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Cardiovascular risk factors and their association with biomarkers in children with chronic kidney disease in Johannesburg, South Africa

Mudi, Abdullahi

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**CARDIOVASCULAR RISK FACTORS AND THEIR ASSOCIATION WITH BIOMARKERS
IN CHILDREN WITH CHRONIC KIDNEY DISEASE IN JOHANNESBURG,
SOUTH AFRICA.**

Abdullahi Mudi



A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand,
in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, 2017

DECLARATION

I, Abdullahi Mudi declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

A handwritten signature in cursive script, appearing to read 'Mudi', is written above a horizontal line.

Signature

2nd day of November 2017

Dedicated to children

with kidney diseases

PUBLICATIONS AND PRESENTATIONS ARISING FROM THE STUDY

Publications:

The following manuscripts have been published in peer-reviewed journals

1. Mudi A, Dickens C, Levy C, Ballot D. Cardiovascular risk factors and mortality in children with chronic kidney disease. **SAMJ**. 2017;107(8):710-714.
2. Mudi A, Ntsinjana H, Dickens C, Levy C, Ballot D. Cardiac changes and their association with Fetuin-A and Fibroblast growth factor-23 in children with chronic kidney disease. **Nephron**. 2017;136(3):233-242.

The remaining two manuscripts have been submitted to peer-reviewed journals and are currently under review

1. Mudi A, Holland Z, Dickens C, Levy C and Ballot D. Carotid intima media thickness in South African children with chronic kidney disease
2. Mudi A, Dickens C, Levy C and Ballot D. Fibroblast growth factor-23 and Fetuin-A gene relationship in black South African children with chronic kidney disease

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2. Mudi A, Holland Z, Dickens C, Levy C and Ballot D. Carotid Intima Media Thickness in Children with Chronic Kidney Disease. The American Society of Nephrology (ASN) Kidney week 2016, Chicago, USA, November 2016, TH-PO602, Abstract Supplement, *J Am Soc Nephrol* 2016; 27: 231A
3. Mudi A, Dickens C, Ballot D and Levy C. Fetuin-A and fibroblast growth factor-23 relationship in children with chronic kidney disease. The World Congress of Nephrology (WCN) 2017, Mexico city, Mexico. WCN17-0617, abstract booklet. (Poster accepted for presentation in April 2017)
4. Mudi A, Dickens C, Ballot D and Levy C. Alpha-2 Heremans-Schmid glycoprotein (AHSG) gene polymorphism in children with chronic kidney disease. The World Congress of Nephrology (WCN) 2017, Mexico City, Mexico. WCN17-1025, abstract booklet. (Poster accepted for presentation in April 2017)

ABSTRACT

Background: In spite of the contributions of cardiovascular disease (CVD) to morbidity and mortality in chronic kidney disease (CKD) worldwide, there are no studies that have looked at cardiovascular risk factors (CVRFs) and their association with cardiovascular changes in African children with CKD. Several CVRFs have been implicated in the initiation and progression of cardiovascular changes in children with CKD, and these changes have been reported even in early CKD. This study investigated CVRFs and their association with cardiovascular changes in South African children with CKD.

Method: This comparative cross sectional study recruited children (5-18 years) with CKD being followed up at the Division of Paediatric Nephrology of the Charlotte Maxeke Johannesburg Hospital and the Chris Hani Baragwanath Academic Hospital. One hundred and six children with a spectrum of CKD including those on chronic dialysis (34 CKD I, 36 CKD II-IV and 36 CKD V-dialysis) were enrolled over a 12 month study period. All patients had a short history taken along with a physical examination. Blood samples for serum creatinine, urea, albumin, calcium, phosphorus, parathyroid hormone (PTH), alkaline phosphatase, total cholesterol, haemoglobin and C-reactive protein, Vitamin D, Fibroblast growth factor-23 (FGF-23), Fetuin-A and genomic DNA studies were taken. Where feasible, transthoracic echocardiography and high resolution ultrasonography of the common carotid artery was performed.

Results: The overall median age of the patients was 11 years (8-14 years), with a male female ratio of 2.1:1. Several CVRFs detected include hypertension, proteinuria, anaemia, hypercholesterolaemia and dysregulated mineral bone metabolism. The most common CVRF detected was anaemia (39.6%) and its

prevalence was highest in the dialysis group when compared with the other CKD groups. The overall median (range) cIMT was 0.505mm (0.380-0.675), and was highest in patients with dialysis dependant CKD ($p=0.003$). The distribution of left atrial diameter (LAD) and left ventricular mass (LVM) differed significantly ($p<0.05$) across the different CKD groups. Abnormal LAD was seen in 10% of patients; left ventricular hypertrophy (LVH) in 27%; left ventricular systolic dysfunction in 6% and diastolic dysfunction in one patient. Mean arterial pressure and haemoglobin levels were independently associated with cIMT; hypertension was independently associated with concentric LVH; and age and hypoalbuminaemia were independently associated with eccentric LVH. Overall, the dialysis group had the highest prevalence of vascular changes, cardiac changes and associated risk factors.

A skewed pattern of Fetuin-A and FGF-23 levels with medians (range) of 57.7 (0.9-225.2) mg/dL and 28.9 (0-3893.0) pg/ml respectively, were observed. The levels of these two biomarkers varied significantly between the different CKD groups ($p<0.05$). Fetuin-A was independently associated with abnormal LAD but no similar relationship with other cardiovascular changes and plasma levels of Fetuin-A and FGF-23 was found. Plasma FGF-23 levels correlated better with markers of bone mineralization than Fetuin-A. Eight Fetuin-A SNPs were analysed; rs2248690, rs6787344, rs4831, rs4917, rs4918, rs2070633, rs2070634 and rs2070635. We found an association between log-transformed Fetuin-A levels and the SNP rs4918 G-allele compared to the rs4918 C-allele ($p=0.046$) and the rs2070633 T-allele when compared to the rs2070633 C-allele ($p=0.015$). Markers of MBD such as phosphate and PTH levels were associated with Fetuin-A SNPs. The rs6787344 G-allele was

significantly associated with phosphate levels (0.042), and the rs4918 G-allele with PTH ($p=0.044$).

Seven deaths were recorded in the dialysis group during the study period and severe hypertension and intracranial bleed were the most common causes of death. Modifiable risk factors such as increased total cholesterol (TC) and decreased albumin levels were more commonly seen among the deceased dialysis patients.

Conclusion: A high prevalence of CVRFs and cardiovascular changes were observed in the study groups, even in those with mild to moderate disease. Information obtained from the study highlights the need to address modifiable CVRFs such as hypertension, anaemia and hypoalbuminaemia in children with CKD and also the need to determine new, population specific, paediatric reference values for cIMT in healthy African children. Finally, the study was able to demonstrate differences in the relationship between Fetuin A SNPs and Fetuin-A levels and cardiovascular changes in our study population when compared with previously published data. We postulate that these differences may be due to genetic differences between our population and other population groups previously studied.

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NOMENCLATURE

ABPM	Ambulatory blood pressure monitoring
AHSG	Alpha-2 Heremans-Schmid glycoprotein
BMI	Body mass index
BP	Blood pressure
BSA	Body surface area
CAD	Coronary artery disease
CAKUT	Congenital anomalies of the kidney and urinary tract
CaxP	Calcium-phosphate product
CCA	Common carotid artery
CDC	Centre for disease control
CH	Concentric hypertrophy
cIMT	Carotid intima media thickness
CKD	Chronic kidney disease
CKiD	Chronic kidney disease in children study
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CRP	C-reactive protein
CVD	Cardiovascular disease
CVRF	Cardiovascular risk factors
DNA	Deoxyribonucleic acid
DBP	Diastolic blood pressure
E/A	Trans-mitral flow velocity ratio
EDTA	Ethylenediaminetetraacetic acid
E/E'	Index for LV filling pressure
EF	Ejection fraction
EH	Eccentric hypertrophy
EPO	Erythropoietin
ESA	Erythropoiesis stimulating agent
ESR	Erythrocyte sedimentation rate
ESRD	End stage renal disease
FGF-23	Fibroblast growth factor-23
FS	Fractional shortening
GFR	Glomerular filtration rate
Hb	Haemoglobin
HD	Haemodialysis
HDL	High density lipoprotein
IDWG	Inter-dialytic weight gain
IMT	Intima media thickness
IQR	Inter-quartile range
IVSD	Intraventricular septum thickness at end diastole

KDIGO	The Kidney Disease: Improving Global Outcomes
KDOQI	Kidney Disease Outcomes Quality Initiative
LAD	Left atrial diameter
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein-cholesterol
LV	Left ventricle
LVEDD	Left ventricular end diastolic diameter
LVESD	Left ventricular end systolic diameter
LVH	Left ventricular hypertrophy
LVM	Left ventricular mass
LVMi	Left ventricular mass index
LVPWD	Left ventricular posterior wall thickness at end diastole
MAP	Mean arterial pressure
MBD	Mineral bone disease
NAPRTCS	North American Pediatric Renal Trials and Collaborative Studies
NHLS	National Health Laboratory Service
PCR	Polymerase chain reaction
PD	Peritoneal dialysis
PI	Pulsatility index
PP	Pulse pressure
PTH	Parathyroid hormone
PUJ	Pelvic-ureteric junction
PUV	Posterior urethral valve
REDCap	Research electronic data capture
RFLP	Restriction fragment length polymorphism
RI	Resistance index
RRT	Renal replacement therapy
RWT	Relative wall thickness
SBP	Systolic blood pressure
SNP	Single nucleotide polymorphism
TC	Total cholesterol
TNF- α	Tissue necrotic factor-alpha
TRL	Triglyceride rich lipoprotein
uPCR	Urine protein-creatinine ratio
VUJ	Vesico-ureteric junction
VUR	Vesico-ureteric reflux
WHO	World Health Organisation
25(OH)D	25-Hydroxyvitamin D
1,25(OH)D	1, 25-Hydroxyvitamin D

PREFACE

This study was undertaken to determine the prevalence of cardiovascular risk factors (CVRFs) and their association with cardiovascular changes in children with chronic kidney disease. In addition, this study also determined the relationship between Fetuin A SNPs with Fetuin-A levels and cardiovascular changes in the study group.

The researcher was involved in patient recruitment, clinical examination, Fetuin-A and FGF-23 assays, DNA extraction, RFLPs, and likewise analysis and interpretation of all results. Where feasible, the researcher was also involved with echocardiogram and cIMT measurements.

This PhD is presented in a divided block format consisting of seven chapters. In this format, the results are presented in the form of submissible manuscripts (chapters 3 to 6).

Chapter 1-2 addresses the literature review and the methods

Chapter 3 addresses the results on the prevalence of cardiovascular risk factors and their association with mortality

Chapter 4 addresses the results on the prevalence of increased cIMT and their association with CVRFs including Fetuin-A and FGF-23.

Chapter 5 addresses the results on prevalence and types of cardiac changes and their association with CVRFs including Fetuin-A and FGF-23.

Chapter 6 addresses the results on the Fetuin-A and FGF-23 relationship in CKD and the relationship between Fetuin-A gene polymorphisms and their association with Fetuin-A levels and CVRFs.

Chapter 7 addresses the summary of the findings, the recommendations and the study limitations

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

The prevalence of Chronic Kidney Disease (CKD) continues to increase worldwide, and it has been implicated to be a major risk factor for cardiovascular disease (CVD).(1)

The true incidence and prevalence of CKD in Africa in general and in South Africa in particular is unknown. Even in the first world, given that CKD in its initial stages is asymptomatic, most reports in children focus on patients with ESRD and requiring renal replacement therapy (RRT) and are therefore likely to underestimate the true prevalence of CKD.(2) This makes it difficult to compare the prevalence of CKD in adults and children.

The Italkid study, probably the largest report of its kind, puts the mean incidence of CKD in children to be 12.1 cases per million age-related population (pmarp) with a prevalence of 74.7 pmarp,(3) and in the most recent registry of The European Society for Paediatric Nephrology, The European Renal Association and European Dialysis and Transplantation Association, the overall incidence of end stage renal disease (ESRD) in children in Europe was reported as 5.2 pmarp.(4)

In Africa, poor data quality from most countries and limited RRT restricts interpretations of the prevalence of CKD.(5, 6) Except for Sudan and South Africa, the RRT rate is <20 pmarp for most African countries.(6)

There are no reliable statistics about the overall prevalence of CKD in children in South Africa.(6) The South African renal registry only provides details about patients on RRT indicating the burden of end stage renal disease (ESRD) among the South

African population and does not differentiate the prevalence in children from adults. In its most recent report, the registry highlights the increase in the prevalence of patients on RRT from 167 to 178 pmarp.(7) The increase in prevalence was attributed to the increase in number of treatment centres that contributed to the data and this may not necessarily reflect a true increase in the prevalence of patients with ESRD.

Bhimma *et al.* found the incidence of ESRD in KwaZulu-Natal to be 1–2 pmarp.(8) This is less than half the reported incidence in Europe and possible explanations for this discrepancy include a lack of adequate clinical skills, adequate laboratory services and radiography facilities when it comes to diagnosing and reporting CKD in children in South Africa.

Data from North America demonstrates that there are fewer children with ESRD compared to adults and, although no such studies exist in South Africa, it is likely that the patient load will be approximately the same.(9)

The Division of Paediatric Nephrology of the Department of Paediatrics of the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), from which the majority of the patients were recruited for the study, provides the largest paediatric dialysis service in the country. The Division of Paediatric Nephrology at Chris Hani Baragwanath Academic Hospital (CHBAH), the third largest hospital in the world, runs a very large inpatient service, as well as a large outpatient service. However, for historical reasons, the Division of Paediatric Nephrology at Chris Hani Baragwanath Academic Hospital has a very limited capacity to provide long term paediatric dialysis, and so most of the children with ESRD from CHBAH end up being managed, dialysed and transplanted at CMJAH. At CMJAH, in addition to a large

outpatient service, in which around 250 children are seen per year, the division has 10 haemodialysis machines dedicated for paediatric use.

At the time of the study the division was dialysing 35 children with chronic ESRD. Unpublished data from our unit has shown that, of all the children with ESRD on chronic dialysis managed by the unit, up to 60% are no longer transplantable! One of the major reasons for having to be delisted from our transplant program was cardiovascular disease in the form of irreversible myocardial dysfunction.

Cardiovascular disease is thought to begin early in renal failure and progress rapidly on dialysis.(10, 11) Cardiovascular disease was found to be the most common cause of death among paediatric patients with end stage renal disease (ESRD). (12, 13) Various cardiovascular risk factors (CVRFs) including FGF-23 and Fibroblast growth factor-23 (FGF-23) have been implicated in the initiation and progression of cardiovascular changes observed in children with CKD.(14-18) The prevalence of these CVRFs in children with CKD has been on the increase and hypertension remains the single most important risk factor as it accounts for left ventricular hypertrophy (LVH), vascular damage and vascular remodelling.(13) Dysregulated bone metabolism and chronic inflammation have been associated with vascular changes and calcification especially in children on dialysis.(19, 20)

There are no documented African studies that have looked into these cardiovascular risk factors in children with CKD. For this reason, this study looked at risk factors for CVD in children with CKD and determined whether these risk factors are associated with cardiovascular changes.

1.2 Background on CKD

Chronic kidney disease (CKD) simply refers to a state of irreversible kidney damage and/or reduction of kidney function that can lead to a future decrease in kidney function. The Kidney Disease: Improving Global Outcomes (KDIGO) 2012 clinical practice guideline for evaluation and management of CKD defines children with CKD based on fulfilling one of the following clinical criteria:(1)

- Glomerular filtration rate (GFR) of less than 60 mL/min per 1.73 m² for greater than three months with implications for health regardless of whether other CKD markers are present.
- GFR greater than 60 mL/min per 1.73 m² that is accompanied by evidence of structural damage or other markers of functional kidney abnormalities including proteinuria, albuminuria, renal tubular disorders, or pathologic abnormalities detected by histology or inferred by imaging

The KDIGO guideline classifies the severity of CKD for patients greater than two years of age based on GFR as seen in Table 1.1

Clinically, the GFR is usually estimated from the serum creatinine rather than by the more difficult technique of timed urine collection for creatinine clearance, especially in children. There are various equations recommended for estimation of GFR and each one has its own limitations.(1)

Table 1.1. KDIGO classification for CKD (1)

Grade	GFR (mL/min/1.73m ²)	Terms
1	≥ 90	Normal or high
2	60 – 89	Mildly decreased
3a	45 – 59	Mildly to moderately decreased
3b	30 – 44	Moderately to severely decreased
4	15 – 29	Severely decreased
5	< 15	Kidney failure

In children, CKD is often asymptomatic especially in the early stages and hence it is felt that, in many reports, a large number of children have not been included in the epidemiological data for CKD. (3, 21)

In children, CKD may progress leading to end-stage renal disease (ESRD).(3, 21) This group of patients with ESRD will require renal replacement therapy (RRT), either in the form of dialysis or renal transplantation. Slowing the progression of CKD is of utmost importance in terms of improving quality of life and minimising the risk of complications.

Various complications occur in children with CKD. These include volume overload, hyperkalaemia, metabolic acidosis, renal osteodystrophy, hypertension, accelerated atherosclerosis, anaemia, dyslipidaemia, growth failure and stunting, neurodevelopmental impairment and psychosocial disturbances.

In low income countries where RRT is not readily available due to high cost, shortage of skilled personnel and donor organs, death often complicates CKD.(6) Besides the high mortality rate in low income settings, modifiable CVRFs in patients

on RRT such as anaemia, fluid overload, hypertension, infection, growth failure and stunting also contribute to the morbidity in paediatric CKD due to poor compliance and adherence to medication, prolonged hospitalisation, scarce resources and low socio-economic status.(8)

1.3 Chronic kidney disease and the cardiovascular system

Chronic kidney disease is a major risk factor for cardiovascular disease (CVD) and CVD is thought to begin early in renal failure and progress rapidly on dialysis.(10, 11, 22) Cardiovascular disease has been reported to be the most common cause of death among paediatric patients with end stage renal disease (ESRD).(12, 13) There are no local data in children to support the contribution of CVD to CKD associated morbidity and mortality in South Africa.

In older adults with ESRD, coronary artery disease (CAD) and cardiomyopathy are the leading cause of CVD mortality. This is not the case in children. CVD related causes of death in children that have been described up until now include cardiac arrest, arrhythmia, cardiomyopathy, cerebrovascular disease and rarely myocardial disease.(20) Atrial fibrillation (AF) is one of the most common cardiac arrhythmias seen in patients with CKD. Dialysis patients are at highest risk for AF especially those with congestive heart failure or severe dilated cardiomyopathy.(23, 24)

In our clinic, an unpublished observation indicated that cardiovascular related problems were the major reason that patients get removed from the kidney transplant list. In addition, cardiovascular related issues also contributed significantly to mortality. Cardiomyopathy associated with poor ejection fraction is the most common cardiovascular related problem observed in our patients on chronic dialysis. Less frequently, cerebrovascular disease, arrhythmias and poor vascular access have

also been noted. The above reasons prompted the researcher to investigate and provide evidence for these observations.

Various vascular changes have been reported in children with CKD.(18, 21) Some of these vascular changes include atherosclerosis, arteriosclerotic lesions, including fibrous or fibro-elastic intimal thickening, disruption of the internal elastic lamella, and vascular calcification. These vascular changes increase the risk for symptomatic CVD later in life.(25)

1.4 Cardiovascular risk factors in children with CKD

The prevalence of traditional cardiovascular risk factors (CVRFs) such as hypertension, dyslipidaemia, obesity and hyperglycaemia are increased in children with even early stages of CKD. (21, 22) Non-traditional (CKD-related) CVRFs are more evident in children with moderate to severe CKD.(10, 13, 18, 21, 25, 26) These include anaemia, fluid overload, dysregulated mineral bone metabolism (hyperparathyroidism, increased calcium-phosphate product), proteinuria, hypo-albuminaemia, inflammation (increased C-reactive protein and cytokines) and oxidative stress. Other potential risk factors are treatment-related such as calcium overload from dialysate, calcium-based phosphate binders and vitamin D therapy.

The single most important risk factor for CVD is hypertension.(21, 22, 27, 28) Nevertheless, dysregulated bone metabolism is responsible for much of the vascular calcification typical of CKD.(18, 29, 30) It is believed that calcification is widespread and begins in the pre-dialysis stage and rapidly accelerates on dialysis.(31) There is no doubt that individual CVRF play a vital role in CVD either independently or

amplified by the presence of multiple other CVRFs.(21, 25) In spite of the above information, there is a large knowledge gap due to the lack of local data on CVRFs in South African children with CKD.

Identifying these CVRFs in our population is of vital importance if we are to plan interventions to manage these CVRFs so as to prevent CVD in our young patients with CKD.

1.4.1 Hypertension

Hypertension on its own accounts for left ventricular hypertrophy (LVH), vascular damage and vascular remodelling.(21, 22, 27, 28) Hypertension in children with CKD can be as a result of complication of the disease and/or treatment, and may also be due to other causes such as essential hypertension, vascular disease (arteritis and vascular stenosis), endocrine causes and tumours.(32, 33) In children with CKD, sodium retention, fluid overload and increased renin activity are recognised causes of hypertension.(33)

Autonomic dysfunction and increased sympathetic activity have also been shown to cause hypertension in children with CKD.(34-36) In addition, the use of glucocorticoids, erythropoietin and cyclosporine-A, among other medications used in the management of children with CKD, may also cause hypertension in children with CKD.(37-39)

Hypertension remains the most prevalent CVRF reported in children with CKD. Data from the CKD in children (CKiD) study and North American Pediatric Renal Transplant Cooperative Study (NAPRTCS) demonstrated a high rate of 54% and 48% respectively, and this was seen to increase to a higher rate in ESRD (50-75%).(21, 40)

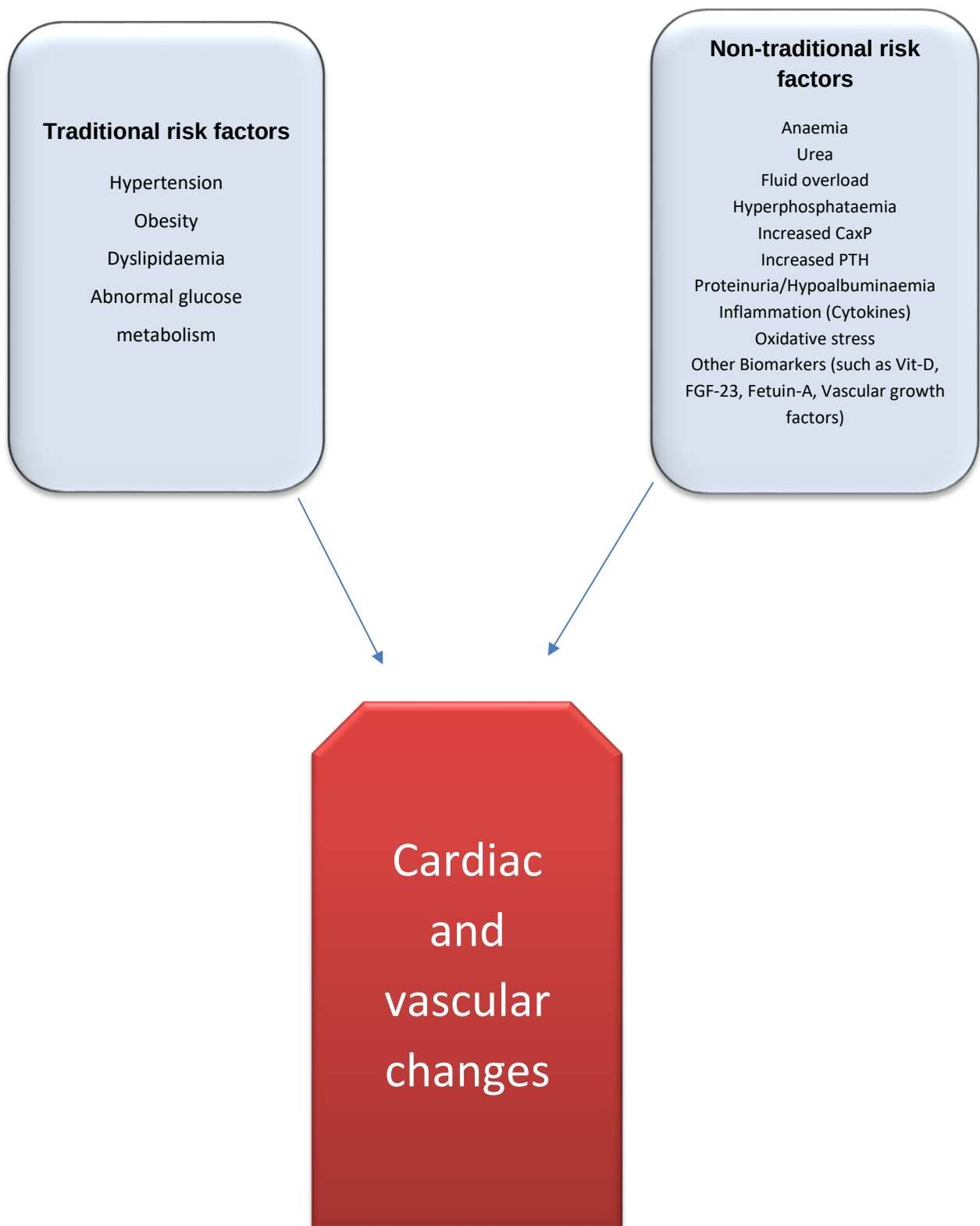


Figure 1.1: Illustrative summary of cardiovascular risk factors

Masked hypertension, which is best detected via ambulatory blood pressure monitoring (ABMP), is common among pre-dialysis CKD patients. A high prevalence of 20% was reported by Mitsnefes *et al* and was found to be associated with LVH.(41) This indicates the importance of ABMP in early CKD rather than routine BP measurement.

Various characteristics have been associated with hypertension in children with CKD. (21, 42) These include black race, glomerular cause of CKD, shorter duration of CKD, obesity, elevated serum potassium and nephrotic range proteinuria.

1.4.2 Dyslipidaemia

In children, dyslipidaemia plays an important role in the atherogenesis which is seen in CVD.(43, 44) Dyslipidaemia in CKD results from an abnormal lipoprotein metabolism and this has been reported in both pre-dialysis and dialysis CKD patients.(45, 46) Common changes observed include moderate hypertriglyceridemia, increased triglyceride-rich lipoproteins (TRL) and reduced high-density lipoproteins (HDL). Total and low-density lipoprotein cholesterol (LDL-C) remain normal or modestly increased.(47) Studies in children with CKD have reported high rates of dyslipidaemia, as high as up to 44-45%, in children with CKD and, in addition, dyslipidaemia has been documented as the second most common CVRF seen in children with CKD.(22, 46). It is therefore very important to screen for dyslipidaemia in children with CKD especially with the added risk of other associated CVRF.

1.4.3 Obesity and Under-nutrition

Obesity is an independent risk factor for CVD.(48, 49) This is a major concern as there has been a general increase in the trend of obesity all over the world, (50, 51) even though under nutrition may be seen more in our setting as confirmed by the Birth to 20 study.(52) Overweight and obesity in children are classified based on the

body mass index (BMI) for age and sex and the Centre for Disease Control (CDC) and World Health Organization (WHO) BMI centile charts are the most commonly used references when assessing childhood BMI. (53, 54)

Even though growth retardation is more often seen in children with advanced CKD, and has been reported to contribute to mortality,(55, 56) obesity has also been reported in pre-dialysis CKD children.(22) In addition, data has shown that being overweight or obese increases the risk of having other CVRF (hypertension, dyslipidaemia and abnormal glucose metabolism) when compared to lean patients.(22)

The relationship between under-nutrition in CKD and risk of cardiovascular disease is not well understood but has been attributed to chronic inflammation. Under-nutrition and inflammation are usually present in patients on maintenance dialysis.(57, 58) Chronic inflammation via several mechanisms attract pro-fibrotic factors leading to renal damage and progression of CKD.(59) Chronic Inflammation also contributes to atherosclerotic changes and endothelial injury via several mechanisms and one of these complex process is known as the malnutrition inflammation atherosclerosis (MIA) syndrome leading to cardiovascular disease.(57, 58, 60)

1.4.4 Anaemia

The decline in the production of erythropoietin (EPO) by the kidney is known to play the major role in the pathogenesis of anaemia in CKD.(61) The kidney takes over the production of EPO from the liver soon after birth and remains the major site of production.(62). Other factors that contribute to anaemia in CKD include iron deficiency, inflammation, severe secondary hyperparathyroidism leading to

myelofibrosis, marrow hypo-responsiveness, aluminium toxicity from long term haemodialysis, poor nutrition, the use of myelosuppressive agents and infection.(63)

Anaemia has been associated with poor cardiovascular outcome and an increase in morbidity and mortality in CKD.(64, 65) Studies have shown that there is an increase in quality of life with treatment of anaemia in children with CKD.(66, 67)

In the CKiD study, a high rate (45%) of anaemia was reported. They also reported that haemoglobin (Hb) levels were significantly associated with a decline in the glomerular filtration rate (GFR) in pre-dialysis CKD.(68) Similarly, other studies have shown a sharp rise in the rate of anaemia in advanced CKD children when compared to children with early or mild CKD.(65, 69)

1.4.5 Fluid overload

Fluid overload is of major concern in children with advanced CKD especially in oligo-anuric patients on chronic dialysis. In these patients, there is a decreased ability to excrete sodium and water which leads to an increase in extracellular fluid. This increase in extracellular fluid then results in an increase in cardiac output and subsequent increase in BP.(70) Fluid overload contributes to hypertension and increased strain on the heart.(33, 71) However, both fluid overload and aggressive fluid removal can lead to circulatory stress and multi-organ injury on chronic dialysis.(26) In adults on chronic dialysis, fluid overload has been reported to be an important independent predictor of mortality.(72)

Several methods have been described for the assessment of volume status in dialysis patients, but they only allow for crude detection of volume overload.(73) Inter-dialytic weight gain (IDWG) differs in physiology from the chronic overload seen in dialysis patients.(73) Inter-dialytic weight gain is also a marker of nutrition besides volume status in chronic dialysis patients and the use of IDWG alone in regulating

salt and water regulation may not be appropriate in controlling fluid status of dialysis patients.(74, 75) Furthermore, studies have shown that it is chronic overload that increases mortality and not IDWG.(73)

1.4.6 Dysregulated mineral bone metabolism

The kidney regulates calcium and phosphate excretion in the body through the help of parathyroid hormone (PTH).(76) It excretes phosphate and retains calcium in response to PTH. Parathyroid hormone also causes resorption of mineralized bone with release of calcium alongside phosphate into circulation. Active vitamin D is also produced in the kidney and causes calcium absorption from the gut.(77) A decline in renal function will lead to a decline in active vitamin D production, retention of phosphate and subsequent secondary hyperparathyroidism.(78) In addition to secondary hyperparathyroidism, long term uraemia, metabolic acidosis and FGF-23 have been found to contribute to mineral bone disorder seen in CKD. (79, 80)

A detailed definition of mineral and bone disorder (MBD) seen in CKD was recommended by KDIGO to accommodate ectopic calcification including vascular calcification;(81)

The KDIGO defined this disorder of MBD seen in CKD as a systemic disorder of mineral and bone metabolism due to CKD manifested by either one or a combination of the following:

- i. Abnormalities of calcium, phosphorus, PTH or vitamin D metabolism
- ii. Abnormalities in bone turnover, mineralization, volume, linear growth or strength
- iii. Vascular or other soft tissue calcification

The KDIGO recommends monitoring serum levels of calcium, phosphate, PTH and alkaline phosphatase for dysregulated mineral bone metabolism from CKD stage 2 in children.(82)

a. Hyperphosphataemia

Hyperphosphataemia is an independent predictor of mortality in CKD and has also been associated with an increase in carotid artery intima media thickness (cIMT), vascular stiffness, coronary calcification and left ventricular hypertrophy.(19, 83-86) A report in adult CKD patients showed that higher serum phosphate levels, even if they fell within the recommended normal ranges for phosphate levels, were associated with CVD.(87)

Studies have reported an increasing rate of Hyperphosphataemia as the GFR declines in children with CKD with rates of Hyperphosphataemia increasing from, 8.51% in CKD I all the way up to the highest rate of 43.5% in CKD V.(88, 89)

b. Increased calcium phosphate product (CaxP)

Both adult and paediatric studies have reported a strong association between increased calcium phosphate product (CaxP) and CVD in patients with end stage renal disease (ESRD).(90-92) Increased CaxP has also been associated with an increase in FGF-23, with a positive significant relationship.(93)

c. Hyperparathyroidism

Parathyroid hormone is a surrogate marker of bone turn over and levels are seen to increase in early CKD as dysfunctional mineral bone metabolism sets in. Parathyroid hormone levels increase as GFR declines(89, 94) and increased PTH levels have been associated with LVH and vascular calcification in children with CKD.(19, 64, 92)

The role of FGF-23 and vitamin D as CVRF will be discussed in the segment 'Biomarkers and CVD' (section 1.7) below.

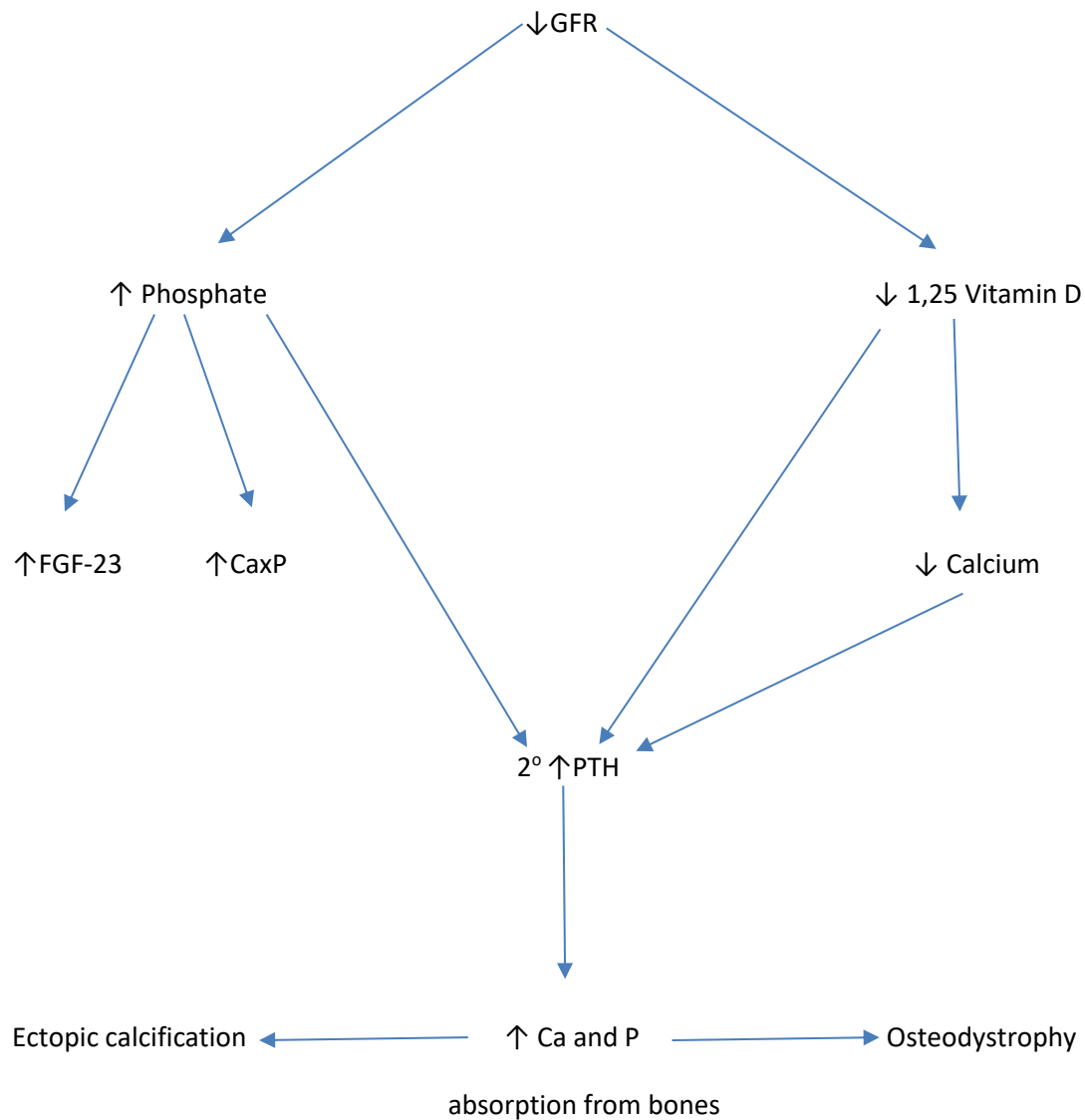


Figure 1.2: Dysfunctional mineral bone metabolism in CKD. GFR-Glomerular filtration rate, CaxP-calcium phosphate product, PTH-parathyroid hormone, Ca-calcium, P-Phosphate.

1.4.7 Proteinuria

Proteinuria is a marker of kidney injury and an important risk factor for CVD in both patients with and without CKD.(95-97) Proteinuria is thought to contribute to impaired endothelial function and atherosclerotic events in patients with CKD.(97) The risk for cardiovascular morbidity and mortality in patients with proteinuria is higher in those with low GFR ($<60 \text{ mL/min/1.73m}^2$). (98, 99)

1.4.8 Hypoalbuminaemia

Hypoalbuminaemia results from increased degradation and reduced synthesis of albumin in CKD patients, especially those with ESRD.(100) Malnutrition arising as a result of anorexia and dietary restriction is the major cause of nutritional hypoalbuminaemia in ESRD. Albumin loss also occurs in patients with nephrotic syndrome and during dialysis.(100) Inflammation also reduces albumin synthesis and increases its catabolism in dialysis patients.(101)

Adult studies have reported hypoalbuminaemia as an independent predictor of CVD in both pre-dialysis and dialysis CKD patients.(102) Hypoalbuminaemia has also been associated with poor outcome and mortality in patients with ESRD.(103, 104) Wong *et al* reported that for every decrease in albumin by 1g/dl, mortality increases by 54% in paediatric ESRD.(103)

1.4.9 Inflammation

Inflammation is an important cardiovascular risk factor in CKD, and various factors have been postulated to cause inflammation in this condition. These include cytokines, acidosis, oxidative stress, infection, dialysate contamination and an incompatible response to the dialysate membrane.(105) A decline in GFR also appears to cause a rise in the levels of circulatory cytokines and this may be because the kidney is the major site for excretion of circulating cytokines.(106, 107)

Inflammation has been implicated in the vascular injury and left ventricular abnormalities seen in CKD.(59, 105) In addition, Fetuin-A, a circulatory inhibitor of calcification, is down regulated by the inflammatory state.(108, 109)

Various markers of inflammation have been described in CVD. These include:(110, 111)

- a. Circulatory cytokines such as interleukin-1 β , -6, -8, -10 and tumour necrosis factor- α (TNF- α).
- b. Acute phase reactants such as C-reactive protein (CRP), fibrinogen and serum amyloid A (SAA)
- c. Adhesion molecules such as E-selectin, P-selectin, intracellular adhesion molecule-1 (ICAM-1), vascular and cell adhesion molecule-1 (VCAM-1).
- d. Others such as white cell count (WCC), erythrocyte sedimentation rate (ESR)

1.4.10 Oxidative stress

Oxidative stress has been defined as a “state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them.”(112) Oxidative stress has been associated with several human diseases including CVD.(113) A number of biomarkers of oxidative stress of research importance in CVD have been described.(114)

The oxidative modification of low density lipoprotein (LDL) contributes to the atherosclerotic changes seen in CVD.(115-118) A recent study in children with CKD showed a significant association between oxidised LDL (oxLDL) and left ventricular hypertrophy, hypertension and dyslipidaemia.(119)

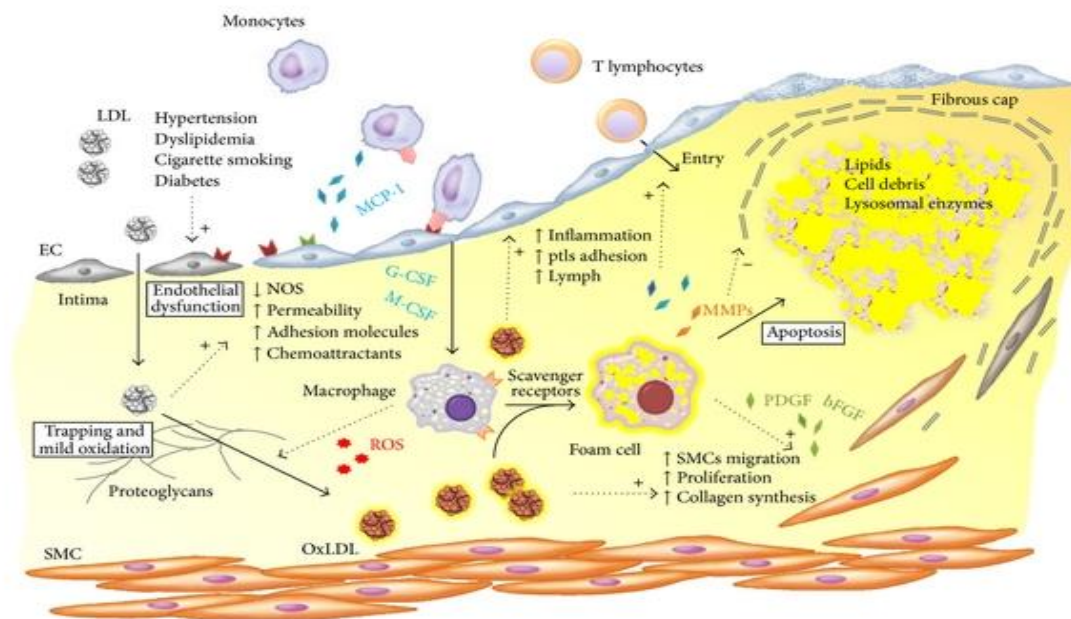


Figure 1.3: Putative pathway of oxidized low-density lipoprotein (oxLDL) in the atherogenetic process according to the oxidative hypothesis of atherosclerosis. (Reproduced under the 'Creative Commons Attribution License' from Maiolino G, Rossitto G, Caielli P, Bisogni V, Rossi GP, Calo LA. The role of oxidized low-density lipoproteins in atherosclerosis: the myths and the facts. *Mediators Inflamm.* 2013;2013:714653.) (118)

1.5 Pathogenesis of CVD in CKD

Cardiac remodelling and vascular injury have been described as the most important processes in the pathogenesis of CVD in CKD.(120, 121) A combination of cardiovascular risk factors plays an important role in the initiation and progression of these changes.(25, 122) Cardiac remodelling leads to left ventricular hypertrophy (LVH) in response to mechanical or haemodynamic overload.(121) Concentric LVH has been attributed to pressure overload due to hypertension. This pressure induced LVH results in an increase in the LV wall thickness with a less evident increase in the LV cavity. The result is an elevated relative wall thickness and concentric LVH.

Eccentric LVH is essentially thought to be related to volume overload, although sodium retention, anaemia, hypoalbuminaemia and arteriovenous shunt have also been implicated in its development.(120, 121) In eccentric LVH, the LV cavity increases together with a symmetric increase in wall thickness. The ratio between the LV transversal ratio and the wall thickness (the relative wall thickness) is maintained and the result is the development of eccentric LVH.(121, 122)

Vascular changes include plaque formation (atherosclerosis), arterial stiffening (arteriosclerosis) and vascular calcification. Atherosclerotic changes occur due to atheroma formation in the vascular intima with subsequent penetration of the vascular wall.(121) These atheromatous plaques consist of lipids, smooth muscle cells and collagen fibres. Calcification may also be part of the process and it usually involves the intima.(18, 121) Atherosclerotic changes occur in a patchy pattern along the length of the artery causing stenosis and occlusion. Arteriosclerosis involves both intimal and medial thickening along the entire arterial tree affecting arterial elasticity.(121) This process is associated with vascular remodelling resulting in an increased vessel wall thickness and lumen enlargement, ultimately leading to an increase in systolic blood pressure, pulse pressure and arterial stiffness.(86, 121) The process of vascular calcification is complex.(18) Dysfunctional mineral bone metabolism has been implicated. Hyperphosphataemia and increased calcium-phosphate products are central to the formation of this vascular calcification, and a decrease in the inhibitors of calcification such as Fetuin-A, matrix Gla-protein and osteoprotegerin, may also contribute to the whole process. (18, 86) Other metabolic, mechanical and inflammatory processes may also contribute to these changes. (115, 120, 122)

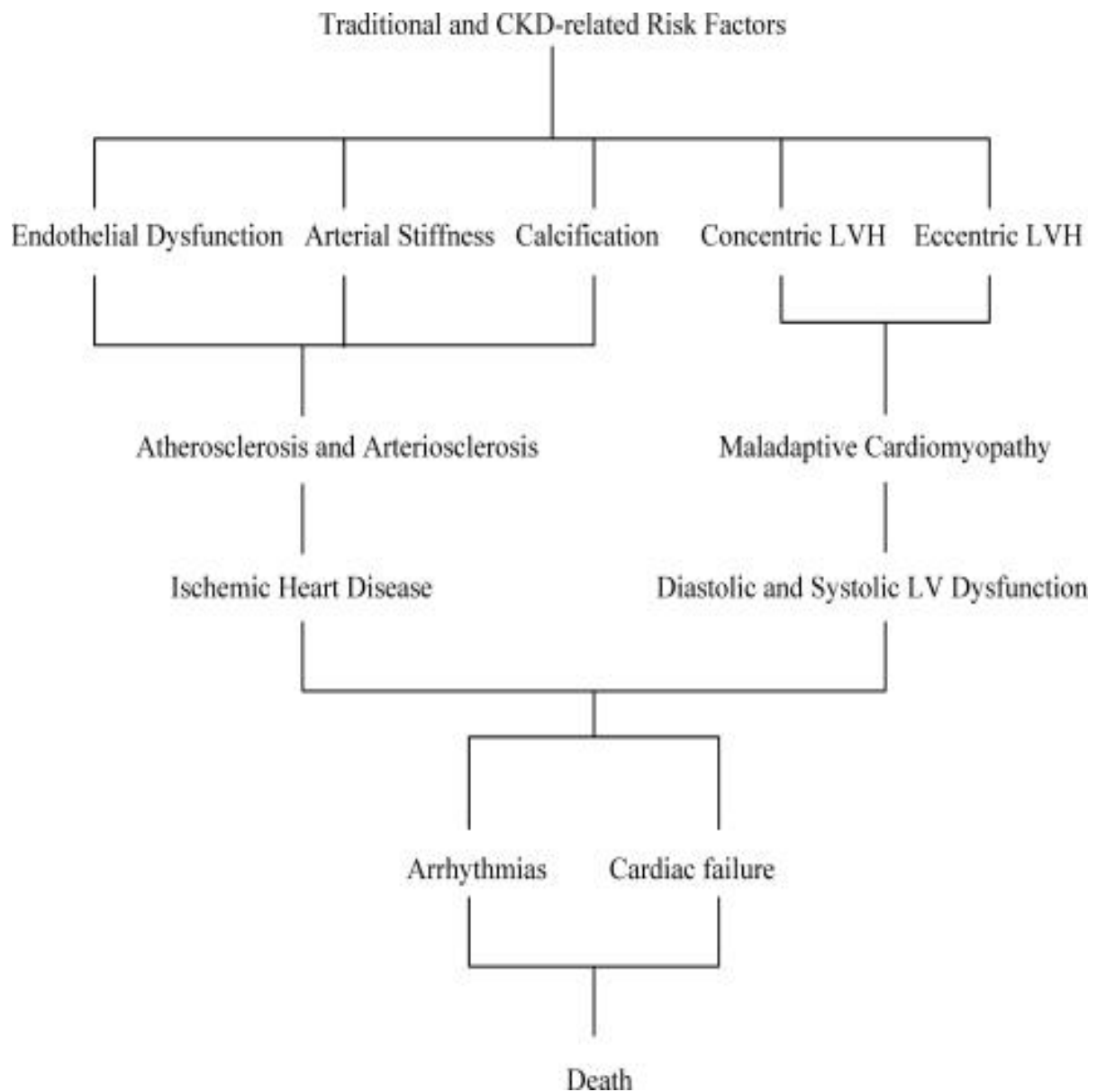


Figure 1.4: Cardiovascular disease in chronic kidney disease. (Reproduced with permission from Mitsnefes MM. Cardiovascular complications of pediatric chronic kidney disease. *Pediatr Nephrol.* 2008;23(1):27-39.)

1.6 Early markers of CVD in children with CKD

Recent research has focused attention on the detection of early cardiovascular abnormalities in children with CKD.(21, 27) Early markers of cardiomyopathy and atherosclerosis such as left ventricular (LV) abnormalities (LV hypertrophy-LVH, LV dysfunction), damage to large arteries such as stiffness, and increased intima media thickness (IMT) are said to be strong independent predictors of cardiac morbidity and mortality.(21) These changes have also been shown to begin early in pre-dialysis CKD patients and are worst in children on chronic dialysis.(21, 31, 123)

Transthoracic echocardiography is a non-invasive method that has been reliably used in the assessment of heart structure and function. Traditionally, the M-mode and the two-dimensional doppler echocardiogram have allowed for an adequate assessment of ventricular mass and volumes, the diagnosis of hypertrophy, the definition of ventricular geometric pattern and systolic and diastolic function estimation. (124-126) However, with the availability of new technology, such as magnetic resonance imaging (MRI), there is ongoing discussion about the best way to assess LVH and LV dysfunction in children.(127-131)

The methods used in this study for assessing vascular changes in CKD are discussed in the segment 'Vascular changes and CKD' below.

1.6.1 Left ventricular hypertrophy

Left ventricular hypertrophy (LVH) has been reported even in the initial stages of CKD in children and progresses as kidney function deteriorates.(64) This LVH is thought to be a compensatory mechanism by the heart to maintain function.(132) Hypertension is the main cause of LVH in both children and adults.(27, 28) However, elevated parathyroid hormone (PTH) has also been implicated in the progression of

LVH in children with stage 2-4 CKD.(133) Of note, volume overload has been associated with LVH in children on dialysis.(26)

In children, the presence of LVH implies an increase in the mass of the left ventricle and this is generally defined using the left ventricular mass index (LVMI) by which the left ventricular mass is indexed for body size by different methods related to height, weight, body surface area (BSA) and lean body mass (LBM).(129, 134) However, there are some concerns about the accuracy of this method in children, (129, 135, 136) although normal values in children have been reported.(129, 137, 138)

Left ventricular mass (LVM) is usually calculated from the following formula:(139)

$$LVM (g) = 0.8 (1.04 [(LVEDD + PWT + IVSD)^3 - (LVEDD)^3] + 0.6).$$

LVEDD-Left ventricular end diastolic diameter

PWT-Left ventricular posterior wall thickness at end diastole

IVSD-Intraventricular septum thickness at end diastole

Relative wall thickness (RWT) is a measure of concentricity and is calculated as the ratio of the posterior and septal wall to the LV diastolic diameter.(140)

$$RWT = [(IVSD + PWT) / LVEDD]$$

Four types of left ventricular geometric patterns have been described based on the ratio of the wall thickness to the cavity diameter:(140-144)

- a. Normal LV geometry-normal LV mass with normal RWT
- b. Concentric remodelling (CR)-normal LV mass with elevated RWT
- c. Concentric hypertrophy (CH)-increased LV mass and elevated RWT
- d. Eccentric hypertrophy (EH)-increased LV mass and normal RWT

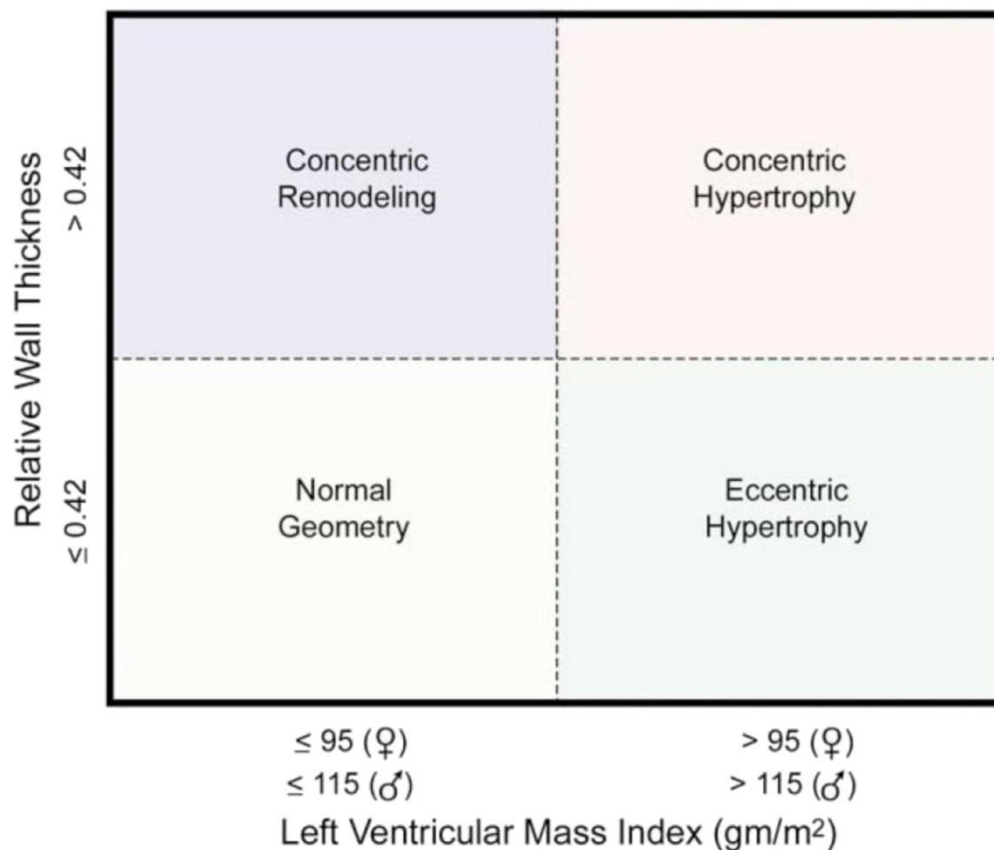


Figure 1.5: Geometric patterns of left ventricular hypertrophy based on linear measurements. (Reproduced with permission from Lang RM *et al.* Recommendations for chamber quantification: a report from the American Society of Echocardiography's Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. *J Am Soc Echocardiogr* 2005; **18**: 1440-146) (144)

1.6.2 Left ventricular (LV) dysfunction

Left ventricular dysfunction can be systolic, due to a reduction of the ejection fraction (EF) with an enlarged LV chamber. or diastolic with preserved EF and increased resistance to filling.(145) Both types of LV dysfunction can be diagnosed non-invasively with echocardiography.

The highest risk for LV dysfunction in children with CKD is seen in children on maintenance dialysis,(13, 146) and several studies have documented subtle alterations in LV wall mechanics in these children.(146, 147) Studies in children with CKD have reported the presence of systolic dysfunction in pre dialysis CKD patients

with LVH and also in dialysis CKD patients.(146-148) Mitsnefes *et al* reported diastolic dysfunction in mild to moderate CKD patients and also showed that their dialysis CKD group had a worsened dysfunction when compared to their other groups.(149)

Measured parameters, such as ejection fraction and fractional shortening, may serve as an indicator of LV systolic function, but they may be insensitive in milder degrees of systolic dysfunction or in heart failure with a preserved ejection fraction (HFEPF).(150-152) The use of trans-mitral flow velocity ratio (E/A) and the index for LV filling pressure (E/E') can be used as a measure of LV diastolic dysfunction.(153)

1.6.3 Vascular changes in CKD.

Various methods have been documented for assessing vascular anatomy and function especially in patients with CKD. Some of these methods include; measuring common carotid artery(CCA) intima media thickness (cIMT), carotid distensibility, flow mediated dilatation (FMD), and pulse wave velocity.(18) Some studies have even attempted to define paediatric reference values for these measurements.(154, 155)

a. Intima media thickness

The carotid intima media thickness (cIMT) is a validated morphological parameter reflecting vascular structural changes.(155-158) The measurement of cIMT can be achieved by high resolution doppler ultrasound of the common carotid artery (CCA).(155, 159) The values for cIMT increase with age and body dimensions.(155) It is thought that the early increase in cIMT seen in stage 2-4 CKD is due to hypertension and dyslipidaemia, while in late pre-

dialysis it is due to abnormal mineral bone metabolism.(160) Normal values in children have been reported.(155, 161-163)

b. Resistance index and pulsatility index

Changes in resistance index and pulsatility index are determined by blood flow velocity and both of them reflect downstream flow resistance.(164, 165) In addition, they are also affected by arterial stiffness and compliance.(165, 166) Resistance index has been reported to be an indirect marker of atherosclerosis similar to the cIMT.(167)

1.7 Biomarkers of CVD in CKD.

Various substances in the circulation may act as predictors of CVD risk and future CVD.(11, 18, 30, 168) These include 25-hydroxyvitamin D (25[OH]D), fibroblast growth factor 23 (FGF-23) and physiological calcification inhibitors such as Fetuin-A, Osteoprotegerin (OPG), Matrix Gla-protein (MGP) and Pyrophosphate. Other biomarkers include vascular/endothelial growth factors such as angiopoietin-1 and -2.(14, 16)

In the current study, the researcher assessed 25(OH)D, FGF-23 and Fetuin-A and how they relate to CVD risk and future CVD. The biomarkers FGF-23 and Fetuin-A were selected based on their important role in cardiovascular disease, mortality and mineral and bone disorder (MBD). In CKD patients, studies have shown that FGF-23 contributes to left ventricular hypertrophy, mortality and MBD,(80, 169, 170) while Fetuin-A has been described as the most important circulatory inhibitor of ectopic calcification and also associated with mortality.(18, 171) Although MGP and OPG are also inhibitors of calcification, Fetuin-A contributes to over 50% of circulatory inhibition of ectopic calcification.(172)

1.7.1 Vitamin D

Both 25(OH)D and 1,25(OH)₂D can have a direct effect on the vascular smooth muscle cells (VSMC).(30, 173) Studies in children have shown that a high vitamin D dose adversely affects cIMT and calcification. (23-25) In addition, children on maintenance dialysis have been shown to have a bimodal association of vitamin D levels with vascular change measurements, such that both low and high doses of vitamin D are associated with abnormal cIMT and CAC.(173)

Plasma concentrations of 25(OH)D can be measured by various methods such as liquid chromatography/tandem mass spectrometry (LC-MS/MS), high performance liquid chromatography(HPLC), ELISA, chemiluminescence and other immunoassays.(174, 175) Even though studies have compared the other methods against LC-MS/MS and found a positive correlation in terms of precision and sensitivity with other techniques (161-164), Liquid chromatography/tandem mass spectrometry is currently considered to be the gold standard for the measurement of plasma concentrations of 25(OH)D. In addition, it also has the advantage of being able to measure all the known vitamin D metabolites.(176, 177)

1.7.2 Fibroblast growth factor 23

Fibroblast growth factor 23 (FGF-23) is a phosphaturic hormone produced by osteocytes and osteoblasts, and the use of an ELISA assay to measure the plasma concentration of the carboxy-terminal (C-Term) of FGF-23 is well documented.(71, 168, 170, 171, 173). FGF-23 binds to its receptors alongside Klotho (an FGF-23 co-receptor) and causes an increase in the urinary excretion of phosphate and the inhibition of the renal production of 1,25(OH)₂D. Studies have shown that FGF-23 is independently associated with LVH, LVMI and increased cardiovascular mortality in

adult dialysis patients. (169, 170, 178). Research has also implicated FGF-23 in mineral bone disease seen in children with CKD.(80, 179)

1.7.3 Fetuin-A

Fetuin-A (α 2-Heremans-Schmid glycoprotein-AHSG) is a multifunctional glycoprotein produced in the liver, and the use of an ELISA technique to measure plasma concentrations of Fetuin-A is well documented. (11, 177-180) Other methods used to measure plasma Fetuin-A include immunoturbidimetry and nephelometry.(96, 181) Fetuin-A plays a very important role in mineral bone metabolism, inflammation and metabolic disease.(17, 180, 181) It is an important inhibitor of calcification, and contributes significantly to the calcification inhibitory capacity of human plasma.(18, 180) Studies have reported low Fetuin-A levels in dialysis patients, possibly as a combined result of reduced Fetuin-A production in a pro-inflammatory uremic milieu and an increased Fetuin-A consumption in a pro-calcific environment.(108, 171) Increased cardiovascular mortality in dialysis patients has also been linked to low Fetuin-A levels and this has been confirmed in two large multicentre trials which were appropriately designed and powered.(108, 171)

1.7.4 Fetuin-A gene polymorphism

Functional polymorphisms of the AHSG gene can lead to the alteration of the levels of Fetuin-A in the plasma. The altered levels of Fetuin-A will ultimately affect the control of ectopic vascular calcification. Several single nucleotide polymorphisms (SNPs) of the AHSG gene have been implicated in vascular calcification and stiffness seen in CKD and other diseases. (109, 182) Furthermore, other diseases that involve tissue calcification such as Alzheimer's disease and pseudoxanthoma elasticum (PXE), that involves wide spread brain calcification, have also been associated with Fetuin A gene polymorphism.(183)

Eight SNPs of interest (rs2248690, rs6787344, rs4831, rs4917, rs4918, rs2070633, rs2070634 and rs2070635) were identified based on their reported relationship with Fetuin-A levels, CVD and markers of bone mineralisation in both CKD and non-CKD patients.(109, 182, 184-189)

1.8 Summary of study and research gap

Cardiovascular disease is the most common cause of mortality and morbidity in CKD worldwide and several cardiovascular risk factors (CVRF) that contribute to the pathogenesis of CVD have been reported in both adults and children with CKD from other parts of the world. Despite the burden of disease imposed by paediatric CKD in Africa, there are no documented studies that have specifically looked into these CVRFs in African children with CKD. This knowledge gap needs to be addressed in order to prevent the initiation and progression of CVD and its associated complications in African children with CKD.

This study aimed to provide an insight into the types and prevalence of CVRFs, and their role in cardiovascular changes seen in a South African cohort of children with CKD.

1.9 Research hypothesis

1. The prevalence of CVRFs is higher in South African children with severe CKD (stage V on dialysis) when compared to children with mild (stage 1) and moderate disease (stage 2-4).
2. The prevalence of cardiovascular abnormalities is higher in South African children with severe CKD (stage V on dialysis) when compared to children with mild (stage 1) and moderate disease (stage 2-4).
3. Fetuin-A and FGF-23 are associated with CVRFs and cardiovascular abnormalities in South African children with CKD.
4. Fetuin-A gene polymorphisms are negatively associated with plasma Fetuin-A levels in South African children with CKD.

1.10 Study aim

The study aimed to determine and compare the prevalence of cardiovascular risk factors, cardiovascular abnormalities, and their association with Fetuin-A and FGF-23 in a cohort of South African children with mild (stage 1), moderate (stage 2-4) and severe (stage 5 on dialysis) CKD.

1.10.1 Objectives

1. To determine and compare the prevalence of traditional and non-traditional CVRFs (including FGF-23 and Fetuin-A) in these children.
2. To determine and compare cardiovascular abnormalities (where feasible) in these children.
3. To determine and compare the association between the risk factors and cardiovascular abnormalities identified in these children.
4. To determine and compare common Fetuin-A gene polymorphisms using polymerase chain reaction and their association with plasma Fetuin-A levels.
5. To determine the association of Fetuin-A gene polymorphisms, cardiovascular risk factors and cardiovascular abnormalities.

CHAPTER 2: MATERIALS AND METHODS

2.1 Study design and population

This was a comparative cross-sectional study that recruited 106 children with CKD who were being followed up at the Division of Paediatric Nephrology of the Charlotte Maxeke Johannesburg Academic Hospital and Chris Hani Baragwanath Academic Hospital, Johannesburg, South Africa.

2.1.1 Sample size

Sample size was calculated using a sample size formula for proportions in a comparative cross sectional study.(190) In order to detect a difference in the prevalence of cardiovascular risk factors among pre-dialysis (CKD II-IV) and dialysis (CKD V) children with CKD a sample error of 0.05, and a power of 90%, were considered.

$$\text{Number of participants required per group} = \frac{P_1(1-P_1) + P_2(1-P_2)}{(P_2-P_1)^2} \times f(\alpha, \beta)$$

P_1 = prevalence of hypercholesterolemia in children with pre-dialysis CKD, 21% (46)

P_2 = prevalence of hypercholesterolemia in children with dialysis CKD, 60.9% (89)

$f(\alpha, \beta) = 10.5$ for a power of 90%

A minimum of 27 participants in each comparison group was needed to detect this difference and thus the final sample size was aimed to be a minimum of 30 participants per comparison group.

2.1.2 Inclusion criteria

- a. Children aged between 5 and 18 years with CKD ($<90\text{mL/min/1.73m}^2$) being followed up by the Division of Paediatric Nephrology at CMJAH and CHBAH.
- b. Children whose parents/caregivers consented to the study
- c. Children ≥ 7 years who assent to the study

2.1.3 Exclusion criteria

- a. Children with known congenital heart disease, diabetes mellitus, liver disease, active infection, systemic lupus erythematosus (SLE) and malignancies
- b. Children with a kidney transplant
- c. Children who were febrile (Temperature $\geq 38^\circ\text{C}$)

2.1.4 The reference group

Due to the need for study participants to be subjected to venepuncture it was difficult to obtain ethical clearance to recruit a healthy control group (i.e. apparently well children) for this study. It was therefore decided that CKD I patients with an eGFR $>90\text{mL/min/1.73m}^2$, a normal blood pressure and with no proteinuria would be used for the reference/comparison group. The reference group patients were those seen in the clinic who had evidence of structural abnormalities [e.g pelvi-ureteric junction (PUJ) obstruction, vesico-ureteric junction (VUJ) obstruction, vesico-ureteric reflux (VUR), posterior urethral valve (PUV)] or other markers of functional kidney abnormalities including: isolated haematuria, pathologic abnormalities detected by histology and/or inferred by imaging but who had no proteinuria, normal blood pressure and GFR. The exclusion criteria previously mentioned were also applied when recruiting these participants.

2.2 Ethics and permission

The study was approved by the University of the Witwatersrand, Human Research Ethics Committee (Protocol M150312) and was conducted in accordance with the Helsinki Declaration, Good Clinical Practice and within the laws and regulations of South Africa.

Written consent was obtained for each parent/guardian of participating child, with assent being obtained from children >8 years old.

To maintain patient confidentiality and anonymity, study numbers and not names were allocated to the participants and anonymous data without potential identifiers such as date of birth were used in the data analyses. Access to raw data was restricted to the researcher and supervisors.

2.3 Methods

Recruitment of participants for the study commenced in August 2015 and ended in July 2016. Participants were recruited consecutively into each of the groups mentioned above during the study period. Patients on both peritoneal dialysis (PD) and haemodialysis (HD) were recruited into the dialysis group.

Participants' medical records were reviewed for age; gender; race; dialysis modality; cause of CKD; duration of CKD (since diagnosis); duration of dialysis; and pertinent medications, including antihypertensive agents, calcium-based phosphate binders (P-binders), and calcitriol.

All recruited participants had their history taken and a standard physical examination was done. Their height, weight, temperature and blood pressure (BP) were

measured. Body mass index (BMI) was calculated from the weight and height. Blood pressure was measured by auscultation using an appropriately sized cuff and a mercury sphygmomanometer and with the patient in the sitting position. For the haemodialysis CKD participants, BP was measured prior to the dialysis session. All other participants had their BP measured during a routine clinic visit. Blood pressure readings were indexed to the age-, gender-, and height-specific percentile for each participant according to the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents.(19) Pulse pressure (PP) was calculated as the difference between the systolic blood pressure (SBP) and diastolic blood pressure (DBP), while mean arterial blood pressure (MAP) was calculated as the sum of DBP and a third of PP.

The racial composition of the different CKD groups recruited is shown in Table 2.1.

The CKD V patients were patients on maintenance dialysis; 26 on haemodialysis (HD) and 10 on peritoneal dialysis (PD). (Table 2.1) The majority (31/36) of these patients were on erythropoietin (22 on HD and 9 on PD). The range of the dose of erythropoietin per week was 1500 – 18000 IU depending on the patients' age and weight and their response to treatment.

Table 2.1: Racial distribution of the CKD groups

	CKD groups			Total
Racial groups	I	II-IV	V-Dialysis	
Black	32	30	31	93
White	2	3	1	6
Asian	0	2	2	4
Mixed	0	1	2	3
Total	34	36	36	106

2.3.1 Sample collection and processing

Blood samples were drawn from each participant and sent for measurement of the serum creatinine, albumin, calcium (Ca), phosphorus (P), parathyroid hormone (PTH), alkaline phosphatase (ALP), total cholesterol, haemoglobin (Hb), C-reactive protein (CRP), iron, transferrin, transferrin saturation and ferritin levels. An extra 10mL of blood was drawn during the same venepuncture and sent for measurement of the blood vitamin D, FGF-23 and Fetuin-A levels. Genetic testing was also performed on this blood sample. Where applicable, participants also had their urine sample collected for measurement of the urine protein to creatinine ratio. The blood samples and urine samples were sent to the hospital's National Health Laboratory Service (NHLS) for analysis and the results retrieved. The extra blood samples were taken to the Department of Internal Medicine Research Laboratory of the University of the Witwatersrand for further study.

Ethylene-di-amine-tetra-acetic acid (EDTA) tubes were used to collect an extra 10mL of blood (for the biomarker assay and genetic testing). Plasma was used to run the specific biomarker assay. In order to obtain plasma, 5mL of the extra 10mL of blood

was centrifuged for 15 minutes at 1,500 x g within 30 minutes of collection. Where there was a delay in processing the samples, the samples were stored on ice and processed within the same day. The plasma obtained was then divided into equal aliquots, decanted into micro-containers and stored in the Department of Internal Medicine Research Laboratory of the University of the Witwatersrand at -80°C for subsequent assay.

The remaining 5mL of the extra blood sample was stored at -20°C in the Department of Internal Medicine Research Laboratory of the University of the Witwatersrand for subsequent DNA extraction.

2.3.2 Carotid artery ultrasound

For the purpose of this study, the researcher used carotid intima media thickness (cIMT), the common carotid artery (CCA) resistance index (RI) and pulsatility index (PI) to assess the vascular changes in CKD.

Common carotid artery ultrasound was performed using high resolution B-mode ultrasonography with the aid of L3-11 MHz linear array transducer (Phillips Corporation USA) as has been previously described elsewhere.(164, 165, 191) This was carried out by a single research sonographer who was blinded to the clinical details of the participants.

The sonographer visualised and focused on the far walls of the common carotid artery, 1 cm proximal to the bifurcation of the CCA in the longitudinal plane (Figure 2.1), and then acquired the cIMT measurements automatically. A similar process was followed for both right and left CCA and the mean of right and left common cIMT was used for analysis.

The doppler mode of the same machine was used to record the flow velocities of both the left and the right CCA and to determine the PI and the RI. The PI was calculated as $(\text{systolic velocity} - \text{diastolic velocity}) / (\text{time averaged mean velocity} - 0)$ and the RI was calculated as $(\text{systolic velocity} - \text{diastolic velocity}) / (\text{systolic velocity})$.(165)

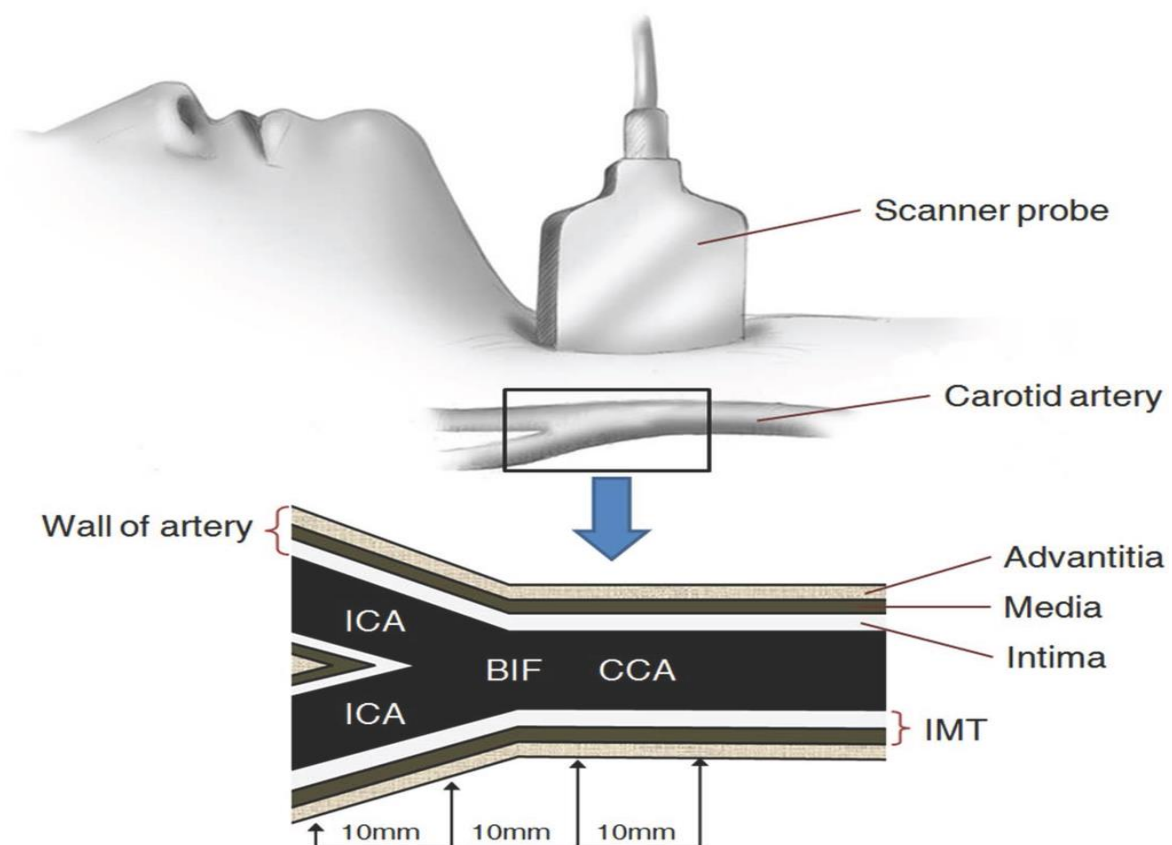


Fig 2.1: The measurement of intima-media thickness in the carotid artery using ultrasonograph. IMT: intima-media thickness, CCA: common carotid artery, BIF: bifurcation, ICA: internal carotid artery. (Reproduced under the 'Creative Commons Attribution License' from Kim H, Ishag M, Piao M, Kwon T, Ryu K. A data mining approach for cardiovascular disease diagnosis using heart rate variability and images of carotid arteries. Symmetry. 2016;8(6):47.)(192)

2.3.3 Echocardiography

A once off echocardiography to evaluate cardiac structure and function was performed by a single experienced research echocardiography technician. Echocardiography was performed by the use of the Phillips iE33 machine equipped with a S5-1 1-5 MHz transducer, allowing for M-mode, two dimensional and colour doppler measurements (Phillips Corporation USA). All examinations were carried out with the participant at rest, according to the American Society of Echocardiography recommendations.(144, 193, 194)

The following parameters were determined: left atrium diameter (LAD), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameters (LVESD), left ventricular posterior wall thickness at end diastole (LPWD), interventricular septal thickness at end diastole (IVSD), ejection fraction (EF), fraction shortening (FS), trans-mitral flow velocity ratio (E/A) and the index for LV filling pressure (E/E').

Left ventricular mass (LVM) was calculated according to the equation described previously.(139, 195) Left ventricular mass was then indexed (LVMI) for sex and body surface area .(196) Relative wall thickness (RWT) was also calculated in order to determine the pattern of the left ventricular geometry.(140-143)

Where abnormalities were found, participants were referred to the paediatric cardiology service at CMJAH for further evaluation.

Because of time and budgetary constraints and other logistical problems, only 68% and 83% of the study participants had cIMT measurements and echocardiography respectively.

2.3.4 Biochemical assay

a. Routine blood samples

Blood samples were assayed at the hospital laboratory serviced by the NHLS, using standard operating procedures. Specifically, serum creatinine and intact parathyroid hormone (PTH) were assayed using the Siemens Advia enzymatic technique and Siemens Advia Centaur chemiluminometric immunoassay technique.

b. Vitamin D assay

Plasma 25(OH)D was quantitatively measured using The ARCHITECT 25-OH Vitamin D assay method which employs the chemiluminescence micro-particle immunoassay (CMIA) technique.

d. FGF-23 assay

FGF-23 was assayed using Human FGF-23 ELISA Kit by Merck Millipore (Merck group, Massachusetts, USA).

e. Fetuin-A assay

Fetuin-A was assayed using the EDI™ Human Fetuin-A ELISA kit (EPITOPE Diagnostics, Inc, CA, USA).

2.3.5 DNA extraction and Fetuin-A genotyping

a. DNA extraction

The extraction of DNA was carried out by an automated method facilitated by the Maxwell platform for DNA extraction using commercially available Maxwell® DNA purification kits (Promega corporation, WI, USA) as per manufacturer's protocol. The DNA concentrations were determined by the NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA) with A260/280

ratios, and then the DNA samples were stored at -80°C for subsequent genotyping.

Eight SNPs of interest (rs2248690, rs6787344, rs4831, rs4917, rs4918, rs2070633, rs2070634 and rs2070635) were identified based on their reported relationship with Fetuin-A levels, CVD and markers of bone mineralisation in both CKD and non-CKD patients.(109, 182, 184-189)

b. Polymerase chain reaction (PCR) set up and DNA amplification

In preparation for genotyping, polymerase chain reaction (PCR) was set up for DNA amplification. The PCR reaction consisted of water, 2 x master mix (KAPA2G Robust Hot Start Ready-Mix PCR kit, Kapa Bio systems, USA), 1.25µl of 10µM DNA primers and 50 ng DNA diluted to 25ng/µl in a total reaction volume of 25µl. The reactions were amplified on the MJ Mini™ Thermal cycler (Bio-Rad, USA) with initial denaturation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 20 seconds and a final extension of 72°C for 1 minute. A higher annealing temperature of 66°C was used for rs6787344, while the remaining four SNPs were annealed at 60°C.

c. Fetuin-A gene restriction fragment length polymorphism (RFLP)

Genotypes for rs2248690, rs6787344, rs4831, rs4917 and rs4918 in Fetuin-A were determined by restriction fragment length polymorphism (RFLP). Samples were incubated with their respective restriction enzymes (Table 2.1) overnight at 37°C. To prevent evaporation, each reaction was overlaid with 15 µl of mineral oil. The following day, the reactions were terminated by adding an EDTA-containing gel dye.

Table 2.2: Primers and product lengths for the different SNPs

SNP	Primers	PCR product	Restriction Enzyme	RFLP Allelic discrimination	
				Allele	Size
rs2248690	Fwd: 5' - GAA CCC AGA GCT GTG TCA TA - 3'	150 bp	NdeI	A	150bp
	Rev: 5' - TCC TTC TCC AGA CCT CAC T - 3'			T	132bp and 18bp
rs6787344	Fwd: 5' – TAC CGA GGT AAG GAG GGA TTG - 3'	145 bp	BsaI	C	147bp
	Rev: 5' – CCT TAA AAT AGA TTG GCT AGG GAGA - 3'			G	125bp and 20bp
rs4831	Fwd: 5' – GGC AGG CTC CAA CAG ATA AA - 3'	361 bp	PvuII	C	361bp
	Rev: 5' – CAT AGA CAG CAG GTC CAC TTAC - 3'			G	199bp and 162bp
rs4917	Fwd: 5' – TCT CTG TGG GCA GCA ATA TG - 3'	284 bp	NlaIII	C	284bp
	Rev: 5' – GGA GGG AAA GGC ATA GCT AAA - 3'			T	202bp and 82bp
rs4918	Fwd: 5' – GGG AGG AGG AAG CAA ACT AAC - 3'	264 bp	SacI	C	264bp
	Rev: 5' – CAA TGA GAC CAC ACC CAT GAA - 3'			G	209bp and 55bp
rs2070633, rs2070634 and rs2070635	Fwd: 5' - GCT CTA TGA AAC AGG TGG AAG A - 3' Rev: 5' - GGG CTG AGA AGA GTA CAT GAA A - 3'	439 bp	-	-	-

Three closely positioned SNPs (rs2070633, rs2070634 and rs2070635) were genotyped by direct sequencing at a private laboratory (Inqaba biotech).

d. Gel electrophoresis

Restricted products were resolved on 10% Tris-Boric Acid-EDTA polyacrylamide gels. Polyacrylamide gel electrophoresis was performed for rs2248690, rs6787344, rs4831, rs4917 and rs4918.

e. Gel imaging

Visualization of the restricted PCR products representing Fetuin-A genotypes performed using Gel Doc™ EZ imager (Bio-Rad systems, USA).

2.4 Definition of terms

- Hypertension: defined as the need for antihypertensive treatment and/or according to the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents.(19)
- Body mass index (BMI): interpreted according to the World Health Organization (WHO) BMI centile for gender and age in children.(54)
- Proteinuria: urine protein/creatinine ratio >0.02g/mmol.(197, 198)
- Hypercholesterolaemia: total cholesterol >5.18mmol/L (>200mg/dL).(46)
- Anaemia: defined based on age according to the Kidney Disease Improving Global Outcome (KDIGO) clinical practice guidelines for anaemia in CKD.(199)
- Hyperphosphatemia, hypocalcaemia, hypercalcaemia, elevated calcium product and elevated alkaline phosphatase: defined based on age according to the Kidney Disease Outcomes Quality Initiative (KDOQI) clinical practice guidelines for bone metabolism and disease in children with chronic kidney disease.(200)

- Hyperparathyroidism: parathyroid hormone (PTH) levels above laboratory normal limit ($>7.6\text{pmol/L}$) in pre-dialysis patients and above nine times the upper normal limit ($>68.4\text{pmol/L}$) in dialysis patients as recommended by KDIGO.(82)
- Hypoalbuminaemia: serum albumin $<35\text{g/L}$.(103)
- Elevated CRP: $>10\text{mg/L}$.(201)
- Low 25 OH Vitamin D: $<30\text{ng/ml}$.(200)
- Increased cIMT: $>95^{\text{th}}$ percentile for age, height and gender. (155)
- Abnormal LAD: $>$ normal for age.(202)
- Abnormal LVMI defined based on body surface area for gender.(196)
- Ejection Fraction: low ($<40\%$), borderline (41-50%), normal (51-70%), high ($>70\%$)
- Abnormal E/A: <1 .(203)

2.5 Data analysis

Study data were collected and managed using Research Electronic Data Capture (REDCap) tools hosted at the University of the Witwatersrand.(204) Research Electronic Data Capture-REDCap is a secure, web-based application designed to support data capture for research studies, providing 1) an intuitive interface for validated data entry; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for importing data from external sources.

The statistical analyses used are described in the respective result chapters.

The results are presented in the form of manuscripts as chapters 3 to 6.

CHAPTER 3 (MANUSCRIPT 1): CARDIOVASCULAR RISK FACTORS AND MORTALITY IN CHILDREN WITH CHRONIC KIDNEY DISEASE

3.1 Abstract

Background: Cardiovascular disease (CVD) begins early in children with chronic kidney disease (CKD), and its progression is determined by the presence of single or multiple cardiovascular risk factors (CVRFs). This study determined the prevalence of CVRFs in children with CKD and their association with mortality in children on chronic dialysis.

Methods: This comparative cross sectional study recruited children (5-18 years) with all stages of CKD. All patients had a short history taken along with a physical examination and their blood samples assessed for serum creatinine, urea, albumin, calcium, phosphorus, parathyroid hormone, alkaline phosphatase, total cholesterol, haemoglobin and C-reactive protein. Patients' urine samples were also assessed for proteinuria.

Results: One hundred and six children who met the study criteria were recruited, 34 CKD I, 36 CKD II-IV and 36 CKD V (dialysis). The overall median age of the patients was 11 years (8-14 years) with a male female ratio of 2.1:1. The most common CVRF was anaemia (39.6%). The rate of anaemia was highest in the dialysis group when compared with the CKD II-IV group and the CKD I group (77.8%, 33.3% and 5.9%). Other CVRFs detected include hypertension, proteinuria, hypercholesterolaemia and dysregulated mineral bone metabolism. Seven deaths were recorded in the dialysis group during the study period. Severe hypertension and intracranial bleed were the most common causes of death. Modifiable risk factors such as increased total cholesterol (TC) and decreased albumin levels were more among the deceased dialysis patients.

Conclusion: Cardiovascular risk factors may be present in early CKD, even before the decline in GFR. Routine screening for CVRFs, along with timely intervention, may prevent the progression of CVD and mortality later in life.

Keywords: Cardiovascular disease, Risk factors, Mortality, Children, Chronic kidney disease.

3.2 Introduction

Cardiovascular disease (CVD) is thought to begin early in chronic kidney disease (CKD) and to progress rapidly as renal function declines especially on dialysis.(10, 11, 22) Cardiovascular disease is the most common cause of death among paediatric patients with end stage renal disease (ESRD). (12, 13)

Traditional and non-traditional cardiovascular risk factors (CVRFs) play an important role in the initiation and progression of CVD in children with CKD. Combinations of these risk factors could cause accelerated manifestations of cardiac and vascular changes in children.(21, 25) Traditional CVRFs such as hypertension, dyslipidaemia, obesity and hyperglycaemia have been shown to be increased in children even with early stages of CKD.(21, 22) Non-traditional CVRFs including anaemia, fluid overload, dysregulated mineral bone metabolism (hyperparathyroidism, increased calcium-phosphate product), hypoalbuminaemia, inflammation (increased C-reactive protein and cytokines) and oxidative stress are more evident in children with moderate to severe CKD. (10, 13, 18, 21, 25, 26) Other risk factors for CVD are potentially treatment-related such as calcium overload from dialysate, calcium-based phosphate binders and vitamin D therapy.(29, 92, 173)

In adults with ESRD, coronary artery disease (CAD) and cardiomyopathy are the leading cause of CVD mortality. Children however die from cardiac arrest, arrhythmia, cardiomyopathy and, rarely, myocardial disease.(205, 206) Various vascular changes such as atherosclerosis, arteriosclerotic lesions (including fibrous or fibro-elastic intimal thickening), disruption of the internal elastic lamella, and atheromatous plaques have also been reported in children with CKD.(18, 21) These vascular changes increase the risk of symptomatic CVD later in life.(25)

This study looked at the prevalence of CVRFs and their association with mortality in children with CKD.

3.3 Methods

This comparative cross sectional study recruited 106 children with CKD being followed up by the Division of Paediatric Nephrology of the Charlotte Maxeke Johannesburg Academic Hospital and Chris Hani Baragwanath Academic Hospital, Johannesburg, South Africa.

Thirty-four CKD I, 36 CKD II-IV and 36 CKD V (dialysis) were recruited consecutively over a 12-month period (August 2015 – July 2016). The CKD I group were children with a glomerular filtration (GFR) of >90 ml/min/1.73m² (with either structural abnormalities or isolated haematuria) with normal blood pressure and no proteinuria, CKD II-IV were those with GFR of 15-90 ml/min/1.73m² and CKD V were those children on maintenance haemodialysis and peritoneal dialysis.

Children with known congenital heart disease, diabetes mellitus, liver disease, active infection, systemic lupus erythematosus, malignancies and renal transplant were excluded from the study.

All patients had a short demographic and clinical history taken along with a physical examination. Routine blood samples for serum creatinine, urea, albumin, calcium (Ca), phosphorus (P), parathyroid hormone (PTH), alkaline phosphatase (ALP), random total cholesterol, haemoglobin (Hb) and C-reactive protein (CRP) were taken, results retrieved and analysed.

3.3.1 Definition of terms

- Hypertension: defined as the need for antihypertensive treatment and/or according to the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents.(207)
- Body mass index (BMI): interpreted according to the World Health Organization (WHO) BMI centile for sex and age in children.(54)
- Proteinuria: urine protein/creatinine ratio >0.02g/mmol.(197, 198)
- Hypercholesterolaemia: total cholesterol >5.18mmol/L (>200mg/dL).(46)
- Anaemia: defined based on age according to the Kidney Disease Improving Global Outcome (KDIGO) clinical practice guidelines for anaemia in CKD.(199)
- Hyperphosphataemia, hypocalcaemia, hypercalcaemia, elevated calcium-phosphate product (CaXP) and elevated alkaline phosphatase: defined based on age according to the Kidney Disease Outcomes Quality Initiative (KDOQI) clinical practice guidelines for bone metabolism and disease in children with chronic kidney disease.(200)

- Hyperparathyroidism: parathyroid hormone (PTH) levels above laboratory normal limit ($>7.6\text{pmol/L}$) in pre-dialysis patients and above nine times the upper normal limit ($>68.4\text{pmol/L}$) in dialysis patients as recommended by KDIGO.(82)
- Hypoalbuminaemia: serum albumin $<35\text{g/L}$.(103)
- Elevated CRP: $>10\text{mg/L}$.(201)

3.3.2 Data analysis

All data were collected and managed using Research Electronic Data Capture (REDCap).(204) Computer based statistical package STATA 13.1 was used for the analysis. Continuous variables were described using means and standard deviations for data normally distributed, and medians and inter-quartile ranges used for skewed data. Categorical variables were presented as percentages and frequencies. Statistical significance in the prevalence of risk factors was tested for using Chi-square (χ^2) tests or Fisher exact test where appropriate. Mean/median values of the different groups were compared using ANOVA, Kruskal-Wallis test, T-test and Mann-Whitney U test depending on the distribution of the data. To compensate for multiple testing, Bonferroni type correction was used to adjust for significant levels for the CVRFs as appropriate. Logistic regression was used to determine the relationship between mortality and CVRFs. All CVRFs were tested using univariate logistic regression and only significant CVRFs ($p<0.05$) are presented in Table 3 and included in the multivariate regression model. A confidence interval of 95% was used and $p<0.05$ was regarded as significant.

3.3.3 Ethics and permission

The study was approved by the University of the Witwatersrand, Human Research Ethics Committee (Protocol M150312) and was conducted in conformance with the Helsinki Declaration, Good Clinical Practice and within the laws and regulations of South Africa. Informed written consent/assent was obtained from participants where appropriate.

3.4 Results

The overall median age of the patients was 11 years (8-14 years), with a male:female ratio of 2.1:1 (Table 3.1).

Congenital anomaly of the kidney and urinary tract (CAKUT) was the most common cause of CKD across all groups, with a total rate of 47.2% (50/106) observed (Table 3.1).

A breakdown of the nutritional status of the different groups of patients is illustrated in Figure 3.1. The majority of the patients (79/106; 74.8%) were well nourished. Undernutrition was seen only in the dialysis group (6/36; 5.6%). Seventeen (16.0%) of the patients were classified as overweight (10 CKD I, 7 CKD II-IV) and three (2.8%) as being obese (1 CKD I, 1 CKD II-IV, 1 CKD V).

Anaemia (42/106; 39.6%) was the most common CVRF as shown in Table 3.1. Furthermore, anaemia differed significantly between the groups and was observed in 2/34 (5.9%) patients in CKD I group, 9/36 (25%) of CKD II-IV patients and 31/36 (86.1%) of CKD V patients ($p<0.001$). Hypertension was the second most common CVRF and was significantly higher in the dialysis group compared with the CKD II-IV

group (28/36 vs 12/36; $p<0.001$). Elevated CaXP was the CVRF with the overall lowest rate (6.5%) and was found in the dialysis group only. Similar trends were observed when the absolute values of the various CVRFs were compared across groups (Table 3.1). The majority of these CVRFs remain significant after Bonferroni correction, where $p<0.004$ was considered significant. (Table 3.1)

Over the 12-month duration of the study, we recorded seven deaths all of whom were in the dialysis (CKD V) group. Cerebrovascular accident (intracranial bleed) associated with severe hypertension accounted for the majority (4/7; 57.1%) of the deaths (Figure 3.2). Further analysis of the dialysis group showed a statistically significant difference in the presence of hypercholesterolaemia and hypoalbuminaemia between the deceased patients and the surviving patients (Table 2). Similarly, the deceased group had significantly higher mean levels of total cholesterol (TC) and lower mean levels of albumin, when compared to the surviving group (Table 3.2). After Bonferroni correction, where $p<0.004$ was considered significant, only Cholesterol remained significant in Table 3.2. Cholesterol and albumin levels were not associated with proteinuria in this sub group. Univariate logistic regression among the dialysis group showed an association of mortality with age, serum TC and albumin (Table 3.3). After adjusting for these three in a regression model, we found age to be the most important associated factor for mortality in our group of patients (Table 3.3).

Table 3.1: Cardiovascular risk factors in all CKD patients

	CKD I (n=34)	CKD II-IV (n=36)	Dialysis CKD (n=36)	p value
Age (years) [#]	9.48 (2.85)	11.28 (3.52)	11.86 (3.77)	0.012
Sex (M:F)	25:9	26:10	21:15	0.314
Diagnosis				
• CAKUT	13 (38.2%)	19 (52.8%)	18 (50.0%)	
• Glomerular disease	12 (35.3%)	13 (36.1%)	11 (30.6%)	
• Others	9 (26.5%)	4 (11.1%)	7 (19.4%)	0.515
MAP (mmHg) [*]	71 (70-80)	80 (70-86)	91 (81-102)	<0.001^α
Hypertension	0	12 (33.3%)	28 (77.8%)	<0.001^α
BMI (Kg/m ²) [*]	17 (15-18)	18 (16-19)	16 (15-18)	0.089
Increased BMI	11 (32.4%)	8 (22.2%)	1(2.8%)	0.002^α
Haemoglobin (g/dl) [*]	13.2 (12.5-14.4)	13.4 (12.1-14.3)	9.3 (8.1-10.6)	<0.001^α
Anaemia	2 (5.9%)	9 (25.0%)	31 (86.1%)	<0.001^α
CRP (mg/L) [*]	10 (-)	10 (-)	10 (10-14)	0.003^α
Increased CRP	0	4 (11.1)	10 (27.8%)	0.002^α
Cholesterol (mmol/L) [*]	3.7 (3.2-4.3)	4.2 (3.3-4.7)	4.2 (3.3-5.7)	0.242
Hypercholesterolaemia	4 (11.8%)	3 (8.3%)	13 (36.1%)	0.007
Albumin (mg/dl) [*]	44 (42-45)	44 (41-45)	36 (30-39)	<0.001^α
Hypoalbuminaemia	0	2 (5.6%)	15 (41.7%)	<0.001^α
Phosphate (mmol/L) [#]	1.46 (0.23)	1.37 (0.29)	1.64 (0.52)	0.014
Hyperphosphatemia	4 (11.8%)	4 (11.1%)	17 (47.2%)	<0.001^α
CaXP [*]	3.3 (3.0-3.6)	3.3 (2.8-3.6)	3.5 (2.5-4.2)	0.329
Increased CaXP	0	0	7 (19.45)	-
Alphos (U/L) [*]	245 (212-328)	263 (184-313)	250 (184-484)	0.773
Increased Alphos	4 (11.8%)	9 (25.0%)	19 (52.8%)	0.001^α
PTH (pmol/L) [*]	3.5 (2.7-5.3)	6.6 (4.0-10.9)	77.9 (17.4-144.5)	<0.001^α
Increased PTH	4/34 (11.8%)	15/35 (42.9%)	19/36 (52.8%)	0.001^α
PCR (g/mmol) [*]	0.004 (0.001-0.007)	0.030 (0.007-0.084)	0.235 (0.050-0.930)	<0.001^α
Proteinuria	0	20/32 (62.5%)	8/10 (80%)	<0.001^α

#-Mean ± SD, *-Median (IQR), α-significant after Bonferroni correction, CAKUT-congenital anomalies of the kidney and urinary tract, MAP-mean arterial pressure, BMI-body mass index, CRP-C reactive protein, TC-total cholesterol, CaXP-calcium-phosphate product, Alphos-alkaline phosphatase, PTH-parathyroid hormone, PCR-urine protein-creatinine ratio

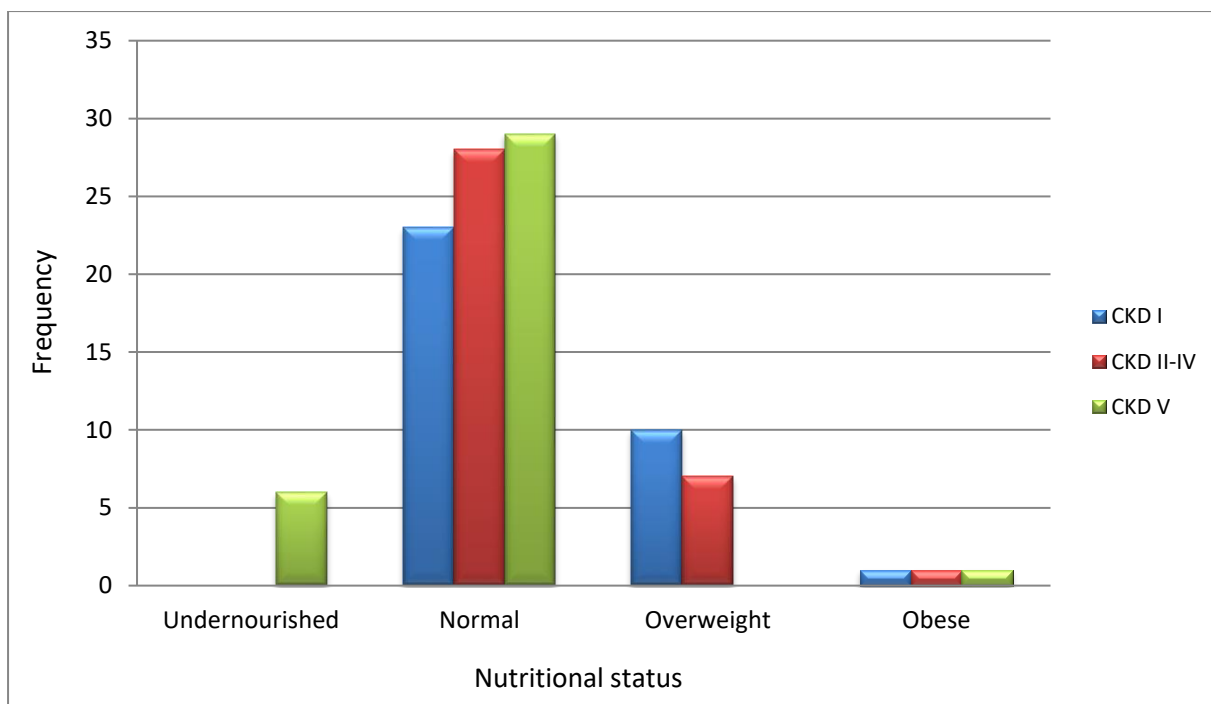


Figure 3.1: Nutritional status of the patients. CKD-chronic kidney disease

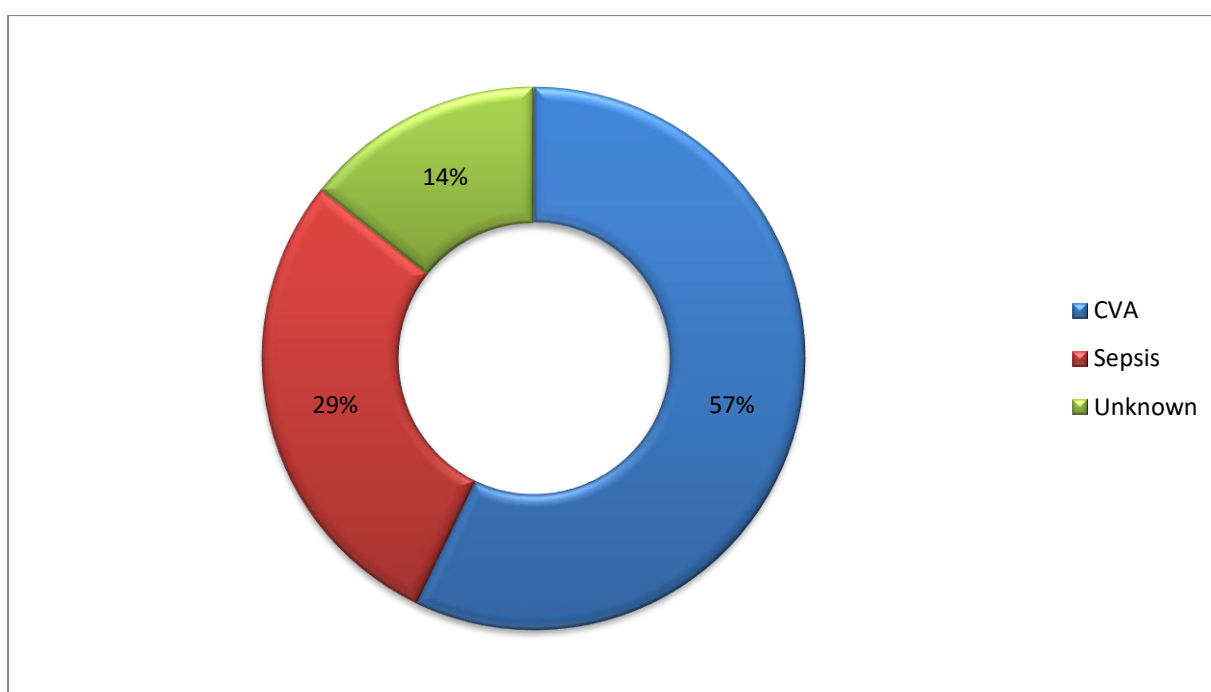


Figure 3.2: Causes of death. CVA-cerebrovascular accident

Table 3.2: Mortality and cardiovascular risk factors among dialysis patients

	Mortality		P value
	Yes (n=7)	No (n=29)	
Age (years) [#]	7.71 (2.81)	12.86 (3.28)	0.005
Duration of dialysis (months) [#]	11.43 (9.20)	25.72 (36.50)	0.309
BMI (Kg/m ²) [*]	15 (14-16)	17 (15-18)	0.031
MAP (mmHg) [#]	85.86 (16.69)	93.66 (18.61)	0.435
Hypertension	5 (71.4%)	23 (79.3%)	0.639
Haemoglobin (g/dL) [#]	8.30 (2.65)	9.46 (1.96)	0.200
Anaemia	6 (85.7%)	25 (86.2%)	1.000
CRP (mg/L) [*]	10 (10-29)	10 (10-14)	0.441
Increased CRP	2 (28.6%)	8 (27.6%)	1.000
Cholesterol (mmol/L) [#]	6.17 (1.44)	4.13 (1.29)	0.001^α
Hypercholesterolemia	6 (85.7%)	7 (24.1%)	0.005
Albumin (g/L) [#]	29.57 (4.50)	36.04 (5.62)	0.008
Hypoalbuminaemia	6 (85.7%)	9 (31.0%)	0.013
Phosphate (mmol/L) [#]	1.57 (0.52)	1.66 (0.53)	0.695
Hyperphosphatemia	3 (42.9%)	14 (48.3%)	1.000
CaXP [#]	3.31 (1.26)	3.59 (1.37)	0.622
Increased CaXP	1 (14.3%)	6 (20.7%)	1.000
PTH (pmol/L) [#]	71.49 (69.42)	89.19 (74.36)	0.571
Increased PTH	3 (42.9%)	15 (51.7%)	1.000
PCR (g/mmol) [*]	1.46 (1.00-1.91)	0.11 (0.03-0.35)	0.116
Proteinuria	2/2	6/8	0.622

[#]-Mean ± SD, ^{*}-Median (IQR), ^α-significant after Bonferroni correction, SBP-systolic blood pressure, DBP-diastolic blood pressure, MAP-mean arterial pressure, BMI-body mass index, Hb-haemoglobin, CRP-C reactive protein, CaXP-calcium-phosphate product, PTH-parathyroid hormone

Table 3.3: Logistic regression for mortality among dialysis patients

	Univariate			Multivariate		
	OR	p value	95% CI	OR	p value	95% CI
Age (years)	0.62	0.008	0.44-0.88	0.67	0.058	0.44-1.01
Cholesterol (mmol/L)	2.83	0.008	1.31-6.14	2.32	0.119	0.81-6.68
Albumin (mg/dl)	0.81	0.021	0.68-0.97	0.85	0.194	0.66-1.09

3.5 Discussion

The initiation and progression of CVD in children with CKD is determined by the presence of single or multiple CVRFs, and this is thought to begin early and then worsen as the renal function declines. Our study looked at traditional and non-traditional risk factors for CVD, as well as their association with mortality, among children with CKD.

The use of BMI alone in determining the nutritional status of dialysis CKD patients may not be appropriate because of inter-dialytic weight gain and variable dry weight status.(208) Despite these concerns, under-nutrition was exclusively seen in the dialysis group and we recorded only a single case of over-nutrition in the same group. A similar finding of undernutrition in advanced CKD has been reported in several studies.(209, 210) The finding of under-nutrition in advanced CKD may be explained by low appetite, nausea and vomiting, the effect of enforced dietary restriction resulting in caloric deficiency, along with chronic illness, increased metabolic rate, metabolic acidosis and chronic inflammation, in a growing child.(211)

Being overweight or obese has been reported to increase the risk of having other CVRFs (hypertension, dyslipidaemia and abnormal glucose metabolism) when compared to lean patients.(22) It should be emphasized at this point that the CKD I group did not have proteinuria and so it is unlikely that the presence of nephrotic syndrome patients influenced the rate of hypercholesterolaemia in this group. In our study, over-nutrition was most common in the CKD I and CKD II-IV groups and we feel that this is a more likely explanation for the higher rate of hypercholesterolemia among the CKD I group than in the CKD II-IV group.

Even though hypertension has been reported to be the single most important CVRF in CKD,(21, 27, 28) we found anaemia to be the overall most common CVRF in our study, and hypertension to be the overall second most common CVRF. The high rate of anaemia likely reflects the undernutrition reported in the dialysis group in addition to other causes of anaemia in CKD such as declining production of erythropoietin, inflammation, severe secondary hyperparathyroidism leading to myelofibrosis, marrow hypo-responsiveness and infection.(212) In spite of anaemia being a modifiable risk that can be corrected with erythropoiesis stimulating agents (ESA) and iron supplements, poor absorption and compliance to oral iron due to side effects like constipation, diarrhoea and abdominal discomfort might have also contributed to the high rate of anaemia in our patients.

Across the study groups, except for over-nutrition, the rates of recorded CVRF were highest among the dialysis group. Previous studies have documented a similar pattern in children with CKD.(21, 89, 213)

Although less common, increased serum total cholesterol, phosphate, alkaline phosphatase, PTH and anaemia were also seen in the CKD I group. This may be attributed to the presence of early changes of CKD even before a decline in GFR, onset of proteinuria and hypertension are manifest.

The high death rate recorded over the study period is concerning, with severe hypertension and cerebrovascular accident (intracranial bleed) accounting for the most common causes of death. Our observations differ from cardiac related deaths reported in other cohorts of paediatric dialysis CKD patients,(205, 206) where cardiac arrest, arrhythmia, cardiomyopathy, cardiac failure and myocardial infarction/ischemia were the most common causes of death. The reason for this

difference may be attributed to lack of adequate volume and blood pressure control in our dialysis group leading to severe hypertension. Our findings further emphasize the need for adequate blood pressure and fluid volume control in our chronic dialysis patients, in addition to control of other CVRFs.

Younger age at commencement of dialysis, especially being under the age of one year, has been associated with poorer survival compared to those of older age (>5 years) at the start of dialysis.(205, 206) Similarly, we found age to be an associated factor for mortality in our group of patients.

The finding of a higher rate of increased TC and decreased albumin levels, among the deceased dialysis patients, may be indicative of their role in mortality. Even though both TC and albumin levels were not significantly associated with mortality after adjusting for age in this study, it is nevertheless still important to correct TC and albumin levels in all CKD patients as previous studies have implicated both of these risk factors as predictors of morbidity and mortality in CKD.(44, 102-104)

3.6 Conclusion

Cardiovascular risk factors may be present in early CKD, even before the decline in GFR is detected, and tend to worsen as renal function deteriorates especially in dialysis patients. These risk factors play an important role in morbidity and mortality associated with CVD in children with CKD. Routine screening for these CVRFs, along with timely intervention, may go a long way to prevent the progression of CVD and cardiac related mortality later in life.

3.7 Strength and limitation

This is the first African study to look at traditional and non-traditional risk factors for CVD, as well as their association with mortality, among children with CKD.

The major limitation is the small number of patients in this study and the lack of a disease free control group for comparison. Another limitation is that random and not fasting cholesterol was measured.

3.8 Acknowledgement

The authors acknowledge the contribution of all team members who provided care for these children and assisted with this study. We would also like to thank the Carnegie Corporation of New York and the University of the Witwatersrand for funding the research.

3.9 Conflict of Interest

The authors declare no conflict of interest.

CHAPTER 4 (MANUSCRIPT 2): CAROTID INTIMA MEDIA THICKNESS IN SOUTH AFRICAN CHILDREN WITH CHRONIC KIDNEY DISEASE

4.1 Abstract

Background: Increased carotid intima media thickness (cIMT) is one of the early changes seen in chronic kidney disease (CKD) associated cardiovascular disease. This study aimed to determine cIMT measurements and its association with cardiovascular risk factors, including FGF-23 and Fetuin-A, in African children with CKD.

Methods: Seventy-two children (5-18 years) with CKD; 20 with CKD I, 23 with CKD II-IV, 29 with CKD V (on dialysis) were recruited. Each patient had a clinical examination and blood samples assessed for creatinine, urea, albumin, calcium, phosphorus, parathyroid hormone, alkaline phosphatase, total cholesterol, haemoglobin, C-reactive protein, Vitamin D, Fetuin-A and FGF-23. Carotid intima media thickness was measured with high resolution ultrasound.

Results: The mean age was 10.8 (3.5) years and there were 49 males and 23 females (2:1). The overall median (range) cIMT was 0.505mm (0.380-0.675), and was highest in patients with dialysis dependant CKD ($p=0.003$). Mean arterial pressure (MAP), haemoglobin and PTH showed a significant correlation with cIMT ($p<0.001$, $p=0.034$ and $p=0.002$ respectively). After adjusting for confounders in a multivariable analysis, MAP and haemoglobin levels were independently associated with cIMT, $p<0.050$. No significant relationship between cIMT and plasma levels of Fetuin-A and FGF-23 was found.

Conclusion: This study reports an unexpectedly high cIMT measurements in African children with CKD and also the uncommon finding of haemoglobin as an independently associated factor for cIMT in children with CKD.

4.2 Introduction

Increased carotid intima media thickness (cIMT) is one of the early changes seen in cardiovascular disease (CVD) associated with chronic kidney disease (CKD).(13) Other reported vascular changes include increased arterial wall stiffness and vascular calcification.(2, 13, 132) These changes begin early in disease and tend to progress with advancing disease, especially during dialysis.(18, 214) High resolution ultrasound of the common carotid artery (CCA) is used in the assessment of cIMT, and paediatric reference values have been defined.(155)

Several modifiable risk factors such as hypertension, dyslipidaemia, mineral bone disease (MBD), uraemia, anaemia and inflammation have been implicated in cardiovascular disease and mortality.(18, 21, 215) Mineral bone disease presents as dysregulated phosphate, calcium, parathyroid hormone and Vitamin D levels, which may eventually lead to the vascular changes seen in CKD.(86)

In this study, the biomarkers FGF-23 and Fetuin-A were selected base on their important role in cardiovascular disease, mortality and mineral and bone disorder (MBD). Fibroblast growth factor-23 (FGF-23), produced by the osteocytes, is a phosphaturic hormone that regulates phosphate levels by suppressing phosphate reabsorption and suppressing 1,25-Hydroxyvitamin D production in the kidney.(18) Studies have shown that FGF-23 is implicated in vascular calcification and the risk of cardiovascular events in CKD. (216-218) Fetuin-A, which is predominantly produced

in the liver, has been described as one of the key circulatory inhibitors of calcification.(18, 219) Although MGP and OPG are also inhibitors of calcification, Fetuin-A contributes to over 50% of circulatory inhibition of ectopic calcification.(172) Low levels of Fetuin-A have been associated with vascular calcification, arterial stiffness and other cardiovascular events.(171, 184, 220, 221)

This study aimed to determine cIMT measurements and its association with cardiovascular risk factors including FGF-23 and Fetuin-A in African children with CKD.

4.3 Methods

Fifty-two children (5-18 years) with a glomerular filtration (GFR) of <90 ml/min/1.73m² (23 CKD II-IV, 29 CKD V on dialysis) and 20 CKD I children (5-18 years) with a GFR>90 ml/min/1.73m² (with either structural abnormalities, or isolated haematuria but with a with normal blood pressure and no proteinuria) were recruited consecutively over a 12-month period (August 2015 – July 2016). Children with known congenital heart disease, diabetes mellitus, liver disease, active infection, systemic lupus erythematosus, malignancies and renal transplant were excluded from the study.

In this comparative study, participants were recruited out of over 250 children with CKD who are being followed up by the Divisions of Paediatric Nephrology of the Charlotte Maxeke Johannesburg Academic Hospital and the Chris Hani Baragwanath Academic Hospital, Johannesburg, South Africa for various paediatric renal pathologies. Due to the difficulty in obtaining ethical clearance for blood sampling in healthy children, a disease free control group was not included.

All patients had a short demographic and clinical history taken together with a physical examination. Blood samples were taken and assessed for serum creatinine, urea, albumin, calcium, phosphorus, parathyroid hormone (PTH), alkaline phosphatase, total cholesterol, haemoglobin and C-reactive protein (CRP) in the National Health Laboratory Service of CMJAH. Specifically, serum creatinine and intact parathyroid hormone (PTH) were assayed using the Siemens Advia enzymatic technique and the Siemens Advia Centaur chemiluminometric immunoassay technique respectively. From the same blood samples, plasma 25-Hydroxyvitamin D [25(OH)D] was measured using the Chemiluminescence Micro-particle Immunoassay (CMIA) using the ARCHITECT 25-OH Vitamin D method in a private laboratory (Lancet Laboratories), while intact Plasma FGF-23 was measured using the Human FGF-23 ELISA Kit (Merck Millipore, Merck group, Massachusetts, USA) and plasma Fetuin-A levels using the EDI™ Human Fetuin-A ELISA kit (EPITOPE Diagnostics, Inc, CA, USA) in the Medical Research Laboratory of the Department of Internal Medicine of the University of the Witwatersrand.

Each patient had a high-resolution ultrasound of the common carotid artery (CCA) performed as previously described.(191) This was carried out by a single research sonographer who was blinded to the clinical details of the participants. Both the right and left cIMT were measured and the mean was used for analysis. The Doppler mode of the same machine was used to record the flow velocities of both left and right CCA and to determine pulsatility index (PI) and the resistivity index (RI) as previously described.(165)

4.3.1 Definition of terms

- Glomerular filtration rate (GFR) was estimated by the use of the modified Schwartz formula.(222)
- Hypertension: the need for antihypertensive treatment and/or according to the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents.(19)
- Hypercholesterolaemia: total cholesterol >5.18mmol/L (>200mg/dl).(46)
- Anaemia: defined based on age according to the Kidney Disease Improving Global Outcome (KDIGO) clinical practice guidelines for anaemia in CKD.(199)
- Hyperphosphatemia and elevated calcium phosphate product: defined based on age according to the Kidney Disease Outcomes Quality Initiative (KDOQI) and KDIGO clinical practice guidelines for bone metabolism and disease in children with chronic kidney disease.(82, 200)
- Hyperparathyroidism: parathyroid hormone (PTH) levels above laboratory normal limit (>7.6pmol/L), in pre-dialysis patients and above nine times the upper normal limit (>68.4pmol/L) in dialysis patients as recommended by KDIGO.(82)
- Hypoalbuminaemia: serum albumin <35mg/dl.(103)
- Elevated C-reactive protein (CRP): >10mg/L.(201)
- Low 25(OH)D: <30ng/ml.(200)

4.3.2 Data analysis

All data were collected and managed using Research Electronic Data Capture (REDCap) tools hosted at the University of the Witwatersrand (204) and STATA 13.1

used for the analysis. Data was tested for normal distribution or skewedness using the STATA programme. Continuous variables were described using means (standard deviations) for data normally distributed, and medians (inter-quartile ranges) for skewed data. Categorical variables were presented as percentages and frequencies. Standard deviation scores (SDS) expressed as z scores for the individual cIMT measurements for age and height were calculated using the reference values by Doyon et al.(155)

Mean values of the different groups were compared using the student t and ANOVA test, while median values were compared using the Mann-Whitney U and Kruskal-Wallis tests. Spearman's correlation was used to determine the correlates of cIMT. Proportions were tested using the Chi square test and the Fisher exact test, where appropriate. Multivariate analysis was used to determine independently associated factors for increased cIMT. All risk factors in the univariable analysis were included in the multivariable analysis. A p value of <0.05 was regarded as statistically significant for all analyses.

4.3.3 Ethics and Consent

The study was approved by the University of the Witwatersrand, Human Research Ethics Committee (Protocol M150312) and was conducted in conformance with the Helsinki Declaration, Good Clinical Practice and within the laws and regulations of South Africa.

Written consent was obtained for each parent/guardian of participating child, with assent being obtained from children >8 years old.

4.4 Results

The distribution of age and gender for the different CKD groups can be seen in Table 4.1. The mean age was 10.8 (3.5) years and there were 49 males and 23 females (2:1). The racial distribution of the cohort was Black race 79 (90%), White race 3(4%), Asian race 3(4%) and the Mixed race 2(2%).

The overall median cIMT was 0.505mm (0.380-0.675), and the median cIMT was higher in patients with dialysis dependant CKD compared to the other CKD groups ($p=0.003$). (Table 4.1) Thirty-three patients (46%) had cIMT greater than the mean cIMT of 0.506mm. The Z scores for cIMT for both age and height were highest in the dialysis group, and four patients (6%) had cIMT Z score greater than two. (Table 4.1). We did not find any patients with vascular calcification. (Table 4.1)

The mean pulsatility index (PI) and median (IQR) resistive index (RI) were 1.68 ± 0.42 and 0.72 (0.68-0.76) respectively, and did not differ significantly between the different CKD groups. (Table 4.1)

The CKD V group had the highest proportion of hypertension and abnormal biochemical parameters when compared to the other CKD groups. Among these parameters, haemoglobin showed a negative correlation with cIMT ($\rho=-0.43$, $p<0.001$), while MAP and PTH showed a positive correlation with cIMT ($\rho=0.251$, $p=0.034$ and $\rho=0.37$, $p=0.002$ respectively). No significant relationship was found between cIMT and plasma levels of Fetuin-A and FGF-23.

Table 4.1: Comparison among the different study groups

	CKD groups			p value	Overall (n=72)
	I (n=20)	II-IV (n=23)	V-Dialysis (n=29)		
Age (years) ^a	8.9 (2.4)	10.8 (3.6)	12.1 (3.6)	0.006	10.8 (3.5)
Sex (M/F)	15/5	16/7	18/11	0.628	49/23
Disease duration (years) ^b	7 (0-10.0)	6 (0-15)	4 (0-18)	0.0347	6.0 (0-18.0)
Height (m) ^a	1.30 (0.12)	1.38 (0.21)	1.34 (0.18)	0.411	1.34 (0.18)
Weight (kg) ^b	26.8 (16.0-52.5)	33.7 (14.5-72.9)	27.4 (9.5-46.0)	0.659	27.5 (9.5-72.9)
BMI ^b	17 (14-28)	17 (13-23)	16 (11-30)	0.085	17 (11-30)
MAP (mmHg) ^b	74 (60-100)	76 (63-113)	94 (66-163)	<0.001	81 (60-163)
Hypertension (y/n)	0/20	7/16	22/7	<0.001	29/43
Haemoglobin (g/dl) ^a	13.3 (1.4)	13.2 (1.7)	9.4 (2.1)	<0.001	11.7 (2.6)
Anaemia (y/n)	1/19	5/18	24/5	<0.001	30/42
CRP (mg/l) ^b	10 (-)	10 (10-37)	10 (10-38)	0.377	10 (10-38)
Elevated CRP (y/n)	0/20	3/20	7/22	0.050	10/62
Albumin (g/L) ^b	44 (39-49)	42 (26-48)	36 (22-48)	<0.001	41 (22-49)
Hypoalbuminaemia (y/n)	0/20	2/21	11/18	0.001	13/59
Cholesterol (mmol/l) ^b	3.8 (2.0-6.2)	4.5 (2.0-13.5)	4.1 (2.3-8.1)	0.517	4.1 (2.0-13.5)
Hypercholesterolaemia (y/n)	3/17	3/20	9/20	0.265	15/57
Calcium (mmol/l) ^b	2.22 (2.16-2.57)	2.31 (2.04-2.46)	2.23 (1.29-2.72)	0.018	2.28 (1.29-2.72)
Phosphate (mmol/l) ^a	1.49 (0.23)	1.41 (0.31)	1.60 (0.54)	0.222	1.52 (0.57-2.81)
Hyperphosphataemia (y/n)	2/18	4/19	13/16	0.013	19/53
CaXP ^a	3.46 (0.52)	3.21 (0.68)	3.45 (1.39)	0.642	3.41 (1.36-6.60)
Elevated CaXP	0/20	0/23	6/23	-	6/66
Alkaline phosphatase (U/l) ^b	256 (159-438)	262 (110-482)	248 (101-1352)	0.929	250 (101-1352)
PTH (pmol/l) ^b	4.0 (1.1-43.0)	6.9 (1.5-19.9)	40.8 (1.7-201.0)	<0.001	7.4 (1.1-201.0)
Elevated PTH (y/n)	1/19	9/13	14/15	0.003	24/48
25(OH)D (ng/ml) ^b	22.2 (9.9-43.5)	24.8 (8.0-46.1)	18.6 (8.0-33.3)	0.037	21.7 (8.0-46.1)
Low 25(OH)D (y/n)	18/2	19/4	28/1	0.241	65/7
FGF-23 (pg/ml) ^b	15.7 (1.5-90.0)	21.2 (0-66.1)	264.1 (13.1-3893.0)	<0.001	32.2 (0-3893.0)
Fetuin-A (mg/dl) ^b	60.0 (11.1-96.6)	57.7 (26.6-126.0)	39.8 (0.9-225.2)	0.055	55.8 (0.9-225.2)
cIMT (mm) ^b	0.508 (0.425-0.647)	0.470 (0.380-0.555)	0.525 (0.405-0.675)	0.003	0.505 (0.380-0.675)
Z score cIMT for age and sex ^a	1.17 (0.48)	0.77 (0.39)	1.26 (0.54)	0.002	1.08 (0.52)
- Patients with Z score > 2	2 (10%)	0 (-)	2 (7%)	0.371	4 (6%)
Z score cIMT for height and sex ^a	1.19 (0.48)	0.81 (0.39)	1.34 (0.58)	0.002	1.12 (0.54)
- Patients with Z score > 2	2 (10%)	0 (-)	4 (14%)	0.196	6 (8%)
RI, n=72 ^b	0.72 (0.63-0.78)	0.74 (0.62-0.83)	0.70 (0.48-0.86)	0.081	0.72 (0.48-0.86)
PI, n=72 ^a	1.66 (0.31)	1.83 (0.43)	1.57 (0.45)	0.083	1.67 (0.42)

#-Mean (Standard deviation), *-Median (Range), BMI-Body mass index, MAP-Mean arterial pressure, CRP-C reactive protein, CaXP-Calcium phosphate product, PTH-Parathyroid hormone, 25(OH)D-25-Hydroxyvitamin D, FGF-23-Fibroblast growth factor-23, cIMT-Carotid intima media thickness, RI-Resistance index, PI-pulsatility index.

Table 4.2. Regression analysis for log transformed carotid intima media thickness

	Univariable			Multivariable		
	Coefficient	P value	95% CI	Coefficient	P value	95% CI
Age (years)	0.002	0.558	-0.006; 0.010	-0.007	0.395	-0.025; 0.010
Sex	-0.032	0.297	-0.092; 0.029	-0.017	0.601	-0.084; 0.049
Log duration of illness (years)	0.022	0.215	-0.013; 0.058	0.043	0.050	-0.001; 0.086
Height (m)	-0.079	0.325	-0.237; 0.080	-0.057	0.693	-0.347; 0.233
Log BMI	-0.010	0.904	-0.180; 0.160	-0.074	0.504	-0.293; 0.147
Log MAP	0.149	0.054	-0.003; 0.300	0.324	0.008	0.090; 0.557
Haemoglobin (g/dL)	-0.020	<0.001	-0.030; -0.010	-0.022	0.035	-0.042; -0.002
Log CRP	0.028	0.552	-0.066; 0.122	0.057	0.313	-0.056; 0.169
Log Albumin	-0.072	0.345	-0.237; 0.084	0.072	0.588	-0.195; 0.340
Log Cholesterol	0.015	0.731	-0.069; 0.098	0.077	0.158	-0.031; 0.185
Log Calcium	-0.202	0.108	-0.450; 0.046	-0.038	0.956	-1.425; 1.349
Phosphate (mmol/L)	0.002	0.945	-0.066; 0.072	-0.206	0.665	-1.158; 0.746
CaXP	-0.007	0.631	-0.036; 0.022	0.046	0.830	-0.386; 0.478
Log Alkaline phosphate	0.066	0.028	0.006; 0.105	0.045	0.176	-0.021; 0.055
Log PTH	0.033	<0.001	0.015; 0.051	0.021	0.210	-0.012; 0.055
Log 25(OH)D	-0.016	0.629	-0.082; 0.050	0.012	0.791	-0.082; 0.107
Log FGF-23	0.008	0.343	-0.008; 0.024	-0.001	0.940	-0.032; 0.030
Log Fetuin-A	-0.009	0.538	-0.038; 0.020	-0.013	0.581	-0.059; 0.033
CKD						
Stage I	Reference	-	-	Reference	-	-
Stage II-IV	-0.083	0.017	-0.150; -0.016	-0.112	0.006	-0.190; -0.034
Stage V (Dialysis)	0.030	0.347	-0.033; 0.094	-0.123	0.084	-0.265; 0.017

BMI-Body mass index, MAP-Mean arterial pressure, CRP-C reactive protein, CaXP-Calcium phosphate product, PTH-Parathyroid hormone, 25(OH)D-25-Hydroxyvitamin D, FGF-23-Fibroblast growth factor-23, CKD-Chronic kidney disease, CI-Confidence interval

Regression analysis for cIMT in relation to the clinical and biochemical parameters showed that only haemoglobin levels, log transformed Alkaline phosphatase and log transformed PTH were significantly associated with log transformed cIMT in a univariable model, $p < 0.050$. In the multivariable analysis, log transformed MAP showed a positive independent association with log transformed cIMT ($p = 0.008$) and haemoglobin showed a negative independent association with log transformed cIMT (0.035). (Table 4.2)

4.5 Discussion

Our study results highlighted four findings which are at odds with the current literature. We observed a higher overall median cIMT (even in our CKD 1 group) than previously reported, (19, 29, 223) we demonstrated a negative association between cIMT and low levels of haemoglobin which has generally not been described before, we found no association between cIMT and FGF-23 and we also found no association with cIMT and Fetuin A.

It is difficult to compare our data with that from other papers. Many papers demonstrating lower mean levels of cIMT had a normal distribution of cIMT data around the mean, (29-34) while our data showed a skewed distribution of cIMT. In addition, of the papers which showed lower levels of cIMT only recruited patients with mild to moderate CKD, while our study recruited patients with advanced disease in addition to those with mild to moderate disease. On the other hand, a study by Poyrazoğlu *et al* which reported a high median cIMT was carried out in older children and young adults who had a much higher mean age than our study patients.(224)

Despite this, all of the above mentioned studies reported higher cIMT values in patients with advanced CKD when compared to healthy controls or with those with mild to moderate CKD, in keeping with our data.

It is possible that our observations are a true reflection of the current vascular state of our patients although, keeping in mind that it is likely that vascular calcification takes time to develop, we did not find any evidence of vascular calcification in any of our cohort. Given the high cIMT observed in our study population and also that our CKD 1 group was noted to have high cIMT, we wonder if the reference values

provided by Doyon et al (6) are appropriate for our cohort. Unfortunately, the absence of a control group in our study makes it difficult to assess this issue.

Several studies have reported an association of cIMT with various cardiovascular risk factors such as duration of dialysis, hypertension, phosphate levels, calcium-phosphate product levels and PTH levels,(19, 20, 29, 225) but the independent association of cIMT we noted was with MAP and haemoglobin level. This is interesting for two reasons; firstly, in the past, haemoglobin has been associated more with cardiac changes than with vascular changes in patients with CKD(64, 226) and, secondly, it may explain why we saw high cIMTs despite the absence of vascular calcification which is usually associated with MBD.

The relationship between cIMT and anaemia is a surprising finding and the reasons are unclear. It has not been reported elsewhere and could possibly be the topic of further research

In spite of previous reports of the association between cIMT with FGF-23 in adult CKD patients,(227-229) we did not find a similar association in our cohort. This is in keeping with findings from another paediatric study in CKD patients where no association between FGF-23 and cIMT in children on peritoneal dialysis was seen.(230) We also did not find any association between Fetuin-A and cIMT as previously reported in both adults and children with CKD.(231, 232)

4.6 Strength and limitations

This is the first African study looking at cIMT in groups of children with different spectra of CKD and also determined the association of cIMT with cardiovascular risk factors, Fetuin-A and FGF-23. Given our findings we would suggest that population

specific cIMT levels need to be defined for our group of patients, and we would like to see a prospective study performed specifically looking at the effect of level of haemoglobin on the development, and regression, of cIMT.

The major limitation of our work is the lack of a disease free control group for comparison, and also the relatively small sample size. However, we do not believe that these limitations impact on the overall strength of the study and hence we believe that the findings still remain significant.

4.7 Conclusion

This study reports an unexpectedly high cIMT in African children with a wide range of CKD and also the uncommon finding of haemoglobin as an independently associated factor for cIMT in African children with CKD. Mean arterial pressure was independently associated with cIMT. Also, contrary to previous reports, we did not find any independent associations of cIMT with Fetuin-A, FGF-23 or markers of MBD. We believe that our findings highlight the need to address modifiable risk factors, especially anaemia and hypertension, in our group of CKD patients, and also the need to establish paediatric reference values for cIMT in healthy African children.

4.8 Acknowledgement

The authors acknowledge the contribution of all team members who provided care for these children and assisted with this study.

4.9 Conflict of interest

The authors declare no conflict of interest

CHAPTER 5 (MANUSCRIPT 3): CARDIAC CHANGES AND THEIR ASSOCIATION WITH FETUIN-A AND FIBROBLAST GROWTH FACTOR-23 IN CHILDREN WITH CHRONIC KIDNEY DISEASE

5.1 Abstract

Aims: In children with chronic kidney disease (CKD), Fetuin-A and Fibroblast growth factor-23 (FGF-23) have been implicated in the mechanism and progression of several cardiac changes. This study aimed to determine the types and rates of cardiac changes in children with CKD and their association with Fetuin-A, FGF-23 and other cardiovascular risk factors (CVRFs).

Methods: This comparative cross sectional study recruited 88 children (5-18 years); 27 CKD I with a GFR>90 ml/min/1.73m², 61 with a glomerular filtration (GFR) of <90 ml/min/1.73m² (29 CKD II-IV, 32 CKD V-Dialysis). Each patient had a short demographic and clinical history taken along with a physical examination. Blood was taken and sent for routine tests and for Fetuin-A and FGF-23 assay. All patients had an echocardiogram to evaluate cardiac structure and function.

Results: The distribution of left atrial diameter (LAD) and left ventricular mass (LVM) differed significantly ($p<0.05$) across the different CKD groups. Abnormal LAD was seen in 10% of patients; left ventricular hypertrophy (LVH) in 27%; left ventricular systolic dysfunction in 6% and diastolic dysfunction in one patient. Fetuin-A was the only independent predictor for abnormal LAD; mean arterial pressure was independently associated with concentric LVH, and age and hypoalbuminaemia with eccentric LVH. Overall, the dialysis group had the highest rate of cardiac changes and associated risk factors.

Conclusion: Though not common, the importance of left atrial changes in children with CKD is highlighted along with the need to address modifiable CVRFs such as hypertension and hypoalbuminaemia.

5.2 Introduction

Cardiovascular disease (CVD) is a major cause of death in children with chronic kidney disease (CKD), and children with CKD have the highest cardiovascular risk in the paediatric population.(13, 18) Several cardiac changes have been reported such as left ventricular hypertrophy, cardiomyopathies, systolic and diastolic dysfunctions, coronary artery disease, arrhythmias and myocardial ischemia.(89, 148, 149) These changes can begin early in CKD and worsen as the disease progresses.(132)

Cardiovascular risk factors (CVRFs) have been reported even in children with early CKD.(132) In addition to risk factors such as anaemia, dyslipidaemia, hypertension, inflammation and dysfunctional mineral bone metabolism, cardiovascular risk assessment using biomarkers such as Fibroblast growth factor-23 (FGF-23) and Fetuin-A have been recommended for early detection and intervention of subclinical CVD.(18)

Transthoracic echocardiography is a non-invasive method that can be used to assess heart structure and function. Traditionally, the M-mode and the two-dimensional doppler echocardiogram allow assessment of ventricular mass and volumes with good accuracy for the diagnosis of hypertrophy, definition of ventricular geometric pattern and systolic and diastolic function estimate.(124, 125)

In a recent unpublished study in our centre, we observed that more than 60% of the children with CKD awaiting transplant get delisted due to various cardiovascular

related complications. Cardiomyopathy, associated with poor ejection fraction, is the most common cardiovascular related problem observed in our patients on chronic dialysis and, less frequently, cerebrovascular disease, arrhythmia and poor vascular access have also been noted. We have also observed that some of these children with dialysis CKD die from cardiovascular causes. We therefore felt that it was essential to identify and manage these cardiovascular complications to improve the outcome of these children.

This study determined the different types and rates of cardiac changes seen in children with different spectrum of CKD, and their association with Fetuin-A, FGF-23 and other CVRFs.

5.3 Methods

This comparative cross sectional study recruited 88 children (5-18 years) with CKD being followed up by the Divisions of Paediatric Nephrology of the Charlotte Maxeke Johannesburg Academic Hospital and Chris Hani Baragwanath Academic Hospital, Johannesburg, South Africa.

Sixty-one children with a glomerular filtration (GFR) of <90 ml/min/1.73m² (29 CKD II-IV, 32 CKD V-Dialysis) and 27 CKD I with a GFR >90 ml/min/1.73m² (with either structural abnormalities or isolated haematuria) with normal blood pressure and no proteinuria were recruited consecutively over a 12-month period (August 2015 – July 2016). Twenty three of the patients on maintenance dialysis were on haemodialysis and the remaining nine on peritoneal dialysis.

Children with known congenital heart disease, diabetes mellitus, liver disease, active infection, systemic lupus erythematosus, malignancies and renal transplant were excluded from the study.

All patients had a short demographic and clinical history taken along with a physical examination. Blood samples were taken for serum creatinine, urea, albumin, calcium, phosphorus, parathyroid hormone (PTH), alkaline phosphatase, total cholesterol, haemoglobin and C-reactive protein (CRP). Blood samples for 25-Hydroxy Vitamin D [25(OH)D], Fibroblast growth factor-23 (FGF-23) and Fetuin-A were also taken and sent to a research laboratory for assay.

Blood pressure (BP) was measured by auscultation using an appropriately sized cuff and a mercury sphygmomanometer and with the patient in the sitting position. For the haemodialysis CKD participants, BP was measured prior to the dialysis session. All other participants had their BP measured during a routine clinic visit. Blood pressure readings were indexed to the age-, gender-, and height-specific percentile for each participant according to the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents.(19) Pulse pressure (PP) was calculated as the difference between the systolic blood pressure (SBP) and diastolic blood pressure (DBP), while mean arterial blood pressure (MAP) was calculated as the sum of DBP and a third of PP.

Serum creatinine and intact parathyroid hormone (PTH) were assayed using the Siemens Advia enzymatic technique and Siemens Advia Centaur chemiluminometric immunoassay technique. Plasma 25(OH)D was assayed by Chemiluminescence Micro-particle Immunoassay (CMIA) using the ARCHITECT 25-OH Vitamin D method. Plasma intact FGF-23 was assayed using Human FGF-23 ELISA Kit (Merck

Millipore, Merck group, Massachusetts, USA). Plasma AHSG level was assayed using EDI™ Human Fetuin-A ELISA kit (EPITOPE Diagnostics, Inc, CA, USA).

Cardiac structure and function was evaluated using an echocardiogram administered by a single experienced research echocardiogram technician using a Phillips iE33 machine equipped with a S5-1 1-5 MHz transducer, allowing for M-mode, two dimensional and colour doppler measurements (Phillips Corporation USA). All examinations were carried out according to the American Society of Echocardiography recommendations.(144, 193, 194) The following parameters were determined: left atrial diameter (LAD), left ventricular dimensions including, left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameters (LVESD), left ventricular posterior wall thickness at end diastole (LPWD), interventricular septal thickness at end diastole (IVSD). Subsequently, ejection fraction (EF) and fractional shortening (FS) were calculated. Traditional pulsed wave doppler indices of peak early (E) and late (A) trans-mitral inflow velocities were measured to obtain the (E/A) ratio. Tissue doppler imaging (TDI) was used to obtain the index for LV filling pressure (E/E').

Left ventricular mass (LVM) was calculated according to the equation described by Devereux *et al.*(139, 195) Left ventricular mass was indexed (LVMI) for gender and body surface area and abnormal LVMI graded into mild, moderate and severe.(134, 196) Relative wall thickness (RWT) was also calculated in order to determine the pattern of the left ventricular geometry.(144) Left ventricular (LV) geometry was classified using LVMI and RWT into normal, concentric remodelling (CR), concentric hypertrophy (CH) and eccentric hypertrophy (EH).

Where abnormalities were found, patients were referred to a paediatric cardiologist for further evaluation.

5.3.1 Definition of terms

- Glomerular filtration rate (GFR) was estimated by the use of the modified Schwartz formula.(222)
- Hypertension: the need for antihypertensive treatment and/or according to the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents.(19)
- Proteinuria: urine protein/creatinine ratio >0.02g/mmol.(197, 198)
- Hypercholesterolaemia: total cholesterol >5.18mmol/L (>200mg/dl).(46)
- Anaemia: defined based on age according to the Kidney Disease Improving Global Outcome (KDIGO) clinical practice guidelines for anaemia in CKD.(199)
- Hyperparathyroidism: parathyroid hormone (PTH) levels above laboratory normal limit (>7.6pmol/L), in pre-dialysis patients and above nine times the upper normal limit (>68.4pmol/L) in dialysis patients as recommended by KDIGO.(82)
- Hypoalbuminaemia: serum albumin <35mg/dl.(103)
- Elevated C-reactive protein (CRP): >10mg/L.(201)
- Low 25 OH Vitamin D: <30ng/ml.(200)
- Abnormal LAD: > normal for age.(202)
- Abnormal LVMI and its severity defined based on body surface area for sex.(196)

- Ejection Fraction: low (<40%), borderline (41-50%), normal (51-70%), high (>70%)
- Abnormal E/A: <1.(203)

5.3.2 Data analysis

All data were collected and managed using Research Electronic Data Capture (REDCap) tools hosted at the University of the Witwatersrand (204). STATA 13.1 was used for the analysis. Continuous variables were described using means and standard deviations for data normally distributed, and medians and inter-quartile ranges for skewed data. Categorical variables were presented as percentages and frequencies. Mean/median values of the different groups were compared using Student's t-test, Mann-Whitney U test, ANOVA and Kruskal-Wallis test, depending on the distribution of the data. Statistical significance in the proportions of cardiac changes and risk factors was tested for using Chi square test and Fisher exact test, where appropriate. Regression analysis was used to determine independent associated factors for abnormal LAD and LVH. A p value <0.05 was regarded as statistically significant for all analyses.

5.3.3 Ethics and Consent

The study was approved by the University of the Witwatersrand, Human Research Ethics Committee (Protocol M150312) and was conducted in conformance with the Helsinki Declaration, Good Clinical Practice and within the laws and regulations of South Africa.

Written consent was obtained for each participating parent/guardian, with assent from the children >8 years old.

5.4 Results

The mean age of patients was 10.85 years $\pm$ 3.14. There were 58 males and 30 females giving a male to female ratio of 2:1. The median (interquartile range-IQR) age at diagnosis and duration of illness was 4 years (0-7) and 6 years (2-9) respectively. The majority of the children were from the Black racial group (79/88). Children from the White (3/88), Asian (2/88) and the Mixed racial groups (4/88) made up the rest of the cohort.

The overall median (IQR) left atrial diameter (LAD) was 26mm (22-29). The median LAD was seen to increase significantly across the study groups with advancing disease ($p=0.017$). Nine patients had abnormal LAD (>95 centile for age), and the dialysis group had the highest number of patients (6/9; 67%) with abnormal LAD. (Table 5.1)

The overall absolute LVM median (IQR) was 81g (56-134). The dialysis group had the highest absolute LVM median when compared to the other study groups and there was a statistically significant difference in the median comparison of absolute LVM by the different CKD groups ($p<0.001$). A skewed LVMI pattern was observed with a median (IQR) of 74.5g/m² (61.5-117.5). Similarly, the dialysis group had the highest median and there was a statistically significant difference in the median LVMI of the study groups ($p<0.001$). (Table 5.1) There was a strong positive correlation between LAD and LVMI, $p=0.54$, $p<0.001$.

Abnormal LVMI was defined based on gender and BSA. The majority (21/32; 66%) of the patients on dialysis had an abnormal LVMI, while none of the patients in the CKD I group had an abnormal LVMI. The majority (12/24; 50%) of patients with LVMI

abnormality had a severe form of this abnormality and this was most common in the dialysis group. (Table 5.1)

More than half of the patients (46/88; 52%) had abnormal geometry. Left ventricular hypertrophy (LVH) was seen in 27% (24/88) of the patients, with the majority (21/24; 88%) of patients with LVH seen in the dialysis group. (Table 5.1) The CKD I group had the least number of patients (9/88; 10%) with abnormal LV geometry and this was only in the form of concentric remodelling.

Left ventricular systolic function was determined using LV ejection fraction (EF) and fractional shortening (FS). The median (IQR) EF and FS were 66% (60-73) and 36% (32-41) respectively. There was no statistically significant difference in EF and FS when compared across the study groups, $p=0.071$ and $p=0.535$ respectively. Ejection fraction was further categorised into low (<40%), borderline (40%-50%), normal (50%-70%) and high (>70%). About 58% (51/88) of the patients had a normal EF and about 6% (5/88) had low EF. Four (80%) of the five patients with low EF belonged to the dialysis group. (Table 5.1) Low EF was only seen in patients with abnormal LVMI and abnormal LV geometry. (Figure 5.1 and 5.2)

Diastolic function was determined using the trans-mitral flow velocity ratio (E/A) and the index for LV filling pressure (E/E'). The median (IQR) E/A and E/E' was 1.59 (1.30-1.90) and 10.1 (8.3-13.2) respectively. There was a statistically significant difference in the median E/A and E/E' when compared across the different study groups, $p=0.031$ and $p<0.001$ respectively. (Table 5.1) Only one of the dialysis patients was found to have an abnormal E/A (<1).

Several CVRFs described previously were identified in the study groups. (Table 5.1) The CKD dialysis group had the highest rate of all the risk factors identified. There

was a statistically significant difference in the rates of the majority of the risk factors when compared by the different CKD groups ($p<0.05$).

These risk factors mentioned above were compared for abnormal LAD. Patients with abnormal LAD had a higher proportion of these risk factors compared to their counterparts with a normal LAD. (Table 5.2) Mean arterial pressure, haemoglobin and CRP were significantly associated with abnormal LAD in a univariable regression model but only Fetuin-A was identified as an independent predictor of abnormal LAD, $p=0.034$. (Table 5.3)

Left ventricular hypertrophy showed a significant association with age, MAP, haemoglobin, albumin, PTH and FGF-23 in a univariable model ($p<0.050$). (Table 5.2) After adjusting for these risk factors in a multivariable model only MAP was identified as an independent associated factor for LVH, $p=0.020$. (Table 5.3) Concentric LVH was also found to have MAP as an independent associated factor ($p=0.018$), while Eccentric LVH was found to have age and albumin levels as independent associated factors, $p=0.026$ and $p=0.011$ respectively. (Table 5.4)

We did not find any association between mode of dialysis and cardiac changes and, similarly, duration of dialysis was not associated with cardiac changes.

Table 5.1: Comparison among the different study groups

	CKD I (n=27)	CKD II-IV (n=29)	CKD V-Dialysis (n=32)	P value [@]
Demographics				
Age (years) [#]	9.33 ± 2.40	11.10 ± 3.55	11.88 ± 3.73	0.015
Sex (M/F)	19/8	20/9	19/13	0.616
Height (m) [#]	131.36 ± 14.33	137.22 ± 20.03	133.24 ± 19.50	0.471
Weight (kg) [#]	30.22 ± 11.78	34.21 ± 13.73	29.73 ± 11.10	0.309
CVRFs				
MAP (mm/Hg) [*]	70 (70-80)	76 (70-83)	93 (82-103)	<0.001
Hypertension	0/27	9/29	24/32	<0.001
Haemoglobin (g/dl) [*]	13.2 (12.5-14.4)	13.4 (11.9-14.1)	9.4 (8.3-10.6)	<0.001
Anaemia	2/27	8/29	27/32	<0.001
CRP (mg/l) [*]	10 (-)	10 (-)	10 (10-13.5)	0.008
Elevated CRP	0/27	3/29	9/32	0.004
Albumin (mg/dl) [*]	44 (42-45)	43 (39-44)	36 (30-40)	<0.001
Low Albumin	0/27	2/29	12/32	<0.001
Cholesterol (mmol/l) [*]	3.7 (3.2-4.5)	4.5 (3.5-4.7)	4.2 (3.3-5.7)	0.479
Hypercholesterolaemia	4/27	3/29	11/32	0.057
PTH (pmol/l), n=87 [*]	3.5 (2.8-5.3)	7.3 (4.1-11.1)	50.5 (17.4-136.6)	<0.001
Elevated PTH, n=87	3/27	13/28	16/32	0.002
Vitamin D (ng/ml) [*]	22.7 (19.3-27.3)	24.8 (18.3-29.3)	18.9 (12.9-23.0)	0.025
Low Vitamin D	23/27	24/29	31/32	0.157
FGF-23 (pg/ml) [*]	12.0 (6.7-25.3)	21.2 (8.0-42.5)	265.1 (120.7-463.2)	<0.001
Fetuin-A (mg/dl) [*]	61.1 (44.2-73.6)	55.1 (47.8-74.7)	40.5 (11.9-70.1)	0.049
PCR (g/mmol), n=63 [*]	0.004 (0.001-0.008)	0.030 (0.006-0.080)	0.310 (0.060-0.930)	<0.001
Proteinuria, n=63	0/27	15/27	8/9	<0.001
ECHO				
LAD (mm) [*]	24 (20-28)	25 (22-29)	28 (23-32)	0.017
Abnormal LAD	1/27	2/29	6/32	0.159
LVM (g) [*]	60 (44-83)	84 (65-119)	127 (78-200)	<0.001
LVMi (g/m ²) [*]	63 (51-70)	72 (62-92)	122 (91-168)	<0.001
Abnormal LVMi	0/27	3/29	21/32	<0.001
Severity of abnormal LVMi				
Mild	0	0/3	7/21	0.564
Moderate	0	1/3	4/21	
Severe	0	2/3	10/21	
LV geometry				
Normal	18/27	18/29	6/32	<0.001
Concentric remodelling	9/27	8/29	5/32	
Concentric hypertrophy	0/27	1/29	16/32	
Eccentric hypertrophy	0/27	2/29	5/32	
EF (%) [*]	68 (63-74)	66 (61-68)	67 (55-73)	0.374
EF pattern				
Low (<40%)	0/27	1/29	4/32	0.071
Borderline (41-50%)	1/27	1/29	1/32	
Normal (51-70%)	14/27	22/29	15/32	
High (>70%)	12/27	5/29	12/32	
FS (%) [#]	37.57 ± 7.11	35.35 ± 8.67	35.13 ± 10.34	0.525
e/a (m/s) [*]	1.6 (1.3-2.0)	1.6 (1.5-2.0)	1.3 (1.2-1.7)	0.031
e/e' (m/s) [*]	8.6 (7.1-9.3)	9.1 (8.3-10.9)	13.4 (10.9-15.8)	<0.001

[@]-Comparison across CKD groups, ANOVA test for means, Kruskal Wallis test for medians, Chi square/Fisher exact test for proportions, ^{*}-Median (Interquartile range), [#]-Mean (Standard deviation), CVRFs-Cardiovascular risk factors, ECHO- Echocardiogram, MAP-mean arterial pressure, CRP-C-reactive protein, PTH-Parathyroid hormone, PCR-Protein creatinine ratio, LAD-Left atrial diameter, LVM-Left ventricular mass, LVMi-Left ventricular mass index, EF-Ejection fraction, FS-Fractional shortening, e/a-Trans mitral flow velocity ratio, e/e'- left ventricular filling pressure

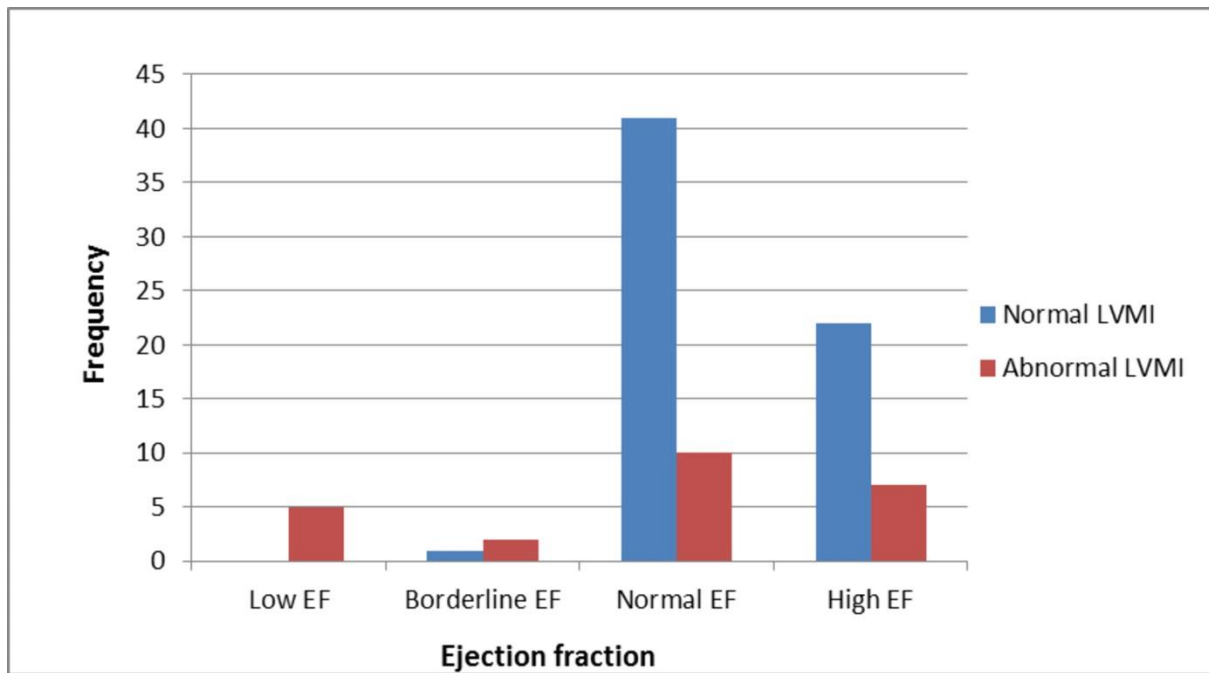


Figure 5.1 Ejection fraction by left ventricular mass index. EF-ejection fraction, LVMI-left ventricular mass index

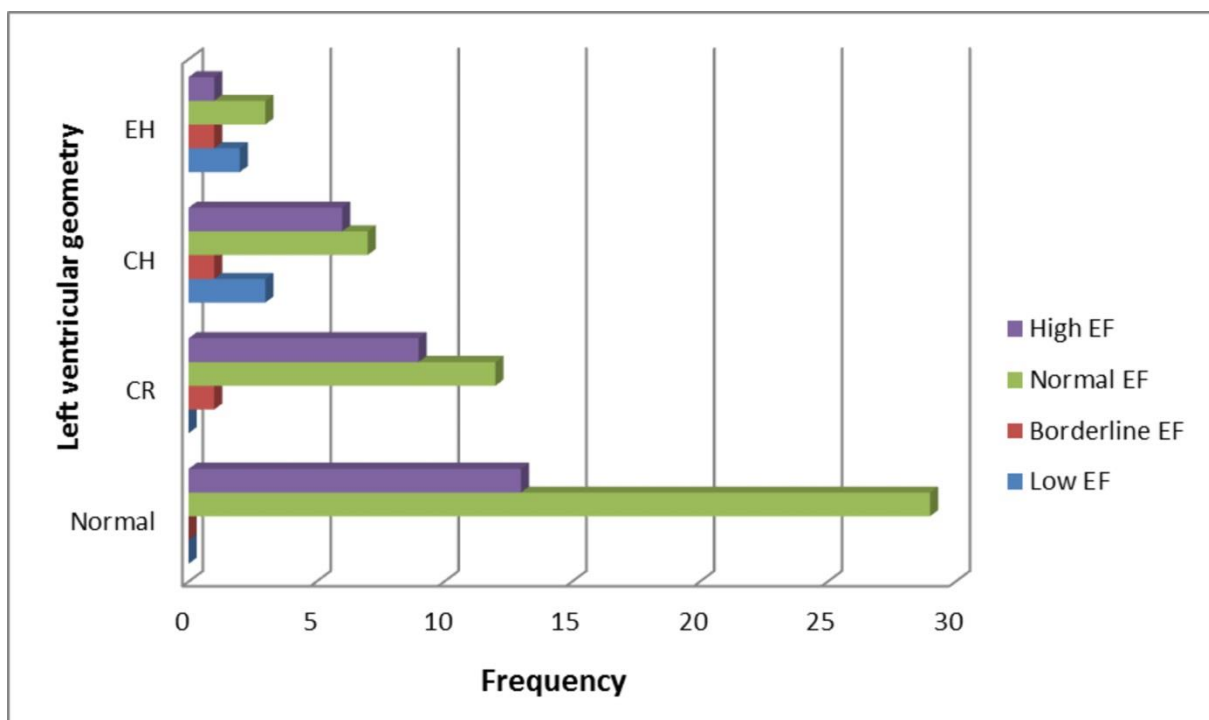


Figure 5.2 Ejection fraction by left ventricular geometry. EH-eccentric hypertrophy, CH-concentric hypertrophy, CR-concentric remodelling, EF-ejection fraction.

Table 5.2: Risk factors for abnormal left atrial diameter (LAD) and LVH

	Abnormal LAD		p value [@]	LVH		p value [@]
	Yes (n=9)	No (n=79)		Yes (n=24)	No (n=64)	
Age (years) [#]	12.56 ± 3.40	10.65 ± 3.42	0.115	12.25 ± 3.87	10.31 ± 3.14	0.018
Sex (M/F)	5/4	53/26	0.483	13/11	45/19	0.155
MAP (mm/Hg) [*]	100 (79-105)	80 (70-88)	0.016	97 (82-106)	74 (70-83)	<0.001
Hypertension	7/9	26/79	0.013	22/24	11/64	<0.001
Haemoglobin (g/dl) [*]	9.5 (8.4-11.2)	12.7 (10.1-13.7)	0.029	9.3 (8.4-11.0)	13.1 (12.0-14.1)	<0.001
Anaemia	7/9	30/79	0.032	21/24	16/64	<0.001
CRP (mg/l) [*]	10 (10-19)	10 (-)	0.004	10 (10-14)	10 (-)	0.012
Elevated CRP	4/9	8/79	0.018	7/24	5/64	0.015
Albumin (mg/dl) [*]	36 (29-45)	42 (38-44)	0.238	36 (29-39)	42 (39-45)	<0.001
Low Albumin	4/9	10/79	0.033	10/24	11/64	<0.001
Cholesterol (mmol/l) [*]	3.6 (3.0-4.2)	4.2 (3.4-5.0)	0.218	4.2 (3.3-5.5)	4.2 (3.4-4.9)	0.859
Hypercholesterolaemia	1/9	17/79	0.679	7/24	11/64	0.243
PTH (pmol/l), n=87 [*]	18 (4.1-60.1)	7.3 (3.8-25.7)	0.299	36.1 (15.7-136.6)	5.3 (3.1-11)	<0.001
Elevated PTH, n=87	3/9	29/78	1.000	13/24	19/63	0.038
Vitamin D (ng/ml) [*]	20.8 (10.6-27.1)	22.3 (15.5-27.3)	0.457	18.9 (14.4-23.0)	23.1 (15.8-27.8)	0.109
Low Vitamin D	8/9	70/79	1.000	22/24	56/64	0.721
FGF-23 (pg/ml) [*]	266.1 (52.9-384.1)	25.3 (9.0-102.4)	0.011	265.1 (120.7-579.5)	19.4 (7.9-43.2)	<0.001
Fetuin-A (mg/dl) [*]	24.1 (4.0-44.2)	59.0 (39.8-74.7)	0.003	37.7 (13.8-79.1)	58.8 (44.1-71.4)	0.120
PCR (g/mmol), n=63 [*]	0.046 (0.007-0.693)	0.009 (0.003-0.048)	0.230	0.432 (0.105-1.422)	0.01 (0.001-0.030)	<0.001
Proteinuria, n=63	2/3	21/60	0.548	7/8	16/55	0.003

[@]-t-test for means, Mann-Whitney U test for medians, Chi square/Fisher exact test for proportions, ^{*}-Median (Interquartile range), [#]-Mean (Standard deviation), LAD-left atrial diameter, LVH-left ventricular hypertrophy, MAP-mean arterial pressure, CRP-C-reactive protein, PTH-Parathyroid hormone, PCR-Protein creatinine ratio

Table 5.3: Regression analysis for abnormal left atrial diameter and left ventricular hypertrophy

Abnormal LAD (n=88)						
Univariable				Multivariable		
	OR	p value	95% CI	OR	p value	95% CI
Age (years)	1.18	0.123	0.96-1.47	1.08	0.653	0.78-1.50
MAP (mmHg)	1.04	0.036	1.00-1.08	1.06	0.096	0.99-1.15
Haemoglobin (g/dl)	0.76	0.035	0.58–0.98	0.84	0.503	0.49-1.42
Albumin (mg/dl)	0.92	0.098	0.83-1.02	0.97	0.689	0.83-1.13
CRP (mg/l)	1.06	0.083	0.99-1.13	1.07	0.115	0.99-1.15
PTH (pmol/l), n=87	1.01	0.217	1.00-1.02	0.994	0.531	0.975-1.013
FGF-23 (pg/ml)	1.000	0.229	0.999-1.001	1.000	0.744	0.999-1.001
Fetuin-A (mg/dl)	0.956	0.005	0.927-0.987	0.955	0.034	0.916-0.997
CKD						
I	Reference	-	-	Reference	-	-
II-IV	1.93	0.602	0.17-22.55	1.11	0.944	0.06-22.21
V	6.00	0.108	0.67-53.38	0.30	0.582	0.003-22.412
LVH (n=88)						
Univariable				Multivariable		
Age (years)	1.19	0.022	1.03-1.38	1.19	0.286	0.87-1.63
MAP (mmHg)	1.14	<0.001	1.07-1.20	1.10	0.020	1.02-1.19
Haemoglobin (g/dl)	0.55	<0.001	0.42-0.72	0.60	0.058	0.35-1.02
Albumin (mg/dl)	0.82	<0.001	0.74-0.91	0.93	0.396	0.78-1.10
CRP (mg/l)	1.03	0.321	0.97-1.09	1.03	0.517	0.95-1.12
PTH (pmol/l), n=87	1.02	<0.001	1.01-1.03	1.006	0.504	0.988-1.025
FGF-23 (pg/ml)	1.001	0.044	1.000-1.002	1.001	0.089	1.000-1.002
Fetuin-A (mg/dl)	1.00	0.530	0.980-1.010	1.030	0.080	0.997-1.064
CKD ^a						
II-IV	Reference	-	-	Reference	-	-
V	16.55	<0.001	4.08-67.10	0.62	0.738	0.04-10.36

LAD-left atrial diameter, LVH-left ventricular hypertrophy, MAP-mean arterial pressure, CRP-C-reactive protein, PTH-Parathyroid hormone, OR-Odds ratio, CI-Confidence interval

^a- None of the patients in the CKD I group had LVH and as such the model automatically drops this group from the regression analyses for LVH.

Table 5.4: Regression analysis for Concentric and Eccentric left ventricular hypertrophy

Concentric LVH (n=88)						
	Univariable			Multivariable		
	OR	p value	95% CI	OR	p value	95% CI
Age (years)	1.11	0.193	0.95-1.30	0.78	0.139	0.56-1.08
MAP (mmHg)	1.10	<0.001	1.05-1.16	1.12	0.018	1.02-1.23
Haemoglobin (g/dl)	0.57	<0.001	0.44-0.75	0.70	0.166	0.42-1.16
Albumin (mg/dl)	0.90	0.017	0.83-0.98	1.21	0.070	0.99-1.48
CRP (mg/l)	1.03	0.388	0.97-1.09	1.02	0.705	0.93-1.11
PTH (pmol/l), n=87	1.018	<0.001	1.008-1.027	1.014	0.129	0.996-1.031
FGF-23 (pg/ml)	1.0006	<0.001	1.0000-1.0012	1.001	0.227	1.000-1.002
Fetuin-A (mg/dl)	0.996	0.668	0.980-1.013	1.017	0.266	0.988-1.047
CKD ^α						
II-IV	Reference	-	-	Reference	-	-
V	28.0	0.002	3.39-231.32	4.91	0.422	0.10-238.05
Eccentric LVH (n=88)						
	Univariable			Multivariable		
	OR	p value	95% CI	OR	p value	95% CI
Age (years),	1.28	0.062	0.99-1.66	1.96	0.026	1.08-3.53
MAP (mmHg)	1.04	0.062	1.00-1.08	0.99	0.853	0.92-1.08
Haemoglobin (g/dl)	0.79	0.097	0.59-1.05	0.83	0.614	0.39-1.75
Albumin (mg/dl)	0.81	0.001	0.71-0.92	0.72	0.011	0.56-0.93
CRP (mg/l)	1.02	0.689	0.94-1.10	0.97	0.718	0.81-1.16
PTH (pmol/l), n=87	1.002	0.779	0.989-1.015	0.985	0.259	0.959-1.011
FGF-23 (pg/ml)	1.000	0.131	0.999-1.001	1.001	0.276	0.999-1.002
Fetuin-A (mg/dl)	0.995	0.680	0.970-1.020	1.017	0.354	0.981-1.055
CKD ^α						
II-IV	Reference	-	-	Reference	-	-
V	2.5	0.298	0.45-14.02	0.43	0.731	0.004-50.325

LVH-left ventricular hypertrophy, MAP-Mean arterial pressure, CRP-C-reactive protein, PTH-Parathyroid hormone, OR-Odds ratio, CI-Confidence interval

α- None of the patients in the CKD I group had LVH and as such the model automatically drops this group from the regression analyses for LVH.

5.5 Discussion

Cardiovascular disease remains a major cause of morbidity and mortality among children with CKD. This study compared the type and rates of cardiac changes in three distinct groups of CKD patients; CKD I, CKD II-IV and CKD V-Dialysis. Cardiac changes observed include abnormal LAD, abnormal LVMI, abnormal LV geometry, LVH and changes in LV systolic and diastolic function. Cardiovascular risk factors in these patients were also compared and their association with abnormal LAD and LVH was determined.

Even though LAD has been described as an independent predictor of cardiovascular events in adults,(233, 234) abnormal LAD is not frequently reported as one of the cardiac changes seen in children with CKD. Our study observed a 10% rate of abnormal LAD in our cohort. Of these patients with LAD, 67% of them were of the dialysis group. The LAD measurement by CKD groups, when compared with a previous study that looked at LAD, showed a similar pattern, where the highest measurements were observed in the dialysis group.(149) The influence of blood pressure on atrial size has been previously reported,(235) but in our study plasma Fetuin-A level was the only independent associated factor for abnormal LAD observed.

We are not sure how to explain the presence of an abnormal LAD in the one patient in the CKD 1 group. We hypothesize that the child might have had an underlying cardiac problem, such as mitral valve pathology or a congenital heart condition, which was missed during the study. Alternatively, although this child was in the CKD 1 group, they might have already started to develop cardiac changes even though the deterioration of their renal function is not clinically apparent.

Left ventricular hypertrophy has been reported as the most common cardiac abnormality observed in children with CKD.(28, 148) A wide range of rates of LVH (10-50%) in children with various stages of CKD has been reported,(28, 71, 89, 131, 134, 148, 236) and this disparity has been attributed to the different methods of indexation of LVMI which ultimately affects the categorization of LVH in children.(134) The rate of LVH observed in our study (27%) falls within this range but was found to be higher than the rate reported by Simpson *et al*,(134) where BSA was also used for indexation of LVMI. This difference may be explained by the fact that their study did not include dialysis CKD patients while our study did include them. Another similar study by Adiele *et al* reported a higher rate of 50% for LVH but the smaller nature of their sample size may be the reason for the variance when compared to our study.(148)

Various risk factors have been implicated in LVH but hypertension has been described as the most important risk factor.(28) In our study MAP was identified as overall independent associated factor for LVH. Even though FGF-23 has been associated with LVH in both adults and children with CKD,(237-239) we didn't find a similar association after adjusting for other risk factors. The reason for this may be that the majority of these studies were carried out in adult patients, and the few studies performed in paediatric patients were conducted in children with advanced disease.

Concentric LVH (CH) has been attributed to hypertension,(71, 240, 241) while eccentric LVH (EH) has been attributed to anaemia.(226, 242) Our findings also suggest that hypertension, plays a vital role in CH, while age and hypoalbuminaemia are independent associated factors for EH.

It is interesting that, although Fetuin A was associated with the development of LAD, there was no correlation between Fetuin A and the development of LVH. As far as we are aware this is a novel finding which has not been previously described. Further research on the relationship between Fetuin A and cardiac changes in paediatric would need to be undertaken to confirm, and explain, this finding.

In contrast to adult patients, left ventricular systolic function (often assessed by the use of left ventricular EF and FS) has been observed to occur in lower rates when compared to LV diastolic dysfunction in children with CKD.(89, 121, 148) Our study reports a rate of a low left ventricular EF of 6%. Diastolic dysfunction was observed only in one patient in our study; this is certainly much lower than what has been described previously.(89, 148, 149) No clear reason could be identified for this finding.

5.6 Strength and limitations

This is a study from an African setting that looked at three groups of children with different spectrum of CKD. It also looked at the association between early cardiac changes and biomarkers such as Fetuin-A and FGF-23 that are not routinely assessed in clinical practice.

The major limitations are the small sample size and the lack of a control group for comparison. Another limitation is that left atrial diameter, rather than left atrial volume which correlates better with cardiovascular disease, was measured.

5.7 Conclusion

This comparative study provides new information on the types and rates of cardiac changes seen in a spectrum of South African children with CKD. It also highlights the possible role played by biomarkers such as plasma Fetuin-A and FGF-23 in the development of early changes seen in cardiovascular disease. Although Fetuin A is associated with the development of an increased LAD it does not appear to be associated with the development of LVH. We also found no independent association between FGF-23 and cardiac changes. Left ventricular hypertrophy remains the most common cardiac change seen in children with CKD and, although less common, it is important to monitor for left atrial changes in these children. Hypoalbuminaemia, in addition to adequate blood pressure control, need to be addressed in order to retard the progression of cardiovascular disease in children with CKD. A prospective study, with a larger sample size and a control group, looking at these biochemical markers and cardiac changes alongside progression of CKD, may provide more information on interventions which might halt or slow the risk of cardiovascular disease in these children.

5.8 Acknowledgement

The authors acknowledge the contribution of all team members who provided care for these children and assisted with this study.

5.9 Conflict of interest

The authors declare no conflict of interest

CHAPTER 6 (MANUSCRIPT 4): FIBROBLAST GROWTH FACTOR-23 AND FETUIN-A GENE RELATIONSHIP IN BLACK SOUTH AFRICAN CHILDREN WITH CHRONIC KIDNEY DISEASE

6.1 Abstract

Background: Both Fibroblast growth factor-23 (FGF-23) and Fetuin-A levels have been implicated in mineral and bone disorder associated with chronic kidney disease (CKD), and several single nucleotide polymorphisms (SNPs) of the Fetuin-A gene have also been associated with Fetuin-A levels. This study aimed to determine the relationship between FGF-23 and Fetuin-A and also to determine the role of Fetuin-A SNPs with respect to Fetuin-A levels and markers of bone mineralisation in black South African children.

Methods: Blood samples from 93 children (5-18 years) with various stages of CKD were assessed for C-reactive protein, calcium, phosphate, parathyroid hormone, 25-hydroxyvitamin D, FGF-23 and Fetuin-A levels. Genomic DNA was extracted from whole blood and regions of the Fetuin-A gene amplified by polymerase chain reaction. Single nucleotide polymorphisms (SNPs) were genotyped by restriction fragment length polymorphism analysis or by direct sequencing.

Results: The median FGF-23 and Fetuin-A levels were 28.9 (0-3893) pg/ml and 57.7 (0.9-225.2) mg/dl respectively. A significant negative relationship between Fetuin-A and FGF-23 was only observed in the CKD V group ($\rho=-0.60$, $p<0.001$). Plasma FGF-23 levels correlated better with markers of bone mineralization than Fetuin-A. Eight SNPs were analysed; rs2248690, rs6787344, rs4831, rs4917, rs4918, rs2070633, rs2070634 and rs2070635. We found significant association of the Fetuin-A SNPs rs4918-G and rs2070633-T alleles with log-transformed Fetuin-A

levels. Serum phosphate and parathyroid hormone levels were also associated with Fetuin-A SNPs.

Conclusion: An inverse relationship between FGF-23 and Fetuin-A is more likely to be observed in children with advanced CKD, and patients with rs4918-G and rs2070633-T alleles are more likely to have altered Fetuin-A levels.

6.2 Introduction

Fibroblast growth factor-23 (FGF-23), produced by the osteocytes, is a phosphaturic hormone that regulates phosphate levels by suppressing phosphate reabsorption and suppressing 1,25-Hydroxyvitamin D production in the kidney.(18) This effect is exerted when FGF-23 binds to its receptors alongside Klotho (an FGF-23 co-receptor).(243) Studies have shown that FGF-23 is associated with progression of chronic kidney disease (CKD) as well as various CKD complications such as mineral and bone disorder (MBD), cardiovascular disease (CVD) and Mortality.(218, 237, 244, 245)

Fetuin-A also known as α 2-Heremans-Schmid glycoprotein (AHSG) is a multifunctional glycoprotein predominantly produced in the liver and has been described as one of the key circulatory inhibitors of calcification.(18, 219) Low levels of Fetuin-A have been associated with MBD, vascular calcification, arterial stiffness, atherosclerosis and other cardiovascular events.(171, 184, 185, 220, 221)

A rise in FGF-23 is often observed in response to hypophosphataemia observed with declining renal function in CKD patients.(246) On the other hand, studies have reported low Fetuin-A levels with declining renal function, possibly as a combined

result of reduced Fetuin-A production in a pro-inflammatory uraemic environment and increased Fetuin-consumption in a pro-calcific environment.(108, 171) Considering the roles of FGF-23 and Fetuin-A, it may be assumed that these biomarkers maintain an inverse relationship in patients with CKD.

The biomarkers FGF-23 and Fetuin-A were selected based on their important role in cardiovascular disease, mortality and mineral and bone disorder (MBD). In CKD patients, studies have shown that FGF-23 contributes to left ventricular hypertrophy, mortality and MBD,(80, 169, 170) while Fetuin-A has been described as the most important circulatory inhibitor of ectopic calcification and also associated with mortality.(18, 171) Although MGP and OPG are also inhibitors of calcification, Fetuin-A contributes to over 50% of circulatory inhibition of ectopic calcification.(172)

Fetuin-A gene polymorphisms have been associated with Fetuin-A levels, ultimately affecting cardiovascular injury in both CKD and non-CKD patients. Several single nucleotide polymorphisms (SNPs) of the Fetuin-A gene have been implicated in this process. (182, 184-186, 247) Eight SNPs of interest (rs2248690, rs6787344, rs4831, rs4917, rs4918, rs2070633, rs2070634 and rs2070635) were identified based on their reported relationship with Fetuin-A levels and markers of bone mineralisation in both CKD and non-CKD patients.(109, 182, 184-189)

This study aimed to determine the relationship between FGF-23 and Fetuin-A in children with CKD and also to determine the role of Fetuin-A SNPs with respect to Fetuin-A levels and biochemical parameters in these children.

6.3 Methods

Thirty-two CKD I, 30 CKD II-IV and 31 CKD V (dialysis) black South African children (aged 5-18) were recruited consecutively over a 12-month period (August 2015 – July 2016). The CKD I group were children with a glomerular filtration (GFR) of >90 ml/min/1.73m² (with either structural abnormalities, or isolated haematuria) with normal blood pressure and no proteinuria, CKD II-IV were those with GFR of 15-90 ml/min/1.73m² and CKD V were those children on maintenance haemodialysis and peritoneal dialysis. Children with known congenital heart disease, diabetes mellitus, liver disease, active infection, systemic lupus erythematosus, malignancies and renal transplant were excluded from the study.

Blood samples were drawn for biochemical assay and genomic DNA. Serum creatinine, phosphate, calcium and intact parathyroid hormone (PTH) levels were assayed using the Siemens Advia system. Plasma 25-hydroxyvitamin D or 25(OH)D was assayed using the ARCHITECT 25(OH)D method. Plasma intact FGF-23 was assayed using Human FGF-23 ELISA Kit (Merck Millipore, Merck group, Massachusetts, USA). Plasma AHSG level was assayed using EDI™ Human Fetuin-A ELISA kit (EPITOPE Diagnostics, Inc, CA, USA).

Glomerular filtration rate (GFR) was estimated by the use of the modified Schwartz formula.(222) Hyperphosphatemia, hypocalcaemia, elevated calcium product and elevated alkaline phosphatase were defined based on age according to the Kidney Disease Outcomes Quality Initiative (KDOQI) and the Kidney Disease Improving Global Outcomes (KDIGO) clinical practice guidelines for MBD in children with CKD.(200) Hyperparathyroidism was defined as PTH levels above laboratory normal limit (>7.6 pmol/L) in pre-dialysis patients and above nine times the upper

normal limit ($>68.4\text{pmol/L}$) in dialysis patients as recommended by KDIGO.(82) Low 25(OH)D was defined as $<30\text{ng/ml}$ and elevated CRP as $>10\text{mg/L}$.(200, 201)

Genomic DNA was extracted using the automated Maxwell platform and commercially available Maxwell® DNA purification kits (Promega corporation, WI, USA). DNA concentrations were determined by the NanoDrop™ 2000 spectrophotometer (Thermo Scientific, USA), and the DNA samples stored at -80°C . Regions of the AHSG gene covering the SNPs of interest (rs2248690, rs6787344, rs4831, rs4917, rs4918, rs2070633, rs2070634 and rs2070635) were amplified by polymerase chain reaction (PCR) on the MJ Mini™ Thermal cycler (Bio-Rad, USA). Primers were designed using the IDT PrimerQuest software (<http://eu.idtdna.com/PrimerQuest/Home/Index>) with sequences and product lengths shown in table 1. For SNPs rs2248690 and rs6787344, primers were modified to induce a restriction site for restriction fragment length polymorphism (RFLP) analysis. The PCR amplification was carried out using the KAPA2G Robust HotStart ReadyMix PCR Kit using $\times 50\text{ ng}$ DNA and $1.25\text{ }\mu\text{l}$ of each of the forward and reverse primers and the recommended thermocycling conditions.

Genotypes for rs2248690, rs6787344, rs4831, rs4917 and rs4918 in Fetuin-A were determined by RFLP. Samples were incubated with their respective restriction enzymes overnight at 37°C . (Table 6.1) To prevent evaporation, each reaction was overlaid with $15\text{ }\mu\text{l}$ of mineral oil. The following day, the reactions were terminated by adding an EDTA-containing gel dye. Fragments were resolved on 10% polyacrylamide gels and visualized using a Gel Doc™ EZ imager (Bio-Rad systems, USA). Three closely positioned SNPs (rs2070633, rs2070634 and rs2070635) were genotyped by direct sequencing at a private laboratory (Inqaba biotech).

6.3.1 Data analysis

All data were collected and managed using Research Electronic Data Capture (REDCap) tools hosted at the University of the Witwatersrand and STATA 13.1 used for the analysis.(204) Continuous variables from the biochemical parameters were described using means (standard deviations) for data normally distributed, and medians (ranges or inter-quartile ranges; IQR) used for skewed data. Categorical variables were presented as proportions. Statistical significance in proportions was tested for using Chi-square (χ^2) tests or Fisher exact test where appropriate. Mean/median values of the different groups were compared using ANOVA or Kruskal-Wallis test depending on the distribution of the data. To compensate for multiple testing, Bonferroni type correction was used to adjust for significant levels for the biochemical parameters as appropriate.

Genotype frequencies for each SNP distribution were calculated and tested for Hardy-Weinberg equilibrium. In addition to the Fisher exact test or the Chi square test, the Trend test of association were used to determine significant difference in the distribution of the genotypes in the CKD groups. Median values of Fetuin-A for each of the different SNP genotypes were compared using Kruskal-Wallis tests. To test for relationships between SNPs and Fetuin-A levels and markers of mineral bone disease, linear regression was used. Skewed data was log transformed before inclusion into the regression model. Only the allele and not the genotype patterns were used to determine these relationships due to the low numbers in some of the genotype categories. A p value of <0.05 was regarded as statistically significant for all analyses.

Table 6.1: Primers and product lengths for the different SNPs

SNP	Primers	PCR product	Restriction Enzyme	RFLP Allelic discrimination	
				Allele	Size
rs2248690	Fwd: 5' - GAA CCC AGA GCT GTG TCA TA - 3'	150 bp	NdeI	A	150bp
	Rev: 5' - TCC TTC TCC AGA CCT CAC T - 3'			T	132bp and 18bp
rs6787344	Fwd: 5' – TAC CGA GGT AAG GAG GGA TTG - 3'	145 bp	BsaI	C	147bp
	Rev: 5' – CCT TAA AAT AGA TTG GCT AGG GAGA - 3'			G	125bp and 20bp
rs4831	Fwd: 5' – GGC AGG CTC CAA CAG ATA AA - 3'	361 bp	PvuII	C	361bp
	Rev: 5' – CAT AGA CAG CAG GTC CAC TTAC - 3'			G	199bp and 162bp
rs4917	Fwd: 5' – TCT CTG TGG GCA GCA ATA TG - 3'	284 bp	NlaIII	C	284bp
	Rev: 5' – GGA GGG AAA GGC ATA GCT AAA - 3'			T	202bp and 82bp
rs4918	Fwd: 5' – GGG AGG AGG AAG CAA ACT AAC - 3'	264 bp	SacI	C	264bp
	Rev: 5' – CAA TGA GAC CAC ACC CAT GAA - 3'			G	209bp and 55bp
rs2070633, rs2070634 and rs2070635	Fwd: 5' - GCT CTA TGA AAC AGG TGG AAG A - 3' Rev: 5' - GGG CTG AGA AGA GTA CAT GAA A - 3'	439 bp	-	-	-

6.3.2 Ethics and Consent

The study was approved by the University of the Witwatersrand, Human Research Ethics Committee (Protocol M150312) and was conducted in conformance with the Helsinki Declaration, Good Clinical Practice and within the laws and regulations of South Africa.

Written consent was obtained for each parent/guardian of participating child, with assent being obtained from children >8 years old.

6.4 Results

The overall mean age of the patients was 10.7 (3.6) years with a male female ratio of 2.3:1. The biochemical parameters of the patients are shown in Table 6.2. The CKD V group had the highest proportion of abnormal parameters.

The median FGF-23 and Fetuin-A levels were 28.9 (0-3893) pg/ml and 57.7 (0.9-225.2) mg/dl respectively. Both FGF-23 and Fetuin-A levels varied between the different CKD groups. (Table 6.2) Median plasma levels of FGF-23 were lowest in the CKD I group and highest in the CKD V group and differed significantly between the three groups ($p < 0.001$). Fetuin-A levels were almost similar in the CKD I and CKD II-IV groups but much lower in the dialysis dependant group although range was wider ($p = 0.006$).

There was no significant linear relationship between total Fetuin-A and FGF-23 levels ($p = -0.18$, $p = 0.088$), but a sub-group analysis showed a significant negative relationship between Fetuin-A and FGF-23 in the CKD V group ($p = -0.60$, $p < 0.001$). Plasma FGF-23 levels correlated better with markers of bone mineralization than Fetuin-A, and no correlation was observed between Fetuin-A and CRP (a marker of inflammation). (Table 6.3)

Table 6.2: Biochemical parameters of the patients

	CKD I (n=32)	CKD II-IV (n=30)	Dialysis CKD (n=31)	p value ^a
Age (years) ^c	9.5 (2.9)	11.0 (3.6)	11.6 (4.0)	0.051
Sex (M/F)	23/9	24/6	18/13	0.167
CRP (mg/L) ^d	10 (-)	10 (10-61)	10 (10-62)	0.024
Elevated CRP (y/n)	0/32	4/26	7/24	0.008
Calcium (mmol/L) ^d	2.32 (2.22-2.38)	2.32 (2.21-2.37)	2.22 (1.99-2.35)	0.005
Hypocalcaemia (y/n)	17/15	15/15	18/13	0.816
Phosphate (mmol/L) ^c	1.47 (0.26)	1.42 (0.25)	1.55 (0.49)	0.333
Hyperphosphatemia (y/n)	4/28	4/26	12/19	0.023
CaXP ^c	3.42 (0.65)	3.23 (0.52)	3.35 (1.28)	0.722
Increased CaXP	0	0	7/24	-
Alkaline phosphatase (U/L) ^d	249 (209-333)	263 (199-310)	252 (184-517)	0.669
Increased Alkaline phosphatase (y/n)	4/28	8/22	16/15	0.003^b
PTH (pmol/L) ^d	3.8 (2.9-5.4)	6.6 (3.8-10.9)	60.1 (18.0-157.4)	<0.001^b
Increased PTH (y/n)	4/28	13/16	16/15	0.002^b
25(OH)D ^c	22.7 (7.4)	25.5 (9.1)	18.1 (6.7)	<0.002^b
Low 25(OH)D (y/n)	28/4	23/7	30/1	0.059
FGF-23 (pg/ml) ^e	15.0 (1.5-90.0)	19.02 (0-219.0)	264.1 (2.4-3893.0)	<0.001^b
Fetuin-A (mg/dl) ^e	65.5 (11.1-96.6)	56.4 (26.6-126.0)	34.2 (0.9-225.2)	0.006

CKD-Chronic kidney disease, CRP-C reactive protein, CaXP-Calcium-phosphorous product, PTH-Parathyroid hormone, 25(OH)D-25Hydroxyvitamin D, FGF-23-Fibroblast growth factor-23

a-Anova, Kruskal Wallis or Chi square test as appropriate

b-Significant after Bonferroni correction (p<0.005)

c- Mean (standard deviation)

d- Median presented with interquartile ranges

e-Median presented with ranges

Table 6.3: Fetuin-A and FGF-23 association with markers of inflammation and bone mineralisation

	FGF-23 Correlation (p value)	Fetuin A Correlation (p value)
CRP (mg/L)	0.082 (0.435)	-0.165 (0.113)
Calcium (mmol/L)	-0.258 (0.012)	0.129 (0.219)
Phosphate (mmol/L)	0.192 (0.065)	-0.044 (0.676)
Alkaline Phosphatase (U/L)	-0.125 (0.234)	0.193 (0.064)
PTH (pmol/l)	0.485 (<0.001)	-0.253 (0.015)
25(OH)D (ng/ml)	-0.370 (<0.001)	0.103 (0.328)

CRP-C reactive protein, PTH-Parathyroid hormone

Table 6.4: Distribution of SNPs by CKD groups

		CKD I (n=32)	CKD II-IV (n=30)	CKD V-Dialysis (n=31)	p value ^b	p value ^c
rs2248690	AA	15	15	18	0.866	0.402
	AT	13	10	10		
	TT	4	5	3		
rs6787344	CC	4	3	5	0.864	0.673
	CG	28	27	26		
	GG	0	0	0		
rs4831	CC	19	15	12	0.481	0.121
	CG	11	11	16		
	GG	2	4	3		
rs4917	CC	20	16	19	0.940	0.918
	CT	11	13	11		
	TT	1	1	1		
rs4918^a	CC	0	0	1	0.327	0.038
	CG	30	29	30		
	GG	2	0	0		
rs2070633^a	TT	12	13	17	0.128	0.135
	TC	10	5	10		
	CC	9	12	4		
rs2070634^a	TT	12	13	16	0.495	0.138
	TG	12	13	13		
	GG	7	4	2		
rs2070635^a	AA	31	28	31	0.104	1.000
	AG	0	2	0		
	GG	0	0	0		

CKD-chronic kidney disease

^a Total sample size <106,

^b Fisher's exact or Chi square test as appropriate,

^c trend test for association

Table 6.5: Regression analysis for CVRFs and individual SNP alleles

	β coefficient	p value	95% CI
log Fetuin-A			
rs4918_C	-0.40	0.568	-1.78; 0.98
rs4918_G	1.94	0.046	0.03; 3.85
rs2070633_T	-0.55	0.015	-0.98; -0.11
rs2070633_C	-0.10	0.616	-0.51; 0.30
Phosphate			
rs6787344_C	-0.16	0.568	-0.71; 0.39
rs6787344_G	0.22	0.042	0.01; 0.43
log PTH			
rs4918_C	1.57	0.135	-0.50; 3.65
rs4918_G	-2.97	0.044	-5.86; -0.08

PTH-Parathyroid hormone

Eight SNPs were analysed; rs2248690, rs6787344, rs4831, rs4917, rs4918, rs2070633, rs2070634 and rs2070635. Four of these SNPs (rs2248690, rs6787344, rs4918 and rs2070633) did not follow the Hardy-Weinberg law ($p \leq 0.05$) but were not excluded from the analysis consequent to the small sample size. (Table 6.4)

We found no significant difference in Fetuin-A levels in the different SNP genotype distributions, but we found significant association between log transformed Fetuin-A levels and the rs4918 G-allele compared to the rs4918 C-allele ($p=0.046$) and the rs2070633 T-allele when compared to the rs2070633 C-allele ($p=0.015$). (Table 6.5)

Markers of MBD such as phosphate and PTH levels were associated with Fetuin-A SNPs. The rs6787344 G-allele was significantly associated with phosphate levels (0.042), and the rs4918 G-allele with PTH ($p=0.044$). (Table 6.5)

6.5 Discussion

This comparative study demonstrates the changes in the levels of Fetuin-A and FGF-23 seen in the different spectra of CKD in black South African children. As expected, given the roles of these two biomarkers in MBD,(246, 248, 249) we identified a strong negative relationship between Fetuin-A and FGF-23 in the dialysis group. It is interesting to observe that we did not demonstrate a similar relationship in the other CKD groups. This seems to suggest that, although changes of MBD are seen in early CKD,(250, 251) a clearer relationship between these two biomarkers is only seen in advanced disease. Fetuin-A levels varied across the disease spectrum but did not decrease linearly with disease progression, while FGF-23 levels did show a linear increase with progression of disease. Our study also showed that FGF-23 correlated better with markers of bone mineralization than with Fetuin-A.

The near similar levels of Fetuin-A in the CKD I and CKD II-IV groups despite advancing disease and the wider range in the dialysis group cannot be attributed solely to disease progression, other factors such as Fetuin-A gene polymorphisms might have contributed to these differences.

Previous studies have reported a link between various Fetuin-A SNPs and Fetuin-A levels with the most widely reported SNPs associated with Fetuin-A levels being rs4917, rs4918, rs2248690, rs2070633 and rs2070635.(109, 184-189) In this study, there was a similar association of the rs4918 and rs2070633 SNPs and Fetuin-A levels. The rs4918 G-allele showed a positive association with Fetuin-A levels, while the rs2070633 T-allele showed a negative association with Fetuin-A levels.

Contrary to what has been previously reported, where having the SNP genotypes such as rs4918 CG or GG genotypes were associated with Fetuin-A levels,(109,

184, 185, 187, 188) we did not observe a similar trend. This variation may be attributed to the small size of our study group which affected the distribution of the SNP genotypes and alleles. The younger age of our patients and their genetic makeup of our patients might have also contributed to the difference.

There is a dearth of studies that have looked at the association between Fetuin-A SNPs and markers bone mineralization implicated in vascular injury such as phosphate, calcium-phosphate product and parathyroid hormone (PTH). A study by Osawa et al reported a significant difference in phosphate levels among Fetuin-A genotypes.(188) Our study observed a positive relationship of phosphate levels with the Fetuin-A SNP rs6787344 G-allele. Serum PTH levels were also found to be negatively associated with rs4918 G-allele.

6.6 Strength and limitations

This is a study that described the relationship between FGF-23 and Fetuin-A, and also explored the influence of several Fetuin-A SNPs on Fetuin-A levels and markers of bone mineralization in black South African children with different spectra of CKD. The major limitations are the small sample size and the lack of a disease free control for comparison.

6.7 Conclusion

In spite of the limitations, this study was able to demonstrate the relationship between FGF-23 and Fetuin-A and the association of Fetuin-A SNPs with serum Fetuin-A, phosphate and PTH levels in children with CKD. The study suggests that an inverse relationship between FGF-23 and Fetuin-A is more likely to be observed in children with advanced CKD, and that FGF-23 correlates better with markers of

bone mineralisation children when compared to Fetuin-A. The study also suggests that children with the rs4918-G allele and rs2070633_T allele are more likely to have altered Fetuin-A levels.

6.8 Acknowledgement

The authors acknowledge the contribution of all team members who provided care for these children and assisted with this study.

6.9 Conflict of interest

The authors declare no conflict of interest.

CHAPTER 7: CONCLUSION

The initiation and progression of CVD in children with CKD is determined by the presence of single or multiple CVRFs, and this is thought to begin early and then worsens as the renal function declines. There is a shortage of studies from Africa that have looked at CVD in paediatric CKD. This comparative multifaceted cross sectional study tested the following hypotheses; that the prevalence of CVRFs was higher in South African children with CKD when compared to results reported in those from developed countries, that the prevalence of cardiovascular changes was higher in South African children with CKD when compared results reported in those from developed countries, that Fetuin-A and FGF-23 were associated with CVRFs and cardiovascular changes in children with CKD and that Fetuin-A gene polymorphisms were negatively associated with plasma Fetuin-A levels in South African children with CKD.

7.1 Summary of study findings

The results revealed the following:

1. There is a high prevalence of mortality in CKD-Dialysis South African children when compared with developed countries and this is most likely due to a younger age at commencement of dialysis and the high prevalence of modifiable CVRFs in our cohort.
2. The overall prevalence of CVRFs is higher in South African children with severe CKD (stage V on dialysis) when compared to children with mild (stage 1) and moderate disease (stage 2-4).

3. Anaemia is the most prevalent cardiovascular risk factor in South African children with CKD, and not hypertension as has been reported from developed countries. The high prevalence of anaemia likely reflects the undernutrition reported in the dialysis group in addition to other causes of anaemia in CKD such as declining production of erythropoietin, inflammation, severe secondary hyperparathyroidism leading to myelofibrosis, marrow hypo-responsiveness and infection.(63) Poor absorption and adherence to oral iron due to side effects like constipation, diarrhoea and abdominal discomfort might have also contributed to the high rate of anaemia in our patients.
4. There was a high prevalence of hypoalbuminaemia and hypercholesterolaemia, however these high prevalences were not due to nephrotic range proteinuria. There were a total of 22 children with nephrotic syndrome, but only 17 had a urine protein assessment (1-congenital NS, 4-FSGS and 12-MCD). All the patients with MCD were in remission (no proteinuria), and only 2 patients had nephrotic range proteinuria and the remaining of the patients had non-nephrotic range proteinuria. Therefore, it is unlikely that the hypercholesterolemia was due to nephrotic range proteinuria.
5. The carotid intima media thickness (cIMT) measurements and the prevalence of cardiovascular changes such as abnormal left atrial dimension (LAD) and left ventricular hypertrophy, are higher in South African children with severe CKD (stage V on dialysis) when compared to children with mild (stage 1) and moderate disease (stage 2-4).

6. Cardiovascular risk factors including Fetuin-A, but not FGF-23, are associated with cardiovascular changes, and FGF-23 correlates better with CVRF including markers of mineral bone metabolism when compared to Fetuin-A
7. Fetuin-A gene SNPs rs4918 G-allele is positively associated with Fetuin-A levels, while the rs2070633 T-allele showed a negative association with Fetuin-A levels. This differs from previously published findings and it is possible that genetic variations due to the African descent of the majority of our patients, and also the background CKD, could explain these differences. Serum phosphate and parathyroid hormone levels are also associated with Fetuin-A SNPs.

7.2 Significance of the study

This is the first study to specifically look at CVRFs, cardiovascular changes as well as mortality in African children with CKD and we found significant differences in our cohort of patients when compared with previously reported data from elsewhere. Our results highlight a higher cIMT measurements than has been previously described, and also the uncommon finding of haemoglobin as an independent associated factor for cIMT in children with CKD. Our results also highlight the need for us to determine our own, population specific, paediatric reference values for cIMT in healthy children.

The study also provides information on the types and prevalence of cardiac changes seen in a spectrum of South African children with CKD. Left ventricular hypertrophy remains the most common cardiac change seen in children with CKD and, although less common, it is also important to monitor for left atrial changes in these children.

The study also provides more insight into the role of CVRFs in morbidity and mortality in children with CKD. Modifiable risk factors, such as anaemia and hypoalbuminaemia, in addition to adequate blood pressure control, need to be addressed in order to retard the progression of cardiovascular disease in these children.

The study also demonstrated the possible role of genetic variations in Fetuin-A gene expression and its relationship with Fetuin-A levels and CVRFs.

7.3 Future research and recommendations

Based on our results we would recommend the following going forward:

1. The establishment of large multicentre study to determine paediatric reference values for cardiovascular parameters in African children
2. The establishment of a large, prospective, multicentre Southern African study (with a control group) looking at CVRFs and cardiac changes as CKD progresses
3. The establishment of a screening program to detect, and address, CVRFs (especially the modifiable ones) in children with CKD in order to improve their outcome.

7.4 Study limitation

Even though this is the first African study that has highlighted the above important findings, the study was limited by its small sample size and the lack of a formal control group. In spite of the small number of patients due to the selection criteria

and small paediatric population of CKD patients, participants were recruited from two centres; CMJAH and CHBAH. The CKD groups (CKD II-IV) were lumped up together due to the small number of patients in the individual groups which would have made the analysis difficult or void.

Furthermore, the nature of the study did not allow for intervention and observation of the progression of cardiovascular changes. Another limitation was that not all of the study participants had cardiovascular imaging performed due to time constraints and other logistical problems.

The details of the dialysis adequacy particularly fluid control was not available from the clinical records of these patients. However, it was clear from the frequent admissions and clinical records that poor compliance amidst an unfavourable social background could have contributed to the high prevalence of these modifiable risk factors and the mortality observed in the dialysis patients.

7.5 Concluding remarks

The objectives of this study were met and the study confirmed the higher rate of cardiovascular risk factors and cardiovascular changes in South African children with severe CKD (stage V on dialysis) when compared to children with mild (stage 1) and moderate disease (stage 2-4). The study also confirmed the association of these CVRFs and cardiovascular changes and showed that population specific differences need to be taken into account when using standard reference values established on different population groups. The researcher anticipates that this study, when

published, will bridge a significant part of the knowledge gap of CVD in African children with CKD.

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APPENDIX A: ETHICAL CLEARANCE CERTIFICATE



R14/49 Dr Abdullahi Mudi

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150312

NAME: Dr Abdullahi Mudi

(Principal Investigator)

DEPARTMENT: Paediatrics
Charlotte Maxeke Johannesburg Academic Hospital

PROJECT TITLE: Cardiovascular Risk Factors and Their Association
with Biomarkers in Children with Chronic Kidney
Disease in Johannesburg, South Africa

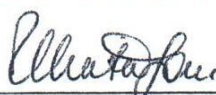
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DECISION: Approved unconditionally

CONDITIONS:

SUPERVISOR: Prof Daynia Ballot, Dr Cecil Levy and Dr Caroline Dickens

APPROVED BY:



Professor P Cleaton-Jones, Chairperson, HREC (Medical)

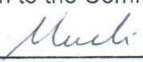
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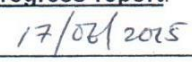
DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**



Principal Investigator Signature



Date

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APPENDIX D: INFORMATION SHEET FOR CONSENT (GENERAL)

Study title: Cardiovascular risk factors and their association with biomarkers in children with chronic kidney disease in Johannesburg, South Africa.

Investigator: Dr Abdullahi Mudi 011 488 3296

Supervisors: Prof D Ballot, Dr CS Levy, Dr C Dickens

Institution: University of the Witwatersrand

Good day. My name is Dr Abdullahi Mudi. I am currently working in the Division of Paediatric Nephrology, Charlotte Maxeke Johannesburg Academic Hospital. Permission to conduct the study has been granted by the WITS Human Research Ethics Committee.

Introduction: We are conducting research on the cardiovascular risk factors and their association with biomarkers in children with chronic kidney disease (CKD). Research is just the process to learning the answer to a question or learn more about something. In this study we want to learn more about the heart and the risk factors for developing heart disease and the association of these risk factors with certain blood markers in children with CKD. This is because children with CKD are at risk of developing heart and vascular problems. The finding of this research may determine if changes should be made in the follow up protocol for children with chronic kidney disease.

Invitation to participate: We are asking for your permission to include your child in this research study.

What is involved in the study: One hundred and twenty five to one hundred and fifty patients are expected to participate in the study. All that is required from you is to answer some questions, your child will be examined and routine blood samples and an extra 10mL of blood (a tablespoon) would be taken for analysis and Genetic testing.

Testing of DNA and genes is called genetic testing. Genetic testing may help in the diagnosis and better understanding of a disease. Genetic testing can also be performed for research. When a gene has changes it is called a mutation.

Results of the blood analysis will be retrieved later and interpreted. The remaining samples will be frozen and stored in a designated freezer for an unlimited period of time for future use in research related to diseases. However, if you decide later that you do not want the specimens collected from your child to be used for future research, please notify the principal investigator in writing and the sample will be discarded in an appropriate and timely manner.

As part of the study, your child will also have an ultrasound of the heart (echo) and carotid (in the neck) vessel.

Participation is entirely voluntary and declining to participate will not affect the treatment of your child in any way. You are also free to opt out at any point in the research.

Risks: There are no anticipated risks for your child.

Benefits of being in the study: Your child has been diagnosed with CKD and CKD has been identified as a major risk factor for heart and blood vessel changes in children with CKD. These changes may lead to heart disease. No one knows exactly when these changes begin to occur and it is believed that early diagnosis is important for early intervention. The potential benefit from your participation in this study is that early changes in the blood vessels may be picked up and treated early. Should we detect any problem with your child's heart and blood vessels we will inform you of this and will refer your child on for appropriate management.

You will be given pertinent information on the study while involved in the project and after the results are available.

Participation is voluntary, refusal to participate will involve no penalty or loss of benefits to which your child is otherwise entitled and that he/she may discontinue participation at any time without penalty loss of benefits to which he/she is otherwise entitled.

There will be no extra cost to you and your family.

Confidentiality: Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Research Ethics Committee.

If results are published, your child will not be identified.

Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, and your right to withdraw your child from the study at any time.

Contact details of researcher – For further information on the study, you can call the following number : **011 488 3296** (Working hours only).

Contact details of REC administrator and chair – for reporting of complaints / problems.

- Professor Peter Cleaton-Jones .
- Phone no: 011-717-2301.

Thank you for taking the time to consider our request.

INFORMED CONSENT FORM (GENERAL)

- I hereby confirm that I have been informed by the study doctor, Dr Mudi, about the nature, conduct, benefit and risks of the clinical study.
- I have also received, read and understood the above written information (Information sheet and Informed Consent) regarding the clinical study.
- I am aware that the results of the study, including personal details regarding my child's sex, age, date of birth, initials, and diagnosis will be anonymously processed into a study report.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

Guardian

Printed Name	Signature / Mark or Thumbprint	Date and Time
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I, Dr Abdullahi Mudi, herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

Researcher:

Printed Name	Signature	Date and Time
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Translator / Other person explaining informed consent..... (Designation):

Printed Name	Signature	Date and Time
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INFORMATION SHEET FOR STORAGE OF SPECIMEN AND GENETIC TESTING

Study title: Cardiovascular risk factors and their association with biomarkers in children with chronic kidney disease in Johannesburg, South Africa.

Investigator: Dr Abdullahi Mudi

Supervisors: Prof D Ballot, Dr CS Levy, Dr C Dickens

Institution: University of the Witwatersrand

Good day. My name is Dr Abdullahi Mudi. I am currently working in the Division of Paediatric Nephrology, Charlotte Maxeke Johannesburg Academic Hospital. Permission to conduct the study has been granted by the WITS Human Research Ethics Committee.

Introduction: In this study we want to learn more about the heart and the risk factors for developing heart disease by carrying out blood tests and genetic testing on your child's blood sample. Storage of collected blood samples is required in order to carry out these testing.

Testing of DNA and genes is called genetic testing. Genetic testing may help in the diagnosis and better understanding of a disease. Genetic testing can also be performed for research. When a gene has changes it is called a mutation and can be associated with a disease.

Invitation to participate: We are asking for your permission to store your child's blood sample for an unlimited period of time and to carry out genetic testing on the sample as part of the study.

What is involved in the study: All that is required from you is to answer some questions, your child will be examined and routine blood samples and an extra 10mL of blood (a tablespoon) would be taken for analysis and Genetic testing.

Results of the blood analysis will be retrieved later and interpreted. The remaining samples will be frozen and stored in a designated freezer for an unlimited period of time for future use in research related to this study and other diseases. However, if you decide later that you do not want the specimens collected from your child to be used for future research, please notify the principal investigator in writing and the sample will be discarded in an appropriate and timely manner.

Participation is entirely voluntary and declining to participate will not affect the treatment of your child in any way. You are also free to opt out at any point in the research.

Risks: There are no anticipated risks for your child.

Benefits of being in the study: The potential benefit from your participation in this study is that risk factors or problems can be picked up and treated early. Should we detect any problem with your child's heart and blood vessels or blood tests we will inform you of this and will refer your child on for appropriate management.

You will be given pertinent information on the study while involved in the project and after the results are available.

Participation is voluntary, refusal to participate will involve no penalty or loss of benefits to which your child is otherwise entitled and that he/she may discontinue participation at any time without penalty loss of benefits to which he/she is otherwise entitled.

There will be no extra cost to you and your family.

Confidentiality: Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Research Ethics Committee.

If results are published, your child will not be identified.

Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, and your right to withdraw your child from the study at any time.

Contact details of researcher – For further information on the study, you can call the following number : **011 488 3296** (Working hours only).

Contact details of REC administrator and chair – for reporting of complaints / problems.

- Professor Peter Cleaton-Jones .
- Phone no: 011-717-2301.

Thank you for taking the time to consider our request.

CONSENT FORM FOR STORAGE OF SPECIMEN AND GENETIC TESTING

- I hereby confirm that I have been informed about the study by Dr. Mudi about the nature, benefits and risks of sample storage and genetic testing.
- I understand that my child's blood sample will be stored for future testing.
- I understand that my child's personal details (any identifying data) will be kept strictly confidential.
- I have had the opportunity to ask questions and I have also received, read and understood the study as explained in the participant information sheet and consent to taking part in this research study.

Guardian

Printed Name	Signature / Mark or Thumbprint	Date and Time
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I, Dr Abdullahi Mudi, herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

Researcher:

Printed Name	Signature	Date and Time
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Translator / Other person explaining informed consent..... (Designation):

Printed Name	Signature	Date and Time
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INFORMATION SHEET FOR ASSENT

Study title: Cardiovascular risk factors and their association with biomarkers in children with chronic kidney disease in Johannesburg, South Africa.

Investigator: Dr Abdullahi Mudi

Supervisor: Prof D Ballot, Dr CS Levy, Dr C Dickens

Institution: University of the Witwatersrand

Good day. My name is Dr. Abdullahi Mudi. I am currently working in the Division of Paediatric Nephrology, Charlotte Maxeke Johannesburg Academic Hospital. Permission to conduct the study has been granted by the WITS Human Research Ethics Committee.

Introduction: We are conducting research on the cardiovascular risk factors and their association with some blood tests in children with chronic kidney disease. In this study we want to learn more about the risk factors for heart disease and their relationship with some blood tests for heart disease.

Invitation to participate: We are asking for your permission to include you in this research study.

What is involved in the study: You will be examined and routine blood samples and an extra 10mL of blood (a tablespoon) would be taken for analysis. Subsequently you will have an ultrasound of the heart and neck vessel.

Participation is entirely voluntary and if you decide not to participate in our study this will not affect your treatment in any way. You are also free to change your mind about being in the study at any time.

Risks: There are no anticipated risks for you.

Benefits of being in the study: We may detect early changes in the heart and blood vessels and refer you for appropriate management.

There will be no extra cost to you and your family.

Confidentiality: We will keep your details confidential which means that we won't allow anyone not involved in the running of the study to know your information.

Contact details of researcher – For further information on the study, you can call the following number : **011 488 3296** (Working hours only).

Contact details of REC administrator and chair – for reporting of complaints / problems.

- Professor Peter Cleaton-Jones.
- Phone no: 011-717-2301.

Thank you for taking the time to consider our request.

INFORMED ASSENT FORM

I hereby confirm that Dr Mudi has explained what the study is about to me and that he has explained that I am free to refuse to take part in the study at any time should I so wish.

Participant

Printed Name	Signature / Mark or Thumbprint	Date and Time
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I, Dr Abdullahi Mudi, herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

Researcher:

Printed Name	Signature	Date and Time
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Translator / Other person explaining informed assent..... (Designation):

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Cardiovascular risk factors and mortality in children with chronic kidney disease

A Madi,^{1,2} MBBS, FWACP, MSc (Med), Cert Nephrol (Paed); C Dickens,³ PhD; C Levy,¹ MB BCh, FCPaed, MMed, Cert Nephrol (Paed); D Ballot,¹ MB BCh, FCPaed, PhD

¹ Department of Paediatrics and Child Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

² Department of Paediatrics, Bayero University, Kano, Nigeria

³ Department of Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

Corresponding author: A Madi (abdelmadi@gmail.com)

Background. Cardiovascular disease (CVD) begins early in children with chronic kidney disease (CKD), and its progression is determined by the presence of single or multiple cardiovascular risk factors (CVRFs).

Objective. To determine the prevalence of CVRFs in children with CKD and their association with mortality in children on chronic dialysis.

Methods. This comparative cross-sectional study recruited children aged 5 - 18 years with all stages of CKD. All patients had a short history taken along with a physical examination, and their blood samples were assessed for serum creatinine, urea, albumin, calcium, phosphorus, parathyroid hormone, alkaline phosphatase, total cholesterol (TC), haemoglobin and C-reactive protein. Urine samples were also assessed for proteinuria.

Results. One hundred and six children who met the study criteria were recruited, 34 with CKD I, 36 with CKD II - IV and 36 with CKD V (dialysis). The overall median age was 11 years (range 8 - 14), and the male/female ratio was 2.1:1. The most common CVRF was anaemia (39.6%). The rate of anaemia was higher in the dialysis group than in the CKD II - IV and CKD I groups (77.8%, 33.3% and 5.9%, respectively). Other CVRFs detected were hypertension, proteinuria, hypercholesterolaemia and dysregulated mineral bone metabolism.

Seven deaths were recorded in the dialysis group during the study period. Severe hypertension and intracranial bleeding were the most common causes of death. Modifiable risk factors such as increased TC and decreased albumin levels were more common than other CVRFs in the dialysis patients who died.

Conclusions. CVRFs may be present in early CKD, even before the decline in GFR. Routine screening for CVRFs, along with timely intervention, may prevent the progression of CVD and mortality later in life.

S Afr Med J 2017;107(8):710-714. DOI:10.7196/SAMJ2017.10708.12271

Cardiovascular disease (CVD) is thought to begin early in chronic kidney disease (CKD) and to progress rapidly as renal function declines, especially on dialysis.^[1-3] CVD is the most common cause of death among paediatric patients with end-stage renal disease (ESRD).^[4]

Traditional and non-traditional cardiovascular risk factors (CVRFs) play an important role in the initiation and progression of CVD in children with CKD. Combinations of these risk factors could cause accelerated manifestations of cardiac and vascular changes in children.^[5,6] Traditional CVRFs such as hypertension, dyslipidaemia, obesity and hyperglycaemia have been shown to be increased even in children with early stages of CKD.^[7-9] Non-traditional CVRFs including anaemia, fluid overload, dysregulated mineral bone metabolism (hyperparathyroidism, increased calcium-phosphate product), hypocalcaemia, inflammation (increased C-reactive protein and cytokines) and oxidative stress are more evident in children with moderate to severe CKD.^[10-15] Other risk factors for CVD are potentially treatment related, such as calcium overload from dialysis, calcium-based phosphate binders and vitamin D therapy.^[16,17]

In adults with ESRD, coronary artery disease and cardiomyopathy are the leading cause of CVD mortality. Children, however, die from cardiac arrest, arrhythmia, cardiomyopathy and, rarely, myocardial disease.^[18-20] Various vascular changes such as atherosclerosis, arteriolestenotic lesions (including fibrous or fibroelastic intimal thickening), disruption of the internal elastic lamella and atheromatous plaques have also been reported in children with

CKD.^[21-23] These vascular changes increase the risk of asymptomatic CVD later in life.^[2]

Objective

To investigate the prevalence of CVRFs and their association with mortality in children with CKD.

Methods

This comparative cross-sectional study recruited 106 children with CKD being followed up by the Division of Paediatric Nephrology at Charlotte Maxeke Johannesburg Academic Hospital and Chris Hani Bangwanath Academic Hospital, Johannesburg, South Africa (SA).

Thirty-four patients with CKD I, 36 with CKD II - IV and 36 with CKD V (dialysis) were recruited consecutively over a 12-month period (August 2015 - July 2016). The CKD I group were children with a glomerular filtration rate (GFR) of >90 mL/min/1.73m² (with either structural abnormalities or isolated haematuria) with normal blood pressure and no proteinuria, the CKD II - IV group were those with GFR of 15 - 90 mL/min/1.73m², and the CKD V group were on maintenance haemodialysis and peritoneal dialysis.

Children with known congenital heart disease, diabetes mellitus, liver disease, active infection, systemic lupus erythematosus and malignancies and those who had had a renal transplant were excluded from the study.

All patients had a short demographic and clinical history taken along with a physical examination. Routine blood samples for serum creatinine, urea, albumin, calcium, phosphorus, parathyroid hormone

Cardiac Changes and Their Association with Fetuin-A and Fibroblast Growth Factor-23 in Children with Chronic Kidney Disease

Abdullahi Mudi^{a,c} Hopewell Ntsinjana^a Caroline Dickens^b Cecil Levy^a
Daynia Ballot^a

^aDepartment of Paediatrics and Child Health, and ^bDepartment of Medicine, University of the Witwatersrand, Johannesburg, South Africa; ^cDepartment of Paediatrics, Bayero University, Kano, Nigeria

Keywords

Cardiovascular disease · Fetuin-A · FGF-23 · Children · Chronic kidney disease

Abstract

Aims: In children with chronic kidney disease (CKD), fetuin-A and fibroblast growth factor-23 (FGF-23) have been implicated in the mechanism and progression of several cardiac changes. This study aimed to determine the types and rates of cardiac changes in children with CKD and their association with fetuin-A, FGF-23, and other cardiovascular risk factors (CVRFs). **Methods:** This comparative cross-sectional study recruited 88 children (5–18 years): 27 CKD I with a glomerular filtration rate (GFR) >90 mL/min/1.73 m² and 61 with a GFR of <90 mL/min/1.73 m² (29 CKD II–IV, 32 CKD V–dialysis). Each patient had a short demographic and clinical history taken along with a physical examination. Blood was taken and sent for routine tests and for fetuin-A and FGF-23 assay. All patients had an echocardiogram to evaluate cardiac structure and function. **Results:** The distribution of left atrial diameter (LAD) and left ventricular (LV) mass differed significantly ($p < 0.05$) across the different CKD groups. Abnormal LAD was seen in 10% of patients; LV hypertrophy (LVH) in 27%; LV systolic dysfunction in 6% and diastolic dysfunction in 1 patient. Fetuin-A was the only independent predictor for abnormal LAD; mean arterial pressure was independently associated with concentric LVH, and age and hypoalbuminemia with eccentric LVH. Overall, the dialysis group had the highest rate of cardiac changes and associated risk factors. **Conclusion:** Though not common, the importance of left atrial changes in children with CKD is highlighted along with the need to address modifiable CVRFs such as hypertension and hypoalbuminemia.

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Introduction

Cardiovascular disease (CVD) is a major cause of death in children with chronic kidney disease (CKD), and children with CKD have the highest cardiovascular risk in the pediatric population [1, 2]. Several cardiac changes have been reported such as left ventricular (LV) hypertrophy (LVH), cardiomyopathies, systolic and diastolic dysfunctions, coronary artery disease, arrhythmias, and myocardial ischemia [3–5]. These changes can begin early in CKD and worsen as the disease progresses [6].

Cardiovascular risk factors (CVRFs) have been reported even in children with early CKD [6]. In addition to risk

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Dr. Abdullahi Mudi
Division of Paediatric Nephrology, Department of Paediatrics and Child Health
University of the Witwatersrand and Charlotte Maxeke Johannesburg Academic Hospital
7 York Road, Parktown, Johannesburg 2093 (South Africa)
E-Mail abdulla@mudi@gmail.com

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