THE IMPACT OF VASCULAR CALCIFICATION AMONG DIALYSIS DEPENDENT SOUTH AFRICAN CKD PATIENTS. A FIVE YEAR FOLLOW UP STUDY.

Cardiovascular mortality and morbidity, ethnic variation and hemodynamic correlates

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DECLARATION

I, KUDAKWASHE SIMBA, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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PREFACE

The dissertation as part of the MMED is organized into two main chapters.

Chapter 1 – It is a preamble and review of literature pertinent to the topic, background of the problem and burden of CKD in South Africa. The mechanisms and pathophysiology of vascular calcification are also explored. Ethnic variations in bone mineral metabolism are reviewed. Concepts and theories regarding survival advantage and impact on mortality among Blacks and non-Blacks counterparts with ESKD is revised. Most of the studies were conducted in Europe and North America. This identified a void which helped in providing the rationale for the study.

Chapter 2 -is presented as a manuscript ready for submission to BMC Nephrology. The Abstract and Chapter 2 are presented in a format following the policies and guidelines as per Editorial Board Guidelines. Chapter 2 is expected to be submitted for publication.

TABLE OF CONTENTS

List of Tables

Chapter 2

List of Figures

Abbreviations

ADMA – Assymetric Dymethyl Arginine

AHSG - α2-Heremans Schmid Glycoprotein

AIDS – Acquired Immunodeficiency Syndrome

AURORA - A Study to Evaluate the Use of Rosuvastatin in Subjects on Regular Hemodialysis. An Assessment of Survival and Cardiovascular Events

CASP – Central Aortic Systolic Pressure

Cbfa1 – Core binding Factor Alpha 1

CCS – Coronary Calcium Score

CKD – Chronic Kidney Disease

CKD 5D - Chronic Kidney Disease Stage 5 Dialysis Dependent

CUA – Calcific Uremic Arteriopathy

CV – Cardiovascular

CVD – Cardiovascular Disease

ECG – Electrocardiogram

ESKD – End Stage Kidney Disease

FGF – 23 Fibroblast Growth Factor 23

GFR – Glomerular Filtration Rate

HIV – Human Immunodeficiency Virus

IQR – Inter Quartile Range

LVH – Left Ventricular Hypertrophy

MBD – Mineral Bone Disease

MDRD – Modification of Diet in Renal Disease

MESA - The Multi Ethnic Study of Atherosclerosis

MGP – Matrix Glutamate Protein

NODAT - New Onset Diabetes After Transplantation

NPT2a - Sodium Dependent Phosphate Co transporter 2a

NPT2c - Sodium Dependent Phosphate Co transporter 2c

PLC – Phospholipase C

PTH – Parathyroid Hormone

PWV – Pulse Wave Velocity

RAAS – Renin Angiotensin Aldosterone System

RANKL – Receptor Activator of Nuclear Factor Kappa – B Ligand

SHARP - Study of Heart and Renal Protection

VSMC – Vascular Smooth Muscle Cells

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ABSTRACT

BACKGROUND

Vascular calcification is a major risk factor for cardiovascular morbidity and mortality in patients with end stage renal disease (ESRD). In Western countries, Blacks with ESRD appear to have lesser degrees of vascular calcification compared to non-Blacks. However, there is no published data on the association of ethnic differences in vascular calcification and survival in ESRD from Sub-Saharan Africa.

METHODS

This study assessed the 5-year change in vascular calcification and mortality in a previously published cohort of patients with ESRD. Vascular calcification was assessed by abdominal aortic calcification score (lateral abdominal radiograph) and vascular stiffness by pulse wave velocity.

RESULTS

Sixty-six of the original 74 participants, studied a baseline, were identified. The median age was 46.6 years (37.6-59.2) and 57.6% were women. Abdominal aortic calcification showed no progression among Blacks [baseline range 0-5, follow up range 0-8 (p=1.00)], but a non-significant trend to progression among non-Blacks [baseline range 0-19, follow up range 0-22 (p=0.066)]. Black participants did not display a survival advantage (p=0.870). Overall, sepsis was the most common cause of mortality (64% of those with an identifiable cause of death). Non-Blacks had higher parathyroidectomy rates than Blacks with 9/30 cases compared to 2/36 (p=0.036). After adjustment for parathyroidectomy at follow up, the odds ratio of having abdominal vascular calcification score of ≥1 amongst non-Blacks was 8.6-fold greater compared to Blacks (p= 0.03). Central aortic systolic pressures (CASP) and pulse wave velocities (PWV) were higher in the study population than age matched normative values. At follow up, a positive correlation (r=0.3) was observed between PWV and abdominal aortic calcification (p=0.04). Elevated baseline coronary artery calcification score and FGF-23 level at baseline were not associated with a difference in mortality.

CONCLUSION

There was no significant progression in vascular calcification among Blacks. After adjusting for increased parathyroidectomy rates, there was a greater progression of vascular calcification amongst non-Blacks compared to Blacks highlighting possible ethnic differences in calcium phosphate metabolism in patients with ESRD. The lack of vascular calcification progression in Blacks was not however associated with improved survival, but the sample size was small.

KEYWORDS

Chronic kidney disease, dialysis, vascular calcification, vascular stiffness, pulse wave velocity, FGF- 23

CHAPTER 1: LITERATURE REVIEW

Words 3309

INTRODUCTION

INTRODUCTION

Background of the Problem

With the increasing burden of end stage renal disease (ESRD) there is a consequent growing demand for renal replacement therapy[1,2,3]. A population based sample study done in Bellville, Cape Town showed a very high prevalence of CKD [4]. Using the Modification of Diet in Renal Disease (MDRD) to calculate the GFR, the prevalence of CKD stage 3 to 5 was 7,6% and 23.9 % respectively with and without correction for ethnicity [4]. In Sub Saharan Africa, the number of deaths from CKD have risen by 82% in 2008 compared to 1990[5]. Globally, end stage renal disease (ESRD) has had the third largest increase in mortality rate behind HIV/AIDS and diabetes [5]. Of great concern is the higher cardiovascular mortality among patients requiring dialysis which include sudden cardiac death due to ventricular dysrhythmias, myocardial infarction, heart failure and peripheral vascular diseases [5, 6]. The National Kidney Foundation's Task Force on Cardiovascular Disease (CVD) in Chronic Renal Disease in United States in 1998 demonstrated that patients on hemodialysis had an approximately 10 to 30 fold greater CVD mortality rate compared to the general population after stratification for race, sex and age [7]. It is thought that the heavy burden of vascular calcification involving the tunica media of blood vessels leads to arterial stiffness and calcification in the intima results in a predilection towards formation of atherosclerotic plaques and consequent cardiac disease[8]. Vascular disease in ESRD is further accelerated by altered metabolism of parathyroid hormone (PTH), calcium, phosphate and vitamin D levels which induce mediators of bone like metabolism and mineralization in the tunica media [9].

Vascular Calcification

Vascular Calcification Pathophysiology

Vascular Calcification pathogenesis is not fully understood and is believed to be multifactorial[10]. Various pathogenic mechanism have been studied and proposed[10].

Several studies have identified the link between both traditional risk factors and uremic specific risk factors in the association between vascular calcification and CKD[11,12]. Previously, vascular calcification was considered as a passive mechanism [13]. However, research in the last decade has shown that it is actually an active process involving ossification of vascular structures in which different factors are involved in the deposition of the calcium phosphate product in the extracellular matrix[14,15]. Several factors are expressed during vascular calcification which include bone-related proteins such as type I collagen, osteopontin, bone morphogenetic protein-2, bone sialoprotein and alkaline phosphatase showing that it is a highly regulated active cellular process[16].

Vascular calcification within arteries occurs in several ways, as: dispersed, very small hydroxyapatite crystals, large calcified deposits and mineralized cartilage and bone-like tissue containing marrow [17]. Several in-vitro studies have shown that vascular smooth muscle cells (VSMC) and pericytes demonstrate characteristics of osteoblasts lineage cells if cultured within a medium mimicking ESRD [9]. In cell cultures VSMC and pericytes have shown the ability to produce similar bone forming transcription factors and proteins and is induced by high concentration of oxidized lipids cytokines, uremic serum, phosphorus, high glucose, and other yet to be discovered factors [9,18].

Osteoblasts and VSMC derive from similar mesenchyme precursor cell [10]. Core binding factor alpha (Cbfa1), which is heterodimeric osteogenic differentiation transcription factor [19] is thought to be switched on by high levels of phosphate and uremic specific toxins which turns the VSMC into an osteoblast [18,20] . Jono et al [20] demonstrated that VSMC calcify in the presence of beta glycophospherate, a donor of phosphate, with increased regulation of Cbfa1. Cbfa1 downstream proteins Osteopontin and Type 1 collagen were also found expressed with Cbfa1 in intimal and medial calcification from CKD patients leading to differentiation of VSMC to osteoblast like cells [10].

Run X2 an osteogenic transcription factor that promotes osteogenic differentiation of vascular smooth muscle cells is induced by high calcium/phosphate, parathyroid hormone, Fibroblast Growth Factor 23 (FGF 23) and increased oxidative stress in CKD patients [21]. Run X2 induces RANKL expression in VSMC which enhance directly VSMC calcification also contributes to bone resorption in CKD patients through bone resorbing osteoclasts [21].In addition to promotion of differentiation, Run

X2 strongly promotes the expression of type 1 collagen and upregulates expression of VEGF [22]. VSMC expressing RANKL upregulates migration, production of cytokines and osteoclast formation of macrophage accelerating vascular calcification [21].

Hyperphosphatemia also plays an important part in vascular calcification. Giachelli [23] showed that increase in phosphate plays an important part in promoting the vascular phenotype VSMC to osteogenic phenotype predisposing to vascular calcification. The induced changes by phosphate include upregulated expression osteogenic markers osteocalcin and Cbfa1 genes which seem to occur after exposure elevated phosphate levels exposure [23].

Moustapha et al [24] showed that elevated homocysteine levels poses an independent risk factor for CVS mortality and morbidity in ESKD. Homocysteine in an atheromatous plaque and its ability to stimulate osteogenic cell differentiation partly explains its link with atherosclerosis [25].

FGF 23 discovery has altered our conceptualization of CKD – mineral and bone disorder (MBD) [26] . Complex interactions have been revealed which include interactions, hormonal feedback loops with the kidney, intestine, bone and parathyroid glands [26]. In the past decade, studies have shown the links between FGF 23 and cardiovascular events [26, 27]. FGF 23 is secreted by osteoblasts and osteoclasts in the bone to the bloodstream where it acts as a phosphaturic hormone [28, 29]. FGF 23 requires α Klotho, a membrane co-receptor to induce specific signaling pathways[30,31]. α Klotho is highly expressed in the kidneys and parathyroids [26]. FGF 23 primary target of is α Klotho- FGF 23 receptor complex which induces urinary phosphate excretion by decreasing expression of type 2a and 2c sodium dependent phosphate co transporters (NPT2a and NPT2c) in the renal proximal tubule [32]. FGF 23 also decreases intestinal absorption of phosphate by inhibiting the renal expression of 1 α hydroxylase and stimulating the catabolic effects of 24 hydroxylase [33]. FGF 23 has also been shown to target parathyroid hormones but the molecular regulation of its synthesis is not clearly defined [34].

FGF 23 increases in CKD with decline in kidney function such that at ESRD, FGF 23 levels are sometimes more than 1000 fold above normal range [35]. The increase is to compensate for phosphate retention in order to promote phosphaturia [36]. As the GFR declines, the kidney has diminished ability to excrete phosphate resulting in phosphate

accumulation despite high levels of FGF 23 [35,37]. The supraphysiological FGF 23 levels have been associated with significantly high mortality risk during dialysis [38] [39].When directly compared with phosphate and PTH, FGF23 has been shown to be the strongest predictor of adverse outcomes[40] Several studies have shown a relation between FGF 23 and Left Ventricular Hypertrophy (LVH)[41,42]. FGF 23 is believed to induce cardiac myocytes hypertrophy via Phosphoinositide-specific Phospholipase C (PCL) – γ signaling dependent of FGF receptor activation independent of α Klotho[26]

Inhibitors of Vascular Calcification

Numerous factors are involved in the inhibition of vascular calcification these include:

1. Matrix Glutamate Protein (MGP)

MGP is a vitamin K-dependent γ-carboxylated protein available in vascular smooth muscle and bone [43]. Medial vascular calcification is observed among rats on treatment with a vitamin K antagonist warfarin [44]. Humans with a mutant form of MGP (Keutel Syndrome) develop medial calcification although not severe suggesting that MGP is an inhibitor of calcification [17,45]. Higher rates of vascular calcification is observed among CKD patients on warfarin because of the blockage of γ carboxylation of MGP by warfarin [46]. There is an association of both aortal stenosis and excessive vascular calcification with Warfarin therapy on patients with ESRD [47].

2. Fetuin A

Serum Fetuin-A an α2-Heremans Schmid glycoprotein (AHSG) is encoded by the *AHSG* gene dominantly expressed in the liver [48]. Serum Fetuin A inhibits the de novo formation of calcium phosphate and deficient mice develop widespread tissue calcification in the kidney, myocardium lung, skin and tongue [49]. Patients on dialysis have low Fetuin A levels which are observed to be associated with elevated calcification of vessels and a higher risk of CVS morbidity and mortality [50].

Pyrophosphate

Pyrophosphate acts as an endogenous inhibitor of vascular calcification by inhibiting hydroxyapatite [6]. In ESRD, plasma levels of pyrophosphate are low correlate inversely with vascular calcification [6].

Osteopontin

Speer et al[51] and others have reported that osteopontin levels are elevated at calcification sites in atherosclerotic plaques in uremic arteriopathy, diabetic vasculopathy, native and prosthetic valves but not in normal arteries [52,-46]. Osteopontin is an acid phosphatase found normally in mineralized tissue like bone and teeth [51]. It is thought that Osteopontin promotes osteoclastic function through the $\alpha_v\beta_3$ integrin and also inhibits apatite growth [23].

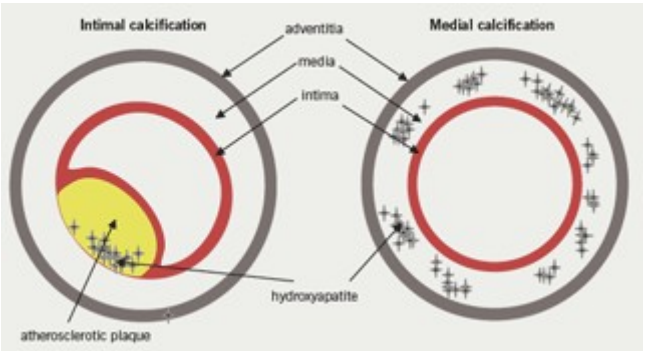
Types of Vascular Calcification

Calcification tends to occur at different sites including the: vascular intima and media, soft tissue, cardiac vasculature and over the mitral and aortic valves.[17].

Intimal Calcification

Inflammation, thickening and calcification of the intimal layer is called atherosclerosis [53]. Atherosclerosis occurs when lipid laden macrophages are trapped in the sub-endothelial layer forming lipid laden plaques in tunica intima of the arteries resulting in micro inflammation of the atherosclerotic plaque [53]. Lamellar bone formation, osteogenesis and osteoblast induction is evident as the atheromatous lesion progresses [54]. The distribution of atherosclerosis is patchy, focal affecting the medium sized arteries such as the epicardial coronary, carotid, iliac and femoral arteries [55]. Muscular arteries such as the internal mammary and radial are spared [55]. Tunica intima vascular calcification is associated with lipid-laden atherosclerotic plaques that are flow-limiting and mainly occurs at arterial branch points increasing the risk of acute coronary syndromes [55,56].

Diagrammatic representation showing the difference between medial and intimal calcification.

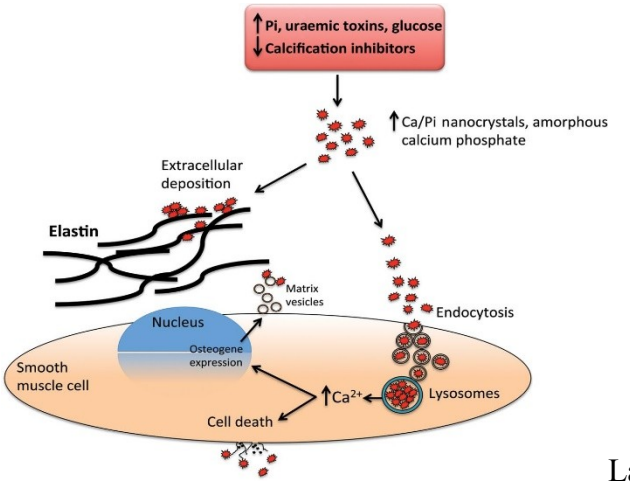


A cross section of an arterial wall Sinha et al 2008[17]

Medial Calcification

Medial calcification also known as Monckeberg sclerosis occurs when circumferentially amorphous mineral forms within or along elastic lamelle of the tunica media [54]. A simplified suggested pathogenesis of medial calcification is shown below

Figure 2 - Diagrammatic representation of pathogenesis of medial calcification



Lanzer et al [47]

Calcium phosphate nanocrystals are endocytosed or deposited on extracellular matrix elastin fibers [47]. Namocrystals endocytosed are dissolved at low pH and calcium is released into the cytoplasm [47]. Osteochodrotic transdifferentiation or cellular death of VSMC or pericytes occurs after a rise in calcium levels [47]. Medial calcification of arteries is observed among renal and diabetic aging populations [53,57]. Predominantly it occurs in large arteries and tributaries [58]. Medial calcification tends occur in younger patients compared to intimal calcification in CKD, but are also more likely to have a prolonged hemodialysis history increasing the possible time for calcification [59]

Calciphylaxis/Calcific Uraemic Arteriolopathy (CUA)

Calciphylaxis is formed by arteriolar medial calcification associated with occlusive thrombotic cutaneous ischemia, tissue death leading to ulceration, secondary infection and high mortality rates [60]. The full pathophysiology of calciphylaxis is poorly

understood [60]. Intimal fibrosis and medial calcification of superficial arterioles coupled with occlusive thrombosis resulting in skin ischemic necrosis occurs in calciphylaxis [61].

Valvular Calcification

Increased Asymmetric Dymethyl Arginine (ADMA), Reactive Oxygen Species (ROS), hyperphosphatemia, and elevated expression of Receptor Activator of Nuclear Kappa Ligand (RANKL) act as promoters of calcific valve degeneration in patients with ESRD [62]. Lower circulating Fetuin A levels and MGP activation of also favor valvular calcification [62].

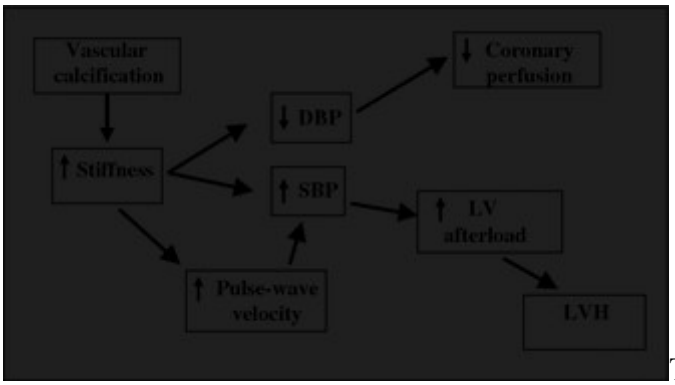
The Link: Pulse Wave Velocity, Vascular Calcification and Mortality.

An important link exists between pulse wave velocity (PWV), vascular calcification and CVS mortality and morbidity. Vessel stiffness due to vascular calcification can be assessed noninvasively using PWV which is the velocity of the pulse wave to travel a given distance between two sites of the arterial system [63]. Vascular stiffness can be measured by PWV which translates to a higher velocity as the blood vessel stiffens [64]. Temmar et al [65] showed that patients with CKD had a higher PWV, regardless of CKD stage compared to controls with preserved kidney function. Patients with ESRD elevated vascular stiffness, determined by aortic PWV measurement, was a robust predictor of all cause, CVS morbidity and mortality [63]. Decreased elasticity due to contributory factors as calcification of vessel walls , atherosclerosis, uremia, changes in vessel quantities of collagen and elastin in CKD results in arterial stiffness [9,59,61].

LVH and its ensuing complications, is a significant cause of mortality and morbidity among CKD 5D patients [26, 66, 67]. LVH is of poor prognostic significance because it is an independent risk factor for arrhythmia development, ischemic heart disease,sudden heart failure and cardiac death [67]. LVH in CKD is both secondary to pressure and volume overload [67]. PWV which is now being widely accepted as the ‘gold standard’ for arterial calcification can be used as a non-invasive way to measure the pressure overload in CKD 5D patients [67,68,69]. The pressure overload is secondary to prior existing hypertension, loss of vessel pliability due to lack of elasticity and to vascular calcification resulting in augmented pulse pressure [67,70]. Vessel stiffness results in increased oxygen consumption and reduced cardiac perfusion pressure [67]. A supply –

demand imbalance occurs resulting in reduced coronary reserve and myocardial ischemia (fig1) [67] .

Fig 1



Taddei et al [67]

Patients with CKD 5D are in a volume and pressure overload states. A combination of CKD 5D factors and sustained overload features such as long standing increased systolic blood pressure, activation of the RAAS system and anemia results in maladaptive LVH causing a uremic cardiomyopathy [71-72]. Certain maladaptive changes of myocytes including accumulation of collagen, myocyte death, calcification, fibrosis are observed in uremic cardiomyopathy causing systolic and diastolic dysfunction [73]. Patients with uremic cardiomyopathy are predisposed to sudden cardiac death due to electrical instability, cardiac failure and reentry tachy - arrythmias [74].

Spectrum of Cardiovascular Disease in CKD

In CKD, nontraditional and traditional risk factors are both involved in the progression of CVS disease [75]. Traditional risk factors include those used to calculate the Frimingham Heart Score in predicting coronary artery disease in the general population [76]. Cardiovascular disease pathophysiology in CKD 5D includes arteriosclerosis, atherosclerosis, coronary artery disease, LVH associated with sudden cardiac death from arrhythmias and electrolyte abnormalities [77]. Current evidence suggests that the severity and aeitiology of atherosclerosis in CKD patients differs from that in the general population [78]. Knowledge of these differences provides a reasoning for identification of treatment and prevention strategies for the management of high burden of CVD in patients with CKD [77]. Weiner et al [79] demonstrated the poor overall accuracy of the Framingham instrument in predicting cardiac events in individuals with CKD and advocated for CKD specific equation.

Observed nontraditional, uremic related, risk factors that worsens as the kidney function declines contribute to the higher risk of CVD observed in CKD patients [5,80]. The risk factors include anemia, arteriosclerosis, atherosclerosis, fluid volume overload, endothelial dysfunction, abnormal calcium, Vitamin D, phosphate metabolism and many more[75]. Dialysis patients are a heterogeneous group. The pathophysiology and dimensions of cardiovascular disease differs markedly [77]. Atherosclerosis is only part of the jig saw puzzle of arterial disease in patients with CKD 5D [77].

In patients on dialysis who have normal cholesterol levels, the treatment with statins in the primary prevention of CVD needs to be investigated further [77]. Three large, randomized, placebo-controlled trials of different statins were done in the dialysis population which were the 4D German Diabetes Dialysis Study [81], AURORA (A Study to Evaluate the Use of Rosuvastatin in Subjects on Regular Hemodialysis. An Assessment of Survival and Cardiovascular Events) [82] and SHARP (Study of Heart and Renal Protection) studies[83]. Results from these studies show that there is a limited role of statins in patients undergoing hemodialysis and peritoneal dialysis, this highlights a different spectrum of risk factors in CKD patients [77].

Ethnic Variations in Vascular Calcification and Mortality of CKD 5D

In mineral metabolism, ethnicity plays a vital role [84]. Fracture risk and osteoporosis is different in various ethnicities [85]. African descendants in the United States have higher bone density and fewer fractures than their Caucasian and Hispanic counterparts [85,86]. Variations in ethnic mineral metabolism of calcium homeostasis, 25-hydroxy vitamin D serum levels, PTH with bone acquisition structure are observed [87].

The Multi Ethnic Study of Atherosclerosis (MESA) showed significant coronary calcification in Caucasians compared to their Black counterparts which was not explained by the traditional coronary risk factors [88]. Whether coronary artery calcification increases the risk of adverse outcomes in ESRD is an important unanswered question [89]. Wilkeinson et al [90] showed that elevated coronary calcium scores in patients with ESRD has a propensity to predict CVS mortality and morbidity but effects were attenuated after age adjustment. A multicenter randomized trial done by Kraus et al [91] of the prevalence of vascular calcification in ESRD patients on hemodialysis showed that the prevalence of aortic vulvular calcification

was higher among whites compared to other races. African Americans also had a lower prevalence of mitral annulus calcification compared to Caucasians and other races [91]. Interestingly radiologically, significant fewer Whites did not have abdominal aortic calcification than African Americans[91]. Proportionally, more Whites had abdominal aortic calcifications in the 'significant' category [91]. Ethnic differences have also been described for the metabolism of calcium and phosphate affecting extracellular calcium- phosphate nanocrystal formation during the initial process of vascular calcification [87].Black individuals have been shown to have lower urinary excretion, increased intestinal absorption of calcium, reduced levels of 25 hydroxy vitamin D and increased levels of 1, 25 hydroxy vitamin D and parathyroid hormone [92,93,94]. The body phosphorus concentration is higher in blacks than whites [95]. Mineral density, bone architecture and bone mass is better in blacks [96]. These differences confer an advantage to blacks who have stronger bones, fewer cardiovascular calcification and fewer risk of fracture [47, 97].

Further, Blacks seem to have a survival advantage in the ESRD population undergoing dialysis [98]. There have been many factors attributed to the survival advantage of blacks which include: a lower pro- inflammatory state among blacks receiving dialysis , dietary intake factors [97], bone mineral metabolism [97] and genetic factors [99]. Kalanter – Zadeh et al [100] showed that Arab Israelis had a faster progression to CKD 5D but showed an overall survival advantage than their Jewish Israelis CKD 5D counterparts. A study done by Frankenfield et al [101] showed that Mexican-American and Hispanics patients undergoing dialysis had survival advantage compared with non-Hispanics counterparts. Wong et al [102] observed that Asian Americans on dialysis had a survival advantage compared to their white counterparts. In their six year follow up study, Mesler et al [103] found that non-white dialysis dependent patients had a survival advantage compared to Whites and that persisted despite case mix adjustment using clinical and demographic information.

Genetic factors have been demonstrated to underlie part of the risk for ESRD in African Americans and are believed to play a role in survival after initiation of dialysis [99]. The APOL 1 gene which has a high frequency among African Americans which is believed to confer resistance against Tryponosomiasis was associated with an increased risk of CKD[104]. The APOL 1 gene has been shown to predict lower age of

dialysis initiation in non-diabetic ESRD [105]. In their historical cohort of 570 808 patients with CKD stages 3 – 5 for 4.7 years non dialysis patients, Kovsedy et al [106] showed that Black patients had a survival advantage compared with White patients. The survival advantage seen in blacks was attenuated with more advanced CKD [106]. It is possible that there are other unknown race differences and genetic factors that causes CKD resulting in different survival rates among patients with ESRD which need to be investigated.

Conclusion

The exact underlying mechanisms of vascular calcification among CKD 5D patients are not entirely clear. The pathophysiology of cardiovascular disease in CKD seems to be multifaceted and poses a unique setting among heath care workers. Identification of at risk groups is crucial in order to attempt, mitigate or reverse the effects of cardiovascular disease in CKD.

Clinical and epidemiological research is required to observe the trends and impact of vascular calcification among CKD 5D patients. The information obtained is important in managing and mitigating the adverse impact of vascular calcification in ESRD patients.

RELAVENCE AND RATIONALE FOR CONDUCTING THE STUDY

There is paucity of data regarding the impact of vascular calcification with regards to cardiovascular mortality, morbidity and the impact of ethnicity in Sub Saharan Africa. Research findings will hopefully advise treatment decisions that will have a positive impact on mortality and morbidity.

OBJECTIVES OF THE STUDY

Primary Objectives

1. Determine the progression of vascular calcification in a cohort of CKD 5D patients using the abdominal x-ray calcification scores.
2. To assess the 5-year clinical outcome of CKD 5D patients in relation to their coronary and abdominal aortic calcification scores measured at baseline by CT angiography and abdominal x-ray.

Secondary Objectives

1. To identify the effects of vascular calcification as assessed by the pulse wave velocity (PWV) on vascular stiffness.
2. To identify if any, differences in progression of vascular calcification among different ethnicities.
3. To examine the contribution of comorbid illnesses and habits in patients with ESRD (e.g. diabetes mellitus, HIV status, hyperlipidemia, smoking) towards progression of vascular calcification.

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CHAPTER 2: JOURNAL READY MANUSCRIPT

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THE IMPACT OF VASCULAR CALCIFICATION AMONG DIALYSIS DEPENDENT SOUTH AFRICAN CKD PATIENTS. A FIVE YEAR FOLLOW UP STUDY.

Cardiovascular mortality and morbidity, ethnic variation and hemodynamic correlates

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ABSTRACT

BACKGROUND

Vascular calcification is a major risk factor for cardiovascular morbidity and mortality in patients with end stage renal disease (ESRD). In Western countries, Blacks with ESRD appear to have lesser degrees of vascular calcification compared to non-Blacks. However, there is no published data on the association of ethnic differences in vascular calcification and survival in ESRD from Sub-Saharan Africa.

METHODS

This study assessed the 5-year change in vascular calcification and mortality in a previously published cohort of patients with ESRD. Vascular calcification was assessed by abdominal aortic calcification score (lateral abdominal radiograph) and vascular stiffness by pulse wave velocity.

RESULTS

Sixty-six of the original 74 participants, studied at baseline, were identified. The median age was 46.6 years (37.6-59.2) and 57.6% were women. Abdominal aortic calcification showed no progression among Blacks [baseline range 0-5, follow up range 0-8 (p=1.00)], but a non-significant trend to progression among non-Blacks [baseline range 0-19, follow up range 0-22 (p=0.066)]. Black participants did not display a survival advantage (p=0.870). Overall, sepsis was the most common cause of mortality (64% of those with an identifiable cause of death). Non-Blacks had higher parathyroidectomy rates than Blacks with 9/30 cases compared to 2/36 (p=0.036). After adjustment for parathyroidectomy at follow up, the odds ratio of having abdominal vascular calcification score of ≥1 amongst non-Blacks was 8.6-fold greater compared to Blacks (p= 0.03). Central aortic systolic pressures (CASP) and pulse wave velocities (PWV) were higher in the study population than age matched normative values. At follow up, a positive correlation (r=0.5) was observed between PWV and abdominal aortic calcification (p=0.047). Elevated baseline coronary artery calcification score and FGF-23 level at baseline were not associated with a difference in mortality.

CONCLUSION

There was no significant progression in vascular calcification among Blacks. After adjusting for increased parathyroidectomy rates, there was a greater progression of vascular calcification amongst non-Blacks compared to Blacks highlighting possible ethnic differences in calcium phosphate metabolism in patients with ESRD. The lack of vascular calcification progression in Blacks was not however associated with improved survival, but the sample size was small.

KEYWORDS

Chronic kidney disease, dialysis, vascular calcification, vascular stiffness, pulse wave velocity, FGF- 23

INTRODUCTION

Chronic Kidney Disease (CKD) is a major public health problem in South Africa (SA) due to the high prevalence of hypertension, diabetes and HIV.[1,2,3] A population based sample study conducted in Bellville, Cape Town using the Modification of Diet in Renal Disease equation to calculate the estimated glomerular filtration rate, demonstrated a high prevalence of CKD stage 3 - 5 of 7.6% (with ethnicity correction) and 23.9 % (without ethnicity correction).[4] CKD has a higher cardiovascular(CV) mortality due to traditional risk clustering of CV as well as non-traditional CV risk factors, including vascular calcification of the tunica media. [5]

Various surrogate markers have been used to assess vascular health in patients with CKD.[6] The extent of coronary artery calcification has shown to be a powerful clinical predictor of long term prognosis in asymptomatic CKD patients .[7] Furthermore, Fibroblast Growth Factor 23 (FGF-23) is positively associated with left ventricular hypertrophy (LVH), endothelial dysfunction, progression of CKD and a higher mortality.[8,9] Pulse wave velocity (PWV) and central aortic systolic pressure (CASP) can be used non-invasively to measure vascular health.[9] In CKD, regardless of the stage, patients have higher PWV level compared to controls with preserved renal function.[10] Blacher et al [11] showed that, in patients with ESRD, increased PWV was a strong independent predictor of CV and all-cause mortality. Vascular calcification, atherosclerosis, changes in collagen and elastin, and uremia are all thought to result in increased arterial stiffness in CKD.[12]

Ethnicity may also play a role in the development of vascular calcification. Numerous studies in developed countries describes slower progression of vascular calcification in Blacks compared to non-Blacks.[13,14]. Freercks et al [15] showed that Black South African dialysis patients appeared to be protected from vascular calcification. The median coronary calcium score among Blacks was 0 (IQR 0) and 66 non-Blacks (IQR 383 $p < 0.01$) [15]. Even after adjustment, Black race

remained a negative predictor for coronary calcification [15]. There is a paucity of other data on ethnic variations in vascular calcification in sub-Saharan Africa. This apparent advantage for Blacks on dialysis may be due to a lower pro-inflammatory state, dietary intake, genetic factors and variations in bone mineral metabolism. [7,10,16] Differences in ethnic bone mineral metabolism regulation, bone mass acquisition and architecture are well described.[16-22] Black subjects with end stage renal disease (ESRD) have been shown to have a more favorable bone density and bone architecture, less calcium renal excretion, elevated gastrointestinal calcium absorption, lower 25-hydroxy vitamin D and higher levels of parathyroid hormone (PTH) than their non-Black counterparts. [17,18,19]

The present study is a five year follow up, single centre study–of patients initially recruited to investigate vascular calcification in dialysis-dependent patients.[15] The study aimed to assess the progression of vascular calcification and ensuing clinical sequelae as well as the possible survival advantage of Blacks on dialysis. The study protocol was approved by the Human Research Ethics Committee of the University of Cape Town (HREC REF: 048/2016).

METHODS

Of the 74 patients recruited in the initial study, 66 were traced. All study participants were over 18 years old and provided written informed consent. Testing was conducted on the dialysis patients after completion of dialysis and on pre-scheduled clinic appointment visits in non-dialysis dependent patients. The cohort's study methods have been described in detail elsewhere [20], but included: baseline anthropometry, electrocardiogram (ECG), abdominal calcification scores (calculated from lateral abdominal radiograph), coronary calcium scores (CCS) calculated from a cardiac computer tomography, and FGF-23 levels. Medical data including smoking habits and dialysis modality were captured. The following investigations were done at the 5-year follow-up visit: anthropometry, lateral abdominal radiograph, ECG, and PWV and CASP measurement using the AtCor Medical SphygmoCor XCEL® device.

All anthropometric measurements were conducted by one investigator (KS). The peritoneal dialysis patient was weighed after drainage of the dialysate. All haemodialysis patients had their weights measured post dialysis. Abdominal truncal obesity was defined using the African normative values of waist circumference of male ≥ 94 cm and females ≥80 cm.[21]

All resting ECGs were performed using standard calibration and analyzed by one investigator (SK). The Sokolow-Lyon (SL) and Cornell Criteria, as per European Society Cardiology guidelines, were used to diagnose left ventricular hypertrophy (LVH)[22] and were corrected for body mass index (BMI).[23]

Abdominal radiographs were taken using a standardized technique. [15] Scoring of calcification was assessed as per 24-point scale using the validated method described by Kaupilla et al. [24] The same investigator (RF) who conducted the abdominal calcification scoring in the initial study was blinded and scored all the radiographs done at follow up.

Statistical Analysis

The data were entered into a RedCap database and cleaned using pivot tables in Microsoft Excel before being exported to STATA 14 (Stata Corp, College Station, Texas) for analysis. Statistical tests were performed according to whether the variable was continuous or categorical. Continuous data are expressed as mean values ± SD or median values (IQR) depending on the normality of data. For categorical data the χ^2 test or Fisher exact test and the Z test were used to test for a statistical difference between variables and proportions, respectively. The Mann – Whitney and Student t test were used to test the association between continuous and categorical variables. Participants of African descent were classified as Blacks. The rest were classified as non-Blacks for analysis.

The survival of Blacks versus non-Blacks at 5 years was analyzed using Kaplan Meir curves. The difference in progression of vascular calcification among Blacks and non-Blacks at baseline and follow-up was analyzed using the paired t-tests and sign-rank paired test based on normality.

PWV measurements were not undertaken in the initial study. Using normative values of PWV published in a South African study [25], the effects of vascular calcification on PWV were identified. A BPro watch device (HealthSTATS, Singapore) was used to measure the CASP in the initial study. The five-year clinical outcome in relation to calcium scores at baseline was analysed using the Student's t-test.

RESULTS

Baseline and follow-up characteristics of the population are presented in table 1. At baseline and follow up 57.6% of participants were women (n=38) and 54.5% were Black (n=36). The majority of patients traced were still alive 63.6% (n=42). Among participants that were alive: 57.1% (n=24) had been transplanted, 40.5% (n=17) were on hemodialysis and one patient was on peritoneal dialysis. Non-Blacks had progressive abdominal calcification approaching significance [baseline range 0-5, follow up range=0-8 (p=0.066)] compared to Blacks who showed no progression [baseline range=0-19, follow up range 0-22 (p=1.00)]. Non-Blacks had higher parathyroidectomy rates of 9/30 cases compared to 2/36 cases of Black patients (p=0.036). The odds ratio of having abdominal vascular calcification score of ≥1 amongst non-Blacks at follow up was 8.6 fold greater than a similar calcification score amongst Blacks, after having adjusted for parathyroidectomy (p=0.031). At follow up, a positive correlation (r=0.3) was observed between PWV and abdominal vascular calcification (p=0.04). Significant weight gain among all participants at follow up compared to baseline was observed (27.1±7.1kg/m^2 versus 24.4±4.2kg/m^2 respectively; p<0.015). There was a regression in LVH using both the SL score corrected for BMI (p<0.001) and Cornell product corrected for BMI (p=0.027). In our study group 12.1% (n=4) of transplanted patients during follow up developed new onset diabetes after transplantation (NODAT). Infections were the most common cause of morbidity (50.8%) followed by: ischemic heart disease (38.5%), gout (10.8%), NODAT (6.0%), peripheral vascular disease (3.1%), cerebrovascular accidents (3.1%), peripheral neuropathy (3.1%), depression (1.5%) and vertebral fractures (1.5%).

There was no difference in survival by ethnicity (p=0.870) (Figure 1). The renal replacement modality of participants who had died at follow-up included: 38% transplant (n=9), 16% peritoneal dialysis (n=4), 38% hemodialysis (n=9) and 8% had no data (n=2). Overall, sepsis was a major cause of mortality in our study group, seen in 9 of 14 participants (64%) in whom a cause of death could be identified. More participants with a CCS≥1 at baseline were on dialysis than had been transplanted at follow up (p=0.035) (Table 3). Participants with coronary calcification had a higher numerical probability of dying (43.3% versus 30.6%; p=0.213).

Table 1: Characteristics of the study population at baseline and follow-up

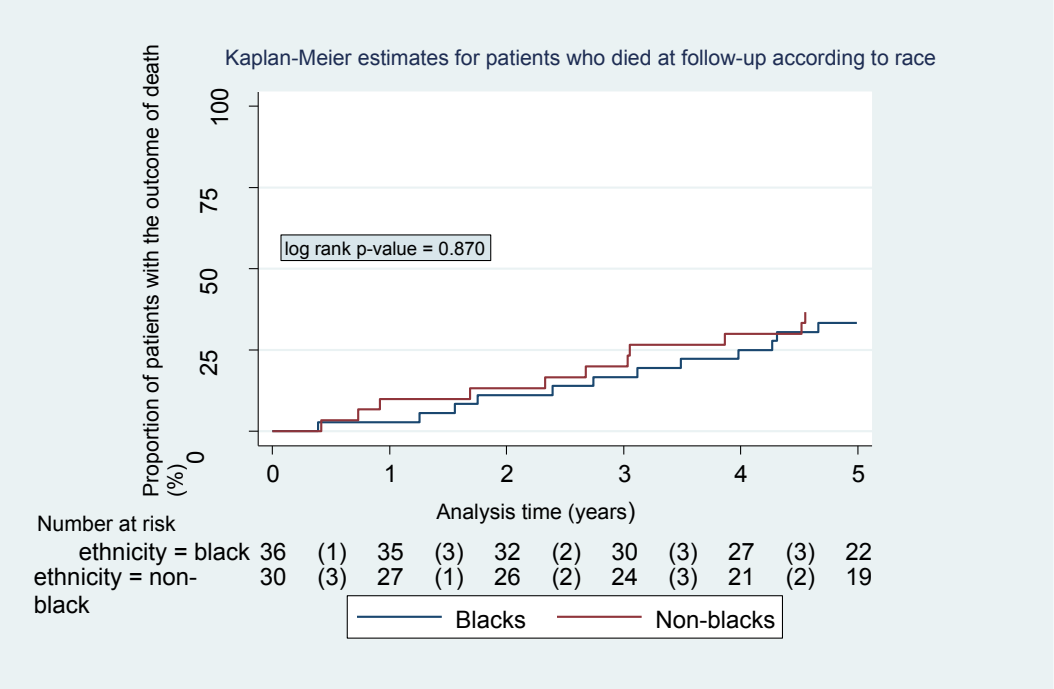
CHARACTERISTIC	BASELINE (74)		FOLLOW-UP (66)		P-VALUE
AGE, median (IQR)	42.1 (32.7 – 49.3)		46.6 (37.6—59.2)		<0.0001 °
WOMEN n, %	42 (56.8)		38 (57.6)		0.922 †
BLACKS n, %	40 (54.1)		36 (54.5)		0.954 †
RENAL REPLACEMENT STATUS, n (%)					
HD			28 (42.4)		
PD			5 (7.6)		
Transplant			33 (50.0)		
ALIVE, n (%)					
All			42 (63.6)		
HD			17 (40.5)		
PD			1 (2.4)		
Transplant			24 (57.1)		
BMI					
All	24.3 (±4.1)		27.1 (±7.1)		0.015 ‡
Transplanted	24.6 (±3.4)		27.4 (±8.3)		0.100 ‡
Dialysis	24.1 (±4.7)		26.6 (±5.1)		0.008 ‡
DM INCIDENCE					
All			4 (6.0)		
Transplant			4 (12.1)		
Dialysis			0		
Blacks			4 (11.1)		0.131 †
Non-Blacks			0		
ABDO CALC (mean, SD)(mode)					
Blacks	0.4 (±1.3)	0 - 5	0.6 (±1.9)	0 - 8	1.000 ‡
Non-Blacks	4.9 (±7.1)	0 - 19	5.6 (±6.9)	0 - 22	0.066 ‡
LVH					
SL Score corrected for BMI ($mm*kg,m^2$)	939 (±376)		671 (±280)		<0.001 ‡
SL Score corrected for BMI Blacks	926 (±364)		696 (±334)		0.068 ‡
SL Score for BMI Non-Blacks	954 (±407)		666 (±215)		0.002 ‡

° Mann-Whitney U test

‡ Student's t-test

† Z-test

Figure 1: Kaplan – Meier estimates of patients who had died at follow-up according to race



All females and 85% of male participants had truncal obesity at follow up. [21] The median abdominal circumference of males was 96.5cm (IQR: 93–102.5cm), 2.7% above the African normative values. Females had a median abdominal circumference of 101.5cm (IQR: 90 -108 cm), 27% above normative African values . There was no difference in abdominal obesity between Blacks and non-Blacks (p=0.254). The median BMI at follow up for participants who developed NODAT was 23.4kg/m^2 (19.5-27.2) compared to those who did not develop NODAT 27.5kg/m^2 (23.7-31.4) (p=0.095). A mean weight gain of 1.1kg (1.5) was observed among

participants who developed NODAT compared to 4.8kg (3.4) (p=0.072) in participants who did not develop NODAT. In our study group, only Black participants developed NODAT (Table 1).

At baseline non-Blacks had a higher numerical median FGF-23 compared to Blacks (p=0.513). Study participants who had higher FGF-23 at baseline had a higher probability of death that approached significance (p=0.075). FGF-23 could not be measured at follow up.

Using normative values among an age-stratified South African population, higher median values of PWV were observed among participants in the age groups below 60 years compared to normative values (Table 2).[24] A positive correlation between PWV and abdominal calcification was observed at follow up (r=0.5, p=0.047) Baseline CASP was not a predictor of outcome at follow up, baseline mean CASP was 134.5mmHg (±24.1) for participants alive at follow up compared to baseline CASP of 132.1mmHg (±29.2) in those who had died at follow up (p=0.723).

Table 2: Pulse wave velocity at follow up compared to African normative values

Pulse wave velocity	Normative Values[24]	Participants	
Males & Females	Value 50th (10th 90th)	Value 50th (n) (10th 90th)	
20-29 years	6.1 (5.3, 7.10)	6.6 (7)	(5.3 ,8.6)
30-39 years	6.4 (5.2,8.00)	7.3 (7)	(4.9, 13.1)
40-49 years	6.9(5.9,8.60)	8.1 (14)	(6.3, 10.4)
50-59 years	8.1(6.3,10.0)	8.8 (5)	(6.5, 12.3)
60-69 years	10.3(9.7,13.1)	8.1 (1)	

Table 3: Patient characteristics at follow up, stratified by baseline coronary calcium scores (CCS)

CHARACTERISTIC	CCS = 0	CCS ≥ 1	P-VALUE
Current status, n (%)			
Haemodialysis	12/36 (33.3)	15/27 (55.6)	0.015 ®
Peritoneal dialysis	1/36 (2.8)	4/27 (14.8)	
Transplant	23/36 (63.9)	8/27 (29.6)	
Vital status, n (%)			
Alive	25/36 (69.4)	14/27 (51.9)	0.155 ¥
Dead	11/36 (30.6)	13/27 (48.1)	
Complications, n (%)			
Cardiovascular diseases	14/36(38.9)	16/30(53.3)	0.241 ¥

Parathyroidectomy	4/36 (11.1)	5/30 (16.7)	0.721 ®
Gout	3/36 (8.3)	4/30 (13.3)	0.511 ®
Infections	16/36 (44.4)	17/30 (56.7)	0.323 ¥
Peripheral neuropathy	1/36 (2.8)	1/30 (3.3)	
Depression	1/36 (2.8)	0	-
Vertebral fracture	1/36 (2.8)	1/30 (3.3	-
SL score corrected for BMI, mean (±SD)	720.3 (±336)	625.1 (±193)	0.289 ‡

‡ Student's t-test, ¥ Chi-square, ® Fishers exact test

Discussion

To our knowledge, this is the first follow up study in sub-Saharan Africa assessing the progression of vascular calcification among ESRD patients. Due to resource limitations, stringent criteria are applied to assess eligibility for state funded dialysis and subsequent transplantation. For example, patients >60 years and diabetics >50 years, morbidly obese patients and diabetics with significant target organ disease are not accepted. This explains our relatively young, non-diabetic, non-obese study population (Table 1).

In this study we found that non-Black patients had a trend to greater progression of vascular calcification. While the sample size is small, this is consistent with numerous studies in the developed world that have shown that ethnicity is a risk factor for the progression of vascular calcification with slower progression among Blacks.[26,27]

Blacks in our study group had lower parathyroidectomy rates. However, studies in the USA have found higher parathyroidectomy rates among Blacks compared to non-Blacks. [28,29] This contrary finding is not explained and could be confounded by the small sample size.

BMI significantly increased during the study period, likely due to the effects of transplantation as well as improved nutrition after the continuation of effective dialysis and amelioration of uremic symptoms. All females and 85% of males met African criteria for abdominal obesity.[21]

Obesity is regarded as a risk factor for cardiovascular disease, reduced patient survival, graft rejection and loss as well as death among patient's organ transplant recipients. [30,31,32] NODAT developed in 12.1% of the patients who had received a kidney transplant and they were all Black (p=0.131). This finding is consistent with published data where NODAT has been reported to occur in 2-53 % of all solid organ transplants and is a serious and common complication of kidney transplantation. [33] [34] In our group, weight gain was not a risk factor of developing NODAT: 1.1kg (±1.5) vs 4.8kg (±3.4) p=0.072. Known risk factors for developing NODAT are multifactorial and include race, age of recipient, male sex, family history of diabetes mellitus, genetics, rejection history and type of immunosuppressant therapy prescribed. [33]

LVH is a poor outcome predictor among patients on hemodialysis and in transplant patients. [35,36] The significant regression in LVH among dialysis and transplant patients at follow up could possibly be ascribed to BP control and management of hyperparathyroidism.[37,38] Left ventricular remodeling is thought to occur after renal transplant, effecting systolic and diastolic function, but the evidence is not uniform. [39] After a follow up of 2 years, Rajan et al observed that kidney transplant had no association with significant regression of left ventricular mass index using cardiac magnetic resonance imaging compared to patients on the waiting list.[40] However, others report that renal transplant is associated with significant LVH regression. [41, 42] The LVH regression among our study participants was not found to be solely due to renal transplantation.

Studies in the developed world have shown a 'survival paradox' in Blacks compared to non-Blacks. [43,44,45] We did not find a difference in survival in Blacks in our cohort despite the absence of progressive vascular calcification in them. Recent studies in the USA have suggested that this apparent survival advantage is less significant in age groups above 40 years.[14] A larger sample size would be needed to accurately assess this possible survival advantage in our predominantly young, Black African dialysis population. However, among participants with a known cause of death, the majority died of sepsis (64%) which is consistent with studies done in Brazil and sub-

Saharan Africa.[46, 47] This is likely to have obscured any difference attributable to cardiovascular causes.

High levels of vascular stiffness are associated with CKD and dialysis dependency [48, 49]. Higher PWV values in participants at follow up, compared to available normative age-stratified ranges, were found.

The study should be viewed in the context of its potential limitations. The sample size is small and medical data was collected from patient files thus some data could have been missing or not documented. The selection criteria for participation in the state dialysis program is rigorous due to limitation of resources and most patients are from a low-socio economic background; this could affect the replicability of these results in other cohorts [50]. Although it was a single center study this was a longitudinal study with a strong follow up rate of 87.8%.The same investigators assessed certain clinical parameters at baseline and follow up which included abdominal X-rays and ECGs to reduce inter-observer variability.

In conclusion, the study describes a majority of less than 50 years old, Black, ESRD population in a resource constrained setting. Increased vascular stiffness and metabolic risk factors, were highlighted and sepsis was a major cause of death among all participants. The differences in coronary calcification progression and parathyroidectomy rates between Blacks and non-Blacks suggest ethnic variances in mineral metabolism among patients with ESRD. Further research, in a larger cohort, is needed to understand these differences and inform and improve management of all patients with CKD.

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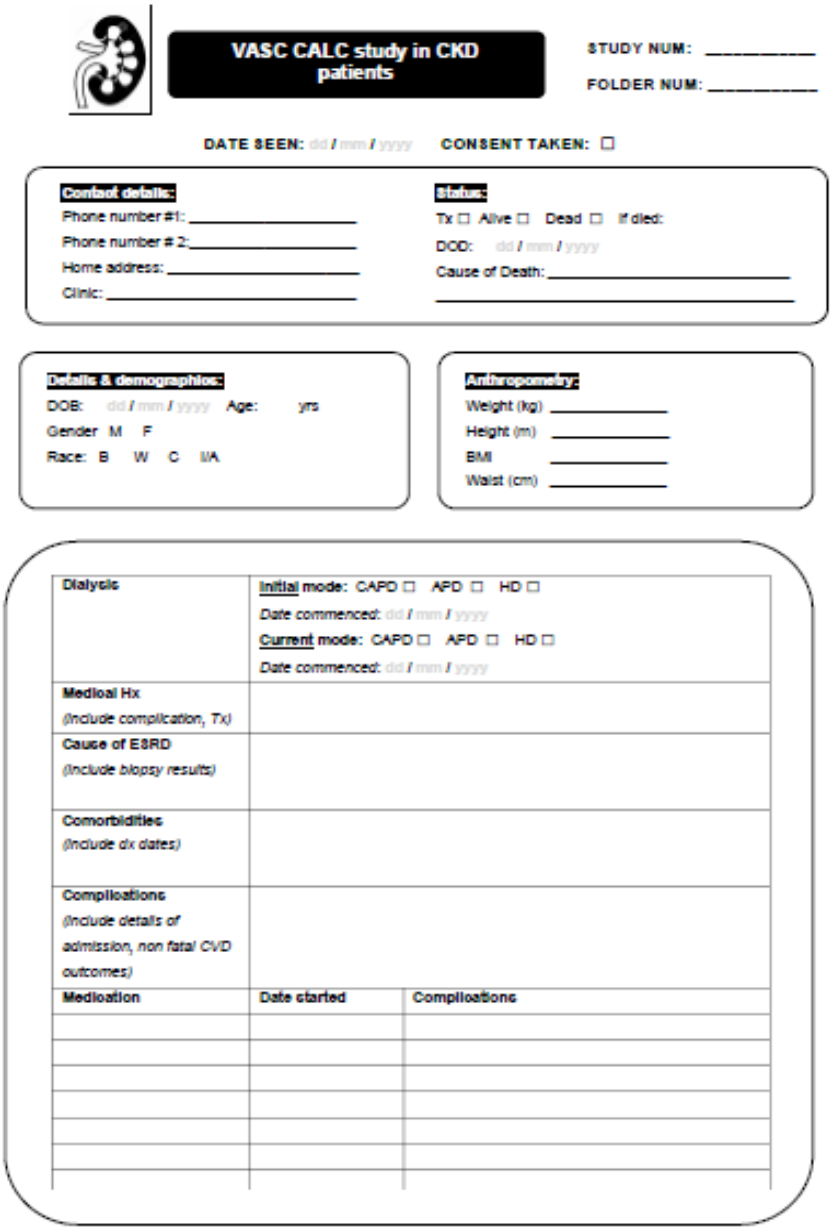
APPENDIX

Appendix 1 - Data Collection Sheet – page 1



VASC CALC study in CKD patients

STUDY NUM: _____

FOLDER NUM: _____

DATE SEEN: dd / mm / yyyy CONSENT TAKEN: ☐

Contact details:
Phone number #1: _____
Phone number # 2: _____
Home address: _____
Clinic: _____

Status:
Tx ☐ Alive ☐ Dead ☐ If died:
DOD: dd / mm / yyyy
Cause of Death: _____
_____

Details & demographics:
DOB: dd / mm / yyyy Age: yrs
Gender M F
Race: B W C IA

Anthropometry:
Weight (kg) _____
Height (m) _____
BMI _____
Waist (cm) _____

Dialysis	**Initial mode:** CAPD ☐ APD ☐ HD ☐ *Date commenced:* dd / mm / yyyy **Current mode:** CAPD ☐ APD ☐ HD ☐ *Date commenced:* dd / mm / yyyy	
Medical Hx *(Include complication, Tx)*		
Cause of ESRD *(Include biopsy results)*		
Comorbidities *(Include dx dates)*		
Complications *(Include details of admission, non fatal CVD outcomes)*		
Medication	**Date started**	**Complications**

Appendix 1 – Data Collection sheet – page 2

Investigations	Initial results	Final results
ECG		
ECH		xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
ABN CALC SCORE		
CARD CT CALC SCORE		xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
PWV	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx	
CASP	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx	

Blood results	Initial results	Final results
HB		
MCV		
PLT		
WCC		
Na		
K		
Creatinine		
GFR (MDRD Formulae)		
Phosphate		
Calcium		
ALP		
PTH		
Cholesterol		

Other tests	
HIV status	CD4: VL:
HbA1c	
Hep B	
Hep C	

Appendix 2 – Ethics Approval letter

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • Facsimile [021] 406 6411
Email: [redacted]
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

10 March 2016

HREC REF: 048/2016

Prof E Rayner
E-13 Renal Unit
NGSH

Dear Prof Rayner

PROJECT TITLE: THE IMPACT OF VASCULAR CALCIFICATION AMONG DIALYSIS DEPENDENT SOUTH AFRICAN CKD PATIENTS. A FIVE YEAR FOLLOW UP COHORT. (CARDIOVASCULAR MORTALITY AND MORBIDITY, ETHNIC VARIATION AND HEMODYNAMIC CORRELATES) (MMed CANDIDATE – Dr K Simba)

Thank you for your response letter dated 06 March 2016, addressing the issues raised by the Human Research Ethics Committee (HREC).

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until the 30th March 2017.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please quote the HREC REF in all your correspondence.

We acknowledge that the student, Dr Kudakwashe Simba will also be involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval before the research may occur.

Yours sincerely

Signature Removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRE00001938

HREC 048/2016