layer and small amounts of connective tissue located just below the endothelium. The second layer is the media, composed mainly of smooth muscle cells and elastin-rich extracellular matrix. Thirdly is the adventitial layer which comprises of large quantities of collagen fibres and fewer elastin fibres [10]. The above-mentioned vascular layers function interactively to maintain adequate blood distribution to the rest of the body [14].

The properties of the arterial wall layers deteriorate with age and this degeneration is accelerated in the presence of conditions such as hypertension, diabetes and chronic renal failure that are associated with arterial stiffening and thickening, as well as with calcium deposits in the arterial wall [10, 15, 16]. Vascular calcification can either occur in the intimal layer or medial layer or both layers simultaneously. The area of localisation of calcification in the arterial wall determines the clinical outcomes, with intimal calcification being associated with atherosclerosis while medial calcification is associated with arteriosclerosis [2, 16].

2.3. Vascular calcification

2.3.1. Pathophysiological mechanisms of vascular calcification

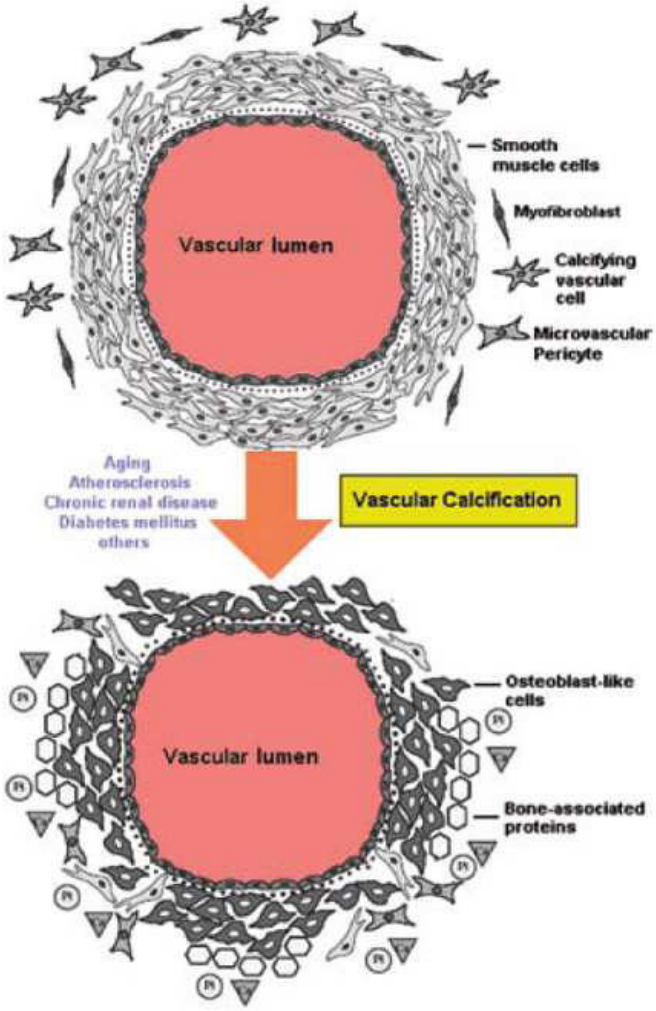

Figure 2: Hypothetical mechanisms of vascular calcification (adapted from Efstratiadis *et al*., 2007) [17].

The processes of vascular calcification and stiffening occur as part of the ageing process in the general population [18]; however vascular calcification may also occur as a response to injury [19]. Previously, vascular calcification was considered a passive, intermittent, degenerative

process involving atherosclerotic lesions [2]. Recent evidence indicates that vascular calcification is, in fact, an active and regulated component of vascular disease processes [3, 20].

Studies using *in vitro* and *in vivo* models discovered various proteins regulating the process of calcification to substantiate that vascular calcification is indeed, an actively regulated process [21, 22]. Dysfunctional vascular smooth muscle cells (VSMCs) orchestrate the mechanisms involved in the initiation and progression of vascular calcification, which involve VSMC apoptosis, microvesicle release by VSMCs, impaired expression of mineralisation inhibitors and, eventually, mineral deposition in the extracellular matrix [23] (Figure 2).

VSMCs can develop osteoblastic characteristics and deposit hydroxyapatite crystals as a result of oxidative proinflammatory activation [24]. Inflammation is one of the initial processes in the development of different types of vascular mineralisation by induction of osteogenesis [25]. The following series of steps may also apply: vascular cells of mesenchymal origin differentiate into osteoblastic-like cells, which is accompanied by expression of alkaline phosphatase (ALP) and mineralisation of the extracellular matrix [26]. In certain disease conditions such as renal dysfunction, diabetes and hypertension, the conversion of VSMCs to bone-like osteoblastic cells is accelerated, favouring early deposition of calcium in the arterial wall [25, 27].

Similarities exist between bone formation and vascular calcification, as indicated by the presence of bone mineralisation proteins such as osteocalcin, osteopontin, matrix Gla protein, Runx2 and fibroblast growth factors-23 (FGF-23) in calcified vascular tissue [4, 28, 29] (Figure 2). In addition, calcification may occur parallel to bone resorption, during which minerals and proteins from bone are deposited into the vascular wall and causes biomineralisation [25, 28]. It is therefore important to study the pathophysiology of vascular calcification in order to determine the potential contributing factors and clinical implications.

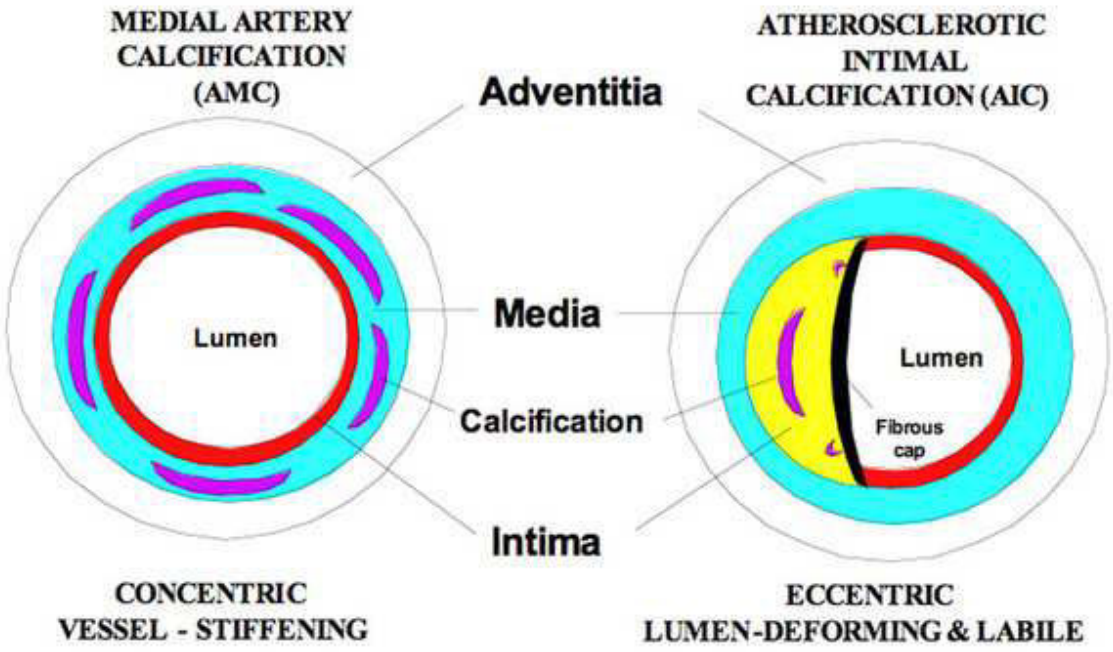

Figure 3: Medial artery calcification and atherosclerotic intimal calcification (adapted from Towler *et al.*, 2008) [30].

Intimal calcification structurally manifest as spotty, disorganised mineral deposition consisting of VSMCs, connective tissue, macrophages, oxidised lipids, and necrotic debris [31, 32] (Figure 3, right). Calcification in the intimal layer is a fundamental part of atherosclerotic plaque which is associated with atheroma and can be used as a surrogate marker for atherosclerosis and as a predictor for cardiovascular outcomes [30, 33, 34].

In contrast to intimal calcification, medial calcification structurally manifests as organised mineral deposition along the elastic lamellae (Figure 3, left) and it involves VSMCs and elastin fibres [31]. Additionally, calcification of the media is common in elderly individuals, and is especially prevalent in renal failure and diabetic patients [33, 35, 36]. The relationship between medial calcification and arteriosclerosis is based on the fact that medial calcification is confined

to areas of elastin degeneration, which is central to arterial stiffening [12, 37]. Therefore, medial calcification is supposedly responsible for the associations between arterial calcification and arterial stiffening [10]. However, it has to be considered that a relationship was also observed between arterial stiffness and intimal calcification [38].

There is a strong association between hypertension and medial calcification. Hypertension serves as a mechanical stressor evoking tensile strain that promotes medial calcification, and reduces arterial elasticity, subsequently increasing vascular stiffness [20, 39]. On the other hand, medial calcification itself can cause hypertension and left ventricular hypertrophy by reducing the elasticity of especially the large vessels, therefore elevating afterload on the heart [40, 41]. Medial calcification can therefore be used as a predictor of arteriosclerosis and a measure for the assessment of cardiovascular risk [42, 43].

2.3.2. Factors involved in calcification

2.3.2.1. Calcium

Calcium is a major component of the skeleton and plays a key role in cell physiology [23]. Its serum levels are tightly regulated by calciotropic hormones including calcitonin, PTH, and calcitriol ($1,25(OH)_2D_3$), the biologically active form of vitamin D [23, 44]. Total calcium exists in three fractions that include 48%-50% as ionised calcium, 40% as protein-bound calcium (80% to albumin), and 10%-12% is calcium compounded with anions such as bicarbonate, lactate and citrate [45-47]. Therefore in this study albumin-corrected calcium will be used to represent free calcium.

High habitual calcium intake causes a decrease in circulating PTH levels and lowers the risk for metabolic and cardiovascular diseases [48, 49]. Calcium dysregulation is also involved in the mechanisms leading to both metabolic syndrome and arterial calcification [50]. Excessive

calcium supplementation is associated with cardiovascular events, and it contributes to vascular calcification in renal failure patients with a marked alteration in calcium regulation [51-53].

In addition, hyperphosphatemia can result in calcium-induced vascular calcification. This is accomplished by incapacitation of the parathyroid gland from detecting changes in serum calcium and interaction of phosphate with free calcium ions, further contributing to secondary hyperparathyroidism [53]. The effects of calcium on vascular disease still need further investigation. Spencer and Weaver recommend that animal models be utilised since causal relationships can be achieved by using feeding protocols that are sufficient for cardiovascular diseases development [54].

2.3.2.2. Magnesium

Magnesium is an essential ion in the body with physiological and clinical roles [55, 56]. It serves as a co-factor for many enzymes, and is crucial for bone metabolism as well as maintenance of normal vascular tone and regulation of blood pressure [57-60]. In the body, magnesium exists in three fractions: 27% to 34% is protein-bound, specifically to albumin, the most abundant extracellular protein in human blood plasma, while 50% to 60% is ionised magnesium and 8% to 12% is bound to ions [61]. Magnesium and calcium compete with each other and other proteins for binding sites on albumin [61], thus albumin-corrected magnesium will be used in the present study as a representative of free magnesium.

Magnesium deficiency could cause arterial calcification, while its supplements reduce accumulation of calcium deposits in the heart and kidney as observed in a Wistar rat model [62]. Sufficient magnesium can inhibit calcification through the suppression of PTH secretion [63], inhibition of both expression of osteogenic proteins and apoptosis of microvesicles [64]. The inhibitory effects of magnesium on vascular calcification are influenced by the presence of

calcium and phosphate [65]. This signifies the interaction between these minerals and the importance of considering the role of each in their relationships with cardiovascular measures.

Negative relationships were observed in uremic patients and the general population between serum magnesium and vascular calcification [66], atherosclerosis [67], cardiovascular and all-cause mortality, supporting the above-mentioned experimental evidence [68]. It has also been indicated that magnesium can delay the progression of intima-media thickening of the carotid arteries and atherosclerosis in haemodialysis patients [67]. In hypertensive women, low serum magnesium was associated with increased intima-media thickness [69]. Therefore magnesium has an overall beneficial effect on the cardiovascular system.

2.3.2.3. Phosphate

Inorganic phosphate (Pi) is essential for cellular function and skeletal metabolism and it is tightly regulated by the renal system [70]. Phosphate interacts with calcium and its concentration increases the ion-bound fraction of calcium [71]. It performs an essential function in altered bone metabolism as presented in patients with chronic kidney disease (CKD) [72]. In pathological conditions calcium and phosphate may combine to form hydroxyapatite crystals, which are then deposited in the arterial wall [73].

A dose-dependent increase in mineralisation of human smooth muscle cells (HSMCs) with an increased dosage of inorganic phosphate was observed in an *in vitro* study and it displayed features similar to bone calcification and pathological vascular calcification [26]. Elevated phosphate levels contribute to calcification through a sodium-dependent phosphate transport mechanism [26], and additionally through degradation of phosphate donors by ALP [74, 75].

2.3.2.4. Alkaline phosphatase

Earlier studies identified alkaline phosphatases as a group of isoenzymes present in most tissues in the human body, such as in the intestines and liver [76, 77]. Its main function

comprises creation of an alkaline environment outside osteoblasts which favours calcium ion deposition and to degrade phosphate containing compounds [77, 78]. In bone metabolism, ALP is an early marker of osteogenic differentiation and osteoblastic activity [2, 79]. Stimuli such as vascular injury result in increased levels of ALP in the vascular wall and induce calcification [80].

In an *in vitro* vascular calcification model it was found that inflammatory cytokines and calcitriol stimulated the up-regulation of ALP and mineralisation [81, 82]. ALP is associated with cardiovascular mortality and hospitalisation in patients with CKD, diabetes and in the general population [26]. Adverse associations between ALP and markers of arterial structure and function were also confirmed in hypertensive and normotensive African men [83, 84].

2.3.2.5. Type I collagen crosslinks

Collagen fibres provide tensile and mechanical strength, in addition to ductility and toughness during bone formation [85]. Type I collagen is the most common and abundant constituent of the extracellular matrix, whilst additional minor collagens include types III and V [86]. The constituents of type I collagen are the amino telopeptide terminal (NTX), the carboxy terminal telopeptide (CTX) and a central triple helical region [87]. Intermolecular crosslinks are formed between the non-helical and the helical domains of adjacent collagen molecules [85]. Breakdown of these bonds produces NTX and CTX markers of bone resorption and are used to investigate osteoporosis [88, 89]. Measurement of NTX and CTX as well as bone matrix proteins in urine and blood can be utilised to evaluate the active changes in bone turnover [90], [91]. CTX as a marker of bone resorption has been linked to vascular calcification, acute myocardial infarction, renal failure, heart failure, morbidity and mortality [92, 93].

2.3.2.6. Calciotropic hormones

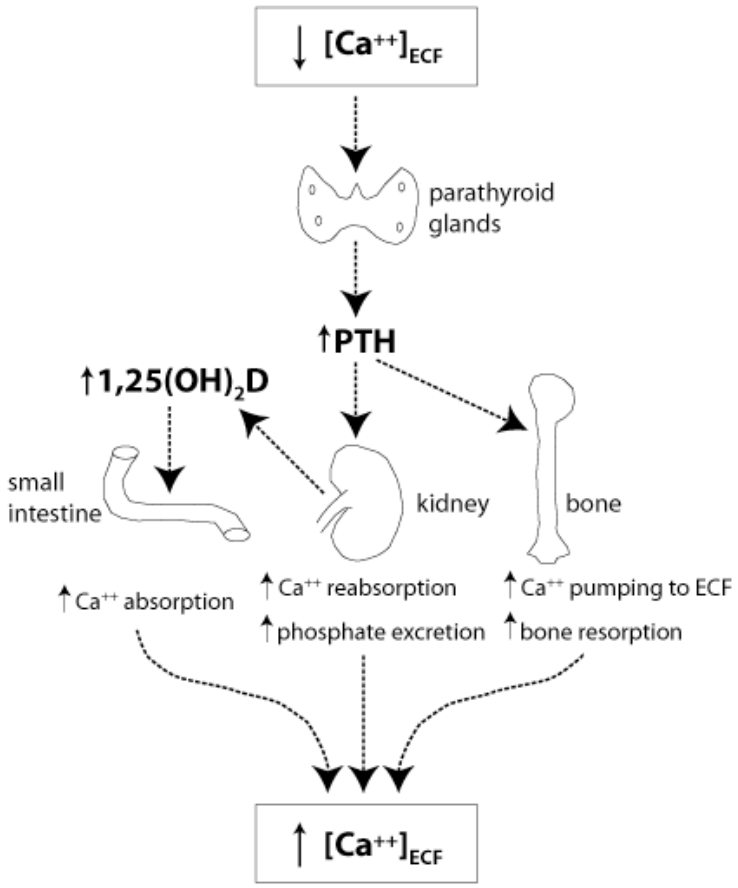

Figure 4: Schematic representation of the roles of PTH and calcitriol in calcium homeostasis (adapted from Washington educational courses) [94]**.**

↑, increase; ↓, decrease; 25(OH)D, calcidiol; $1,25(OH)_2D$, calcitriol; PTH, parathyroid hormone; ECF, extracellular fluid. (both $1,25(OH)_2D$ and $1,25(OH)_2D_3$ refers to calcitriol)

PTH, calcitriol and calcitonin are calciotropic hormones that regulate movement of minerals in and out of cells through their actions on the intestines, kidneys and bone [50, 95]. In the present study we will be focusing on PTH and $25(OH)D_3$ which have been associated with the development of vascular calcification [96, 97]. Calcitriol and PTH interactively regulate urinary

calcium excretion, and constantly keep circulating calcium within the normal concentrations [98] (Figure 4). This is accomplished by equilibrating calcium deposition in bone against gastrointestinal absorption [50]. Increased PTH secretion can be induced by vitamin D deficiency, and decreased levels of circulating calcium and phosphorus [5]. PTH will then cause calcium reabsorption in the kidney, promote conversion of 25(OH)D_3 to calcitriol by the kidney and initiate bone resorption in order to elevate serum calcium to normal levels [99] (Figure 4).

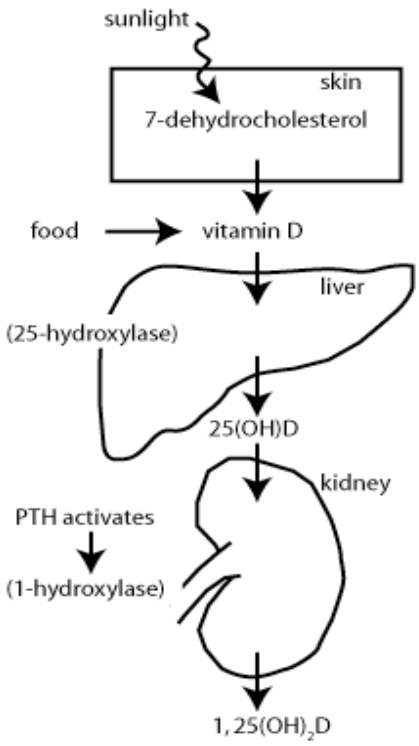

Figure 5: Schematic representation of the mechanism of vitamin D activation to form calcitriol (adapted from Washington educational courses) [94]**.**

25(OH)D, calcidiol; 1,25$(OH)_2$D, calcitriol; PTH, parathyroid hormone. (both 1,25$(OH)_2$D and 1,25$(OH)_2D_3$ refers to calcitriol)

Vitamin D represents cholecalciferol (D3) or ergocalciferol (D2) and calcidiol ($25(OH)D_3$) [100]. Active vitamin D stands for alphacalcitriol (1-hydroxyvitamin D3), doxercalciferol (1-hydroxyvitamin D2) and calcitriol ($1,25(OH)_2D$) [101]. The principal source of vitamin D precursor is the skin, and the diet which only contributes a small percentage (Figure 5) [101]. Cholecalciferol is generated from 7-deoxy-cholesterol by ultraviolet B radiation [8] or from gastrointestinal absorption from food or supplements [102] (Figure 5). Cholecalciferol then undergoes activation by hepatic metabolism to form calcidiol ($25(OH)D_3$) through the activity of 25-hydroxylase [103]. Calcidiol is the metabolite used to determine the amount of vitamin D stored in the body [101]. $25(OH)D_3$ is converted to calcitriol in the proximal renal tubules by 1-alpha-hydroxylase enzyme [104] (Figure 5). This renal metabolism of vitamin D is crucial to the endocrine function of calcitriol and PTH as modulators of calcium homeostasis [8].

The combination of low vitamin D and high PTH is associated with risk factors such as hypertension [105] and hyperlipidaemia [106] and participate in the development of peripheral artery disease [107], diabetes [108], myocardial infarction [109], heart failure [106] and stroke [106]. Insufficient $25(OH)D_3$ is also associated with oxidative stress, arterial stiffness, systemic inflammation and is a predictor of all-cause and cardiovascular mortality [110, 111].

$25(OH)D_3$ has been linked to processes leading to ectopic calcification. It can modulate expression of gamma-carboxyglutamic acid which is a protein capable of protecting against aortic calcification [112, 96]; however, extrarenal stimulation of $25(OH)D_3$ from activated macrophages in the vascular wall can cause opening of calcium channels located in the VSMCs, and accelerate arterial calcification [104]. Low $25(OH)D_3$ may also indirectly result in an elevated PP due to arterial calcification [113].

PTH is a peptide hormone produced by the parathyroid gland and is secreted as a response to decreased levels of circulating $25(OH)D_3$, calcium and phosphorus [5]. Excessive PTH secretion

has adverse effects on the blood vessels due to its prosclerotic effects on VSMCs, which eventually induces vessel thickening and elevated blood pressure [114]. PTH and the PTH:25(OH)D_3 ratio have been associated with CIMT in postmenopausal women and in the general population [5, 50]. In addition, PTH increases calcium mobilisation from bone into soft tissues such as VSMCs [115] and adipocytes [114]. It can also result in calcium influx into smooth muscle cells and induces vasoconstriction, increased vascular resistance and subsequently elevated blood pressure [53, 111]. In renal failure patients, serum PTH is an independent determinant of vascular calcification and its severity has been demonstrated [96, 116].

Calcitonin is also a calciotropic hormone that is involved in calcium regulation [23] and predominantly opposes the effects of PTH by lowering serum calcium levels [117]. Its major effects on calcium homeostasis include inhibition of bone resorption [117], reduction of calcium reabsorption by the kidneys [95], and modulation of calcitriol formation by the kidneys [118]. Its effects on cardiovascular diseases have not been as well studied as PTH and 25(OH)D_3 [99, 116].

2.4. Atherosclerosis and Arteriosclerosis

2.4.1. Pathophysiological mechanisms of atherosclerosis and arteriosclerosis

Arteriosclerosis and atherosclerosis are two distinct disease processes associated with increased cardiovascular morbidity and mortality [119, 120]. The overlapping of their pathological mechanisms remains a challenge when studying their associations [37] with other cardiovascular risk factors such as arterial calcification [32, 121].

Atherosclerosis is characterised by co-occurrence of fatty degeneration (athero) and stiffening (sclerosis) of the arterial wall [122]. The early stages of atherosclerosis involve thickening of the intima of large or medium sized arteries [10]. It is triggered by lipid retention [123], oxidation and enzymatic modification of these lipids that stimulate inflammation [9] that eventually results in thrombosis and stenosis [124, 125].

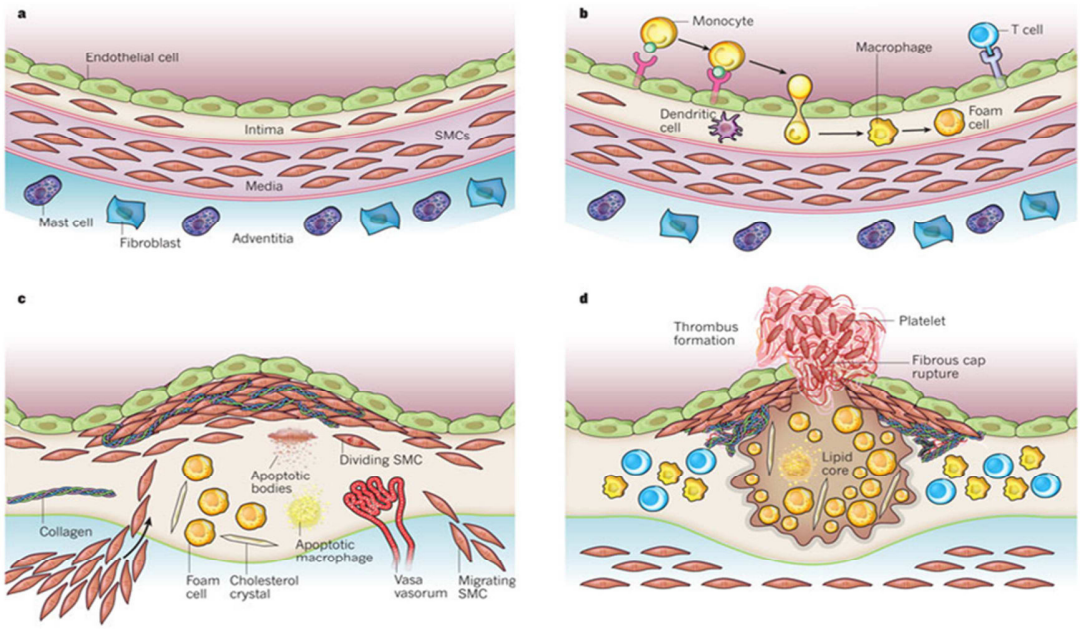

Figure 6: Stages in development of atherosclerotic lesions (adapted from Libby *et al*., 2011) [126]**.**

Initially the low density lipoprotein (LDL) cholesterol molecules enter the intimal layer of the arterial wall from the blood and accumulate [127]. This is followed by enzymatic modification and oxidation into proinflammatory molecules, triggering an innate inflammatory system within the intimal layer [126]. Inflammation starts when monocytes and other inflammatory cells infiltrate the intima and phagocytise the accumulated lipids which will result in formation of foamy macrophages [128] (Figure 4-b). Lymphocytes, neutrophils and basophils also infiltrate the intima [129] and result in formation of an early fatty streak (lesion) [123].

Eventually a fibroatheroma is formed [130] during apoptosis of foamy macrophages and this is accompanied by release of lipids into the interstitium [127]. During this phase, VSMCs migrate from the media and proliferate, and produce more collagen fibres (Figure 4-c) that surround the atheroma [74]. At this stage the deep portion of the fibroatheroma start undergoing calcification [10]. The medial layer and the adventitia become involved in the advanced stages (Figure 4-c-d) [126].

Plaque rupture is the main complication of atherosclerosis that results in cardiovascular events including myocardial infarction [131] and stroke [10]. Studies regarding the effects of calcification on plaque rupture show inconsistent results. Some investigations propose that calcification exerts more biochemical stress on the plaques, predisposing them to rupture [132], while others argue that calcification can in fact have a potentially protective effect on plaques and can provide plaque stability and therefore decrease the risk of rupture [131]. Other investigations indicate that the distribution of calcium in the vascular wall, rather than just the presence of calcium, is the determinant of plaque rupture [130, 133].

Vascular ageing is established as the key element of arteriosclerosis or aortic stiffening [10]. One of the earliest studies to highlight the differences between arteriosclerosis and atherosclerosis was performed by Pickering whom indicated that arteriosclerosis is stiffening of large arteries that is associated with ageing [134]. According to Izzo and Shykoff, arteriosclerosis is generalised stiffening and thickening of the medial layer that is associated with essential hypertension [135]. Arteriosclerosis can also be defined as stiffening and dilation of arteries that is distinct from atherosclerosis [34]. It is noteworthy that evidence exist that black Africans are predisposed to premature vessel alterations such as arterial stiffening [136].

Arteriosclerosis is predominantly characterised by degeneration and sclerosis of the medial layer of the arterial wall [137]. The medial layer of large conduit arteries consists mainly of

VSMCs, elastin and collagen fibres which forms musculoelastic sheets [18, 39]. Mechanical properties of these large arteries are provided by crosslinks between the extracellular matrix and smooth muscle cells [87]. Sustained arterial pulsation in the central arteries can alter arterial properties through rearrangement of elastin [138] and collagen fibres [139]. With ageing, the VSMCs degenerate and their numbers are reduced through apoptosis resulting in degeneration of the medial layer, continuous stiffening and calcification [10, 140]. In addition, the numbers of elastic fibres also decrease as a result of degeneration, thinning and fragmentation [141], while the amounts of collagen fibres increase [121].

Potential risk factors for arteriosclerosis have been identified and include age, elevated blood pressure [34], medial calcification [41], inflammation [142] and accumulation of advanced glycation products (AGEs) [143]. Arteriosclerosis can predispose to cardiovascular diseases by increasing PP and increasing the rate of shear stress [121]. This is further accompanied by an elevated systolic blood pressure (SBP) [18] and low diastolic blood pressure (DBP) that result in myocardial ischemia, fibrosis and heart failure [144]. Elevated central systolic blood pressure (SBP) and central pulse pressure (PP) causes increases in wall stress and left ventricular hypertrophy by increasing the afterload [18, 145]. Central PP is known to be a better measure for assessment of cardiovascular risk than peripheral pulse pressure due to the fact that cPP reflects changes in central hemodynamics [9, 146, 147].

Reports on the interactions between arterial stiffness, atherosclerosis and calcification are inconsistent [34, 148]. Human and animal studies confirm that medial calcification is the direct determinant of aortic stiffness [149, 150]. However, aortic stiffness can also be an indication of both medial and/or intimal calcification, while coronary calcification is indicative of atherosclerosis [151].

2.4.2. Markers of atherosclerosis and arteriosclerosis

2.4.2.1. Carotid Intima Media Thickness (CIMT)

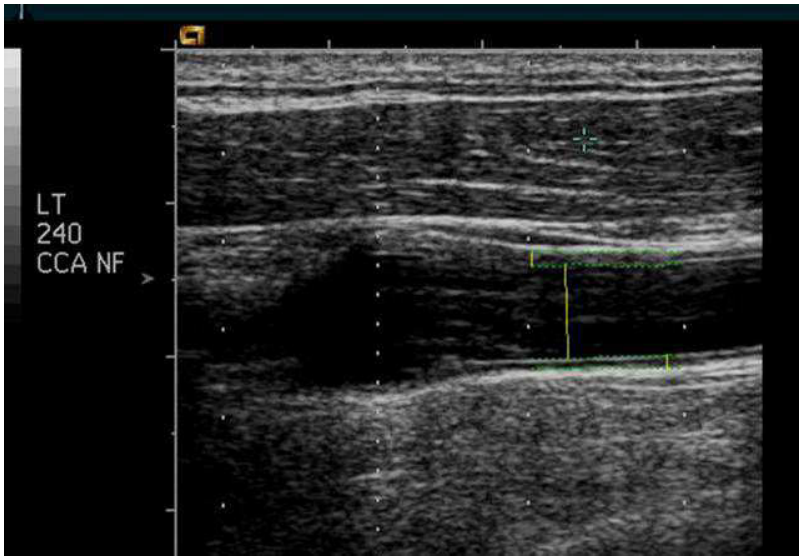

Figure 7: Ultrasound measurement of CIMT (adapted from Meijer and Bots presentation at North-West university, 2007) [152].

CIMT refers to B-mode ultrasound [122, 153] measurements of the thickening of the intima and/or media of the carotid arteries [154, 155]. It is validated as a highly accurate and reproducible method, particularly in large clinical trials [139]. However, recently it was shown that carotid wall thickness (CWT) is more sensitive to changes in the carotid arteries than CIMT [156]. CIMT is a predictor of cardiovascular events such as stroke and myocardial infarction [157] and a reliable measure for cardiovascular risk stratification in hypertensive individuals and the general population and is being extensively used as a marker of target organ damage [158, 159]. CIMT is regarded as a marker of early atherosclerosis [160] and it is a predictor of plaque build-up [161].

Based on the fact that the carotid artery is an elastic artery, increased CIMT may be representative of mainly intimal thickening [162]. Intimal thickening advances with age [163] and autopsy observations showed that thickening occurs mostly in the intimal layer as a result of intimal hyperplasia instead of the load-bearing medial layer [39, 164]. In contrast, the medial layer may undergo insignificant thickening with age, however major changes include thinning and separation of elastin and replacement by nonload-bearing material [165]. Intimal thickening and atherosclerosis accompany ageing in Western populations [37].

Intima-media thickening may also occur as a response to elevated blood pressure and variations in the shear stress pattern that is often observed with ageing [155]. It is the chronic elevated local distending pressure that causes wall thickening of central elastic arteries [166]. This was also confirmed in children with essential hypertension in which CIMT increased potentially as result of vascular abnormalities caused by sustained hypertension at young ages [167]. Hypertension is now considered one of the major risk factors for increased CIMT [153].

Several studies have linked CIMT with lipids, oxidative stress, inflammation and the metabolic syndrome and ethnic differences have been observed. These relationships will be briefly discussed in the following sections.

CIMT has been associated with various *lipid* measures in disease states such as coronary heart disease [168] and *metabolic syndrome* [169]. Hyperlipidaemia and hypercholesterolemia are associated with an increased CIMT in the general healthy population consisting of multiple ethnicities [170]. Lipid levels including elevated triglycerides, ratios of LDL to high density lipoprotein (HDL) are strong predictors of advanced CIMT [168].

Oxidative stress has also been implicated in carotid wall thickening. In hypertensive individuals, low blood glutathione (GSH) increases the risk of increased CIMT and subclinical

atherosclerosis [171] as a result of decreased antioxidant activity [172]. Oxidative stress has additionally been linked to essential hypertension as a result of an increased amount of reactive oxygen species (ROS), subsequently causing endothelial dysfunction, which will ultimately result in augmented vasoconstriction [173].

One of the key aspects that have been associated with plaque rupture is *inflammation* and its mechanism is mediated by inflammatory proteins which degrade the fibrous cap of the plaque [174]. Inflammatory markers implicated include C-reactive protein (CRP), serum amyloid A, interleukin-6 and soluble intercellular adhesion molecule type 1 (s-ICAM1) [175]. C-reactive protein is a nonspecific marker of inflammation and it has been independently associated with increased cardiovascular risk [175, 176]. As described in the previous section, during atherosclerosis, the intima undergoes extreme inflammation [177] which results in thickening [1]. The relationship between CIMT and inflammatory markers has also been reported in individuals undergoing dialysis [178, 179]. Elevated levels of CRP predict new plaque formation in the elderly population in which carotid arteries were without atherosclerotic lesions [180].

In this regard, inflammation may be the link between CIMT, atherosclerosis and vascular calcification. CIMT is an established marker of generalised [181] and subclinical atherosclerosis [155]. It is associated with conventional cardiovascular diseases and cerebrovascular outcomes [182]. Increased carotid wall thickness is linked to the presence and severity of subclinical coronary atherosclerosis as measured by coronary artery calcium (CAC) [156]. In CKD patients, CRP has been associated with serum calcium and CIMT [183].

CIMT was also associated with factors linked to the *metabolic syndrome* development and *vascular calcification* such as $25(OH)D_3$ and PTH [50]. An inverse relationship has been identified between serum $25(OH)D_3$ levels and internal CIMT, but not with the common CIMT [8]. CIMT is also positively related to serum PTH and negatively with $25(OH)D_3$ [5, 184].

Ethnic differences in intima-media thickness have been reported from multi-ethnic studies and it was observed that CIMT is higher in African-Americans as compared to whites [185]. Coronary calcium and CIMT are strongly associated in other population groups, but weaker in black women with increased common CIMT [185]. African-Caribbeans had a high CIMT, which can be explained by genetic polymorphisms, an important aspect of ethnic differences in CIMT [186].

2.4.2.2. Arterial stiffness

Arterial stiffening is the hallmark of arteriosclerosis as well as a predictor of cardiovascular events [187] and all-cause mortality [188]. It can be described as deterioration in the ability of the arteries to dilate and contract with variations in pressure during the cardiac cycle [34]. It becomes prominent with the ageing process even in the absence of vascular diseases [189]. The prevalence of arterial stiffening as result of ageing is common to populations less diagnosed with atherosclerosis [37] such as Africans [190] which substantiate the fact that medial degeneration is central to arterial stiffening [191]. The different proportions of elastin to collagen ratio [192] and VSMCs are responsible for the varying responses of different arterial segments to ageing [189]. As a result, central elastic arteries are more likely to stiffen with age [193] as compared to distal muscular arteries [39].

The relationship between arterial stiffness and blood pressure can be explained in two ways. Firstly, arterial stiffness can increase due to high pressures in the absence of any structural modifications, which is attributable to the engagement of collagen fibres with a higher elastic modulus [9]. On the other hand, chronic high blood pressure can stimulate changes in the structural properties of the arterial wall resulting in increased stiffness [37].

Arterial stiffness can be measured by non-invasive, reproducible and affordable methods which are ideal for large scale studies [188]. Pulse wave velocity (PWV) and augmentation index (AI) are two common methods used [18]. Carotid-femoral PWV is the golden standard for evaluating

central arterial stiffness [194], and is an independent predictor of cardiovascular diseases (CVD) in middle-aged and elderly people [195, 196] and also possesses a significant prognostic value in the general population [197, 198]. According to Cecelja *et al*. arterial stiffness as measured by carotid-femoral PWV relates to arterial calcification, but not to noncalcified atheroma [148].

PP is an established marker of cardiovascular risk in the general population, independent of SBP and DBP [199]. Increased PP is the link between PWV and cardiovascular risks and morbidity [200, 201]. Furthermore, PP is also regarded as an indirect indication of aortic stiffness [139]. In elderly populations [202], an elevated PP is due to isolated systolic hypertension as a result of increased afterload and an unchanged or decreased DBP due to stiffness of the large arteries [146]. Some studies showed PP to be a more powerful predictor of cardiovascular morbidity and mortality than DBP and SBP in the elderly population since its measurement incorporates both the predictive role of elevated SBP and the negative predictive role of decreased DBP [199, 203].

An animal model of atherosclerosis showed no relationship between central arterial stiffness (carotid-femoral PWV) and CIMT [204]. This may indicate that arterial stiffening is not affected by early atherosclerosis [204]. Moreover, no independent association was found between carotid-femoral PWV and CIMT or with noncalcified atheroma in women [148], which suggests that atherosclerotic changes in the arterial wall may not be linked to arterial stiffening [148]. This has been substantiated by atherosclerotic animal models [204], and previous human observations [155]. PWV is thus widely used for assessment of arteriosclerosis, and relates to cardiovascular outcomes [7, 120, 144].

2.5. Contributing factors to vascular calcification

2.5.1. Ethnicity

Vascular calcification has been investigated by making use of *in vitro* models in order to explore the mechanisms involved in CKD patients [205, 206], while information from human studies is mainly focused on populations from European descent and African-Americans [41, 207, 208]. There are differences between ethnic groups regarding the relationships between factors associated with vascular calcification and measures of cardiovascular diseases [7, 209]. It has been indicated that black African men are more susceptible to early development of vascular calcification and premature cardiac overload as compared to their white counterparts [209].

Low 25(OH)D_3 is related to aortic PWV and it may well contribute the differences in PWV observed in different ethnic groups [210]. It is also linked to ethnic differences in BP and the risk of developing hypertension [114]. The factors that determine ethnic variations in vitamin D status include skin pigmentation, limited exposure to sunlight because of clothing habits, less outdoor activities and/or various diets [211]. In the South African context, the main determinants for vitamin D deficiency may include poor dietary intake of vitamin D [212], genetic predisposition [213], increased prevalence of obesity [214] and skin pigmentation [113]. The darker the skin, the more ultraviolet radiation is required to produce a given quantity of vitamin D [215, 216].

South Africa is a developing country with a high prevalence of hypertension [217] and CVD which are enhanced by lifestyle changes associated with urbanisation [190]. It was previously shown in the Heart of Soweto study that urbanisation may be one of the contributors to this health problem [218]. It was indicated that the adverse profile of bone health markers may be a result of urbanisation in black South African women [212]. In the PURE-study, a low calcium and phosphorus dietary intake was common [212]. CTX and 25(OH)D_3 were higher in rural as

compared to urban women and an age-related increase in PTH was observed in both urban and rural women [212]. Urbanisation is one of the possible causes for the increased blood pressures, vascular diseases and other cardiovascular morbidities to increase [219], and be the highest as compared to other ethnic groups [217].

2.5.2. Renal function and its role in vascular calcification

CKD is related to bone and serum mineral dysregulation as indicated by excessive phosphate and PTH [116], which can lead to bone diseases and vascular calcification [96]. In CKD patients, vascular calcification is characterised by conversion of VSMCs into osteoblastic-like cells, apoptosis of VSMCs and formation of matrix vesicles [20]. The converted VSMCs are then modified to initiate adhesion of local factors to the formed matrix, resulting in mineral deposition [92]. However, in the PURE-study, the participants form part of the general population and there is a possibility of only mild renal impairment which can be assessed by urinary albumin excretion [220] and creatinine clearance [221]. Vascular calcification was found to be prevalent in type 2 diabetes patients with normal kidney function [222]. In addition, microalbuminuria is also associated with medial calcification [223].

It is well-known that cardiovascular risk is increased in patients with renal impairment when compared to individuals with normal renal function [224]. Factors that contribute to the progression of vascular disease, including endothelial dysfunction, compromised antioxidant activity and chronic inflammation, have been independently associated with elevated serum creatinine [225, 226]. Additionally, impaired or decreased renal function can contribute to progression of CIMT [227].

Microalbuminuria together with proteinuria has been associated with increased cardiovascular morbidity and mortality [228]. Furthermore, albuminuria is a characteristic of inflammation, a

process crucial in early development of atherosclerosis [229]. Albuminuria was associated with CIMT and coronary artery calcium in type 2 diabetes patients [230].

2.5.3. Osteoporosis and parity

Low bone mineral density (BMD) is a clinical manifestation of osteoporosis and is associated with bone fracture [231]. Osteoporosis and cardiovascular diseases often co-occur in elderly postmenopausal women [28]. Studies have indicated that vascular calcification is prevalent in osteoporotic females [232-234]. There is a positive relationship between cardiovascular risk factors, including arterial stiffness [235], CIMT [236] and osteoporosis as assessed by BMD, which partially explains the epidemiological relationship between osteoporosis with cardiovascular outcomes [237, 238].

Vascular calcification has been reported in older men and women who are at risk of bone fracture and it associates negatively with bone mineral content [115]. Increased PTH secretion due to habitually low calcium intake accelerates bone loss, and as a result calcium deposition in the arterial wall may occur [25, 42]. Additionally, the association between osteoporosis and arterial calcification indicates the significance of the role of calciotropic hormones in the mechanisms leading to mineralisation in the arterial wall [2]. Previously, osteoporosis was not perceived as a problem in African women; however it was shown that increased life expectancy as well as other lifestyle factors associated with urbanisation such as low calcium and vitamin D intake which result in low bone mass increases the risk of osteoporosis in this population [212].

Calcium, PTH and $25(OH)D_3$ have been linked to an increase in markers of bone turnover during pregnancy and breastfeeding [239]. The total number of births and breastfeeding for a period exceeding one year are regarded as risk factors for osteoporosis [240, 241]. In addition, it was found that there is a constant reduction in BMD on the femoral neck resulting from

successive pregnancies, but the probability of this process being an independent risk factor for osteoporosis in multiparous women is low [240].

2.5.4. Age & gender

Vascular damage becomes prominent with ageing [242]. The process includes accumulation of calcium in the arterial wall [242] resulting in stiffening of the large conduit arteries [18]. This causes a high PP to be transferred to other arteries such as the carotid artery, which accelerates arterial remodelling in order to compensate for wall stress, leading to intima-media thickening [18, 243].

Gender is also a determinant of CIMT. It was shown that CIMT is independently related to gender, and is higher in males than in females [220]. Arterial stiffening is common in women during menopause, as indicated by an elevated PWV and augmentation index [18]. A high augmentation index in women is due to a shorter height and therefore their reflection sites are closer to the heart [244].

There is an increasing prevalence of cardiovascular morbidity and mortality in postmenopausal women [245]. Before menopause, women have a lower cardiovascular risk compared to males at similar ages [246]. Therefore their increase in risk is attributable to the declined levels of oestrogen which has a protective effect on the vasculature [247]. Additionally, oestrogen is responsible for maintenance of bone health; its decrease is also associated with increased bone resorption and altered calcium regulation [248].

There are gender and age specific differences in $25(OH)D_3$ and PTH levels [249]. The differences are attributable to biological variations as well as to different behavioural patterns [211]. Decreased $25(OH)D_3$ levels in the elderly result from the skin's low capacity to produce

vitamin D after exposure to sunlight [249]. Furthermore, Kruger *et al*. found that serum PTH increases with age, while 25(OH)D_3 decreases with age in older South African women [212].

2.5.5. Body composition

The contribution of obesity on cardiovascular morbidity and mortality is well documented [250, 251]. Obesity status has been linked to levels of sex hormones and hormones that regulate bone turnover [252]. El Khoudary *et al*. found that the presence of arterial calcification and its association with sex steroid hormones differed between obese and non-obese women [253]. Increased adiposity is also associated with elevated levels of PTH in older healthy adults and kidney failure patients [254, 255]. It has also been suggested that fat mass may be the determinant of the association between increased body weight and primary hyperparathyroidism in postmenopausal women [256].

The role of body weight regarding vascular calcification is not clear. Visceral fat and BMI have been associated with coronary calcium, and visceral fat was found to be most relevant in women regarding the development and progression of atherosclerosis [257, 258]. Abdominal obesity as measured by waist-to-hip ratio has been identified as a determinant of coronary artery calcium in young adults [259]. In addition, there is sufficient evidence linking increased adiposity with low grade and chronic inflammation [260]. These associations may be attributable to the active mediators produced by adipocytes including leptin and resistin, which possesses proinflammatory effects [261]. Therefore, besides calcitropic hormones, inflammation which plays a role in the development of vascular calcification [25] may be one of the mediators in the relationships between obesity and arterial calcification. The relationship between lean body mass and vascular calcification needs further investigation. Currently, the possible explanation may be the calcium paradox [42].

The calcium paradox can be defined as an association of ectopic mineral deposition in the vascular wall with decreased bone mineral density; meaning that during bone resorption the minerals are mobilised from bone into the vascular wall [115]. According to Kovacic *et al*. there is an independent relationship between low body weight and decreased bone mineral density; furthermore low bone mineral density is independently linked to vascular calcification [262]. Therefore, low body weight is linked to vascular calcification. This was confirmed by a negative association between BMI and calcified atherosclerotic lesions in older men and women [262]. It is clear that body weight that includes adipose and muscle tissues as well as distribution of adipose tissue contribute differently to ectopic arterial calcification [257, 262].

2.5.6. Alcohol consumption and smoking

Alcohol consumption is associated with atherosclerotic calcification and CIMT [263, 264]. A dose-response relationship has been observed between the presence and progression of aortic arch calcification and alcohol consumption and the risk increased by 50% in drinkers as compared to non-drinkers [265]. Also, high circulating levels of gamma-glutamyl transferase, a marker of liver function and alcohol consumption, have been independently associated with the presence of coronary artery calcium [265, 266]. In contrast, Ellison *et al*. found no association was reported between calcified atherosclerotic plaque and alcohol consumption [267]. Smoking also increased the risk of aortic arch calcification [268].

2.6. Summary

Vascular calcification is one of the potential risk factors for the development of CVD and it can be investigated by determination of the association between the factors involved in calcification and markers of cardiovascular structure and function. Most of the studies on the impact of vascular calcification on cardiovascular function have been done in CKD patients, populations

from European descent and African-Americans. In previous studies, factors involved in calcification and calcium homeostasis such as ALP, PTH, 25(OH)D_3, magnesium, phosphate, and calcium were associated with measures of arteriosclerosis and atherosclerosis. The relationships seems more pronounced in obese individuals and postmenopausal women and can be influenced by ethnicity as well as lifestyle factors including smoking, alcohol consumption and physical activity. The black South African population has a high risk of developing CVD, and the role of vascular calcification has not been extensively studied.

2.7. Aim, objectives and hypotheses

2.7.1 Aim

The central aim of this study is to investigate the associations of measures of arterial function and structure, namely brachial and central pressures and CIMT, with calciotropic hormones and CTX (a marker of bone resorption) in African women older than 46 years.

2.7.2. Objectives

To determine if CTX, PTH, 25(OH)D_3 and PTH:25(OH)D_3 are associated with:

1. brachial SBP, DBP and PP;
2. central SBP and PP;
3. CIMT and cross-sectional wall area (CSWA).

2.7.3. Hypotheses

Based on the existing literature, the hypotheses are:

All cardiovascular assessments, including

1. Blood pressure (central and brachial);
2. Measures of stiffness (brachial and central PP); and
3. CIMT and CSWA

are positively associated with PTH, PTH:25(OH)D_3, CTX and negatively associated with 25(OH)D_3.

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Chapter 3

Research article

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: The PURE-study

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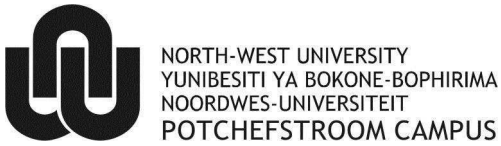

Large artery stiffness and carotid intima-media thickness in relation to markers of calcium and bone mineral metabolism in African women older than 46 years: The PURE-study

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Abstract

Objectives: Increased vascular calcification, cardiovascular morbidity and mortality have been associated with altered bone metabolism, and associated calciotropic hormones. Due to the lack of information on the contribution of altered bone metabolism and calciotropic hormones on cardiovascular disease in Africans, this study aimed to explore the relationships of brachial and central arterial pressures and carotid intima-media thickness (CIMT) with parathyroid hormone to 25-hydroxycholecalciferol ratio (PTH:25(OH)D_3) and C-telopeptide of type I collagen (CTX) in lean and overweight/obese African women older than 46 years.

Methods: The study included 434 African women older than 46 years who were divided into lean and overweight/obese groups. We assessed brachial blood pressure, central pulse pressure (cPP) and CIMT, and determined PTH, 25(OH)D_3 and CTX concentrations.

Results: In the overweight/obese group, we found elevated PTH and PTH:25(OH)D_3 compared to the lean group (both $p<0.001$), while the lean group had higher CTX ($p<0.001$). Single, partial and multiple regression analyses indicated that only in lean women, CIMT was independently associated with PTH:25(OH)D_3 ($R^2=0.22$; $\beta=0.26$; $p=0.003$); whereas in obese women cPP was associated with both PTH:25(OH)D_3 ($R^2=0.20$; $\beta=0.17$; $p=0.017$) and CTX ($R^2=0.20$; $\beta=0.17$; $p=0.025$).

Conclusion: In African women older than 46 years displaying increased adiposity, cPP, as indicator of central arterial stiffness, was positively associated with alterations in bone metabolism and calciotropic hormones, whereas CIMT of lean women was positively associated with PTH:25(OH)D_3. Our results suggest that alterations in bone metabolism and calciotropic hormones may contribute to arterial calcification in African women.

Key words: PTH:25(OH)D_3 , CTX, CIMT, pulse pressure, body weight

Introduction

Cardiovascular morbidity and mortality are associated with altered bone metabolism, particularly in older populations; as well as with calciotropic hormones which has also been observed in young adults [1, 2]. Low bone mineral density (BMD), and the ratio of calciotropic hormones, parathyroid hormone:25-hydroxycholecalciferol (PTH:25(OH)D_3) are implicated in the development of vascular calcification, an emerging cardiovascular risk factor [3-5]. Postmenopausal women are particularly vulnerable due to increased PTH secretion, accelerating bone resorption which is marked by increased c-telopeptide of type I collagen (CTX), and eventually result in osteoporosis [3, 6, 7]. Apart from high bone resorption, increased adiposity is also common among postmenopausal women, resulting in an elevated risk of developing cardiovascular diseases, due to the lack of protective oestrogens [8, 9]. Although increased adiposity is associated with coronary calcification and elevated PTH in renal failure patients [10, 11], low body mass is associated with low bone mineral density, which is linked to arterial calcification [12].

Arterial calcification is an actively regulated process that forms part of aging in the healthy population [13], and is associated with hypertension, arteriosclerosis and atherosclerosis [14, 15]. Medial calcification can result in arterial stiffness, elevated pulse pressure, increased afterload on the left ventricle and eventually hypertrophy [16, 17], while intimal calcification is associated with atherosclerosis and is regarded as a predictor of cardiovascular outcomes [18].

Morbidity and mortality from cardiovascular disease continue to rise in black South Africans [19] ─ a population that is already predisposed to arterial stiffening and carotid intima-media thickness (CIMT) [20, 21]. However, information regarding the relationships of altered bone metabolism and calciotropic hormones with cardiovascular health is limited in this population group. This study therefore aims to investigate the relationships of CIMT, brachial and

central arterial pressures with PTH:25(OH)D_3 and CTX in lean and overweight/obese African women older than 46 years.

Methods

Study design and population

This study forms part of the multi-national Prospective Urban and Rural Epidemiology (PURE) study. The PURE study was initiated to keep track of the development of chronic diseases of lifestyle in low-, middle-, and high-income countries in both urban and rural dwelling participants [22]. The baseline data collection of the South African PURE study in the North West Province was performed in 2005 and the first follow-up collection in 2010. The study population originally consisted of 2010 African volunteers older than 30 years of age from a sample of 6000 randomly selected households in both rural and urban areas. In the present study we will make use of the data collected in 2010 for women older than 46 years of age. Participants who were infected with human immunodeficiency virus (HIV) were excluded from all analyses. A total of 434 women, consisting of 392 postmenopausal and 42 premenopausal women were included for this sub-study.

Participants were given full information regarding the objectives and procedures of the study prior to participation. The information was conveyed in the participant's home language by trained African field workers fluent in English and Tswana. All participants signed an informed consent form. The study complied with all applicable requirements of the international regulations, in particular, the Helsinki declaration of 1975 (as revised in 2008) for investigation of human participants. The Ethics Committee of North-West University (Potchefstroom campus) approved this study.

Questionnaires

African field workers conducted the interviews by making use of structured demographic, socio-economic, lifestyle and physical activity questionnaires that have been developed and standardised for the international PURE study [22].

Anthropometric measurements

Weight, height and waist circumference of the participants were measured using calibrated instruments by accredited anthropometrists according to standardised methods. (Precision Health Scale, A & D Company, Japan; Leicester Height Measure, Seca, Birmingham, UK). Body mass index (BMI) was calculated as weight divided by height squared ($kg/m^{2)}$.

Cardiovascular measurements

After a 10 minute rest period, brachial blood pressure measurements were performed in duplicate (5 minutes apart), on the right upper arm, while the participants were seated upright with the right arm supported at heart level. Systolic blood pressure (SBP), diastolic blood pressure (DBP), pulse pressure (PP) and heart rate (HR) were measured with the validated OMRON HEM-757 (Omron Healthcare, Kyoto, Japan). Appropriate sized cuffs were used for obese participants. Estimated central systolic blood pressure (cSBP) and central pulse pressure (cPP) were measured using the Omron 9000AI (Omron HealthCare, Kyoto, Japan).

CIMT was obtained using a SonoSite Micromaxx ultrasound system (SonoSite Inc., Bothell, WA, USA) and a 6-13 MHz linear array transducer. Images from at least two optimal angles of the left and right common carotid arteries were obtained. A single reader conducted measurements using a semi-automated program, namely the Artery Measurement Systems (AMS) II v1.139 (Chalmers University of Technology, Gothenburg, Sweden). The cross-sectional wall area (CSWA) was calculated to confirm structural and not functional changes in luminal diameter: $CSWA = \pi(d/2 + CIMT)^2 - \pi(d/2)^2$, where d denotes luminal diameter.

Biochemical analysis

Participants were requested to fast overnight by not eating or drinking anything for approximately 8-10 hours prior to sample collection in the mornings. A registered nurse obtained a blood sample by means of a sterile winged infusion set from the antebrachial vein. Samples were prepared according to appropriate methods and stored at -80°C in the

laboratory. In the rural areas, samples were rapidly frozen and stored at -18°C (no longer than five days) until it could be transported to the laboratory facility and was then stored at -80°C until analysis.

Fasting serum samples were utilised to determine standard cholesterol and glucose profiles. A sequential multiple analyser (Cobas Integra 400 plus Roche, Basel, Switzerland) was used to analyse total and high density lipoprotein (HDL) cholesterol, fasting glucose, creatinine, high sensitivity C-reactive protein (CRP), gamma glutamyl-transferase (GGT), alkaline phosphatase (ALP), calcium, phosphorus and magnesium. Percentage glycosylated haemoglobin (HbA1c) was determined by using ion-exchange high-performance liquid chromatography (D-10 Haemoglobin testing system from Bio-Rad laboratories, Hercules, CA). Follicle stimulating hormone (FSH), PTH, 25(OH)D_3, and CTX were measured using the Roche Elecsys 2010 COBAS system (Roche Diagnostics, Indianapolis, USA).

The South African National Department of Health protocol was followed to perform the HIV testing. The participants signed a written consent during the pre-counselling session just before the test. The HIV status of the participant was determined by the use of the First Response (PMC Medical, India) rapid test card and if the first test was positive, confirmation was done with the Pareeshak card test (BHAT Bio-tech, India). The results were provided to the participants by two trained counsellors during individual sessions before departure from the data collection site. Participants who tested positive for HIV were referred to the local clinic or hospital for CD4 cell counts.

Bone mineral density (BMD) measurements

Bone mineral density measurements were performed at the distal site of the non-dominant arm, using DTX 200 peripheral DXA system (Osteometer MediTech, Hawthorn, Carlifonia, USA). All BMD measurements were performed by one qualified radiographer.

Statistical analyses

Statistical analyses were performed using Statistica Version 11 (Stasoft Inc., Tulsa, OK). Due to the reported effects of obesity on CVD and bone mineral density [8, 23], we tested for the interaction with BMI on the associations of CIMT and cPP with PTH:25(OH)D_3. Interactions existed for BMI regarding the relationships of CIMT and cPP with PTH: 25(OH)D_3 (p=0.046 and p=0.027, respectively).Therefore, the study population was divided into lean and overweight/obese groups. Biochemical and bone mineral metabolism variables that displayed a non-Gaussian distribution were logarithmically transformed and represented as the geometric mean and 5^{th} and 95^{th} percentile intervals. Independent T-tests and Chi-square tests were performed to compare means and proportions, respectively. Single, partial and multiple regression analyses were performed to investigate associations between markers of vascular structure and function as dependent variables, and markers of bone mineral metabolism and calciotropic hormones as independent variables.

Results

Characteristics of the study population

The basic characteristics of African women with BMI <25 kg/m^2 (lean group) and African women with BMI ≥25 kg/m^2 (overweight/obese group) are presented in Table 1. In this study, 90% of the women displayed FSH concentration exceeding the cut-off value of 35 mIU/mL [24] indicating a postmenopausal state. The lean group smoked more and consumed more alcohol compared to the overweight/obese group (both p<0.001), but there was a higher use of antihypertensive medication in the overweight/obese group (p=0.001). There was no difference in the self-reported physical activity index between the lean and overweight/obese groups (p=0.96).

Table 1: Comparison of lean and overweight/obese African women

	BMI <25kg/m^2	BMI ≥25kg/m^2	*P*-value
N	173	261	
Age (years)	59.5 ± 7.09	61.6 ± 8.59	0.007
Postmenopausal n (%)	159(92.0)	233(89)	0.36
Anthropometric measurements			
Weight (kg)	50.9 ± 7.74	77.3 ± 13.8	<0.001
Body mass index (kg/m^2)	20.9 ± 2.78	31.8 ± 5.36	<0.001
Waist circumference (cm)	71.6 ± 7.96	90.9 ± 9.87	<0.001
Cardiovascular measurements			
Systolic blood pressure (mmHg)	135 ± 21.6	138 ± 23.7	0.23
Diastolic blood pressure (mmHg)	86.7 ± 12.8	89.8 ± 12.9	0.017
Brachial pulse pressure (mmHg)	48.5 ± 13.9	48.2 ± 16.3	0.83
Central SBP (mmHg)	149 ± 23.4	151 ± 23.0	0.41
Central pulse pressure (mmHg)	67.5 ± 16.3	66.1 ± 16.3	0.42
CIMTf (mm)	0.73 ± 0.16	0.79 ± 0.16	<0.001
CSWA (mm^2)	15.1 ± 4.19	16.5 ± 4.52	0.001
Biochemical measurements			
Glucose (mmol/L)	4.96 (4.03; 6.30)	5.64 (4.30; 11.6)	<0.001
Glycosylated haemoglobin (%)	5.90 (5.20; 6.90)	6.56 (5.50; 10.9)	<0.001
GGT (units)	43.6 (10.8; 359)	34.9 (12.3; 161)	0.012
TC:HDL	3.33 (1.88; 31.7)	4.67 (2.40; 7.88)	<0.001
C-reactive protein (mg/L)	2.62 (0.35; 6.29)	6.21 (0.93; 37.3)	<0.001
Serum creatinine (µmol/L)	57.3 (40.0; 81.0)	63.6 (43.0; 94.0)	0.011
FSH (mIU/ml)	81.6 ± 34.8	65.7 ± 29.8	<0.001
Estimated creatinine clearance (mL/min)	6.9 (3.46; 17.4)	14.1 (0.66; 1.86)	<0.001
Calcium metabolism variables			
Corrected calcium (mmol/L)	2.39 (2.09; 2.80)	2.39 (2.16; 2.80)	0.94
Corrected magnesium (mmol/L)	0.84 (0.69; 1.04)	0.81 (0.70; 1.04)	0.35
Inorganic phosphate (mmol/L)	1.19 ± 0.19	1.14 ± 0.19	0.011
Alkaline phosphatase (U/L)	92.1 (57.1; 175)	91.1 (56.2; 153)	0.73
25(OH)D_3 (ng/mL)	43.8 (23.9; 64.6)	36.6 (20.8; 57.7)	<0.001
Parathyroid hormone (ng/mL)	35.2 (17.6; 79.3)	43.3 (20.6; 86.2)	<0.001
PTH:25(OH)D_3	0.81 (0.35;2.17)	1.19 (0.46; 3.06)	<0.001
CTX (ng/mL)	0.53 (0.22;1.10)	0.44 (0.18; 0.94)	<0.001
Bone mineral density (g/cm^2)	0.37 ± 0.08	0.44 ± 0.10	<0.001
Lifestyle factors			
Smoking n (%)	104 (63.0)	104 (40.9)	<0.001
Alcohol use n (%)	60 (37.0)	45 (18.2)	<0.001
Antihypertensive medication n (%)	44 (25.4)	106 (40.6)	0.001
Physical activity index	6.33 ± 2.10	6.32 ± 1.75	0.96

Values are arithmetic mean ± standard deviation; geometric mean (5th and 95th percentile interval) for logarithmically transformed variables; N, number of participants; BMI, body mass index; CIMTf, carotid intima-media thickness (far wall); CSWA, cross sectional wall area; TC, total cholesterol; HDL, High density lipoprotein; FSH, follicle stimulating hormone; 25(OH)D_3, 25-hydroxycholecalciferol; PTH:25(OH)D_3, parathyroid hormone to 25-hydoxycholecalciferol ratio; CTX, c-telopeptide of type I collagen crosslinks.

When comparing cardiovascular measurements, there was no difference in brachial and central blood pressures or pulse pressures ($p \geq 0.23$), except for DBP which was higher in the overweight/obese group ($p=0.017$). The overweight/obese women displayed higher CIMT ($p<0.001$) and CSWA ($p=0.001$), as well as an unfavourable metabolic profile in comparison to the lean group. Regarding bone mineral metabolism and calciotropic hormones, the lean group had higher phosphate levels ($p=0.011$), CTX, $25(OH)D_3$ (all $p<0.001$); while the overweight/obese group had higher levels of PTH, PTH:$25(OH)D_3$ ratio and a higher BMD, (all $p<0.001$). In addition, 47% of the lean women had a BMD below the cut-off point of 0.371 g/cm^2. Serum calcium, magnesium and ALP concentrations were similar between the groups ($p \geq 0.35$). Within the total group, 17% of the women had vitamin D deficiency namely, $25(OH)D_3$ below 30 ng/mL.

Unadjusted regression analyses

Single regression analyses are shown in Supplementary Table 1 and Figure 1. We found that in the lean group CIMT and CSWA were positively associated with PTH (both $p \leq 0.027$) and PTH:$25(OH)D_3$ ($p \leq 0.013$). In the overweight/obese group brachial and central SBP were positively associated with PTH, PTH:$25(OH)D_3$ and CTX (all $p \leq 0.017$); brachial and central PP were positively associated with PTH, PTH: $25(OH)D_3$ and CTX ($p \leq 0.008$), whereas cPP was also negatively correlated with $25(OH)D_3$ ($p=0.035$).

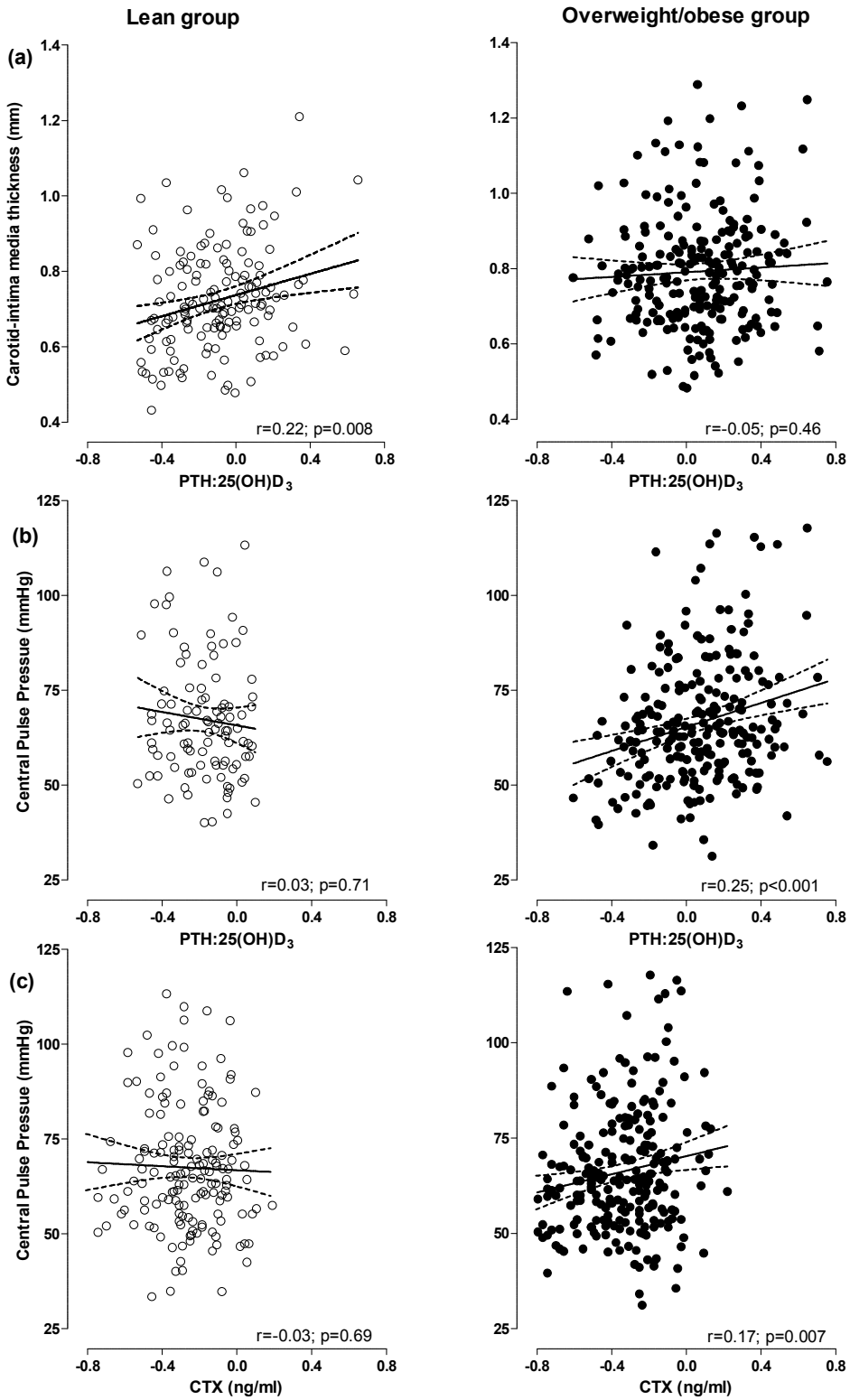

Figure 1 Relationships of markers of vascular structure and function with calciotropic hormones and CTX (a) CIMT as a function of PTH:25(OH)D_3 (b) cPP as a function of PTH:25(OH)D_3 (c) cPP as a function of CTX. Solid and dashed lines represent the regression line and 95% CI boundries respectively.

Adjusted regression analyses

After adjusting for age and BMI (Table 2), the positive correlations between CIMT and CSWA and PTH:25(OH)D_3 (both p≤0.041) remained in the lean group, while in the overweight/obese group, most of the positive associations of brachial pressures with PTH, PTH:25(OH)D_3 and CTX persisted (p≤0.041). cPP also remained positively associated with PTH:25(OH)D_3 (p=0.009) and CTX (p=0.019).

Table 2: Partial correlations of markers of vascular structure and function with calciotropic hormones and CTX in lean and overweight/obese African women

Variables	BMI <25 kg/m^2							
	PTH (ng/mL)		25(OH)D_3 (ng/mL)		PTH:25(OH)D_3		CTX (ng/mL)	
	r	*p*	r	*p*	r	*p*	r	*p*
Brachial SBP (mmHg)	-0.08	0.35	0.14	0.081	-0.12	0.16	-0.07	0.38
Brachial DBP (mmHg)	-0.05	0.52	0.14	0.10	-0.10	0.22	-0.08	0.35
Brachial PP (mmHg)	-0.07	0.38	0.10	0.22	-0.09	0.28	-0.04	0.60
Central SBP (mmHg)	-0.01	0.86	0.12	0.16	-0.06	0.51	-0.06	0.51
Central PP (mmHg)	0.02	0.78	0.05	0.51	-0.01	0.99	-0.03	0.72
CIMTf (mm)	**0.17**	**0.037**	-0.08	0.34	**0.18**	**0.027**	0.04	0.61
CSWA (mm)	0.14	0.093	-0.09	0.30	**0.17**	**0.041**	-0.005	0.95
Variables	**BMI ≥25 kg/m^2**							
	PTH (ng/mL)		**25(OH)D_3 (ng/mL)**		**PTH:25(OH)D_3**		**CTX (ng/mL)**	
	r	*p*	r	*p*	r	*p*	r	*p*
Brachial SBP (mmHg)	**0.18**	**0.006**	0.01	0.86	**0.14**	**0.041**	**0.16**	**0.018**
Brachial DBP (mmHg)	**0.16**	**0.018**	0.07	0.28	0.08	0.22	0.12	0.082
Brachial PP (mmHg)	**0.15**	**0.027**	-0.04	0.51	**0.14**	**0.036**	**0.14**	**0.035**
Central SBP (mmHg)	0.09	0.14	0.004	0.95	0.07	0.30	**0.17**	**0.011**
Central PP (mmHg)	**0.18**	**0.009**	-0.08	0.24	**0.18**	**0.009**	**0.16**	**0.019**
CIMTf (mm)	-0.01	0.90	0.03	0.64	-0.01	0.90	0.01	0.86
CSWA (mm)	-0.02	0.77	0.03	0.68	-0.01	0.85	-0.01	0.91

*Adjusted for age and body mass index; CIMTf, carotid intima-media thickness (far wall); CSWA, cross sectional wall area; PTH, parathyroid hormone; 25(OH)D_3, 25-hydroxycholecalciferol; PTH:25(OH)D_3, parathyroid hormone to 25-hydroxycholecalciferol ratio; CTX, c-telopeptide of type I collagen crosslinks. All bone mineral metabolism variables and calciotropic hormones were logarithmically transformed.

The independent associations of brachial and central pressures as well as CIMT with calciotropic hormones and CTX are presented in Table 3. The relationships of SBP with PTH (p=0.013) and CTX (p=0.038), DBP with PTH (p=0.030), PP and central SBP with CTX (p=0.016 and p=0.024, respectively) were confirmed in the overweight/obese group. In the lean group, the relationship between CIMT and PTH:25(OH)D_3 was also confirmed (β=0.26; p=0.003). In the overweight/obese group, the relationship between cPP and both PTH:25(OH)D_3 (p=0.016) and CTX (p=0.025) was also confirmed.

Table 3: Forward stepwise multiple regression analysis with markers of vascular structure and function as dependent variables

	BMI <25 kg/m^2					
	PTH (ng/mL) (log)		**PTH:25(OH)D_3 (log)**		**CTX (ng/mL) (log)**	
	$^1R^2$	**β (95% C.I)**	**R^2**	**β (95% C.I)**	**R^2**	**β (95% C.I)**
Brachial SBP (mmHg)		–		NS		–
Brachial DBP (mmHg)		–		–		–
Brachial PP (mmHg)		–		NS		–
Central SBP (mmHg)		–		–		–
Central PP (mmHg)		–		–		–
CIMTf (mm)	0.21	0.23 (0.07;0.39) †	0.22	0.26 (0.09;0.42) †		NS
	BMI ≥25 kg/m^2					
	PTH (ng/mL) (log)		**PTH:25(OH)D_3 (log)**		**CTX (ng/mL) (log)**	
	$^1R^2$	**β (95% C.I)**	**R^2**	**β (95% C.I)**	**R^2**	**β (95% C.I)**
Brachial SBP (mmHg)	0.11	0.19 (0.04;0.33) †		NS	0.12	0.16 (0.01;0.31)*
Brachial DBP (mmHg)	0.06	0.17 (0.02;0.31)*		NS		NS
Brachial PP (mmHg)		NS		NS	0.22	0.18 (0.02;0.32) *
Central SBP (mmHg)		NS		NS	0.13	0.17 (0.02;0.32) *
Central PP (mmHg)	0.20	0.17 (0.03;0.31) *	0.20	0.17 (0.03;0.31) *	0.20	0.17 (0.02;0.31) *
CIMTf (mm)		–		–		–

[1], Adjusted R^2; –, did not enter the model; NS, non-significant; *P≤0.05; †, P≤0.01; ‡, P≤0.001. Independent variables included in the model: age, body mass index, physical activity index, smoking, gamma glutamyl transferase, creatinine clearance, total cholesterol, calcium, follicle stimulating hormone, antihypertensive medication, glycosylated haemoglobin, C-reactive protein, and central systolic blood pressure was additionally adjusted for with CIMT as a dependent variable. All independent variables were adjusted for at the same time.

Sensitivity analysis

To investigate whether PTH:25(OH)D_3 and CTX were independent of each other in the overweight/obese group, we included both variables in the same multiple regression model. By doing so, cPP was associated with both PTH:25(OH)D_3 (R^2=0.19; β=0.14; p=0.047) and CTX (R^2=0.19; β=0.14; p=0.049).

To confirm the validity of our associations, correlations were performed between calciotropic hormones, serum minerals and CTX (Supplementary Table 2). In the lean group, PTH:25(OH)D_3 was positively associated with CTX (r=0.34; p<0.001), while the association was weak in the overweight/obese group (r=0.11; p=0.076). A positive association was apparent between PTH:25(OH)D_3 and BMD (r=0.14; p=0.040) in the overweight/obese group.

There were no associations between CIMT and calciotropic hormones as well as between cPP and calciotropic hormones in the total study population after adjustment for age and in multiple regression analyses (results not shown). Since interaction of BMI existed, the group was divided into lean and overweight/obese women and we obtained significant results.

Discussion

We investigated associations of CIMT, brachial and central arterial pressures with PTH:25(OH)D_3 and CTX in older African women. We found different results in lean versus overweight/obese women, namely that carotid wall thickness was independently associated with PTH:25(OH)D_3 in lean women, whereas in overweight/obese women large artery stiffness (cPP) was independently associated with PTH:25(OH)D_3 and CTX, a marker of bone resorption. The associations remained significant even after multiple adjustments for factors that could affect these relationships including age [13], BMI [25], GGT [26], renal function [4] and others.

The first key finding of the positive associations of CIMT with PTH and PTH:25(OH)D_3 in lean women, is consistent with previous findings [27, 28]. CIMT as a marker of subclinical atherosclerosis [29] has been associated with PTH:25(OH)D_3 in the general population [28]. Additionally, serum PTH was found to be an independent determinant of CIMT in postmenopausal women [27]. The prosclerotic effect of PTH on vascular smooth muscle cells (VSMCs) can promote vessel thickening, and eventually elevated blood pressure [30]. In the present study, 47% of the lean women had a BMD below the cut-off point of 0.371 g/cm^2 for central osteoporosis [31] and increased CTX concentration, which indicates accelerated bone resorption [32]. In humans and animal models low bone mass has been linked to low body weight, vascular calcification and atherosclerosis [3, 12, 33]. This occurrence is not limited to osteoporotic individuals only, as it was also observed in populations with minor bone loss, including perimenopausal women and middle-aged healthy men and women [3, 34]. Therefore our results suggest that this population group may be at risk of arterial calcification and cardiovascular diseases.

Our second main finding was evident in women within the overweight and obese ranges, namely a positive association of central arterial stiffness (cPP) with PTH:25(OH)D_3 and CTX. cPP is an established marker of arterial stiffness and a predictor of cardiovascular events and all-cause mortality [35]. An increase in pulse pressure has been associated with arterial calcification [36], particularly medial calcification which leads to arteriosclerosis [37]. The mechanisms involve impaired vitamin D, calcium and phosphate metabolism [37]. PTH is associated with increased blood pressure, while negative associations of blood pressure were observed with 25(OH)D_3 [30, 38]. Furthermore, low 25(OH)D_3 status is associated with arterial stiffness [39]. The potential mechanisms include the direct effect of decreased 25(OH)D_3 on the renin-angiotensin-aldosterone system (RAAS) and vascular wall stiffening induced by elevated PTH [30]. Low 25(OH)D_3 activates RAAS, resulting in increased blood pressure [40]. Vitamin D deficiency also stimulates PTH secretion; however, increased 25(OH)D_3 due to PTH can lead to calcium influx into VSMCs, resulting in contraction, high

vascular resistance and eventually increased blood pressure and stiffness [2, 39]. cPP was also associated with CTX, further indicating the possible contribution of bone resorption to the development of arterial calcification [41]. Sensitivity analysis suggests that different mechanisms maybe involved regarding the contribution of PTH:25(OH)D_3 and CTX to arterial stiffness in women with increased adiposity, since both markers were significantly and independently associated with cPP in the regression model. The combination of PTH:25(OH)D_3 can independently result in increased arterial stiffness as a result of the prosclerotic effects of PTH and extrarenal conversion of 25(OH)D_3 into 1,25(OH)D_3 by macrophages [30, 42]. CTX may independently contribute to arterial stiffness through increased bone resorption, which results in calcium deposition in vascular cells observed in populations at risk of osteoporosis [37].

There is some controversy regarding adiposity and vascular calcification. BMI is known to be positively associated with coronary artery calcium and atherosclerosis [23, 43]. In the present study, however, only the low BMI group showed increased risk of calcified atherosclerosis. Kovacic *et al*. reported a negative association between BMI and calcified atherosclerotic lesions in the elderly population [12], suggesting different mechanisms maybe involved in the development of arterial calcification in lean and obese individuals. For instance, PTH is associated with an increase in BMI and is also responsible for increased bone resorption, while a high BMI is known to increase bone strength and reduce fragility [44, 45]. The results of our study indicate that, besides the relationships obtained between the cardiovascular system and bone resorption and calciotropic hormones; markers of vascular structure and function relate differently to altered bone metabolism and calciotropic hormones in lean and overweight/obese older women. This is validated by the absence of significant results between our main dependent variables and independent variables in the total group.

Our study should be interpreted within the context of its strengths and limitations. Coronary artery calcium (CAC) score was not available to assess vascular calcium deposits. This is a cross-sectional study, therefore causality cannot be implied. Furthermore, the study population cannot be regarded as a representative of the general African population. The main strength of our study is that it is the first to explore the associations of markers of vascular structure and function with PTH:25(OH)D_3 and CTX in the African population. Our results were consistent even after multiple adjustments for known confounders, however residual confounding cannot be excluded. By using the PTH:25(OH)D_3 we could assess the interactive effects of these two calciotropic hormones on arterial structure and function, and possibly on the development of arterial calcification.

In conclusion, we found that in lean African women carotid wall thickness was positively associated with PTH:25(OH)D_3 while in overweight/obese women, large artery stiffness was associated with PTH:25(OH)D_3 and CTX, a marker of bone resorption. These results suggest that older African women may be predisposed to arterial calcification and that different mechanisms may be involved between the lean and overweight/obese women leading to either atherosclerosis or arteriosclerosis or both. These findings confirm the contribution of altered bone metabolism and associated calciotropic hormones to cardiovascular deterioration in older women.

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Disclosure

All authors declared no conflict of interest.

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Supplementary Table 1: Pearson correlations of markers of vascular structure and function with calciotropic hormones and CTX in the lean and overweight/obese African women

Variables	BMI <25 kg/m^2							
	PTH (ng/mL)		25(OH)D_3 (ng/mL)		PTH:25(OH)D_3		CTX (ng/mL)	
	r	*p*	r	*p*	r	*p*	r	*p*
Brachial SBP (mmHg)	-0.45	0.57	0.14	0.08	-0.09	0.28	-0.08	0.33
Brachial DBP (mmHg)	-0.06	0.45	0.14	0.08	-0.11	0.19	-0.08	0.28
Brachial PP (mmHg)	-0.01	0.86	0.09	0.28	-0.04	0.63	-0.04	0.59
Central SBP (mmHg)	-0.001	0.99	0.12	0.15	-0.04	0.61	-0.06	0.44
Central PP (mmHg)	0.06	0.48	0.05	0.56	0.03	0.71	-0.03	0.69
CIMT (mm)	**0.21**	**0.009**	-0.09	0.29	**0.22**	**0.008**	0.04	0.62
CSWA (mm)	**0.18**	**0.027**	-0.09	0.26	**0.20**	**0.013**	-0.002	0.98
Variables	**BMI ≥25 kg/m^2**							
	PTH (ng/mL)		**25(OH)D_3 (ng/mL)**		**PTH:25(OH)D_3**		**CTX (ng/mL)**	
	r	*p*	r	*p*	r	*p*	r	*p*
Brachial SBP (mmHg)	**0.24**	**<0.001**	-0.04	0.53	**0.19**	**0.002**	**0.16**	**0.011**
Brachial DBP (mmHg)	**0.16**	**0.010**	0.04	0.52	0.10	0.11	0.08	0.20
Brachial PP (mmHg)	**0.22**	**0.001**	-0.09	0.15	**0.21**	**0.001**	**0.17**	**0.008**
Central SBP (mmHg)	**0.18**	**0.007**	-0.07	0.32	**0.16**	**0.017**	**0.16**	**0.014**
Central PP (mmHg)	**0.25**	**<0.001**	**-0.14**	**0.035**	**0.25**	**<0.001**	**0.17**	**0.007**
CIMTf (mm)	0.06	0.36	<0.001	0.99	0.05	0.46	0.07	0.29
CSWA (mm)	0.07	0.32	-0.01	0.83	0.06	0.36	0.06	0.39

CIMTf, carotid intima-media thickness (far wall); CSWA, cross sectional wall area; PTH, parathyroid hormone; 25(OH)D_3, 25-hydroxycholecalciferol; PTH:25(OH)D_3, parathyroid hormone to 25-hydoxycholecalciferol ratio; CTX, c-telopeptide of type I collagen crosslinks. All bone mineral metabolism variables and calciotropic hormones were logarithmically transformed.

Supplementary table 2: Pearson correlations between markers of calcium and bone mineral metabolism and calciotropic hormones

Variables	PTH		25(OH)D_3		PTH:25(OH)D_3		CTX		Magnesium		Calcium		Pi		ALP		BMD	
BMI <25 kg/m^2																		
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
PTH (ng/mL)	1.00	-	**-0.18**	**0.023**	**0.87**	**<0.001**	**0.39**	**<0.001**	-0.05	0.56	-0.14	0.069	**-0.15**	**0.047**	-0.02	0.84	-0.08	0.33
25(OH)D_3 (ng/mL)	**-0.18**	**0.023**	1.00	-	**-0.65**	**<0.001**	-0.11	0.15	**0.25**	**0.001**	0.08	0.31	0.04	0.64	0.11	0.15	0.004	0.96
PTH:25(OH)D_3	**0.87**	**<0.001**	**-0.65**	**<0.001**	1.00	-	**0.34**	**<0.001**	**-0.17**	**0.035**	**-0.18**	**0.022**	-0.14	0.074	-0.07	0.36	-0.05	0.57
CTX (ng/mL)	**0.39**	**<0.001**	-0.11	0.15	**0.34**	**<0.001**	1.00	-	-0.02	0.79	-0.10	0.18	0.07	0.39	-0.04	0.61	**-0.18**	**0.032**
Magnesium (mmol/L)	-0.05	0.56	**0.25**	**0.001**	**-0.17**	**0.035**	-0.02	0.79	1.00	-	**0.39**	**<0.001**	**0.20**	**0.007**	0.10	0.19	-0.11	0.21
Calcium (mmol/L)	-0.14	0.069	0.08	0.31	**-0.18**	**0.022**	-0.10	0.18	**0.39**	**<0.001**	1.00	-	**0.32**	**<0.001**	**0.18**	**0.017**	0.11	0.19
Pi (mmol/L)	**-0.15**	**0.047**	0.04	0.64	-0.14	0.074	0.07	0.39	**0.20**	**0.007**	**0.32**	**<0.001**	1.00	-	**0.16**	**0.035**	-0.01	0.93
ALP (U/L)	-0.02	0.84	0.11	0.15	-0.07	0.36	-0.04	0.61	0.10	0.19	**0.18**	**0.017**	**0.16**	**0.035**	1.00	-	-0.11	0.21
BMD (g/cm^2)	-0.08	0.33	0.004	0.96	-0.05	0.57	**-0.18**	**0.032**	-0.11	0.21	0.11	0.19	-0.01	0.93	-0.11	0.21	1.00	-
BMI ≥25 kg/m^2																		
PTH (ng/mL)	1.00	-	**-0.35**	**<0.001**	**0.90**	**<0.001**	**0.17**	**0.008**	-0.12	0.065	**-0.16**	**0.011**	**-0.14**	**0.031**	-0.04	0.53	0.06	0.41
25(OH)D_3 (ng/mL)	**-0.35**	**<0.001**	1.00	-	-0.76	<0.001	-0.02	0.76	0.05	0.48	0.10	0.11	0.03	0.59	0.12	0.069	**-0.21**	**<0.001**
PTH:25(OH)D_3	**0.90**	**<0.001**	**-0.76**	**<0.001**	1.00	-	0.11	0.076	-0.13	0.038	**-0.19**	**0.002**	-0.12	0.066	-0.11	0.091	**0.14**	**0.040**
CTX (ng/mL)	0.17	0.008	-0.02	0.76	0.11	0.076	1.00	-	-0.002	0.97	-0.03	0.61	0.19	0.002	0.12	0.055	**-0.31**	**<0.001**
Magnesium (mmol/L)	-0.12	0.065	0.05	0.48	**-0.13**	**0.038**	-0.002	0.97	1.00	-	**0.79**	**<0.001**	**0.24**	**<0.001**	**0.16**	**0.011**	0.13	0.056
Calcium (mmol/L)	**-0.16**	**0.011**	0.10	0.11	**-0.19**	**0.002**	-0.03	0.61	**0.79**	**0.00**	1.00	-	**0.30**	**<0.001**	**0.30**	**<0.001**	0.11	0.087
Pi (mmol/L)	**-0.14**	**0.031**	0.03	0.59	-0.12	0.066	**0.19**	**0.002**	**0.24**	**<0.001**	**0.30**	**<0.001**	1.00	-	**0.18**	**0.004**	**0.17**	**0.009**
ALP (U/L)	-0.04	0.53	0.12	0.069	-0.11	0.091	0.12	0.055	**0.16**	**0.011**	**0.30**	**<0.001**	**0.18**	**0.004**	1.00	-	-0.06	0.40
BMD (g/cm^2)	0.06	0.41	**-0.21**	**<0.001**	**0.14**	**0.040**	**-0.31**	**<0.001**	0.13	0.056	0.11	0.087	**0.17**	**0.009**	-0.06	0.40	1.00	-

PTH, parathyroid hormone; 25(OH)D_3, 25-hydroxycholecalciferol; CTX, c-telopeptide of type I collagen; Pi, inorganic phosphate; ALP, alkaline phosphate; BMD, bone mineral density

Chapter 4

Summary of main results, limitations, conclusions and recommendations

1. Introduction

This is a summative chapter. It includes an elaborate interpretation and discussion of the main findings of this study. A comparison in light of the original hypotheses as set in Chapter 2 is made with the results of this study as well as with the existing literature and conclusions are drawn. This is followed by recommendations for future research regarding the link between arterial structure and function and bone mineral metabolism and associated calciotropic hormones.

2. Interpretation of the main findings and a comparison with the relevant literature

In this section the findings of this study will be addressed according to the original hypotheses, and with reference to the relevant literature. All of the hypotheses were initially set for the whole population of 434 women. However, after performing statistical analyses it was found that obesity (estimated by body mass index (BMI)) interacted significantly with the relationships of cPP and CIMT with indices of calcium metabolism. All hypotheses will therefore be addressed with reference to lean and overweight/obese groups.

Hypothesis 1: Blood pressure (central and brachial) is positively associated with PTH, PTH:25(OH)D_3, CTX and negatively with 25(OH)D_3.

In this study it was found that in the overweight/obese African women brachial systolic and diastolic blood pressure were independently associated with PTH and CTX, but not with PTH:25(OH)D_3. In addition, central systolic blood pressure was positively associated with CTX, but not with PTH and PTH:25(OH)D_3.

In lean women, no associations were found between brachial and central pressures and calciotropic hormones as well as CTX. There is a lack of specific studies that evaluate the effect of calciotropic hormones and bone resorption on blood pressure in lean women only. The study populations usually consist of people with BMI ≥25 kg/m^2 [1-4].

The results found in the overweight/obese group are consistent with previous studies that indicated that elevated PTH and low 25(OH)D_3 are associated with increased blood pressure in elderly populations [1, 2]. However, the lack of association between 25(OH)D_3 and blood pressure in the present study has also been observed previously in older men and women [2]. PTH increases blood pressure through vessel wall thickening, while vitamin D insufficiency is known to elevate blood pressure through stimulation of the renin-angiotensin-aldosterone system (RAAS) [1, 5]. The reason for the lack of association between blood pressure and 25(OH)D_3 may be the low prevalence of vitamin deficiency [1, 2] which was also observed in the present study (17% of the total group had vitamin D deficiency).

The positive association between blood pressure and CTX can be partly explained by the link between high calcium loss and increased blood pressure. Cappucio *et al*. indicated that elevated blood pressure can result in increased urinary calcium excretion as a result of impaired renal function which alters calcium metabolism, which will result in increased bone resorption to normalise serum calcium levels [4]. Considering that fact that 65% of overweight/obese women in the present study were hypertensive, this mechanism may also apply to our results.

Therefore, the first hypothesis is partially accepted since associations existed between brachial and central pressures and PTH and CTX in the overweight/obese group. Neither the lean nor the overweight/obese group showed association of brachial and central pressures with PTH:25(OH)D_3 or 25(OH)D_3.

Hypothesis 2: Measures of arterial stiffness (brachial and central PP) are positively associated with CTX, PTH, PTH:25(OH)D_3 and negatively with 25(OH)D_3.

In the overweight/obese group, brachial PP was positively associated with bone resorption (CTX), but not associated with PTH and PTH:25(OH)D_3. In addition, cPP which may be a better marker of stiffness in large conduit vessels, was positively associated with PTH, PTH:25(OH)D_3 and CTX.

In lean women no association between brachial and central pulse pressures and calciotropic hormones and CTX existed.

cPP is an established predictor of cardiovascular morbidity and mortality [6] and is also regarded as a more reliable marker of large artery stiffness as compared to brachial PP, since cPP represents the central hemodynamics [6, 7]. cPP was negatively associated with $25(OH)D_3$; however, the association disappeared after adjustments were made for age and BMI. Our results are in keeping with the current information indicating that arterial stiffness as a marker of arteriosclerosis is associated with arterial calcification [8, 9]. Calciotropic hormones, namely PTH and calcitriol as well as bone resorption are associated with the development and progression of arterial calcification [10, 11].

Both types of arterial calcification (intimal and medial) reduce the elasticity of large arteries, resulting in stiffening and arteriosclerosis [12]. This is due to elastin degeneration which frequently precedes mineralisation of the medial layer [13]. Another aspect of vascular calcification is inflammation which was found to play a significant role in the initiation of osteogenic differentiation in intimal (atherosclerotic), and medial [14] calcification of large arteries as well as in coronary arteries [15]. Increased adiposity is associated with low grade and chronic inflammation [16]. Active metabolic substances produced by adipocytes mediate the inflammatory processes [17]. These effects of obesity are pronounced in type 2 diabetes, which is also linked to inflammation-induced vascular smooth muscle cells (VSMCs) mineralisation [14]. Therefore, it is speculated that obesity-induced inflammation may have partly mediated arterial calcification and stiffness in the overweight/obese group and can also explain the absence of associations in the lean group.

With regards to the association between arterial stiffening and a marker of bone demineralisation (CTX), the contribution of diminished oestrogen levels (since 90% of the women were in a postmenopausal state) to increased bone resorption should be considered [18]. In experimental animals, inhibition of bone demineralisation alleviates arterial

calcification [19]. The mechanism by which skeletal demineralisation promotes vascular calcification is based on the fact that during bone breakdown, bone matrix proteins which are also functional in the vascular wall are released into the circulation. In the absence of inhibitors ectopic calcification ensues [14].

The second hypothesis is therefore partially accepted since positive associations were found between brachial and central PP and PTH, PTH:25(OH)D_3 and CTX. We did not find any independent associations between measures of arterial stiffness and 25(OH)D_3 in the overweight/obese group, while in the lean group no association existed between measures of arterial stiffness and calciotropic hormones and CTX.

Hypothesis 3: CIMT and CSWA are positively associated with CTX, PTH, PTH:25(OH)D_3 and negatively with 25(OH)D_3.

In the overweight/obese group, we did not find any associations between carotid wall thickness and calciotropic hormones. However, in the lean group, both measures of carotid wall thickness (CIMT and CSWA) were positively associated with PTH and PTH:25(OH)D_3, while no association were observed with 25(OH)D_3 or CTX. The relationships of CIMT with PTH and PTH:25(OH)D_3 were independent of central systolic blood pressure (cSBP) which was adjusted for in the multiple regression model. This indicates that carotid wall thickening may be due to the prosclerotic effects of PTH that induce vascular wall thickening [1].

The results of the lean group are consistent with previous findings linking PTH and PTH:25(OH)D_3 to carotid wall thickness and arterial calcification [20, 21]. In addition, PTH has also been shown to be a determinant of vascular calcification development in renal failure patients [22]. Low body weight is associated with low bone mineral density [23]. Several studies showed that low bone mineral density (47% of the study population had bone mineral density below the cut-off point of 0.371 g/cm^2) is associated with atherosclerotic calcification and increased CIMT in elderly populations who are at risk of osteoporosis [24, 25].

CIMT is a marker of subclinical atherosclerosis which is in many cases linked to intimal calcification [26, 27]. Deposition of calcium crystals in the intima is common in individuals with decreased bone mineral density and with risk factors for atherosclerosis [28]. Smoking is another risk factor for atherosclerosis and is also associated with aortic arch calcification [29]; and 63% of the lean women in the present study smoked. Therefore, one can speculate that the association between carotid wall thickness and calciotropic hormones may implicate that increased wall thickness may be due to intimal calcification related to atherosclerosis; or pathological process within the arterial wall as a result of injury caused by smoking [30], which will result in an augmented immune response and inflammation, the key step in atherosclerosis [31].

The lack of association between intima-media thickness and CTX in both the lean and overweight/obese groups may be due to the fact that bone resorption is not essential in the development of atherosclerotic (intimal) calcification as compared to medial calcification [9]. Calcification in the medial layer is characterised by conversion of VSMCs into osteoblastic cells which promote calcium deposition [9].

The third hypothesis is therefore partially accepted due to associations observed in the lean group between carotid wall thickness and PTH and PTH:25(OH)D_3, but not with 25(OH)D_3 or CTX. In the overweight/obese group, there was no association of CIMT and CSWA with calcitropic hormones and CTX.

3. Discussion of the main findings

In women with increased adiposity PTH, PTH:25(OH)D_3 and CTX were significantly and independently associated with arterial stiffness, which may be indicative of an increased risk for arterial calcification [22, 37] according to the different mechanisms suggested in the previous section. Associations of CIMT with PTH and PTH:25(OH)D_3 in the lean group were independent of central SBP, suggesting that the mechanisms leading to intima-media thickening may involve factors involved in atherosclerotic calcification [39]. These differences

may possibly be due to the influence of body composition (lean mass, and amount and distribution of adipose tissue) on factors involved in calcification, including bone mineral metabolism, calciotropic hormones and inflammation.

It is well known that altered bone metabolism and calciotropic hormones are linked to the development of vascular calcification and is prevalent in patients with osteoporosis, hypertension, diabetes and renal failure [8, 32-34]. Obesity has been associated with coronary and aortic calcification, while low body weight was associated with calcified lesions [23, 35]. According to Jensky *et al*. adipose tissue is positively associated with aortic calcification, while lean muscle mass is protective against vascular calcification [43]. On the other hand, others reported increased atherosclerotic calcification in non-obese postmenopausal women and speculated that the role of sex steroid hormones on calcification differs according to the level of adiposity [20].

Investigations of vascular calcification with specific focus on bone mineral metabolism and associated calciotropic hormones including both lean and obese individuals are scarce. In the present study, the relations of calciotropic hormones and bone resorption with arterial structure and function differed between lean and overweight/obese women. El Khoudary *et al*. found associations between sex hormones and aortic calcification in obese and non-obese women. There was a high prevalence of atherosclerosis of the carotid arteries in non-obese women with low androgen levels as compared to obese women postmenopausal women [36]. The loss of protective oestrogens at menopause is also linked to arterial stiffness, altered calcium metabolism and increased bone resorption [18, 37].

Body composition has a significant influence on bone resorption, calciotropic hormones and arterial calcification [23, 38, 39] and this was evident in the present study. Increased body weight is associated with increased bone mineral density by increasing bone strength and protecting against bone loss, while on the other hand, lean individuals are prone to increased bone resorption and atherosclerotic calcification [23, 40]. PTH is important in

regulation of bone turnover and has been associated with vascular mineralisation [22]. In addition, increased PTH is associated with increased adiposity in postmenopausal women [39]. In this regard the effects of PTH on factors leading to arterial calcification may be different between lean and obese women as it has been observed in the present study.

Fibroblast growth factor-23, which was not measured in the present study, is another key factor that has to be considered in future studies due to its association with adiposity and medial calcification [41, 42].

Based on the results of this study and the existing literature, we can speculate that the lean group is predisposed to intimal calcification, leading to atherosclerosis, while the overweight/obese group may be predisposed to medial calcification which is linked to arterial stiffness and increased blood pressure. However, vascular calcification is a multifactorial disease and its development and progression is determined by mechanisms which differ by age, body weight and hormonal status. Our study also suggests there is a mechanism by which body composition affects vascular and bone mineralisation. Further prospective and experimental studies could shed more light on the different mechanisms involved.

4. Limitations, chance and confounding

It is essential to reflect on certain factors that may have influenced the results of this study. These include the applied methodology, statistical analyses and interpretation of results.

The cross-sectional design of this study only specifies the current state of health and associations found, and therefore cannot imply causality. In addition, a single measurement of PTH, 25(OH)D_3 and CTX may not reflect the long term status, whereas arterial stiffness, intima-media thickness and the arterial calcification progress over time.

Due to the interaction with BMI on the relationships of the main dependent and independent variables, our study population was divided according to BMI cut-offs of the World Health Organisation (WHO) [43]. Therefore, the group sizes differed, with lean women being fewer

than overweight/obese women. The presence of arterial calcification could not be assessed since the widely used coronary artery calcium (CAC) score was not available in this study. However, the associations between PTH, PTH:25(OH)D_3 and CTX could be indicative of at least the risk for the development of arterial calcification [21, 44]. All participants were from the rural and urban areas of the North West Province and as such cannot be regarded as a representative sample of all African women. The study was well designed, followed a strict protocol and was carried out under controlled conditions.

Regarding the results, the possibility of chance should be taken into consideration. Despite using univariate and multivariate regression analyses, there is a possibility that one out of twenty associations may be due to chance. Additionally, multiple adjustments were made for known confounders in regression analyses. These adjustments may have caused over- or underestimation of the associations observed between markers of vascular structure and function, and calciotropic hormones as well as CTX.

5. Conclusion

In older predominantly postmenopausal African women, blood pressure, large artery stiffness and carotid wall thickness were associated with calciotropic hormones and bone resorption, indicating a predisposition to arterial calcification. The different results between the lean and overweight/obese groups may be attributable to adiposity status and its resultant effect on calcium regulation and bone metabolism. These results are particularly relevant in South Africa as a developing country with an alarming prevalence of cardiovascular diseases including heart failure and stroke which are believed to continue to increase over time. The present study provides new information on how altered bone metabolism and associated calciotropic hormones may affect vascular structure and function. Causality should be investigated in prospective and experimental studies.

6. Recommendations

- Larger study groups including men should be used in prospective studies to determine the cause and effect relationships between calciotropic hormones, bone resorption and arterial calcification.
- The golden standard for arterial stiffness measurements, carotid-femoral pulse wave velocity, should be applied to assess central aortic stiffness.
- In the present study, distal arm bone mineral density was used, but complete bone mineral density should be measured in future studies to assess the risk of osteoporosis and its possible association with ectopic calcification.
- Inclusion of bone matrix proteins and other specific biochemical factors such as fibroblast growth factor 23, matrix GLA protein and osteopontin will shed more light on the link between bone and calcium metabolism and vascular calcification.
- Specific inflammatory markers such as interleukin-6 and tumour necrosis factor alpha should also be included.
- Ultrasound measurements should be employed to calculate coronary artery calcium score.

7. References

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